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ORIGINAL ARTICLE

Duplicated *Abd-B* class genes in medaka *hoxAa* and *hoxAb* clusters exhibit differential expression patterns in pectoral fin buds

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Abstract *Hox* genes form clusters. Invertebrates and *Amphioxus* have only one *hox* cluster, but in vertebrates, they are multiple, i.e., four in the basal teleost fish *Polyodon* and tetrapods (*HoxA*, *B*, *C*, *D*), but seven or eight in common teleosts. We earlier completely sequenced the entire *hox* gene loci in medaka fish, showing a total of 46 *hox* genes to be encoded in seven clusters (*hoxAa*, *Ab*, *Ba*, *Bb*, *Ca*, *Da*, *Db*). Among them, *hoxAa*, *hoxAb* and *hoxDa* clusters are presumed to be important for fin-to-limb evolution because of their key role in forelimb and pectoral fin development. In the present study, we compared genome organization and nucleotide sequences of the *hoxAa* and hox*Ab* clusters to these of tetrapod *HoxA* clusters, and found greater similarity in *hoxAa* case. We then analyzed expression of *Abd-B* family genes in the clusters. In the trunk, those from the *hoxAa* cluster, i.e., *hoxA9a*, *hoxA10a*, *hoxA11a* and *hoxA13a*, were expressed in a manner keeping the colinearity rule of the *hox* expression as those of tetrapods, while those from the *hoxAb* cluster, i.e., *hoxA9b*, *hoxA10b*, *hoxA11b* and *hoxA13b*, were not. In the pectoral fins, the *hoxAa* cluster was expressed in split domains and did not obey the rule. By contrast, those from the *hoxAb* and *hoxDa* clusters were expressed in a manner keeping the rule, i.e., an ancestral pattern similar to those of tetrapods. It is plausible that this differential expression of the two clusters is caused by changes occurred in global control regions after cluster duplications.

Keywords *Oryzias latipes* · Pectoral fin bud · *Hox Abd-B* family · Colinearity · Subfunctionalization

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Introduction

Hox genes encode transcription factors that play key roles in body planning (Gehring et al. 1994). In *Drosophila*, the *Hox* cluster is termed the *HOM* complex, containing 11 homeotic genes. The *HOM* genes have the remarkable feature called ‘colinearity’, that is, they are expressed along the body axis corresponding to the chromosomal positions (Levine et al. 1983; Harding et al. 1985). Homologues of the *HOM* genes have been identified in a number of animal species and vertebrates that maintain *HOM* homologues as clusters as well as the colinearity rule (Izpisúa-Belmonte et al. 1991; Krumlauf 1992). Their *Hox* genes have been classified into 13 paralogy groups, numbered *HoxA1* through to *HoxA13* (Scott 1992).

In the evolutionary lineage of vertebrates, *Amphioxus* with archaic vertebrate features carries only one *hox* cluster (Ferrier et al. 2000), whereas mammals and the basal ray-finned fish *Polyodon* possess four *Hox* clusters that are

termed *HoxA*, *B*, *C* and *D* (Duboule and Dollé 1989, Metscher et al. 2005), due to two episodes of genome duplication (Hart et al. 1987). Further, it is known that common ray-finned fishes (teleosts) including zebrafish, pufferfish and medaka fish have experienced an additional duplication resulting in seven or eight clusters (Amores et al. 1998, 2004; Naruse et al. 2000; Kurosawa et al. 1999, 2006). These are termed *hoxAa*, *hoxAb*, *hoxBa*, *hoxBb*, *hoxCa*, *hoxDa* and *hoxDb* in the medaka (Kurosawa et al. 2006).

The duplicated *Hox* clusters have acquired new or modified regulation features, causing an increase in developmental complexity and capacity for diversification (Holland 1999; Holland and Garcia-Fernàndez 1996). In mice, paralogous *Hox* genes have expressional differences, suggesting that duplication allows expression changes depending on *cis*-regulatory regions (Kessel and Gruss 1990; Burke et al. 1995).

We earlier determined the structure of the entire *hox* gene loci in medaka fish, revealing that 46 *hox* genes in total are encoded in seven clusters, and suggested that the third round of duplication of *hox* clusters might have occurred before their divergence from zebrafish (Kurosawa et al. 2006). The *hoxAa*, *hoxAb* and *hoxDa* clusters are important for fin-to-limb evolution, playing key roles in forelimb and pectoral fin development (Dollé et al. 1989; Sordino et al. 1995). In the present study, we first compared genome organization and nucleotide sequences of *hoxAa* and *Ab* clusters with those known to date for tetrapods and teleosts. Then, we analyzed expression patterns of *Abd-B* family genes in the clusters in the trunk and pectoral fin buds to allow speculation on the regulatory basis of differential expression of the duplicated genes.

Materials and methods

Fish strains

Orange–red variety Japanese medaka fish (*Oryzias latipes*) were originally obtained from a vendor in Nagoya. These fish were raised and maintained in our laboratory under standard conditions of 14/10 h day/night cycle at 27–28°C. Embryos at 1, 2, and 3 days past fertilization (dpfs) were sampled and used for in situ hybridization. These dpfs correspond to stages 21–22, 26–27, and 29–30 of the stage map of medaka embryo development published by Iwamatsu (2004).

Sequence analysis

The *Hox* clusters and their DNA sequences used in this study were as follows: mouse *HoxA* cluster (*Mus musculus HoxA* cluster: *Mmu–HoxA*), accession nos. AC015583.34, AC091106.17, and AC113985.11 in GenBank DNA Database; mouse *HoxD* cluster (*Mmu–HoxD*), accession nos. AL928644.12 and AL928733.14; mouse HoxA amino acid sequences, HoxA2, A9, A10, A11, and A13, accession nos. P31245, P09631, P31310, P31311, and Q62424; mouse HoxD9 amino acid sequence, accession no. P28357. Those for *O. latipes hoxAa*, *hoxAb*, *hoxDa*, *hoxDb* clusters (*Ola–HoxAa*, *Ola–HoxAb*, *Ola–HoxDa* and *Ola–HoxDb*) were obtained from AB232918, AB232919, AB232923, and AB232924, respectively (Kurosawa et al. 2006).

