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Evolution of the nitric oxide synthase family in vertebrates and novel insights in gill development

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Nitric oxide (NO) is an ancestral key signalling molecule essential for life and has enormous versatility in biological systems, including cardiovascular homeostasis, neurotransmission and immunity. Although our knowledge of NO synthases (Nos), the enzymes that synthesize NO *in vivo*, is substantial, the origin of a large and diversified repertoire of *nos* gene orthologues in fishes with respect to tetrapods remains a puzzle. The recent identification of *nos3* in the ray-finned fish spotted gar, which was considered lost in this lineage, changed this perspective. This finding prompted us to explore *nos* gene evolution, surveying vertebrate species representing key evolutionary nodes. This study provides noteworthy findings: first, *nos2* experienced several lineage-specific gene duplications and losses. Second, *nos3* was found to be lost independently in two different teleost lineages, Elopomorpha and Clupeocephala. Third, the expression of at least one *nos* paralogue in the gills of developing shark, bichir, sturgeon, and gar, but not in lamprey, suggests that *nos* expression in this organ may have arisen in the last common ancestor of gnathostomes. These results provide a framework for continuing research on *nos* genes' roles, highlighting subfunctionalization and reciprocal loss of function that occurred in different lineages during vertebrate genome duplications.

1. Introduction

Historically classified as a pollutant, nitric oxide (NO) was recognized as 'Molecule of the Year' in 1992 [1] for its important function as a cellular signalling molecule. NO plays a role in a myriad of physiological processes, including cardiovascular

homeostasis [2], neurotransmission [3], immune response [4], and in neurodegenerative diseases [5] and cancer [6].

Nitric oxide synthase (Nos), the enzyme catalysing the biosynthesis of NO *in vivo*, is ubiquitous among organisms [7]. Three *nos* gene paralogues have been described in vertebrates: the constitutively expressed *nos1* and *nos3*, and the inducible *nos2* [8].

Although the availability of current genomic data covers all major ray-finned fish lineages, the evolutionary history of their *nos* gene repertoire remains puzzling. Previous studies reported a variable number of *nos* genes in teleost fishes: *nos1* is always present in a single copy and *nos2* is lost or in one or two copies, while *nos3* has been reported as missing in the genomes of ray-finned fishes. This apparent gene loss contrasts with literature describing a putative Nos3-like protein localized by antibody stains in gills and vascular endothelium of some teleost species [9,10]. The discovery of a *nos3* orthologue in the spotted gar *Lepisosteus oculatus*, a holostean fish (the sister group of teleosts within the ray-finned lineage) [11], and the variable number of teleost *nos2* genes prompted us to study in deep the evolution of this important gene family and *nos3* expression pattern in fishes representing key nodes in vertebrate evolution. In an attempt to answer these questions, we have studied the Nos family repertoire at unprecedented phylogenetic resolution, investigated conserved syntenies in fish genomes, and studied the expression pattern of all three *nos* genes during development in multiple species.

2. Results

(a) Revised evolutionary history of Nos2 and Nos3

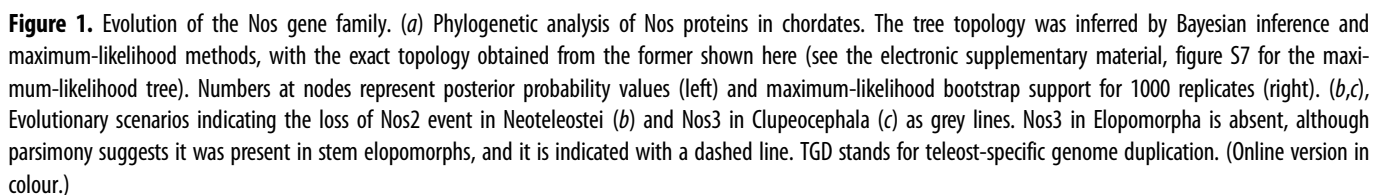
Gaps in our current knowledge of Nos family evolution include the time of origin of the three distinct paralogous *nos* genes and when some of them were secondarily lost in specific lineages. We reconstructed the Nos phylogeny using 116 protein sequences from 54 species (electronic supplementary material, table S1) providing a broad representation of aquatic vertebrates: cyclostomes (modern jawless fishes), chondrichthyans (cartilaginous fishes), and osteichthyes (bony fishes), including ray- and lobe-finned fishes. Lobe-finned fishes include coelacanth, lungfishes, and tetrapods; ray-finned fishes comprise the non-teleost lineages of polypteriformes (e.g. bichir), acipenseriformes (e.g. sterlet sturgeon), holosteans (lepisosteiformes, e.g. spotted gar, and amiiformes, e.g. bowfin), and the teleosts, subdivided into three major living lineages: elopomorphs (e.g. eels and relatives), osteoglossomorphs (e.g. arowana, mooneyes and the freshwater elephantfish), and clupeocephalans (e.g. zebrafish and medaka) [12] (for clarification see the electronic supplementary material, figure S1).

