The subfunctionalization of *shox* and *shox2* paralogs in shark highlights both shared and distinct developmental mechanisms of gill arches and fins

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ABSTRACT

The emergence of novel morphological structures in evolution may be associated with genomic transformations, including the origin of new genes. One of the primary mechanisms for the emergence of new genes is the duplication of ancestral genes, leading to the appearance of multiple paralogs at specific phylogenetic levels. In the early stages of gnathostome evolution, two rounds of whole-genome duplications occurred, which may have played a pivotal role in the reorganization of the body plan and the emergence of novel traits within this group. Among the morphological innovations of gnathostomes are the jaw apparatus, derived from the pharyngeal arches, and the paired appendages, the evolutionary origin of which remains the subject of several hypotheses. The genomes of most gnathostomes contain two paralogs of the *shox* gene - *shox* and *shox2* - both of which are involved in the development of the paired appendage skeleton. Here, we investigated the expression patterns of *shox* and *shox2* paralogs in the gray bamboo shark (*Chiloscyllium griseum*), a representative of Chondrichthyes, which constitute a basally divergent lineage of gnathostomes. Paired fins of cartilaginous fishes are considered a basal model for the gnathostome appendages. Our results reveal spatial subfunctionalization of the *shox* and *shox2* genes. Specifically, *shox* is expressed in the jaw and gill arches, as well as in paired and unpaired fins, indicating shared developmental mechanisms among these structures. In contrast, *shox2* expression is mostly restricted to paired fins, highlighting unique developmental features distinguishing them from the evolutionarily older unpaired fins.

INTRODUCTION

Current understanding suggests that the evolution of the body plan and the emergence of novel morphological structures are underpinned by genomic transformations, which manifest at both regulatory levels and through the gain or loss of specific genes (Rubinstein and de Souza, 2013; Long et al., 2016). For example, the emergence of the novel homeobox gene *Anf/Hesx1* in the ancestors of extant vertebrates established the prerequisites for the development of the telencephalon, a unique region of the vertebrate forebrain (Zaraisky et al., 1992; Kazanskaya et al., 1997; Ermakova et al., 2007; Bayramov et al., 2016). The loss of *actinodin* genes in the ancestors of tetrapods may have facilitated the evolution of limbs adapted for terrestrial locomotion (Zhang et al., 2010). Similarly, the loss of genes such as c-

Answer, *Ag1*, *Ras-dva1*, and *Tfp4* in the ancestors of warm-blooded vertebrates has been proposed as one of the factors contributing to the reduced capacity for limb regeneration in these lineages, a capability that is retained in many extant cold-blooded species possessing these genes (Ivanova et al., 2013, 2015, 2018; Korotkova et al., 2019; Tereshina et al., 2019; Ivanova et al., 2021).

A more widespread evolutionary event than the appearance of entirely novel genes is the duplication of pre-existing ancestral genes, followed by functional divergence among the resulting paralogs (Ohno, 1970; Deem and Brisson, 2024). In the human genome, for instance, over 70% of genes possess at least one paralog (Ibn-Salem et al., 2017). Although most paralogs are typically eliminated post-duplication due to the loss of function, those that undergo subfunctionalization, neofunctionalization, or provide other functional advantages may be retained within the genome (Kuzmin et al., 2022). The considerable increase in morphological complexity observed in vertebrates relative to their ancestral forms may be attributed to two rounds of whole-genome duplications that occurred during the early stages of vertebrate evolution (Ohno, 1970; Bayramov et al., 2021).

Vertebrates comprise two major evolutionary lineages - jawless (agnathans) and jawed (gnathostomes) - which diverged during the Cambrian period, approximately 535–462 million years ago (Janvier, 2006; Kuraku and Kuratani, 2006; Feinberg and Mallatt, 2013; Bayramov et al., 2018). Jawless vertebrates originated in the Cambrian, flourished over the subsequent 150 million years during the Ordovician and Silurian, and experienced extinction of several major groups in the Devonian (Donoghue and Keating, 2014; Johanson, 2020). Extant jawless vertebrates are represented by lampreys and hagfish, comprising approximately 100 species (Shimeld and Donoghue, 2012; Kuraku, 2013). The earliest known jawed vertebrates are dated to the Early Silurian (Zhao and Zhu, 2010), and the Devonian period witnessed a significant diversification of gnathostomes, including the emergence of terrestrial forms by the Late Devonian (~360 million years ago) (Brazeau and Friedman, 2015). Modern gnathostomes, encompassing over 50,000 species, include cartilaginous and bony fishes, amphibians, and terrestrial vertebrates (Kuraku, 2013; Brazeau and Friedman, 2015).

A defining feature of gnathostomes is the presence of both a jaw apparatus and paired appendages (Donoghue and Keating, 2014; Striedter and Northcutt, 2019). In contrast, extant jawless vertebrates lack paired appendages, and the homology between gnathostome limbs and the appendages of fossil jawless vertebrates remains a matter of debate, largely due to incomplete paleontological data on the endoskeletal structure of fins in fossil forms (Tanaka and Onimaru, 2012; Wilson et al., 2007; Bayramov et al., 2024). Consequently, paired appendages in gnathostomes are often regarded as evolutionary novelties, motivating investigations into the genetic mechanisms underlying their origin (Larouche et al., 2019).

Given their phylogenetic position as a basal lineage of jawed vertebrates, cartilaginous fishes serve as a fundamental model for studying the paired appendages of vertebrates (Seixas et al., 2023; Thompson et al., 2021). The pectoral fins of sharks comprise endoskeletal elements (basalia and radials) as well as exoskeletal fin rays. The basal segment is formed by three elements - propterygium, mesopterygium, and metapterygium - which together constitute the tribasal fin architecture (Cass et al., 2021). The appendages of more derived lineages, such as bony fishes and tetrapods, have undergone extensive and divergent modifications, complicating direct comparisons between the developmental mechanisms of bony fish fins and tetrapod limbs, which are common laboratory models (Yano and Tamura, 2013; Hawkins et al., 2021; Thompson et al., 2021). This underscores the importance of studying the appendages of cartilaginous fishes, which represent the first appearance of paired appendages in vertebrate evolution and have largely retained their ancestral structural features (Cole and Currie, 2007).

Beyond the question of the origin of paired appendages in gnathostomes, considerable interest also surrounds the mechanisms driving the evolutionary transformation of ancestral fins into terrestrial limbs. Genetic studies suggest that this transformation may have involved changes in the expression of genes of *Hoxa* and *Hoxd* clusters (Woltering et al., 2020; Leite-Castro et al., 2016). It has been proposed that the potential for these evolutionary innovations did not emerge de novo in terrestrial vertebrates but was at least partially present in their aquatic ancestors (Freitas et al., 2007).

Paralogs of the short stature homeobox genes, *shox* and *shox2*, have been identified as important regulators of proximal limb development in vertebrates (Abassah-Oppong et al., 2024; Decker et al.,

2011). In humans, SHOX plays a critical role in regulating longitudinal growth (Sabherwal et al., 2007). Mutations in *SHOX* are associated with several growth disorders and body disproportions, including Turner syndrome, Léri-Weill dyschondrosteosis (LWD), and Langer mesomelic dysplasia, which are notably characterized by the shortening of zeugopod elements in the limbs (Rao et al., 1997; Shears et al., 1998; Eduful, 2021; Sabherwal et al., 2007). The human *SHOX2* gene encodes a protein with 83% sequence homology to *SHOX* and an identical homeodomain (Blaschke et al., 1998; Yu et al., 2007). Shox2 has been implicated in the development of the stylopod of both fore- and hindlimbs, craniofacial structures such as the temporomandibular joint and secondary palate, the facial motor nucleus and associated facial nerves, as well as neurons of the dorsal root ganglia (Abassah-Oppong et al., 2024; Cobb et al., 2006).