To elucidate similar regions in the two *hox* clusters, we compared them with the Pip-Maker program (Schwartz et al. 2000), which computes alignments of similar regions in two or more DNA sequences. The mouse *HoxA* cluster sequence was employed as the template to which the others were compared with the program MultiPipMaker (http://pipmaker.bx.psu.edu/pipmaker/). The results summarized as "percent identity plots," or "pips" for short.

Probes

Polymerase chain reaction (PCR) primer sequences for production of probes were selected from each gene sequence. To avoid cross hybridization between paralogous genes, we searched for low homology regions by National Center for Biotechnology Information blast and multiple alignment programs, such as ClustalW (http://www.ddbj.nig.ac.jp/search/clustalw-j.html) and GENETYX-MAC (Genetyx, Tokyo). For example, to prepare probes not cross hybridizing between *hoxA9a* and *hoxA9b* that are highly homologous with each other, we identified unique sequences from *hox* coding sequences with accession nos. AB232918 and AB232919 by the alignment software. All *Abd-B* genes in *hoxAa*, *Ab* and *Da* clusters were well amplified by PCR and cloned, but *hoxD9b* gene was missed to amplified. This missing may be caused on very weak or no expressions in 2 and 3 dpfs. Medaka *myoD*, used for the purpose of staining somites in the trunk and muscle mass in the fin buds, was isolated from cDNA library by using PCR, and then sequenced (accession no. AB288366). Medaka *shh* and *dHAND* sequences, posterior markers of pectoral fin buds, were searched from golw_scaffold Hd-rR 200506 in the Medaka genome database (http://dolphin.lab.nig.ac.jp/medaka) based on the partial *shh* mRNA sequence (AB007129) and *dHAND* of other organisms. The primer sequences designed for selective amplification of respective *hox* genes are listed in Table 1.

Total RNA was isolated from medaka 2 and 3 dpf embryos using ISOGEN (Nippon Gene). Total cDNA was generated from total RNA using SuperScript II-Oligo dT, following recommended protocols (Invitrogen), and DNA fragments for probes were amplified by PCR using the primers (Table 1). The products were cloned into pGEM-T

Table 1 PCR primers for probe preparation

Probe name	Primer sequence 5′ → 3′	Probe length (nt)
A9a	TGACCAGCTACTACGTGGAT	551
	CCAGTTGGAAACCGGGTTAT	
A9b	ACCGATGTTCTCCTCAGCTT	440
	CTGGGAAATGGCTTGTCTTC	
A10a	TATGGACTACACAGCTACGG	699
	GTTTTCAGTTTTTGCGCCTC	
A10b	TCAATTTGAGAGGCGACAGC	664
	TTGGCAGCGACTTCAGTTCT	
A11a	TGACATTCAGGGACTACGCA	507
	TTTTGCGGGTTCTCTGTCCA	
A11b	TACTTAAGCCAGAGCCAGTC	382
	TCGTTCTTGAGTGTCTGTCC	
A13a	AAGCAGTGTAGCCCTTGCT	437
	TGCGACACAGCGTCTGATAT	
A13b	AAACAATGCAGCCCCTGTTC	180
	CGAGAACTCCTCACCAGAGA	
D9a	TGTCTTCCAGTGGCACTATC	520
	GGCTCTCCGTGACTCGAAAT	
D10a	CCCGTTTTTAGTGGACTCTT	448
	TCCGTTCGCATATGTTTGGC	
D11a	CCCGATTTTACATCCGTCTC	531
	GGTTGATGAACTCTGTTTGC	
D12a	ATGTGTGAGCGGAATTCTCT	520
	CAAGTTTGTCCGACAGTTCT	
shh	ATCCACTGCTCTGTGAAAGC	667
	GGATCGAGTGGCTGTCCAA	
dHAND	AGTCTGGTGTCGGGATTCC	331
	TCCGCTTCACGGTGCGT	

Easy (Invitrogen) and checked for correct sequences by sequencing, and then transcribed to digoxigenin (DIG)-labeled RNA with T7 or SP6 polymerase (Roche). DIG-labeled antisense RNA probes and sense RNA probes for controls were used for in situ experiments.

Sample fixation, hybridization, and staining

Embryos at 1, 2, and 3 dpf were fixed with 4% paraformaldehyde at 4°C overnight, dehydrated with 100% MeOH and stored at −20°C in 100% MeOH until use. Whole-mount in situ hybridization (WISH) was carried out by a modified Inohaya's method (Inohaya et al. 1995, 1999). Simply, dehydrated embryo samples were washed in phosphate-buffered saline (PBS)-0.1% Tween20 (PBST), and treated with 10 μg/ml proteinase K solution for an appropriate time. After refixing with 4% paraformaldehyde, they were washed three times in PBST, then incubated in prehybridization buffer (50% formamide, 5× saline-sodium citrate (SSC), 50 mg/ml heparin, 0.1% Tween20). 1–2 ml of hybridization buffer with a 1/250 vol of DIG-labeled antisense RNA probe were added to each sample, followed by incubation overnight at 55–58°C. After hybridization, samples were washed four times with 50% formamide-2× SSC—0.1% Tween20, two times with 2× SSC—0.1% Tween20, and two times with 0.2× SSC—0.1% Tween20 at 68°C. After three washes with PBST, samples were blocked with 5% sheep-serum/PBST and bound to DIG-antibody including alkaline phosphatase (Roche) overnight at 4°C. Samples were washed six times with PBST and twice with alkaline phosphatase buffer (0.1 M Tris pH 9.5, 50 mM $MgCl_2$, 0.1 M NaCl, 0.1% Tween20) then stained with NBT/BCIP solution at 4°C or room temperature. When samples were moderately stained, reactions were stopped by PBST washing, and refixation was performed again with 4% paraformaldehyde overnight. Finally, samples were passed through a glycerol series, and stored in 80% glycerol at 4°C until use.

Results

Comparison of mouse *HoxA*, medaka *hoxAa* and *hoxAb* organization

A comparison of the genomic organization of *Ola–hoxAa* and *Ola–hoxAb* with that of *Mmu–HoxA* is illustrated in Fig. 1a. Sizes of *Mmu–HoxA*, *Ola–hoxAa* and *Ola–hoxAb* are ca. 100, 60 and 20 kb, respectively. *Mmu–HoxA* contains 11 *Hox* genes and *Evx1* in the cluster, and *Ola–hoxAa* contains 10 homologous *hox* genes and *evx1* in the same order. The single difference between *Mmu–HoxA* and *Ola–hoxAa* organization is the lack of an *Mmu–HoxA6* homolog in the latter. On the other hand, *Ola–hoxAb* does not contain *hoxA1b*, *hoxA3b*, *hoxA4b*, *hoxA5b*, *hoxA6b* and *hoxA7b* genes, featuring more gene loss than the *Ola–hoxAa* cluster. Thus, genome size, gene order and orientation have been conserved between *Mmu–HoxA* and *Ola–hoxAa*, but not *Ola–hoxAb*.