All Nos proteins considered in the present study showed conservation of canonical domains organization. Here we confirmed the presence of single Nos1 in all jawed vertebrates examined, except for two gene duplicates in cyprinids (*nos1a* and *nos1b*) and salmonids (*nos1α* and *nos1β*) (figure 1a blue shading; electronic supplementary material, figure S2-a). Most fish lineages retained Nos2, including chondrichthyans (*Callorhynchus milii*, *Rhincodon typus*, *Chiloscyllium punctatum*, *Scyliorhinus torazame*), polypteriformes (*Polypterus senegalus*, *Erpetoichthys calabaricus*), acipenseriformes (*Acipenser ruthenus*), holosteans (*Amia calva*, *Lepisosteus oculatus*), elopomorphs

(*Megalops cyprinoides*), osteoglossomorphs (*Paramormyrops kingsleyae*, *Scleropages formosus*) and coelacanthiformes (*Latimeria chalumnae*) (figure 1a, yellow shading), although a *nos2* gene loss event occurred at the stem of Neoteleostei (figure 1b), since it has not been found in any available genomic or transcriptomic data from this clade. On the other hand, our phylogenetic analysis highlights the occurrence of extra *nos2* duplicates in several lineages, for which we adopted a specific nomenclature based on the phylogenetic analysis and synteny conservation: *nos2a* and *nos2b* in the zebrafish *Danio rerio*; *nos2a*, *nos2ba* and *nos2bb* in the goldfish *Carassius auratus*, and the blind golden-line barbel *Sinocyclocheilus anshuiensis* and the common carp *Cyprinus carpio*; *nos2α* and *nos2β* in salmonids (*Salmo salar* and *Oncorhynchus mykiss*); and lastly, *nos2.1* and *nos2.2* in a characid (the Mexican tetra *Astyanax mexicanus*), a gymnotid (the electric eel *Electrophorus electricus*), an ictalurid (the channel catfish *Ictalurus punctatus*), an esocid (the northern pike *Esox lucius*), and a clupeid (the Atlantic herring *Clupea harengus*) (figure 1a, yellow shading).

Nos3 deserves special attention since it was previously believed that a loss event predated the lineage of actinopterygians or alternatively that it represents an innovation of tetrapods [7]. Nevertheless, this hypothesis may have been overinterpreted since few ray-finned genome sequences were originally available. The only actinopterygian *nos3* reported thus far was in the spotted gar [11]. Here we report the identification of *nos3* in genomes of the bichir *Po. senegalus*, the sterlet sturgeon *Ac. ruthenus* [13], the bowfin *Am. calva* [14], and the freshwater elephantfish *Pa. kingsleyae* [15] (figure 1a, red shading). The absence of *nos3* in clupeocephalans indicates a gene loss event at the stem of this group (figure 1c). Furthermore, we did not find *nos3* in the tarpon *M. cyprinoides*, the most complete genome available among Elopomorpha, nor in transcriptomic data of the European eel *Anguilla anguilla*. On the other hand, we did identify a *nos3* orthologue in the cloudy catshark *Scy. torazame*, suggesting its presence in the ancestor of gnathostomes. Previously, two *nos* genes had been found in the lamprey, called *nosA* and *nosB* [7], with unresolved orthology to gnathostome *nos1-nos2-nos3*, and derived from a lineage-specific tandem duplication in the lamprey lineage. Based on this finding, we searched for the presence of *nos* genes in other cyclostomes. We found orthologous genes to *Petromyzon marinus nosA* and *nosB* paralogues in the arctic lamprey *Lethenteron camtschaticum* [16], and a single *nos* gene in the inshore hagfish *Eptatretus burgeri*. Our phylogenetic analysis shows that the hagfish Nos remains outside the lamprey NosA-NosB clade, therefore with no clear orthology relationship to any specific gnathostome Nos1, Nos2, Nos3, and suggesting that the duplication giving rise to the lamprey *nosA-nosB* occurred at least before the last common ancestor of Petromyzontidae.

In order to study the Nos evolution at the protein level and verify if each gene clade is under differential selection pressure, we conducted a branch model (BM) analysis (see the electronic supplementary material). The BM analysis showed significant *p*-value and ω values less than 1 for all Nos proteins: Nos1 (ω 1 = 0.035), Nos2 (ω 1 = 0.092) and Nos3 (ω 1 = 0.082) (electronic supplementary material, table S2). Therefore, they are under purifying (negative) selection, and in particular, the Nos2 and Nos3 evolution resulted slightly more relaxed with respect to Nos1.



translocated to a different position in the genome (figure 2a; electronic supplementary material, figure S2-b). Second, an additional genome duplication event after the TGD specifically occurred independently in several teleost lineages, causing the presence of extra *nos2* paralogues. These include some cyprinids, in which the carp-specific genome duplication event (Cs4R) probably occurred before the divergence of *Ca. auratus*, *Si. anshuiensis* and *Cy. carpio* [17], and salmonids (salmonid-specific genome duplication or Ss4R) [18,19], with *Sa. salar* and *O. mykiss* in this study. These additional tetraploidization events can explain the origin of the two independent sets of *nos2* genes in cyprinid and salmonid species. In the case of cyprinids, both our phylogenetic and synteny analyses clearly show their *nos2b* orthology, and we denote them as *nos2ba* and *nos2bb* (figures 1a and 2a). In the case of salmonids, we name