In human limb development, *SHOX* and *SHOX2* exhibit overlapping yet spatially distinct expression patterns, indicative of spatial subfunctionalization, with *SHOX2* expressed more proximally relative to *SHOX* (Clement-Jones et al., 2000; Yu et al., 2007). A similar spatial distinction is observed in the limbs of the axolotl, where *shox2* is expressed in a restricted proximal-posterior domain of the early limb bud, whereas *shox* is expressed more distally, occupying a broader region of the limb bud (Duerr et al., 2025). Notably, neither gene is expressed in the distal-most regions of the axolotl limb bud. In chicken embryos, *shox* expression is detected in the mesenchyme of the proximal two-thirds of the developing limb bud (Sabherwal et al., 2007).

In mice, the *Shox* gene is absent, and suppression of the remaining paralog *Shox2* results in defective development of the stylopod, the most proximal limb segment (Cobb et al., 2006; Yu et al., 2007). In the fin buds of *Danio* embryos, expression of *shox* and *shox2* has also been documented, however, spatial details of the expression domains for each paralog remain unclear (Thisse and Thisse, 2004; Sawada et al., 2015). Notably, in *Danio* fins, *shox2* has been identified as a downstream target of *shox* (Hoffmann et al., 2021).

Formation of endochondral skeletal elements in vertebrate limbs involves the sequential condensation of mesenchymal cells, the development of cartilage, and its subsequent replacement by bone tissue (Long and Ornitz, 2013; Yu et al., 2007). In *Shox2* knockout mice, mesenchymal condensation occurs normally, however, the subsequent stages of bone development - chondrogenesis and ossification - are disrupted (Yu et al., 2007). Similar defects in endochondral ossification were observed in CRISPR-mediated *shox* knockouts in axolotl, although this effect was limited to the proximal limb segments - the stylopod and zeugopod - while ossification of the autopod elements proceeded normally (Duerr et al., 2025). This suggests that the regulatory mechanisms governing the skeletal elements of proximal versus distal limb segments in tetrapods differ, which is of particular interest in the context of the evolutionary transition from ancestral fins to terrestrial limbs and the development of the autopod, the homology of which with fin elements remains a matter of debate (Cass et al., 2021).

It has been demonstrated that *shox* and *shox2* genes are targets of retinoic acid (RA), a proximal signal involved in vertebrate limb development (Duerr et al., 2025; Feneck and Logan, 2020). It is hypothesized that *shox* expression is activated by the RA-dependent gene *meis1* and repressed by the distally expressed gene *hoxa13* (Duerr et al., 2025).

To elucidate the roles of *shox* and *shox2* in the development of basal vertebrate limb forms, we analyzed their expression in embryos of the grey bamboo shark, *Chiloscyllium griseum*, a representative of cartilaginous fishes, one of the basal groups of gnathostomes. Since, to our knowledge, previous comparative analyses of *shox* and *shox2* expression have been limited to tetrapod species, one of the aims of our study was to determine when the spatial subfunctionalization of *shox* paralogs arose during vertebrate evolution and to explore its possible role as a contributing factor in the evolutionary transformation of ancestral fins into tetrapod limbs.

RESULTS

Shox Genes Phylogeny

To investigate the phylogeny of *Shox* genes in gnathostomes, we performed a search for *shox* homologs in available databases and analyzed the phylogenetic relationships of their encoded protein sequences. In addition to representatives of various gnathostome classes, the analysis included lamprey (a representative jawless vertebrate), amphioxus and ascidians (representatives of invertebrate chordates), as well as species from hemichordates, cnidarians, and several protostome groups (including anthozoans, mollusks, and annelids).

Phylogenetic analysis of Shox protein sequences (Figure 1) revealed that the stable presence of two *Shox* paralogs is unique to gnathostomes, with these paralogs being clearly distinguishable from one another (Supplementary Figure 1). In lampreys, three *shox* paralogs were identified. In the basal chordates - amphioxus and ascidian (*Oikopleura dioica*) - only a single *shox* gene was present. Some other analyzed species possess two paralogs (e.g., *Limulus polyphemus* among arthropods and *Dreissena polymorpha* among mollusks), but this feature was not observed consistently across other representatives within their respective groups. In sterlet (*Acipenser ruthenus*), four *shox* paralogs were identified.