Similarities of hoxA protein sequences in the clusters are summarized in Table 2. Exon2 sequences of the *Abd–B* family show striking similarity between *Mmu–HoxA* and *Ola–hoxAa* proteins (72.5–94.7% identity, 82. 9% overall), while lower similarity is present between *Mmu–HoxA* and *Ola–hoxAb* proteins (53.2–90.7% identity, 73.0% in total). With exon1 protein sequences, the respective figures are 57.2 and 44.3%, again suggesting *Mmu–HoxA* to be more related to *Ola–hoxAa* than to *Ola–hoxAb*.

A comparison of the genomic organization of *Ola–hoxDa* and *Ola–hoxDb* with that of *Mmu–HoxD* is illustrated in Fig. 1a. *D9* orthologues are the only *Abd–B* genes found in both medaka clusters. *D10*, *D11*, and *D12* orthologues are situated in *Ola–hoxDa*, but *D13* genes have not been found in medaka yet. The similarity between *Ola*–hoxD9a and *Mmu*–HoxD9 proteins (exon1, 45.3%; exon2, 88.6% identity) is higher than the similarity between *Ola*–hoxD9b and *Mmu*–HoxD9 (exon1, 18.6%; exon2, 79.7% identity).

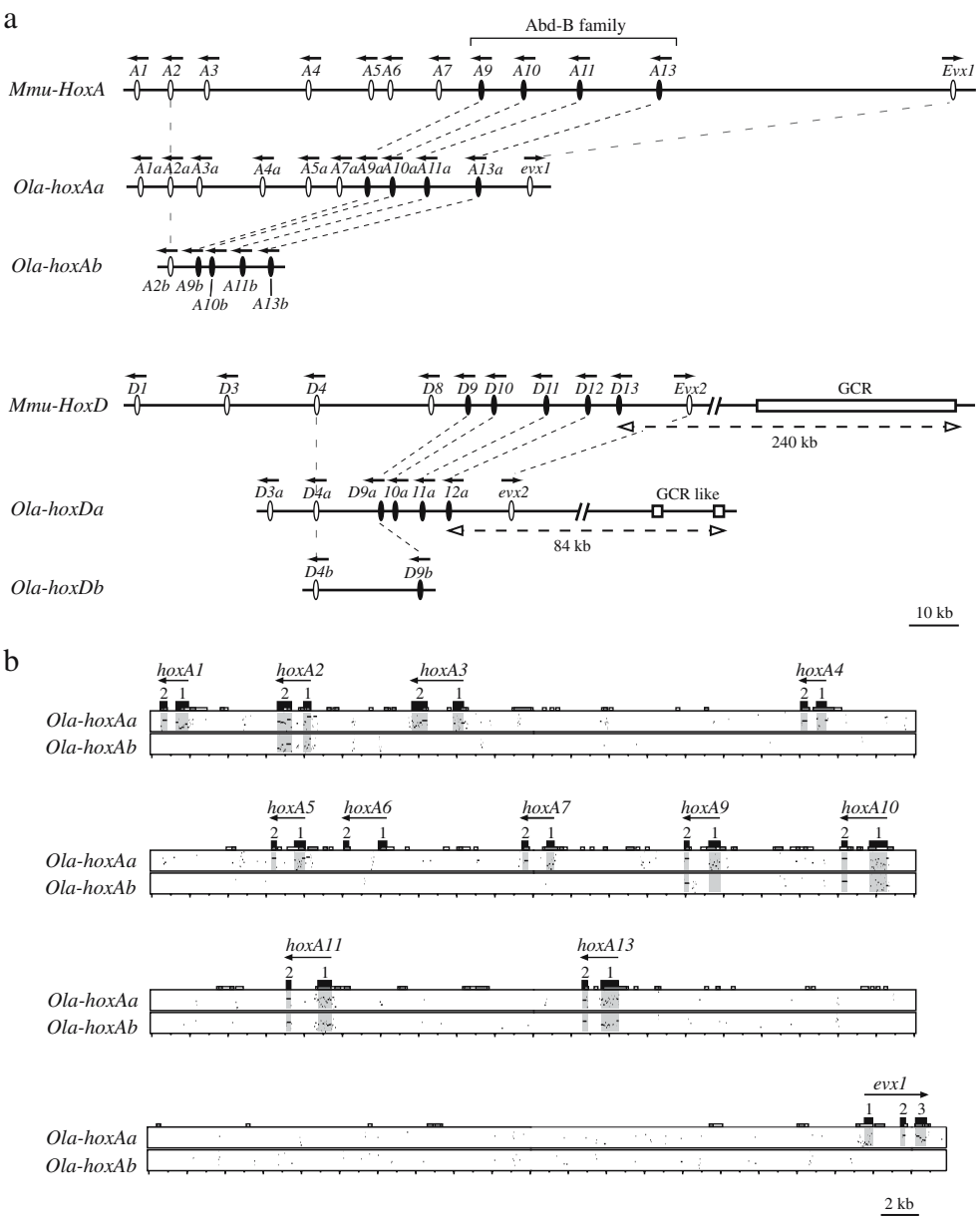


Fig. 1 Comparison of mouse *HoxA/HoxD*, medaka *hoxAa* and *hoxAb/hoxDa* and *hoxDb* clusters. **a** Organization of the *HoxA* and *HoxD* clusters in mouse *Mus musculus* (*Mmu*) and of the *hoxAa/Ab* and *hoxDa/Db* clusters in the medaka *Oryzias latipes* (*Ola*). *Ovals* indicate positions of *hox* and *evx1* genes. *Filled ovals* are for *Abd-B* family genes and *open ovals* are for other genes. *Horizontal arrows* indicate the direction of transcription. A *square* in *Mmu–HoxD* indicates the GCR (Spitz et al. 2003). *Two squares* in *Ola–HoxDa* indicate two blocks of GCR-like region (see the details in the text). **b** Pip output of the comparison of *Mmu–HoxA*, *Ola–hoxAa* and *Ola–hoxAb*. *Shadowed backgrounds* indicate coding regions. *Horizontal arrows* indicate the direction of transcription, and *filled boxes* indicate exons. *Horizontal small open boxes* are for CpG/GpC ratios between 0.6 and 0.75, and *small gray boxes* for ratios over 0.75