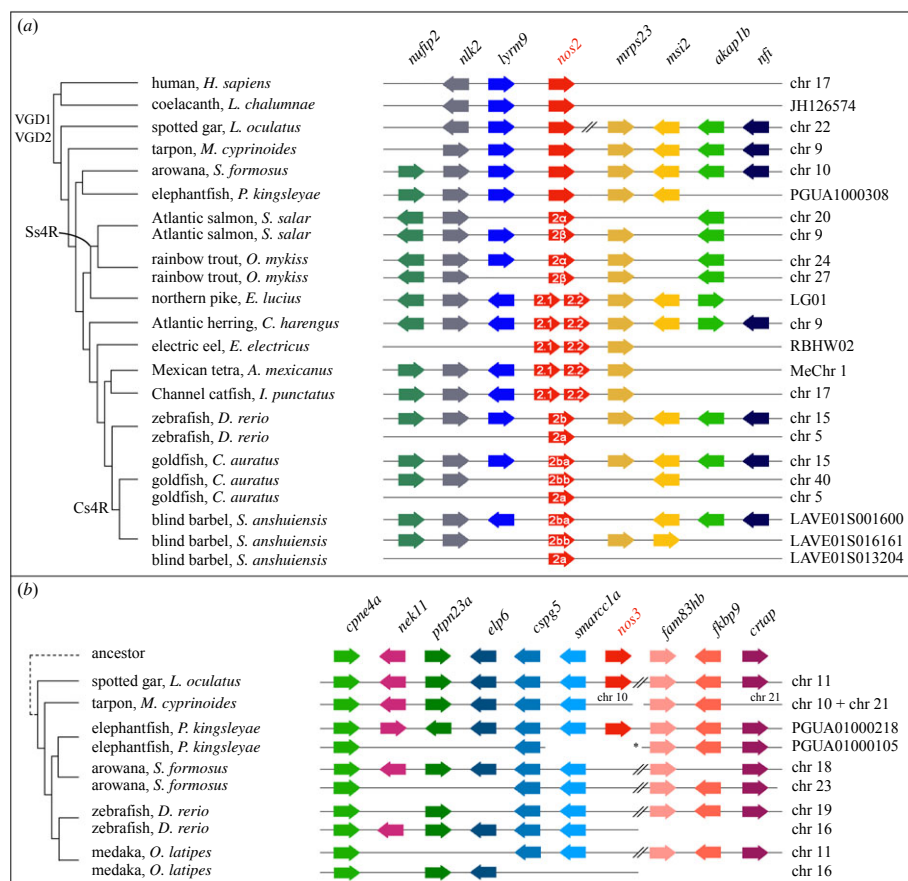


Figure 2. Conserved microsynteny of *nos2* and *nos3*. (a) The *nos2* paralogues derived from different duplication modalities: carp-specific genome duplication (Cs4R) (*nos2ba* and *nos2bb* in the goldfish and blind barbel); salmonid-specific genome duplication (Ss4R) (*nos2α* and *nos2β* in the Atlantic salmon and rainbow trout); tandem gene duplication occurred independently in five lineages (*nos2.1* and *nos2.2* in the northern pike, Atlantic herring, electric eel, Mexican tetra and channel catfish). An additional *nos2* duplicate (*nos2a*) is present in cyprinids (zebrafish, goldfish, and blind barbel) (see the electronic supplementary material, figure S2). (b) A conserved synteny map of genomic regions around the *nos3* gene locus highlights the loss in Clupeocephala (including zebrafish and medaka), and in Osteoglossomorpha (arowana). Consecutive genes are represented as arrows and are colour coded according to their orthology and ohnology. The direction of arrows indicates gene transcription orientation. // indicates long-distance on the chromosome (>600 kb), * indicates scaffold 72 of the freshwater elephantfish genome [15]. (Online version in colour.)

them *nos2α* and *nos2β* to distinguish them from the cyprinid *nos2a* and *nos2b* paralogues, which have a separate origin (see above; figure 2a). Third, independent tandem gene duplications explain the presence of two *nos2* copies, that we named *nos2.1* and *nos2.2*, located next to each other in the same chromosomal fragment in the genomes of the Atlantic herring (*Cl. harengus*), the Mexican tetra (cavefish, *As. mexicanus*), the electric eel (*El. electricus*), the channel catfish (*I. punctatus*) and the northern pike (*Es. lucius*) (figure 2a).

Bichir, reedfish, sterlet, spotted gar, bowfin and freshwater elephantfish are the only ray-finned fishes that retained a *nos3* orthologue. Therefore, we investigated the absence of *nos3* in clupeocephalans. First, we looked for the genomic region containing *nos3* in fishes that represent outgroups to the clupeocephalans. We found one long scaffold of the *Pa. kingsleyae* genome (scaffold 217) [15] showing extensive conserved synteny with the *nos3*-containing segment of the linkage group 11 (LG) in the spotted gar genome (figure 2b). While these appear to correspond to one of the TGD ohnologs (figure 2b), there are two other *Pa. kingsleyae* scaffold segments (from scaffolds 72 and 104) that together seem to represent the second TGD ohnolog, but lacking the expected *nos3* TGD ohnologue (figure 2b). Zebrafish chromosomes 16 and 19 and medaka chromosomes 11 and 16 contain orthologous regions to the two *Pa. kingsleyae* and *Le. oculatus* TGD ohnologs, but lack a *nos3* gene at the expected locations. The one-to-one

relationship between these *Pa. kingsleyae* scaffolds and zebrafish and medaka chromosomes is challenging to determine (figure 2b). Regardless, the most parsimonious explanation for the *nos3* repertoire in ray-finned fishes is that, one of the two *nos3* TGD ohnologs was lost in the teleost common ancestor, while the other was retained and later lost in secondary, independent events in the common ancestor of Clupeocephala and, probably, that of Elopomorpha (figures 1c and 2b).