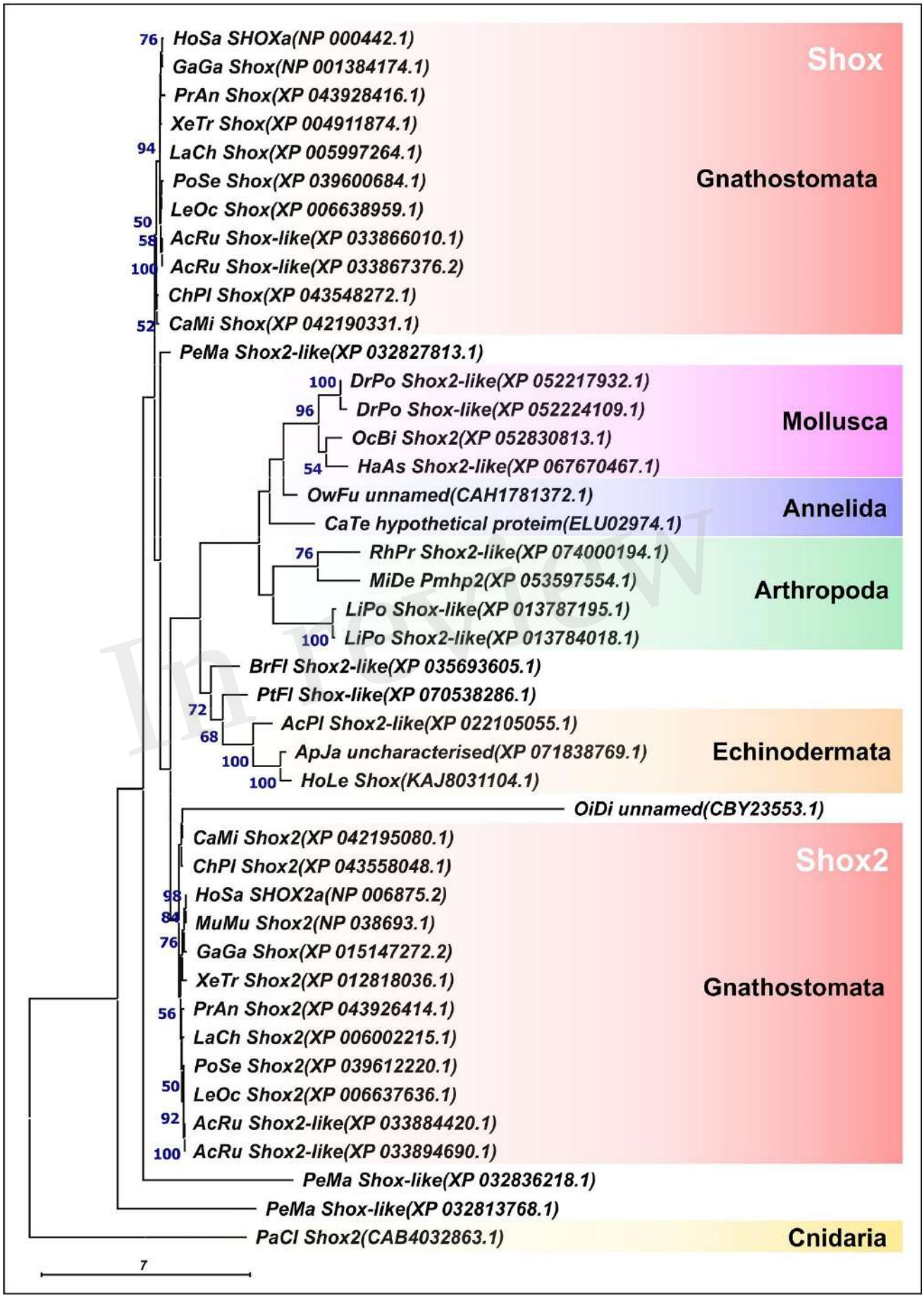
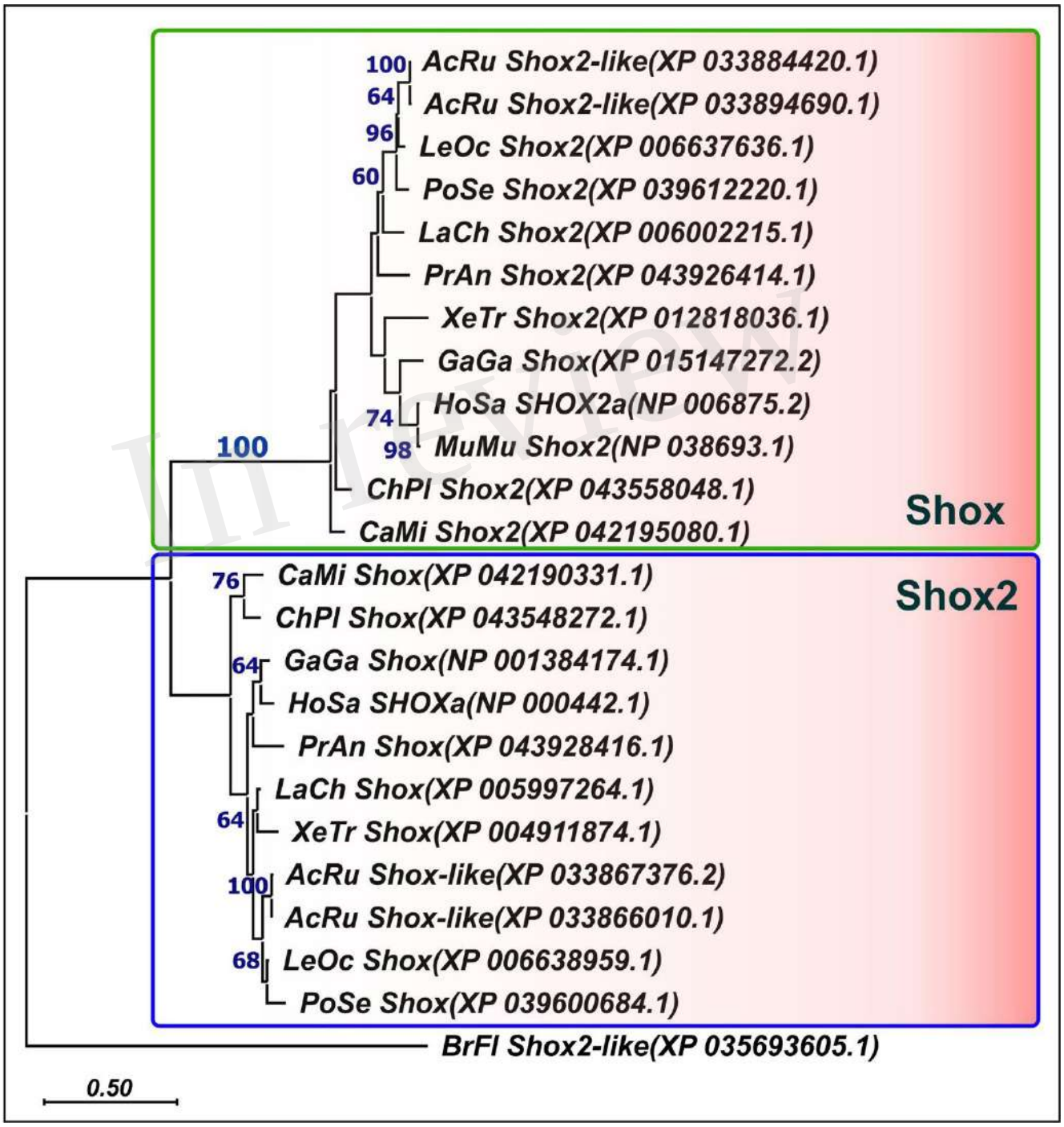


Figure 1 – ML phylogenetic trees of Shox and Shox2 proteins. Bootstraps >50 are shown.

AcPl - Acanthaster planci; AcRu - Acipenser ruthenus; ApJa - Apostichopus japonicus; BrFl – Branchiostoma floridae; CaMi - Callorhinchus milii; CaTe - Capitella teleta; ChPl – Chiloscyllium plagiosum; DrPo - Dreissena polymorpha; GaGa – Gallus gallus; HaAs - Haliotis asinina; HoLe - Holothuria leucospilota; HoSa – Homo sapiens; LaCh – Latimeria chalumnae; LeOc – Lepisosteus oculatus; LiPo - Limulus polyphemus; MiDe - Microplitis demolitor; MuMu – Mus musculus; OcBi - Octopus bimaculoides; OiDi – Oikopleura dioica; OwFu - Owenia fusiformis; PaCl - Paramuricea clavata; PeMa – Petromyzon marinus; PoSe – Polypterus senegalus; PtFl - Ptychodera flava; RhPr - Rhodnius prolixus; XeTr – Xenopus tropicalis.



Supplementary Figure 2 – ML phylogenetic trees of Shox and Shox2 proteins of Gnathostomes. Bootstraps >50 are shown.

Expression of *shox* and *shox2* during the development of the grey bamboo shark *C. griseum*

Two paralogs, *shox* and *shox2*, were identified in *C. griseum* genome. The expression patterns of these genes were analyzed in *C. griseum* embryos using in situ hybridization (ISH).

At the earliest stages examined (stages 24 - 25 after Ballard et al., 1993), when the primordia of paired fins appear as ectodermal crests, *shox* expression was detected in the primordia of the pharyngeal arches, the visceral ganglia of cranial nerves VII and X, and the ganglion of the anterior lateral line (Figure 2 A-C).

By stage 27, when fin buds are already morphologically distinguishable, additional *shox* expression domains appear in the paired (pectoral and pelvic) and unpaired dorsal fins, alongside persistent expression in the mandibular and pharyngeal arches (Figure 2 D-G).