Pip outputs from the comparisons of *Mmu–HoxA*, *Ola–hoxAa* and *Ola–hoxAb* sequences are shown in Fig. 1b. All medaka exons corresponding to mouse exons exhibit high similarity scores. Not only coding regions, but also several conserved noncoding sequences previously described in the literature (Santini et al. 2003) show high similarity, such as proximal 5′ upstream regions of *HoxA2*, *HoxA5* genes and *HoxA10* regions (Fig. 1b). Among them, the *hoxA9b* gene and its intron region are showing high similarity in exon2 (84.6%, Table 2) and intron1 regions (Fig. 1b) but low in exon1 (35.8%, Table 2).

Spitz et al. (2003) identified a global control region (GCR) of *HoxD*, which is highly conserved between a pufferfish *Tetraodon* and *Homo sapiens* (Fig. 1a). The GCR of 40 kb in size is a cluster of global enhancers capable of controlling transcription of several genes, even examples unrelated in structure or function. To confirm the presence of GCR around *Ola–hoxDa*, we compared genomic regions flanking *Mmu–HoxD* and *Ola–hoxDa* with the PipMaker program. Two blocks of high homology regions corresponding to the GCR were thereby detected 84 kb upstream of *Ola–hoxD12a*, at the physical map site of medaka version1.0 scaffold24 in the Medaka genome database (Fig. 1a). In the *Ola–hoxDb* cluster and in its 500 kb upstream region, we could not identify the GCR-like structure.

Table 2 Lengths and similarities (%) of amino-acid sequences between *Mus musculus* (*Mmu*) HoxA and *Oryzias latipes* (*Ola*) hoxAa/Ab proteins

		Length (aa sites)			Similarity	
		Mmu–HoxA	*Ola*–hoxAa	*Ola*–hoxAb	*Ola*–hoxAa	*Ola*–hoxAb
Mmu–HoxA2	exon1	126	122	125	75.8	43.5
	exon2	245	237	222	72.5	53.2
	total	372	379	348	73.9	51.8
Mmu–HoxA9	exon1	192	183	164	42.9	35.8
	exon2	79	80	83	89.7	84.6
	total	271	264	248	57.1	51.4
Mmu–HoxA10	exon1	308	271	229	41.3	42.7
	exon2	91	91	91	90.0	88.9
	total	399	363	321	54.0	56.0
Mmu–HoxA11	exon1	236	223	190	66.2	52.4
	exon2	77	77	76	94.7	90.7
	total	313	301	267	76.3	63.5
Mmu–HoxA13	exon1	306	217	211	61.8	58.9
	exon2	80	82	78	87.5	81.8
	total	386	300	290	74.4	70.6
Abd-B	exon1	1042	894	794	53.0	43.1
	exon2	327	330	328	90.4	86.6
	total	1369	1228	1126	65.0	60.5
Whole	exon1	1168	1016	919	55.7	43.1
	exon2	572	567	550	82.9	73.0
	total	1741	1607	1474	67.0	58.5

Expression of medaka *Abd-B* genes in the trunk

Expression analysis of medaka *Abd-B* genes in embryos at 1, 2, and 3 dpfs was carried out by WISH experiments. The staining pattern of the somites by *myoD* is shown in Fig. 2a–c (i). At 1 dpf, three *Abd-B* genes in *Ola–hoxAa*, i.e., *hoxA9a*, *hoxA10a*, *hoxA11a* but not *hoxA13a*, were found to be clearly expressed in the trunk (Fig. 2a; boxes a, b, c, and d). With *Ola–hoxAb*, two genes *hoxA9b* and *hoxA10b* demonstrated clear expression in the trunk (Fig. 2a; boxes e and f), while *hoxA11b* and *hoxA13b* were lacking (Fig. 2a; boxes g and h).

Figure 2b and c illustrates expression of these genes at 2 and 3 dpf, respectively, *hoxA9a* and *hoxA10a* in *Ola–hoxAa* being strongly (Fig. 2b and c, boxes a and b), but *hoxA11a* only weakly present at both stages (Fig. 2b and c, box c). *HoxA13a* was found in tail ends at both stages (Fig. 2b and c, box d), and strongly in the cloaca area at 3 dpf (Fig. 2c, box d). In *Ola–hoxAb*, *hoxA9b* and *hoxA10b* could be shown to be expressed clearly (Fig. 2b and c, boxes e and f), whereas *hoxA11b* and *hoxA13b* were not expressed or only weakly (Fig. 2b and c, boxes g and h).

The difference between *hoxA9a* and *hoxA9b* expression was seen at all the developmental stages (1, 2, and 3 dpfs) examined. At 2 and 3 dpfs, the anterior boundary of *hoxA9a* was around somite 1 of the spinal cord on the dorsal side (Fig. 2b and c, box a), whereas that for *hoxA9b* was around somite level 5, with a shifting down to the posterior side in comparison with the *hoxA9a* boundary (black arrows in Fig. 2b and c, box e). An equivalent shift was also observed on the ventral side of 2 and 3 dpfs embryos (arrow heads in Fig. 2b and c, boxes a and e).

Dorsal expression boundaries of paralogous group 10 genes, *hoxA10a* and *hoxA10b*, were similar and found around somite level 7 at 2 and 3 dpfs (Fig. 2b and c, boxes b and f).

There was no or weak expression of *hoxA11b* and *hoxA13b* in all stages examined, while paralogous *hoxA11a* and *hoxA13a* were expressed. Because of weak staining, the precise boundaries of the *hoxA11a* expression were difficult to decide at 2 and 3 dpfs. However, these boundaries were more posterior than the boundaries of *hoxA9a/A9b* and *hoxA10a/A10b*. In contrast, the *hoxA13a* expression appeared strong in the tail ends and the cloaca area at 2 and 3 dpfs (Fig. 2a–c, boxes c and d).