(b) Expression of *nos* in vertebrate developing gills

Spotted gar is an important emerging experimental organism representing an evolutionary bridge between teleosts and tetrapods that facilitates cross-species comparisons. The gar genome is slowly evolving compared to that of teleosts and has preserved a more ancient structural organization [20]. Therefore, we examined the expression patterns of *nos* genes during gar development. As expected, *nos1* was expressed in several regions of the developing nervous system (electronic supplementary material, figure S3, and [21]). By contrast, *nos2* expression was not detected during the developmental stages covered in the present study, i.e. from 4 to 14 days post fertilization (dpf). Unexpectedly, the expression of *nos3* was first detected in embryos in the pharyngeal area at 4 dpf (figure 3a,b) and increased at 6 dpf (figure 3c,d). At 7 dpf, embryos showed clear *nos3*

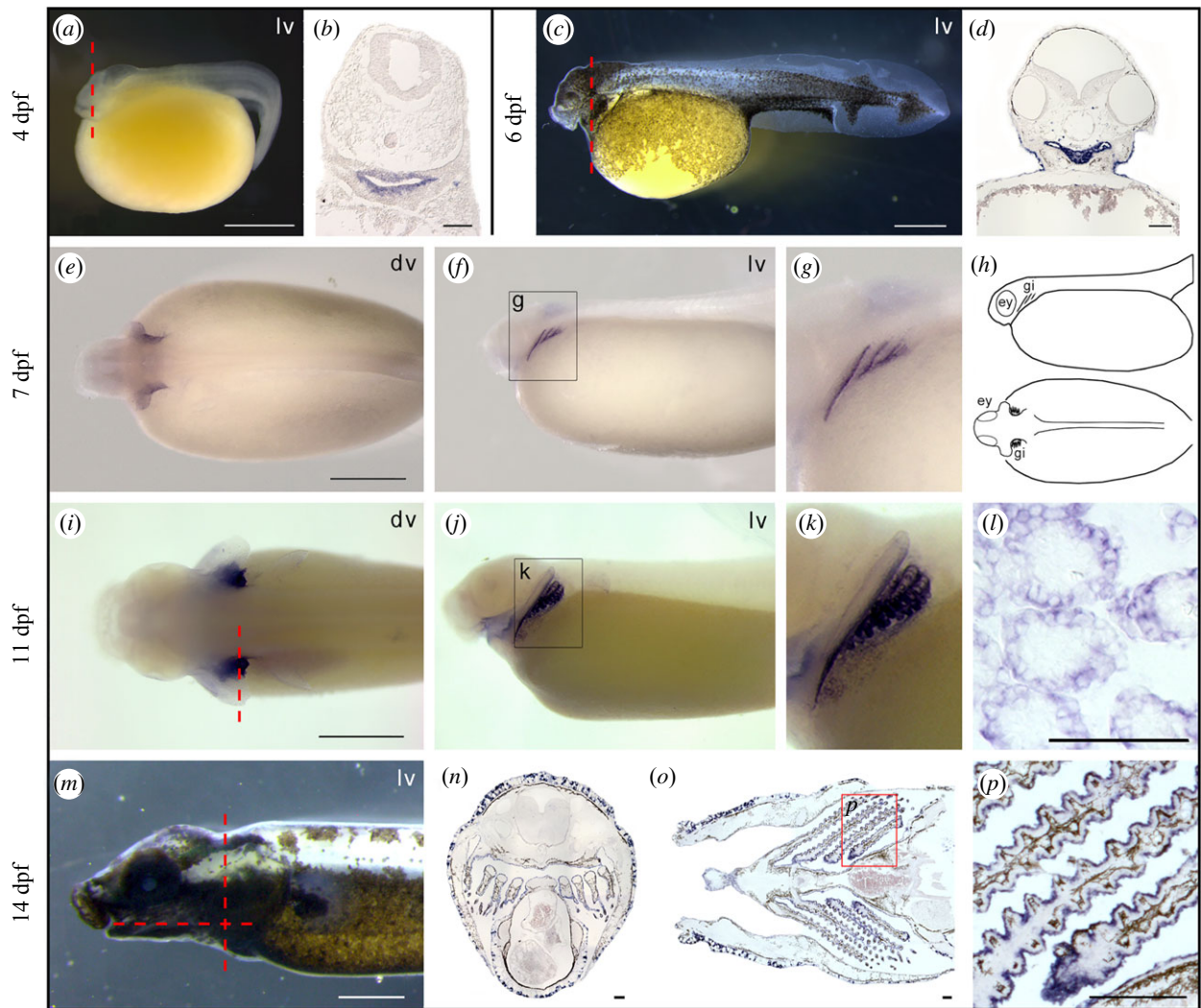


Figure 3. Spotted gar *nos3* localization during development. Expression of *nos3* is localized in the pharyngeal area in 4 dpf (a,b) and 6 dpf (c,d) embryos, in pharyngeal arches in 7 dpf larvae (e–g) schematized in (h), in developing gills in 11 dpf late larvae (i–l), and in gill lamellae in 14 dpf juveniles (m–p). Coronal (n) and transversal section (o) planes are indicated with a red dashed line in (m). ey, eye; gi, gill; dv, dorsal view; lv, lateral view. Scale bar is 1 mm in (a,c,e,i,m); 100 µm in (b,d,f,h,j,l,n,o,p). (Online version in colour.)

expression in developing arches III, IV, and V (figure 3e–g). Later, at 11 dpf, the positive signal is localized in gill filaments (figure 3i–k). Histological sections highlighted the presence of *nos3* in the epithelium of branchial lamellae (figure 3l), also confirmed by the signal in gill structures in an advanced stage of maturation in 14 dpf juveniles (figure 3m–p).