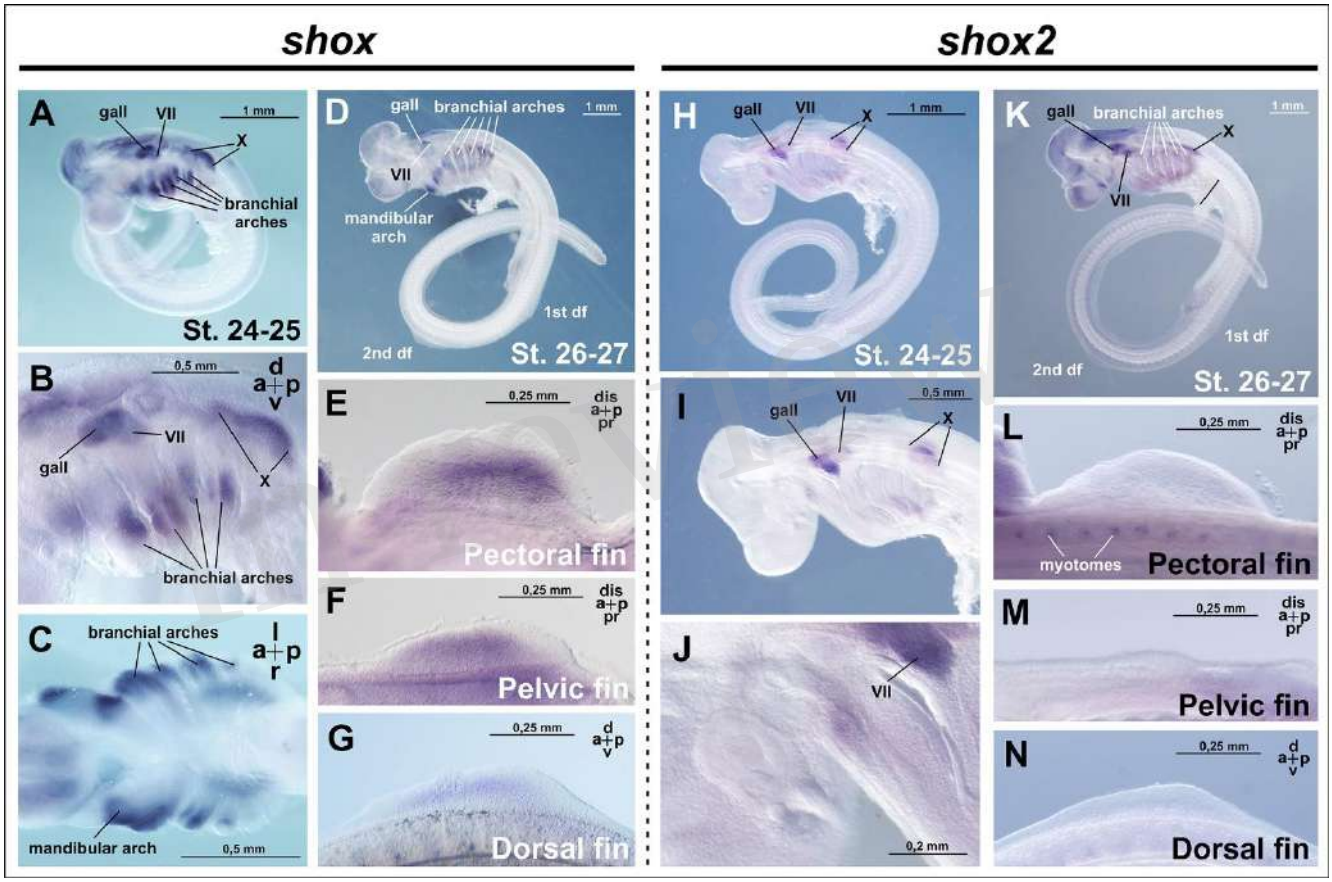


Figure 2. Expression of *shox* and *shox2* in *C. griseum* embryos at stages 24 – 27 (after Ballard et al., 1993). gall – ganglion of the anterior lateral line; VII – ganglion of the VII nerve; X – ganglion of the X nerve.

At stage 28, *shox* expression is observed in the paired (pectoral and pelvic) and unpaired (dorsal and anal) fins (Figure 3 A-E). The expression pattern is highly specific to the fins, as the surrounding trunk tissues show no staining. Within the fin buds, expression is distributed across the entire structure but is not uniform - areas corresponding to the primordia of endoskeletal elements stain less intensely than the surrounding tissues (Figure 3 B-E). In the dorsal fins, expression heterogeneity is more pronounced, with the most intense staining localized to the antero-distal and postero-proximal regions. *Shox* expression persists in the pharyngeal arches, visceral ganglia, and the ganglion of the anterior lateral line (Figure 3 A).

The general pattern of *shox* expression remains consistent at stage 30 (Figure 3 F-M). In addition to fins, *shox* is expressed in the pharyngeal arches and the proximal regions of the developing branchial rays (Figure 3 A, F-I). *Shox* expression is particularly strong in the mandibular arch (Figure 3 A, I).

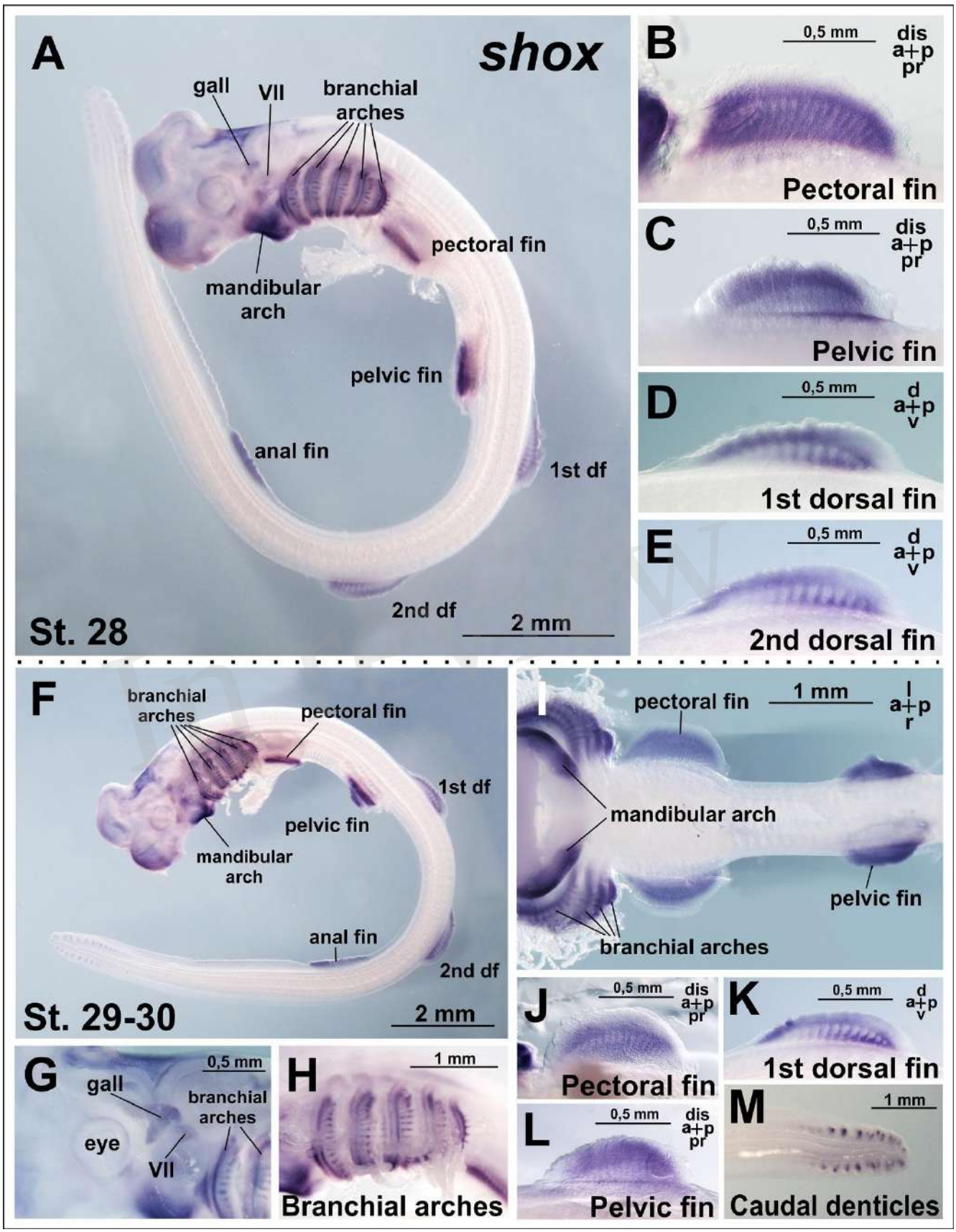


Figure 3. Expression of *shox* in *C. griseum* embryos at stages 28 (A - E) and 30 (F - M).