Expression of medaka *Abd-B* genes in pectoral fin buds

shh and dHAND expression

shh and *dHAND* are typical posterior markers in fin and limb bud development (Riddle et al. 1993; Akimenko and Ekker 1995; Charité et al. 2000) and were found to be expressed in the posterior end of the pectoral fin buds at

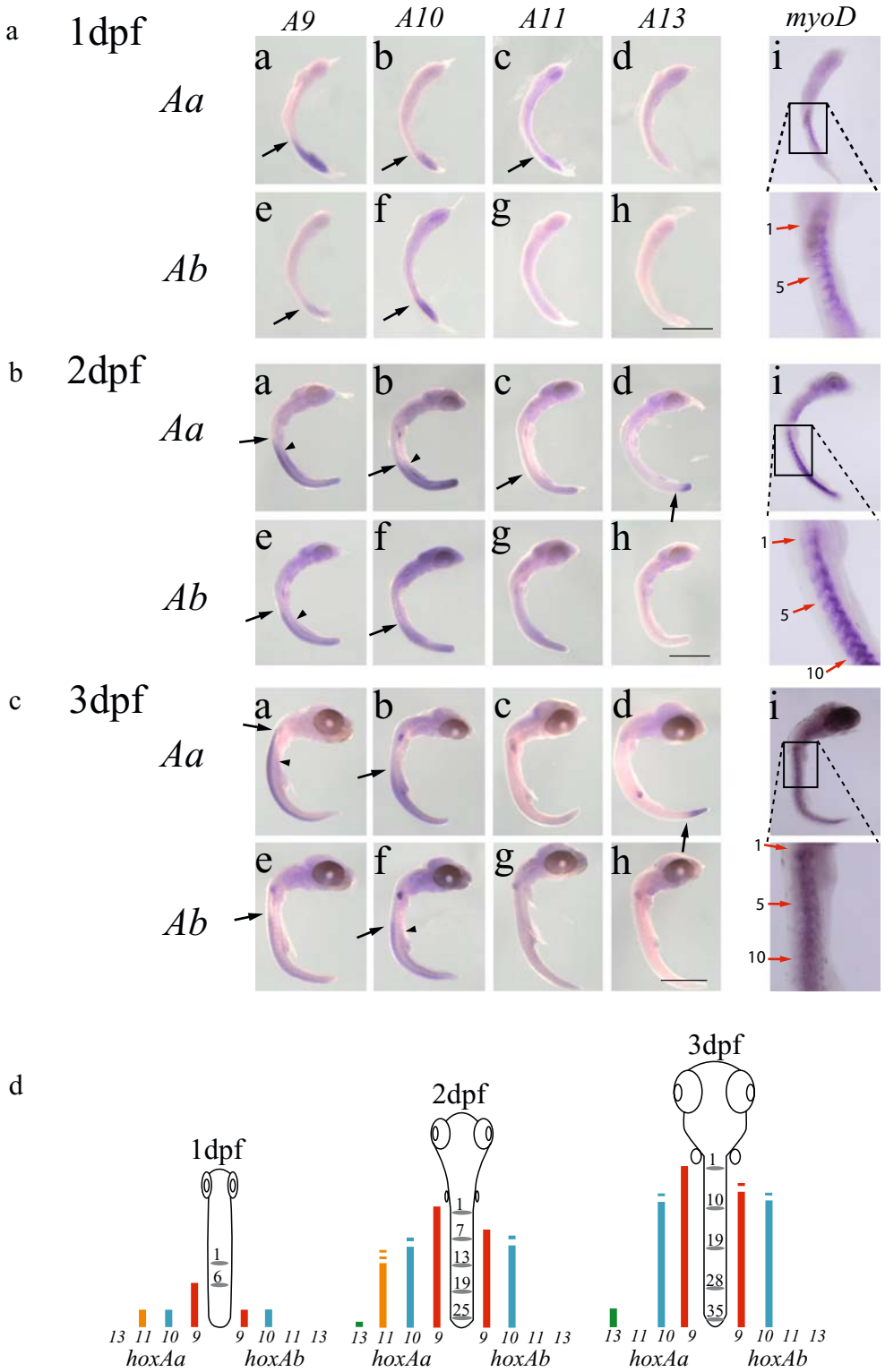


Fig. 2 Expression of medaka *Abd-B* genes in the trunk. Expression of the *Abd-B* genes in medaka embryos were carried out by WISH experiments. *Black arrows* and *arrow heads* indicate the anterior limit of gene expression in the CNS and the paraxial mesoderm, respectively. Scale bars at the *right bottom* (*h*) are for 0.5 mm. Expression at **a** 1 dpf, **b** 2 dpf, and **c** 3 dpf. Figures (*i*) at each dpf are expressions of *myoD*, a somite marker, and *red arrows* in magnified views are applied every five somite steps. **d** Schematic illustration of *Abd-B* gene expression in the trunk. Genes for *hoxAa* are on the left and for *hoxAb* on the right. *Dashed lines* on the top of several bars indicate incomplete boundaries to be caused by the difficulty of precise positions. *Gray ovals* with numbers indicate somite levels

stage 3 dpf (Figs. 3a and 4a), but not before. The fin buds are not well developed at stage 1 and 2 dpfs, and also, *Abd-B* genes in *hox* clusters were not or only weakly expressed (data not shown).

hoxDa expression

Expression patterns of *Abd-B* genes in *Ola–hoxDa* in lateral and dorsal views at 3 dpf are summarized in Figs. 3b and