The detection of *nos3* transcripts in gills of spotted gar and the established involvement of NO gas in osmoregulatory control and vascular motility in gills of numerous teleosts [22–25] prompted us to investigate whether a similar *nos* expression pattern occurred in developing gills of other fish species. We investigated *nos* expression in the sterlet sturgeon and the bichir, members of early branching groups of ray-finned fishes [12]. Moreover, we similarly searched *nos* expression pattern in the chondrichthyan cloudy catshark to infer the ancestral expression condition among gnathostomes. Unlike gar, we discovered that *nos3* was not expressed in gills of other species analysed in this work (electronic supplementary material, figure S3), thus raising questions of whether *nos3* expression in gills represents an oddity of holosteans or gars. Surprisingly, we found a different scenario in which other *nos* genes were expressed in gills of sturgeon, bichir, and shark. In particular, *nos2* was expressed

in the branchial area of the sterlet sturgeon (figure 4a–c) and bichir embryos (figure 4d–f), while *nos1* is expressed in gills of catshark embryos (figure 4g–i).

Our results show that *nos* paralogues are expressed in pharyngeal arches and gills in both actinopterygians and chondrichthyans. These findings lead us to question whether *nos* expression in gills could be a conserved feature also in sarcopterygians, and in particular in amphibians that use gills for gas exchange. Therefore, to investigate the presence of *nos* transcripts in amphibia, we chose the neotenic axolotl *Ambystoma mexicanum* because it retains functional external gills throughout life. Gene expression analysis by quantitative polymerase chain reaction (qPCR) revealed that *nos1* and *nos2* are almost not detectable in adult axolotl gills, while *nos3* is highly expressed in gill structures (electronic supplementary material, figure S4). Therefore, we conclude that *nos* expression in gills is a conserved feature in the neotenic amphibian assayed, previously observed exclusively in fishes.

(c) Expression of *nos* genes in the lamprey

In cyclostomes (jawless vertebrates, including lampreys and hagfish), cartilaginous and bony gnathostomes (jawed