At the earlier stages examined (stages 24 - 27 after Ballard et al., 1993), *shox2* expression is detected in the visceral ganglia of cranial nerves VII and X, the ganglion of the anterior lateral line, and the myotomes (Figure 2 H-N).

From stages 28 to 30, *shox2* is expressed in the proximal-caudal region of the paired (pectoral and pelvic) fins (Figure 4 A-H). In contrast, no *shox2* expression is detected in the unpaired fins (Figure 4 E, I). In the pharyngeal arches, *shox2* is expressed as a thin medial stripe (Figure 4 A, B, F).

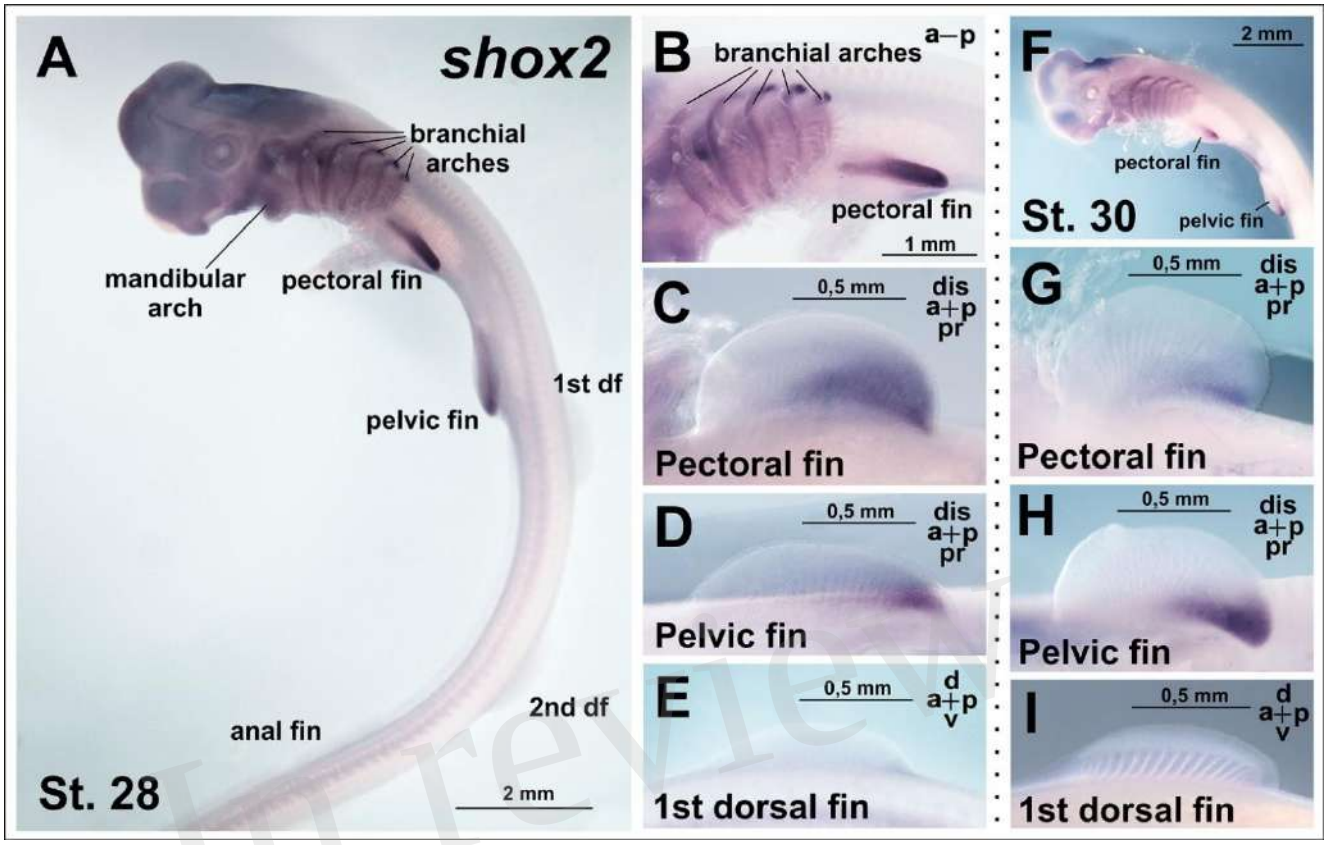
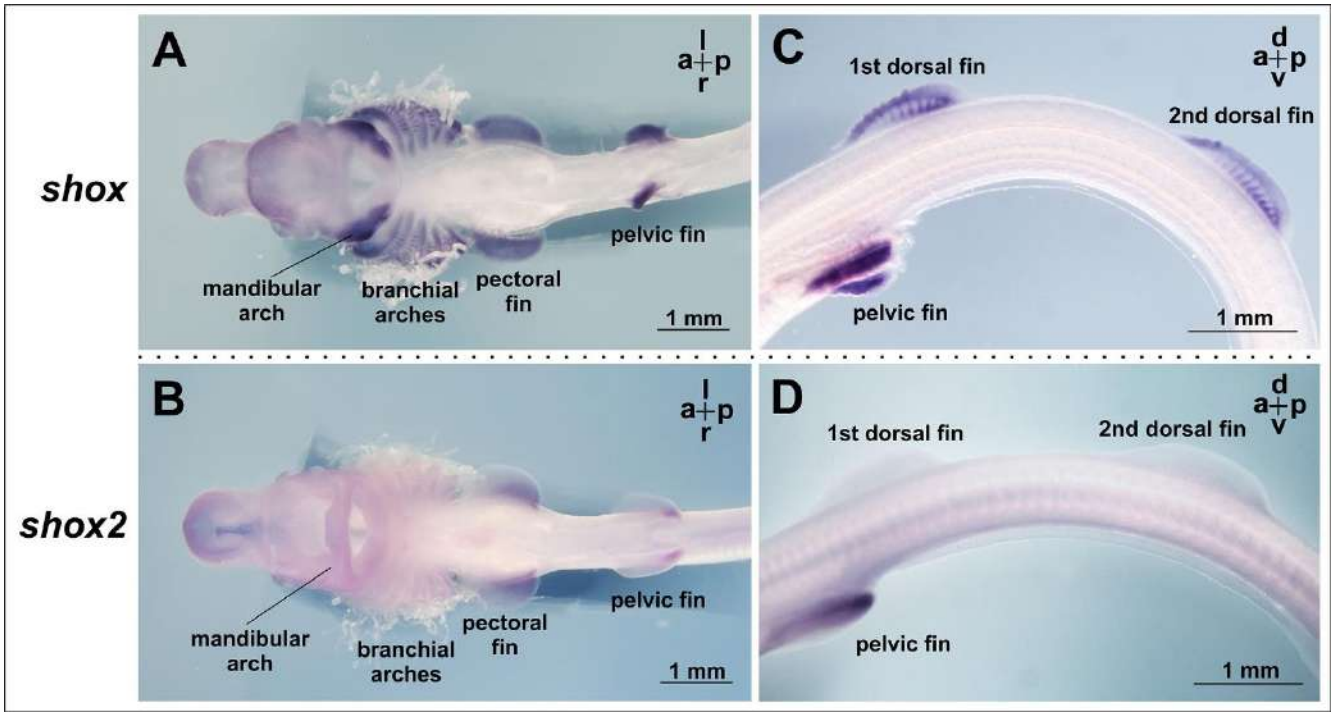