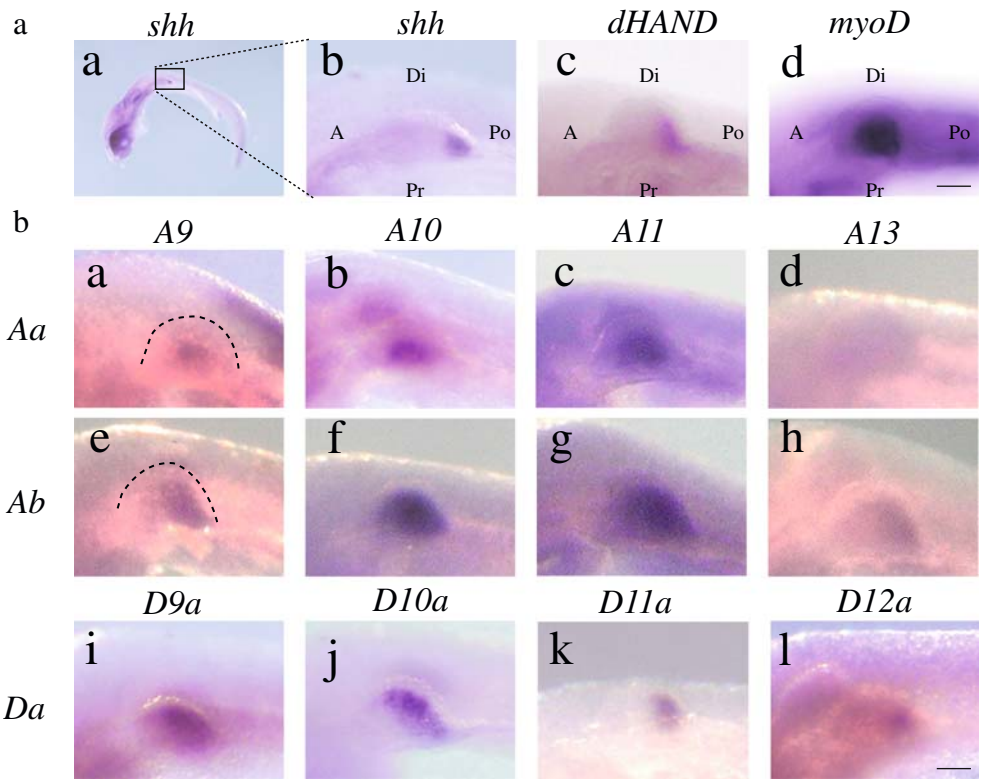


Fig. 3 Expression of medaka *Abd-B* genes in pectoral fin buds—lateral views. Expression of the *Abd-B* genes in medaka embryos at 3 dpf was assessed by WISH experiments. In figure of pectoral fin buds (**a**, *b–d*; **b**, *a–l*), the anterior is to the *left* and the distal end is to the *top*. Scale bars in the *bottom right* of figures (**a**, *d*; **b**, *l*) indicate 0.05 mm. **a** Lateral view of marker gene expression. *shh* and *dHAND* are posterior markers in pectoral fin buds. *myoD* is a muscle mass marker in pectoral fin buds. Total view of *shh* expression (*a*), and magnified views of *shh*, *dHAND*, and *myoD* expression in pectoral fin buds (*b*, *c*, *d*). **b** *hoxAa*, *Ab*, and *Da* expression in pectoral fin buds. *A*, anterior; *Di*, distal; *Pr*, proximal; *Po*, posterior

4b, respectively. When the positions of *shh* and *dHAND* expression were used as posterior markers, *hoxD9a* and *hoxD10a* are expressed in distal–medial portion with moderate posterior restriction (Figs. 3b and 4b, boxes i and j), whereas *hoxD11a* and *hoxD12a* exhibited strong posterior restriction (Figs. 3b and 4b, boxes k and l). Furthermore, genes located more 3′ downstream on the chromosome (Fig. 1a), such as *hoxD9a* and *hoxD10a*, were found to have more advanced boundaries from anterior to posterior sites, clearly following the colinearity rule of *hox* expression. The boundary order was *hoxD9a* > *hoxD10a* > *hoxD11a* > *hoxD12a* (Figs. 3b, 4b, and 5), along the anterior–posterior axis of the fin buds (Fig. 5). Thus expression of the *hoxDa* genes, such as *hoxD9a*, *hoxD10a*, *hoxD11a* and *hoxD12a*, was shown to be similar to those of orthologous genes in mammals (Dollé et al. 1989), aves (Yokouchi et al. 1991) and zebrafish (Sordino et al. 1995), indicating maintenance of "orthodox" *HoxD* expression.

hoxAa and hoxAb expression

Expression of the *Abd-B* genes in *Ola–hoxAa*, such as *hoxA9a*, *hoxA10a*, and *hoxA11a*, was localized in proximal portions of pectoral fin buds, *hoxA13a* being lacking. Lateral and overhead views are shown in Figs. 3b and 4b with a summary in Fig. 5. Thus, the colinearity rule was not followed for *Ola–hoxAa* expression. Note here that genes in *Ola–hoxAa* were found to be in split parts; one in the dorsal and the other in the ventral region (Figs. 4b and 5b). These split expressions were similar to that of *myoD*, a muscle mass marker (Fig. 4a, box d).

Ola–hoxAb forms such as *hoxA9b*, *hoxA10b*, and *hoxA11b* were localized in distal–posterior portions of buds as shown in lateral views (Fig. 3b, boxes e–h). *HoxA13b* exhibited stronger posterior restriction than other *hoxAb* members (Fig. 3b, box h). Overhead views are also summarized in Fig. 5b. The boundaries advanced from posterior to anterior and distal to proximal as recognized in lateral (Figs. 3b and 5a) and overhead views (Figs. 4b and 5b), respectively, in the order of *hoxA10b* > *hoxA9b* > *hoxA11b* > *hoxA13b*.

Discussion

Expressional boundaries of duplicated *Abd-B* genes in the trunk

To facilitate comparison of expression of duplicated *Abd-B* genes in the central nervous system (CNS), we here show schematic diagrams of their expression boundaries on somite levels (Fig. 2d). These diagrams were based on comparisons between *hox* and *myoD* expressions at equivalent stages. It was found that genes more 3′ downstream on the chromosome have more anterior