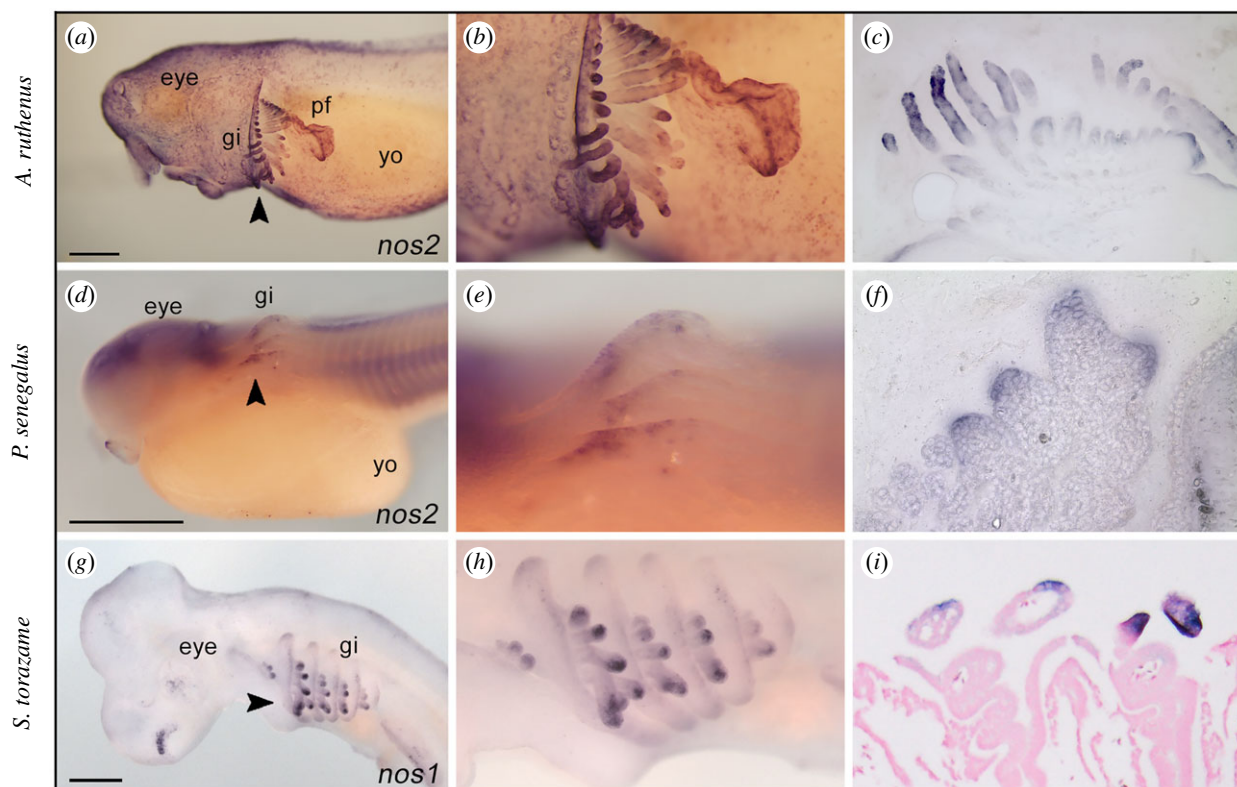


Figure 4. Expression of *nos* genes in developing gills of sturgeon, bichir, and shark embryos. The expression of *nos2* in the gills of sterlet sturgeon *Acipenser ruthenus* (14 mm stage, *a–c*) and bichir *Polypterus senegalus* (stage 31, *d,e*); *nos1* in the shark *Scyliorhinus torazame* (stage 27, *g–i*). Higher magnification views of the gill structure of (*a,d,g*) are shown in (*b,e,h*), respectively. The arrowheads indicate sectioning plane (*a,d,g*): transversal sections (*c,f*, 50 μ m) and frontal section (*i*, 10 μ m). gi, gill; yo, yolk; pf, pectoral fin. Scale bar in (*a,d,g*) is 0.5 mm. (Online version in colour.)

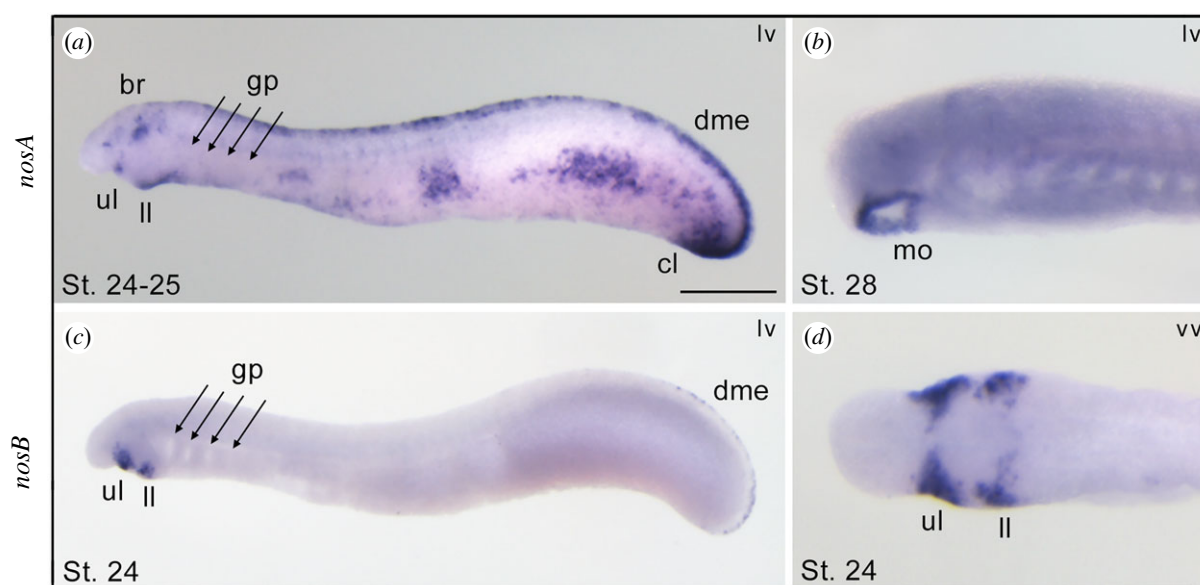


Figure 5. Expression patterns of *nosA* and *nosB* in larvae of the arctic lamprey. At stages 24–25 the *nosA* is expressed in the brain, mouth, upper and lower lip, dorsal midline epidermis, and cloaca (*a*). At stage 28, *nosA* expression is restricted to the mouth (*b*). The *nosB* is exclusively expressed in the cheek process, consisting of upper and lower lips (*c,d*), and faint expression in the dorsal midline epidermis (*c*). br, brain; cl, cloaca; dme, dorsal midline epidermis; gp, gill pouches; mo, mouth; ll, lower lip; ul, upper lip; lv, lateral view; vv, ventral view. Scale bar in (*a*) is 0.5 mm. (Online version in colour.)

vertebrates), gills are endoderm-derived structures, pointing to a single origin of pharyngeal gills before the divergence of these vertebrate lineages [26,27]. To assess whether *nosA* and *nosB* are expressed in gills during embryogenesis, we performed whole-mount *in situ* hybridization experiments at different embryonic stages. We found that lamprey *nosA* was expressed in several tissues, including

the brain, dorsal midline epidermis, tailbud, mouth and cloaca, but not in gills (figure 5*a,b*). Conversely, the lamprey *nosB* paralogue showed restricted expression in the developing mouth, specifically in the cheek process, including upper and lower lip regions (figure 5*c,d*). These results show that in the arctic lamprey, neither of the two *nos* paralogues is expressed in immature or mature gills, suggesting a