Figure 4. Expression of *shox2* in *C. griseum* embryos at stages 28 (A - E) and 30 (F - I).



Supplementary Figure 2. Comparative expression of *shox* and *shox2* in *C. griseum* embryos at stages 28 – 30 (after Ballard et al., 1993).

Comparison of *shox* and *shox2* expression in *C. griseum* embryos at stages 28-30 reveals shared expression in the paired fins (Figure 6) and, to some extent, in the pharyngeal arches (Figure 3 A, F-I; Figure 4 A, B, F), although the expression patterns of the two paralogs differ in these structures. In the mandibular arch, *shox* expression is stronger and detected at earlier stages (Figure 2 A, D), while *shox2* expression is first detected at stage 28 and only after prolonged staining (Figure 4 A). Notably, only *shox* is expressed in the unpaired fins (Figures 3, 4, Supplementary Figure 2).

DISCUSSION

The phylogenetic analysis of *Shox* genes revealed that these genes are not unique to vertebrates but are present in the genomes of various phylogenetic groups, including both vertebrate and invertebrate taxa. This finding refines the earlier hypothesis by Clement-Jones et al. (2000), which suggested a de novo emergence of *Shox* genes in vertebrates linked to the evolution of skeletal structures.

The presence of multiple *Shox* paralogs in jawless vertebrates (three paralogs in the sea lamprey) and in jawed vertebrates (two paralogs in most groups), combined with the presence of a single *shox* paralog in basal chordates such as amphioxus and ascidians, supports the hypothesis that multiple *shox* paralogs arose via whole genome duplications during early vertebrate evolution (Ohno, 1970; Sacerdot et al., 2018; Simakov et al., 2020; Nakatani et al., 2021; Marlétaz et al., 2024; Yu et al., 2024). The presence of four *shox* paralogs in the sterlet (Chondrostei) likely reflects an additional whole genome duplication event that occurred within this lineage (Du et al., 2020; Redmond et al., 2023). The weak clustering of lamprey Shox proteins with those of jawed vertebrates may reflect the distinctive amino acid composition often described as the “lamprey dialect” (Onimaru and Kuraku, 2018).

In jawed vertebrates, the *shox* and *shox2* paralogs are known to exhibit spatial subfunctionalization along the proximodistal axis of limb development (Clement-Jones et al., 2000; Yu et al., 2007). We also observed distinct expression patterns of *shox* and *shox2* in the paired fins of *C. griseum*. The *shox* is expressed broadly throughout the fin bud of both pectoral and pelvic fins, though not uniformly, whereas *shox2* expression is restricted to the proximal-posterior region of paired fins. This suggests that the regulatory mechanisms responsible for the spatial divergence of expression domains between the two *shox* paralogs may have been established early in the evolution of jawed vertebrates. The posterior expression of *shox2* coincides with the area of the future metapterygial basal element, which is thought to have given rise to the tetrapod limb (Cass et al., 2021). The proximal localization of *shox2* expression in the shark fin is similar to its expression in the axolotl limbs and the proximal limb (stylopod) in mammals (Duerr et al., 2025; Cobb et al., 2006). The broader expression of *shox* in shark fins relative to *shox2* parallels the expression of *shox* orthologs in axolotl (Duerr et al., 2025).

A notable feature of *shox* expression in the shark is its activity in the unpaired fins, including both dorsal fins and the anal fin. This may support the hypothesis that the developmental mechanisms and regulatory elements first established in the evolutionarily older unpaired fins were co-opted during the emergence of paired fins (Freitas et al., 2006; Hawkins et al., 2022). However, the absence of *shox2* expression in the unpaired fins indicates that this regulatory co-option did not involve a complete duplication of developmental programs, and that the formation of paired fins involved additional, specific mechanisms.

In addition to fins, *shox* expression in *C. griseum* is observed in the gill arches and the proximal regions of developing gill rays. Gill rays, characteristic of the branchial apparatus in chondrichthyans, develop on the posterior surface of the hyoid and gill arches in elasmobranchs (Gillis et al., 2009). A distal signaling center, the gill arch epithelial ridge (GAER), plays a key role in their development, serving as a source of sonic hedgehog (Shh) signaling (Gillis et al., 2011; Gillis and Hall, 2016; Rees et al., 2023). The presence of gill rays in cartilaginous fishes contributed to Gegenbaur’s hypothesis that paired appendages originated from the posterior branchial arch (Gegenbaur, 1878; Gillis and Hall, 2016). Subsequent studies have revealed shared features of gene expression and regulatory activity between gill arches and fins, involving key signaling pathways such as Shh, Fgf, Wnt, and RA (Akimenko et al., 1994; Gillis et al., 2009; Gillis et al., 2011; Gillis and Hall, 2016; Rees et al., 2023). Additionally, it has

been demonstrated that, alongside neural crest cells, lateral plate mesoderm (LPM) cells, essential for paired fin development, also contribute to gill arch development (Sleight and Gillis, 2020; Prummel et al., 2020). The *shox* expression we observed in the gill arches, including the developing gill rays, and in paired fins of the shark further supports the developmental similarities between these structures.

The expression of *shox* in the mandibular arch reflects its ontogenetic similarity to gill arches (Gillis et al., 2013).

In mammals, including mice and humans, the tissue-specific activity of Shox and Shox2 is regulated by cis-regulatory elements, notably enhancers located within extensive gene deserts downstream (centromeric) of these transcription factor genes (Abassah-Oppong et al., 2024; Rosin et al., 2013). Comparable extended regulatory landscapes have been described for other key genes involved in signaling center formation during paired appendage development, such as Shh and Fgf8 (Symmons et al., 2013; Marinić et al., 2013). It is plausible that such regulatory regions, enabling the expression of *shox* genes in multiple embryonic structures, including the mandibular and gill arches, paired and unpaired fins, and cranial nerve ganglia as observed in the shark, originated early in the evolution of jawed vertebrates and were inherited by more derived lineages.

MATERIALS AND METHODS

Animals and samples preparation

The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry (Moscow, Russia, protocol code IACUC 229 dated February 1, 2018). The study was conducted in accordance with the local legislation and institutional requirements.

C. griseum eggs and embryos were collected in collaboration with the scientific department of the Moskvarium Center for Oceanography and Marine Biology (Moscow, Russia). The embryos of *C. griseum* were staged in accordance with Ballard et al., 1993.