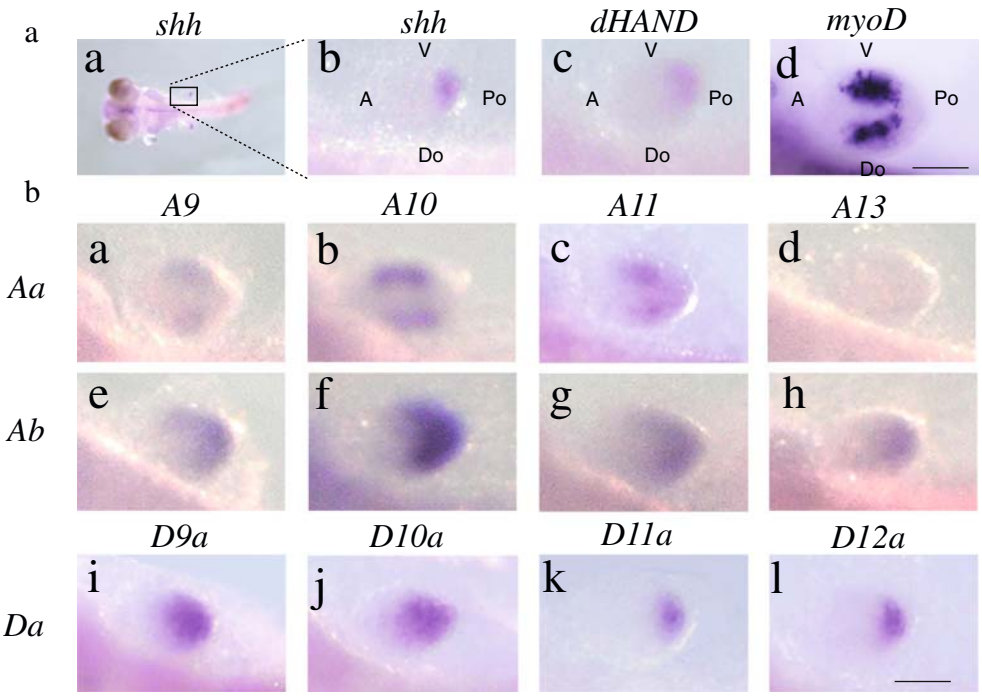


Fig. 4 Expression of medaka *Abd-B* genes in pectoral fin buds—overhead views. Overhead views of the same samples in Fig. 3. The anterior is to the *left* and the ventral side is to the *top*. Scale bars in the *bottom right* of figures (**a**, *d*; **b**, *l*) indicates 0.05 mm. **a** Overhead view of marker gene expression. *shh* and *dHAND* are posterior markers of fin buds. *myoD* is a muscle mass marker in pectoral fin buds. Total view of *shh* expression (*a*), and magnified views of *shh*, *dHAND*, and *myoD* expression in pectoral fin buds (*b*, *c*, *d*). **b** *hoxAa*, *hoxAb*, and *hoxDa* expressions. *A*, anterior; *Do*, dosal; *V*, ventral; *Po*, posterior

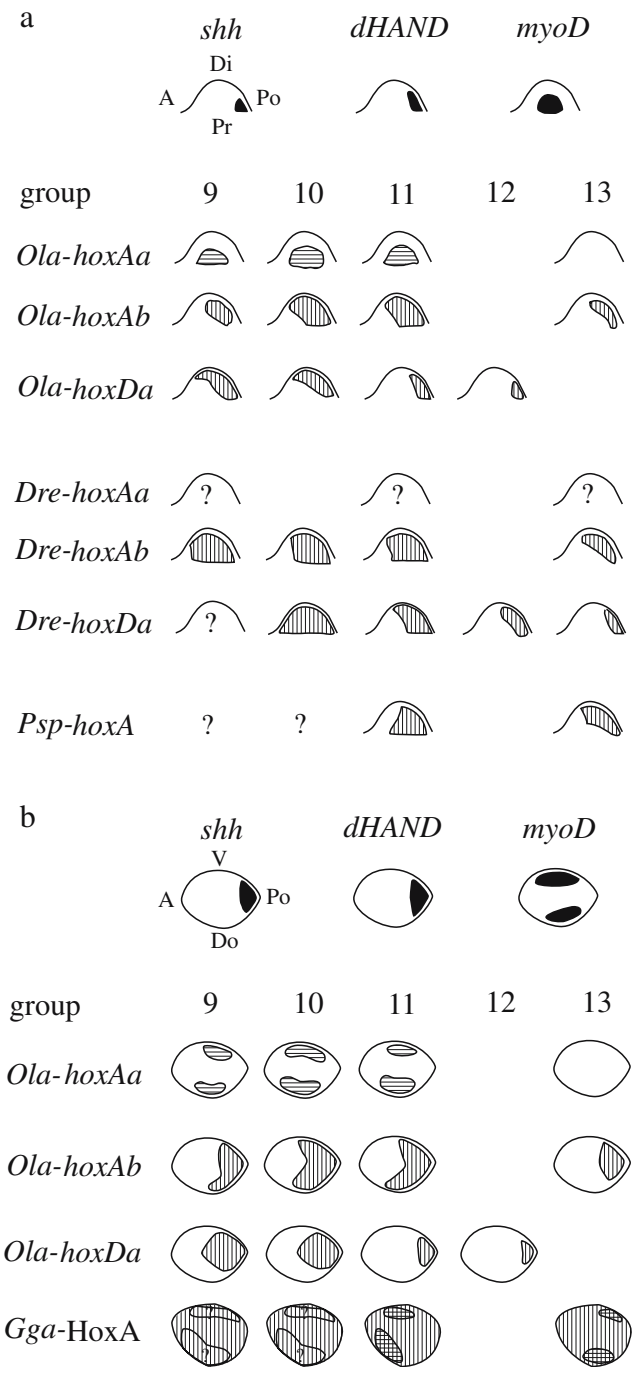
boundaries, especially in the *Ola–hoxAa* case (Fig. 2d). On the other hand, expression of *Ola–hoxAb* does not obey the rule. It is clear that, at least in the trunks, expression of *Ola–hoxAa*, i.e., *hoxA9a*, *hoxA10a*, *hoxA11a* and *hoxA13a*, agrees with the colinearity rule, but that *Ola–hoxAb* is an exception. As described in zebrafish (Sordino et al. 1996), the expression boundaries of medaka *Abd-B hoxA* situate more anteriorly than in mouse. When comparing between medaka and zebrafish, *Ola–hoxA10a* and *A10b* seem to have more anterior expression boundaries than *Dre–hoxA10* (Prince et al. 1998), and *Ola–hoxA9a* expression is found striking more anterior than *Dre–hoxA9a* (accession no. Y14538; there listed as *Dre–hoxx9*). The *Ola–hoxA13a* expressions at the posterior tip and in the cloaca area are similar to the expression of *Dre–hoxA13b* (accession no. Y07699; there listed as *Dre–hoxA13*) and *Dre–hoxD13* (Sordino et al. 1996; van der Hoeven et al. 1996a). In contrast, *Ola–hoxA13b*, the orthologue of the *Dre–hoxA13b* gene, was not expressed in these regions. Based on these differences between medaka and zebrafish, we hypothesize that *hox* duplications allowed expressional differentiation and contributed to teleost evolution.