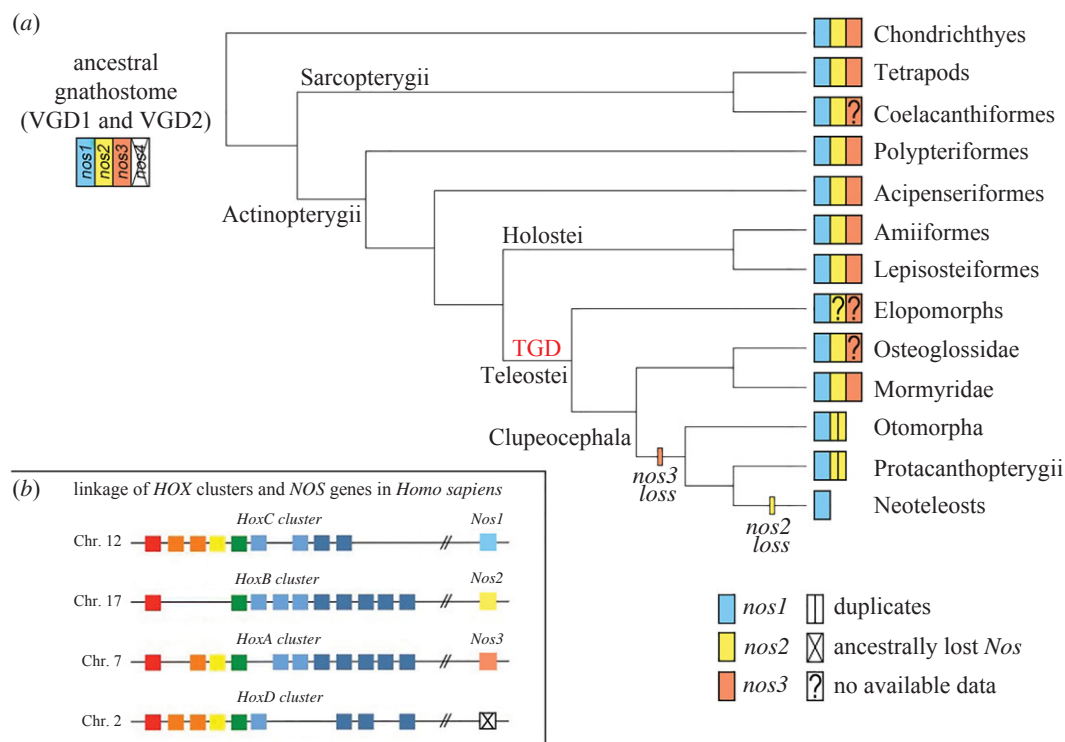


Figure 6. *Nos* evolution in light of recent gene findings in vertebrates. The proposed evolution of *nos* genes in gnathostomes (a) supposes an ancestral loss of a predicted fourth *nos* gene, based on the linkage of human *Nos* and *Hox* clusters (b). Loss of *nos3* occurred in stem Clupeocephala and loss of *nos2* in stem Neoteleostei (a). Species-specific *nos2* duplications occurred in some Otomorpha, including Cyprinidae and Characidae families. (Online version in colour.)

fundamental difference in the role of *nos* genes in jawless and jawed vertebrates.

3. Discussion

Actinopterygians experienced one of the largest radiations in the animal kingdom and their history represents a valuable resource for the formulation of hypotheses regarding the evolution of vertebrate gene families. In this work, we employed data from recent genome projects to clarify and update the evolution of the *Nos* family across vertebrates. Our phylogenetic analysis confirmed that *Nos1* is ubiquitously present as single copy gene across the gnathostome lineage. The only two events of duplication for *nos1* were observed in cyprinids and salmonids, as a consequence of their specific Cs4R and Ss4R tetraploidizations, respectively. Furthermore, our phylogenetic data, complemented with synteny analyses, highlighted for the first time, to our knowledge, a highly complex scenario of *Nos2* evolution, for which we suggest a dedicated nomenclature that attempts to incorporate evolutionary origins into gene names. Previous analyses showed the presence of two *nos2* genes (*nos2a* and *nos2b*) in zebrafish and goldfish [28,29], probably originated from an event of gene duplication that occurred specifically at the stem of the group, and not related to the classic TGD [30,31]. This result is supported by synteny analysis since the chromosomal position of *nos2a* and *nos2b* genes is not conserved, as it would be expected if they were retained after whole-genome duplication. Here we show the presence of a *nos2a* paralogue also in other two cyprinids, *Cy. carpio* and *Si. anshuiensis* (figures 1a and 2a). On the other hand, the cyprinid *nos2b* paralogue independently duplicated in carps after the Cs4R [17], as the conserved synteny suggests (figure 2a). In salmonids, synteny analysis also indicates

that the two *Nos2* paralogues originated secondarily after the Ss4R (figure 2a) [18,19]. Here, we call these genes *nos2ba* and *nos2bb* in carps to emphasize and clarify their relationships to zebrafish genes, and *nos2α* and *nos2β* in salmonids to indicate their distinct evolutionary origin. Additionally, the present work shows that *nos2* has undergone several independent lineage-specific tandem gene duplication events (*nos2.1* and *nos2.2*) (figure 2a). The search of *nos2* in available fish genomes, covering all main groups, failed to find it in any Neoteleostei, and for this reason, we hypothesized a *nos2* gene loss event occurred at the stem of Neoteleostei (figures 1 and 6). Importantly, NO produced upon stimulation of the inducible *nos2* is considered one of the most versatile players of the immune system [4]. For this reason, it would be important in the future to investigate the impact of *Nos2* loss on the immune response in Neoteleostei and if any compensatory mechanisms occurred through the activation of other *nos* paralogues, as well as to understand if *nos2* duplicates underwent neofunctionalization or subfunctionalization, thus providing new functional features to the organism.