For ISH, embryos were fixed in MEMFA solution (3.7 % formaldehyde, 100 mM MOPS, 2 mM EGTA, 1 mM MgSO4), dehydrated in methanol and kept at −20 ºC.

C. griseum total RNA sample was obtained from lysed embryos (3 embryos for probe) by purification with the Analytic Jena innuPREP RNA Mini Kit 2.0 (Berlin, Germany).

Analyses of phylogeny and local genomic synteny

The search for homologs was carried out in Blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&BLAST_SPEC=&LINK_LOC=blasttab&LAST_PAGE=blastn) and tBlastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastn&PAGE_TYPE=BlastSearch&BLAST_SPEC=&LINK_LOC=blasttab&LAST_PAGE=blastn) sections. We checked available Nucleotide collections (nr/nt) and whole genome shotgun contigs (wgs).

Multiple alignment was performed by ClustalW algorhythm in the MEGA11 program.

Phylogenetic analyses of Chordin-like1 protein sequences of vertebrates were performed via the Maximum Likehood (ML) methods using the MEGA11 program (Tamura et al., 2021).

The choosing of optimal model was made in MEGA11. The results are present in Supplementary Table1.

In ML method JTT matrix-based model (Jones et al., 1992) with frequencies and Gamma distribution was used. The percentage of trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 60 amino acid sequences.

The list of the analyzed Shox and Shox2 sequences is attached in Supplementary Info.

Shox and *shox2* cDNA obtaining, ISH

C. griseum shox and shox2 cDNAs were obtained by PCR with following primers:

ChGr_shox_full_Frw1; CAGCGAGCGGGCGAGCTAAC;
ChGr_shox_full_Rev1; CCCCGGCCCGGCTGATTG;
ChGr_shox_full_Frw2; ATTAGATCTGCCACCATGGAGGAGCTAACGGCGTT;
ChGr_shox_full_Rev2; AATGTCGACTCAGAGGCCCAGCGCCTCGG;

ChGr_shox2_full_Frw1; GGGACATATTCCTCCGAACA;
ChGr_shox2_full_Rev1; GATTTGGAATCACTGTTCGG;
ChGr_shox2_full_Frw2; ATTAGATCTGCCACCATGGAAGAACTTACAGCTTT;
ChGr_shox2_full_Rev2; AATCTCGAGTCACAGCCCTAGTGCTGCAG.

Nested PCR (Frw1/Rev1 => Frw2/Rev2) was performed with Encyclo polymerase Evrogen kit (www.evrogen.ru, Moscow).

The resulting cDNA fragments were cloned into the pAL2-T vector (Evrogen, Moscow) and cDNA inserts of 3 clones of each gene were sequenced.

ISH was carried out according to the protocol described by Bayramov et al. (2011), Ermakova et al. (2020) and Ermakova et al. (2024).

Photography was carried out using a Leica M205 stereo microscope.

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AUTHOR CONTRIBUTIONS

GVE – shark embryos collection, *in situ* hybridization, photography, figures preparation, writing the paper; IVM - *C. griseum* embryos; VAL - analysis of *shox* genes phylogeny; AGZ – figures preparation, writing of the paper; AVB – research idea and conceptualization, databases analysis, cDNA cloning, phylogenetic analysis, figures preparation, writing of the paper.

COMPETING INTERESTS

The authors declare no competing interests.

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Figure 1.JPEG

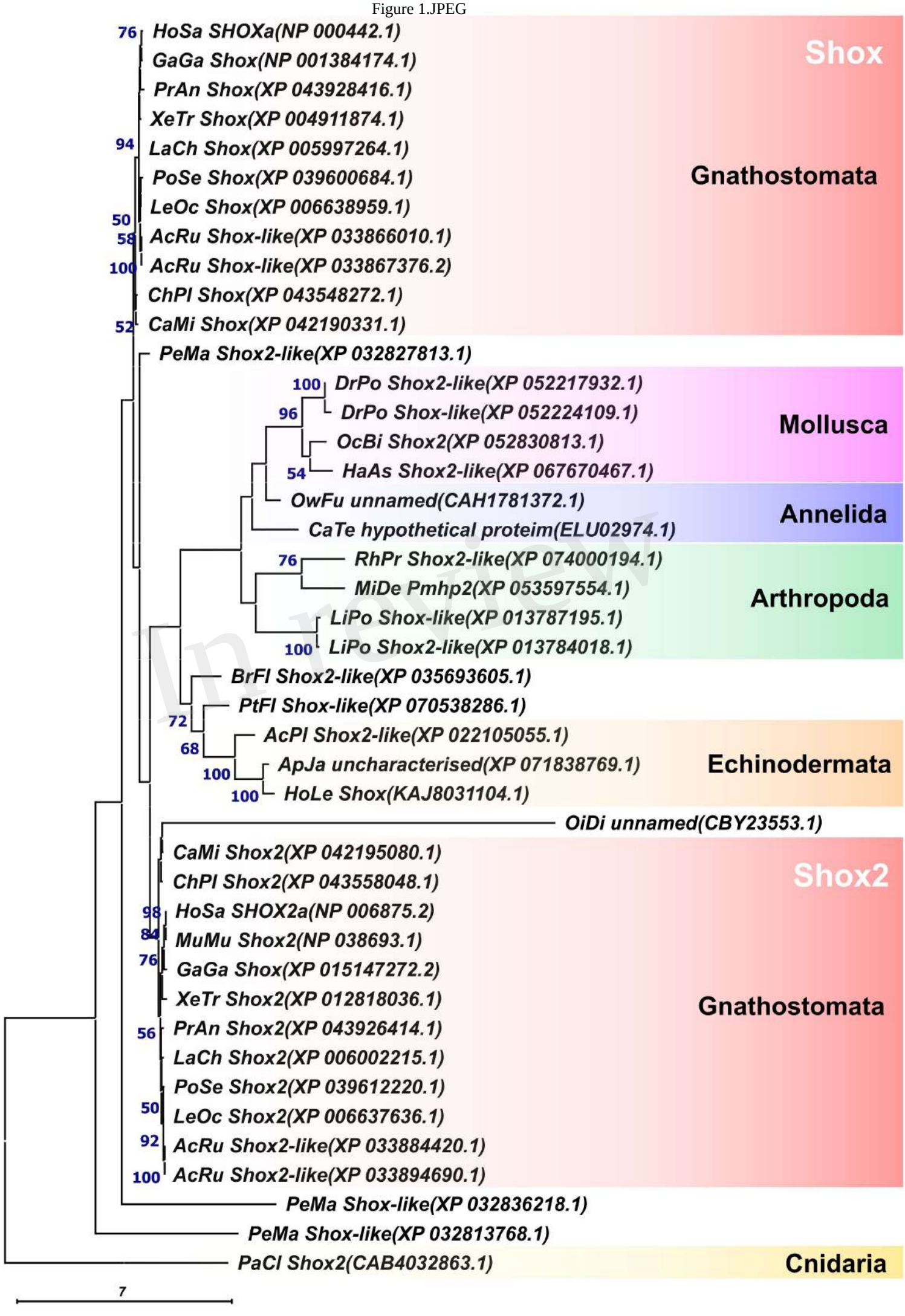
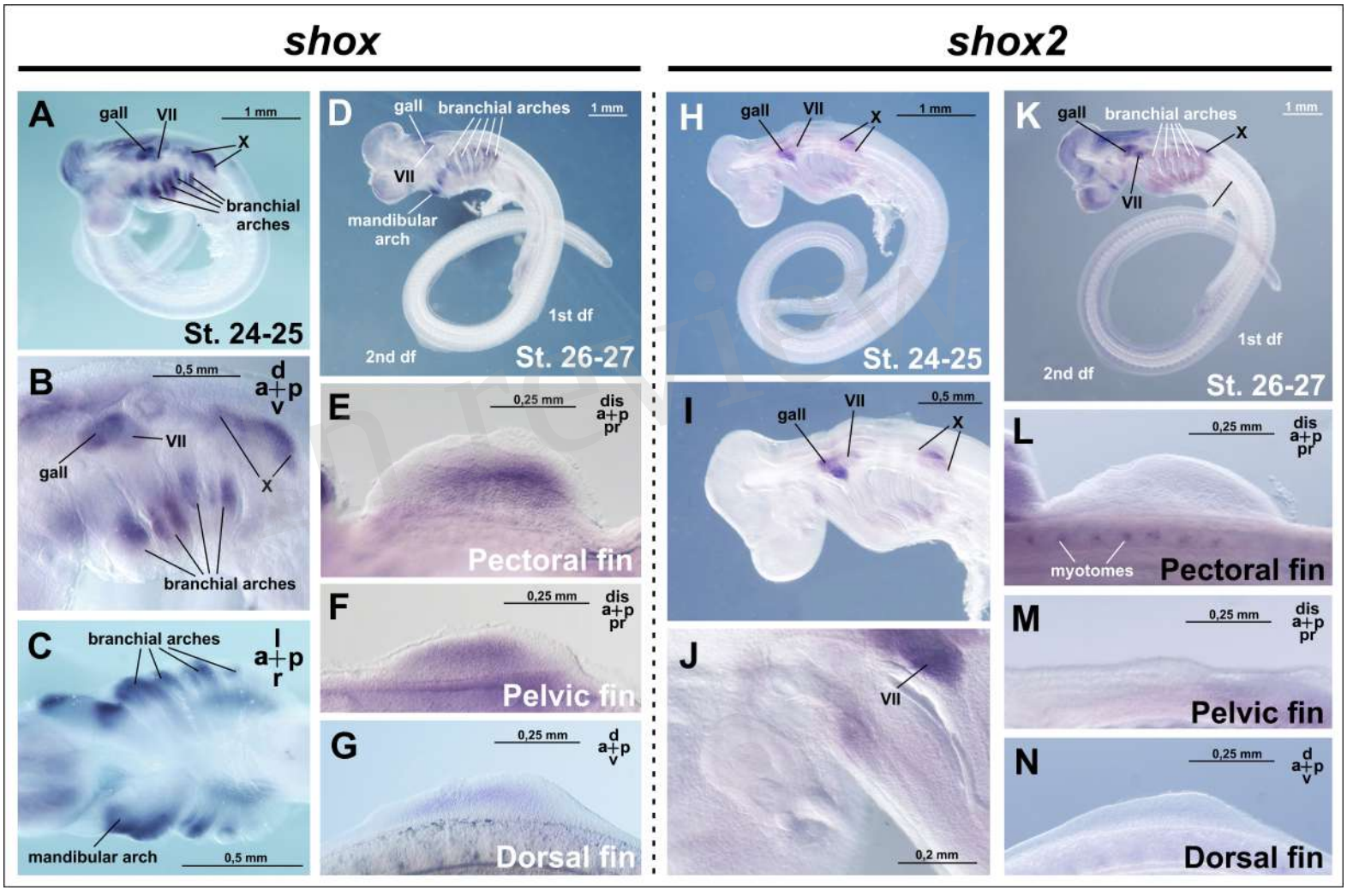