The present study describes differential expression patterns for paralogous genes in medaka *hoxAa* and *hoxAb*. Our finding that the anterior boundary of *hoxA9b* was shifted by 3 or 4 somite levels to the posterior side, compared with that of *hoxA9a*, whereas that of *hoxA10b* seem almost equal to that of *hoxA10a*. Whereas *hoxA11a* and *hoxA13a* were expressed, *hoxA11b* and *hoxA13b* expression was not detectable. These variations in expression of each paralogous genes are probably caused by a local regulatory changes (Gérard et al. 1993; van der Hoeven et al. 1996b). Belting et al. (1998) and Anand et al. (2003) earlier claimed that expressional boundary changes were dependent on differences in local enhancers for the *HoxC8* gene. Furthermore, it is reported in a pufferfish *Takifugu rubripes* that paralogous genes, *hoxA2a* and *hoxA2b*, show differential expression patterns caused by local *cis*-regulatory changes (Tümpel et al. 2006).

The colinearity rule is explained by the presence of the GCR, previously identified in mouse *hoxD* cluster (Spitz et al. 2003). However, the report of *hox* random integration in the genome supports that local control regions make some endogenous aspects of the *hox* expressions (van der Hoeven et al. 1996b). We hypothesize that lack of some parts of the GCR for the trunk caused the colinearity break and that local control regions contribute to the *hoxA9b* and *A10b* expressions. Conservations of group 9–13 in both clusters may be explained by the GCR specificity for fin buds.

Difference of expression patterns between duplicated *Abd-B* genes in pectoral fin buds

In Fig. 5, to facilitate comparisons, WISH results for zebrafish *Danio rerio* (*Dre*) from Sordino et al. (1996) and Neumann et al. (1999), for the basal ray-finned fish *Polyodon spathula* (*Psp*) from Metscher et al. (2005) and expression patterns of Hox proteins from a chick *Gallus gallus* (*Gga*) described by Yokouchi et al. (1991) are included. Note here that *hoxA* expression of the zebrafish in the pectoral fin bud reported by Sordino et al. (1996) and Neumann et al. (1999) should be revised here in their



◀ **Fig. 5** Comparison of expression patterns of *Abd-B* genes in the pectoral fin buds. **a** Schematic lateral view. The anterior is to the *left* and the distal end is to the *top*. In the medaka (*Ola*), *hoxAa* expression is drawn with *horizontal stripes* and *hoxAb* and *Da* expression with *vertical stripes*. Patterns for zebrafish *Danio rerio* (*Dre*) are after Sordino et al. (1996) and Neumann et al. (1999; see the details in the text). Those for the ray-finned fish, *Polyodon spathula* (*Psp*), are after Metscher et al. (2005). *Question marks* indicate expression patterns not yet examined in *Dre* or not identified in *Psp*. *A*, anterior; *Di*, distal; *Pr*, proximal; *Po*, posterior. **b** Schematic overhead view. The anterior is to the *left* and the ventral side to the *top*. In pectoral fin buds of the medaka (*Ola*), regions drawn with *horizontal and vertical stripes* are as in **a**. In wings of the chicken *Gallus gallus* (*Gga*), expression of HoxA proteins examined by Hox-protein-specific antibodies is categorized into two types; the mesenchyme type (*vertical stripes*) and the dorso-ventral muscle mass type (*horizontal stripes*). The latter shows a split pattern (Yamomoto et al. 1998). These two type expressions overlap in overhead views (*cross patterns*). *A*, anterior; *Do*, dosal; *V*, ventral; *Po*, posterior

nomenclature. At the time of their publication, only one *hoxA* cluster in zebrafish was identified, and the extra duplication had not been assumed. Our homology comparisons suggest that the *hoxA* sequences belong to *Dre–hoxAb* (data not shown). Furthermore, it has been reported that the *Dre–hoxAa* cluster lacks not only *hoxA6a* but also *hoxA2a*, *hoxA7a* and *A10a*, with more loss of genes than with the medaka *hoxAa* cluster (Amores et al. 1998; Kurosawa et al. 2006). Thus, in the present paper, we used the results by Sordino et al. (1996) and Neumann et al. (1999) with *Dre–hoxA9b* for *hoxA9*, *Dre–hoxA10b* for *hoxA10*, *Dre–A11b* for *hoxA11*, and *Dre–hoxA13b* for *hoxA13*.

It is well known that muscles in the pectoral fin buds feature distinct two parts, ventral and dorsal muscle masses. Our result of the *myoD* expression exhibited these muscle masses in the medaka (Figs. 4a, box d; and 5b) and was equal to the results of the zebrafish (Neyt et al. 2000; Haines et al. 2004). Haines et al. (2004) proposed that these muscle masses are equal to those shown in chick wing buds by Dietrich et al. (1999). Thus, the dorsal and ventral region *hoxAa* expression documented here reflects the two muscle masses in the pectoral fin buds. In wings of the chicken *G. gallus* (*Gga*), expression of *Gga*–HoxA is also categorized in two types; the mesenchyme type (vertical stripes in Fig. 5) and dorso-ventral muscle mass type (horizontal stripes). The latter shows a split pattern (Yamomoto et al. 1998). As in the case of *Gga*–HoxA, *Ola–hoxAa* expression well corresponds to the positions of ventral and dorsal muscle masses in the pectoral fin buds. On the other hand, *Ola–hoxAb* expression corresponds to the mesenchyme tissue. Bruce et al. (2001) claimed that biochemical functions of duplicate *hoxB5* genes, i.e., *Dre–hoxB5a* and *Dre–hoxB5b*, are not changed, and the ancestral function of *HoxB5* must be divided (subfunctionalization). The *Ola–hoxAa* and *Ola–hoxAb*, which are duplicates of *HoxA*, well share the expression patterns of HoxA in *Gga*, suggesting such subfunctionalization of the ancestral gene. Most importantly, the present study demonstrated this subfunctionalization happened at the level of clusters, not at the level of paralogous genes. Namely, all genes on the *hoxAa* cluster are expressed in muscles and those on the *hoxAb* cluster in mesenchymes. Although GCR-like structures have not been identified in these clusters, the result strongly suggests that the GCR changed its character after duplication, thus leading to this tissue-specific expression of each cluster.

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