Figure 2.JPEG



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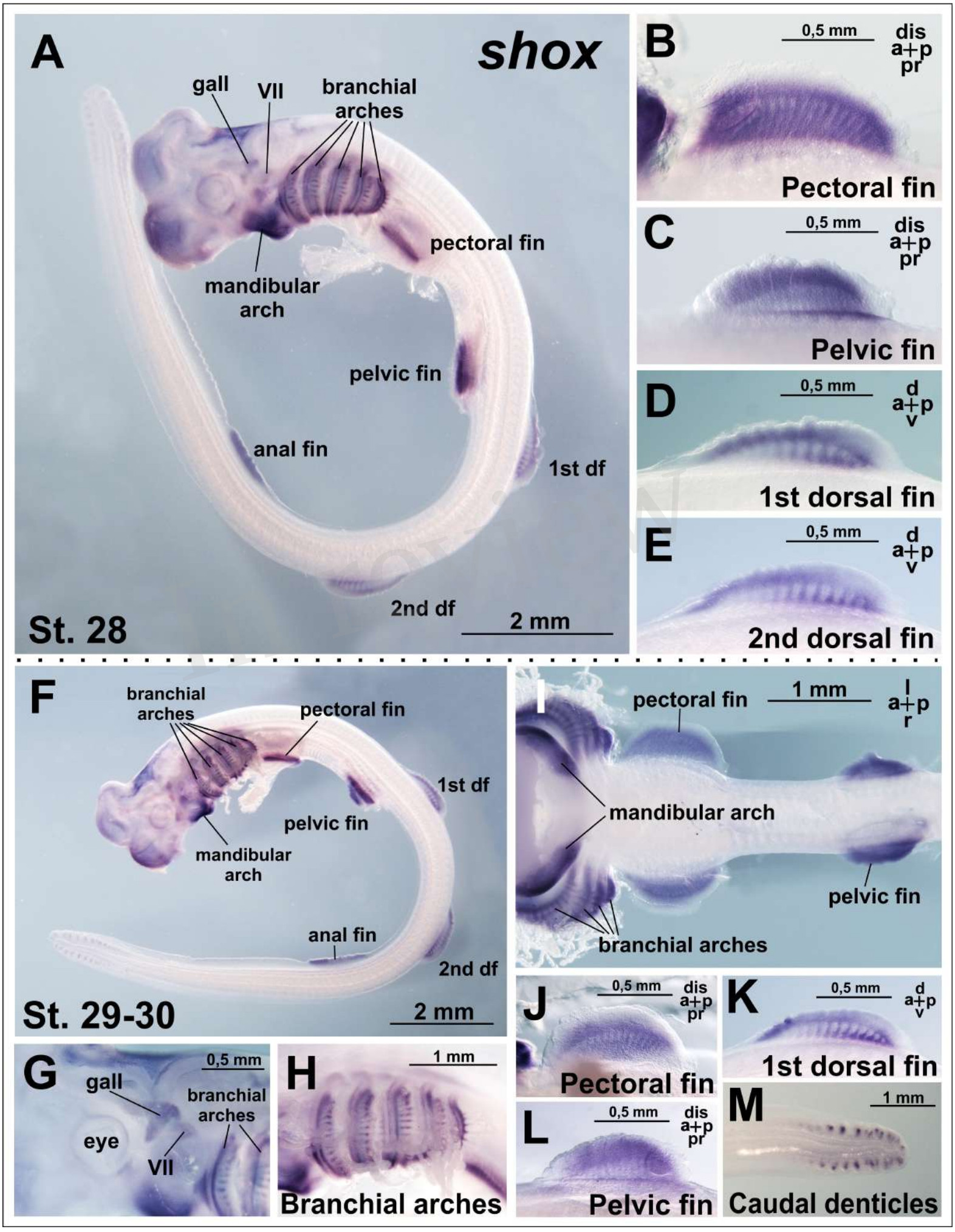

Figure 4.JPEG