Concerning *nos3*, our understanding of its evolutionary history had a twist with the finding of a *nos3* orthologue in the spotted gar genome [11], proving that the previously postulated actinopterygian-specific loss of *nos3* was an incorrect inference. Fostered by this discovery, we specifically searched for the presence of *nos3* orthologues in a wide range of fish species to infer the ancestral condition. We identified a *nos3* gene in bowfin, thus confirming the presence of *nos3* in the other reference genus of the holostean clade, in addition to gar (figure 6). Furthermore, the presence of *nos3* in genomes of bichir and sterlet sturgeon, which diverged prior to the teleostean and holostean split, confirmed the hypothesis that *nos3* was already present in the common ancestor of extant osteichthyes, rather than an innovation of tetrapods [7] or

neopterygians (holosteans plus teleosts) [11] (figure 6). We did not find *nos3* gene in the tarpon *M. cyprinoides* genome (figure 2b), and to date, the limited genomic and transcriptomic data of eels, congers, and morays cannot endorse the presence of a *nos3* in Elopomorpha. Therefore, more genome sequences are necessary to confirm its absence in this key group. We also did not find *nos3* in any Clupeocephala (non-elopomorph and non-osteoglossomorph teleosts) suggesting that a loss event took place in the common ancestor of clupeocephalans. Notably, we found a *nos3* gene in the osteoglossomorph elephantfish *Pa. kingsleyae*, and it allowed us to confirm that the loss of *nos3* did not occur in the last common teleost ancestor, as previously thought [11]. These findings suggest instead the following evolutionary scenario for the *nos3* gene: first, since we only find a maximum of one *nos3* gene in those cases where it is present, we assume that one of the two TGD ohnologues was immediately lost after the TGD, and the other one was retained. This *nos3* gene was then lost in the ancestors of elopomorphs—although further research is needed to confirm this— and clupeocephalans independently in separate events (figure 6).

The discovery of *nos3* in sharks (*Scy. torazame* in this study) suggests that the origin of *nos3* predates the divergence of gnathostomes and that three distinct *nos* paralogues were already present in the last common ancestor of gnathostomes (figure 6), probably originating after the two rounds of whole-genome duplication that took place during early vertebrate evolution (vertebrate gene duplications (VGD) VGD1 and VGD2, 2R hypothesis) [7,32,33]. The origin of *nos* genes is, in fact, supported by the linkage to the evolutionarily conserved *Hox* gene clusters and several other syntenic genes (figure 6b; electronic supplementary material, figure S5). Under this scenario, then a fourth *nos* gene (putative *nos4*) should have existed but was apparently lost early in the gnathostome evolution (figure 6a).

The apparent lack of *nos3* in some vertebrate lineages, such as in coelacanth *La. chalumnae* (an extant basally diverging sarcopterygian), in arowana *Scl. formosus* (an osteoglossomorph), and in elopomorph fishes, remains to be clarified in the future.

The protein evolution analysis highlighted that the three Nos clades show negative selection pressure at different rates, being Nos1 under stronger negative selection, in respect to Nos2 and Nos3 that resulted under more relaxed negative selection based on significant ω values. These results are in agreement with the high degree of conservation of nucleotidic and amino acid sequences during Nos family evolutionary history in vertebrates.

The importance of NO in the ontogeny and function of vertebrate gills has already been documented in the context of physio-pharmacological studies, primarily using inhibitors of Nos activity. In gills, NO acts as a paracrine and endocrine vasoactive modulator and, therefore, plays a crucial role in the distribution of oxygenated blood [34]. Moreover, NO has an osmoregulatory function controlling the movement of ions across the gill epithelium [24,35–37], and represents an important molecular component of the immune system employed by macrophages to attack and destroy pathogens [38]. Nevertheless, documentation of Nos enzymatic activity in fish gills has relied exclusively upon techniques unable to discriminate among individual Nos proteins, such as NADPH-diaphorase activity and immunolocalization with heterologous mammalian antibodies [34,36,37,39]. Therefore, the detected enzymatic activity has for a long time been

indicated generically as ‘Nos-like’. Here, using a specific messenger RNA transcript detection methodology, we showed, for the first time, to our knowledge, that indeed *nos* genes are expressed in gills during development in various vertebrates. Surprisingly different Nos paralogues are expressed in gills in different animals tested: *nos1* in shark, *nos2* in bichir and sterlet sturgeon, and *nos3* in spotted gar. The most parsimonious hypothesis to explain this result is that the ancestral *nos* gene had a number of roles in gills, immune system, brain, and other organs that was controlled by separate regulatory elements and, owing to subfunctionalization after the vertebrate 2R (according to the duplication-degeneration-complementation model) [40], these physiological roles partitioned to different *nos* ohnologues as lineages diverged and reciprocal loss of the gill expression function occurred in a lineage-specific way. Further support for this hypothesis comes from the identification of *nos1*-positive cells in gill of zebrafish at 5 dpf, in addition to brain, eye, periderm and NaK ionocytes, according to the recently released developmental single-cell transcriptome atlas [41] (electronic supplementary material, figure S6).

Additionally, to corroborate the involvement of NO in normal gill physiology, we searched for *nos* expression in gills of a paedomorphic amphibian, the Mexican axolotl, which maintains gill structures in adulthood. Taking into account the different evolutionary and developmental origin of internal and external gills [42], the conservation of *nos3* expression in gills indicated that the NO signalling system could be fundamental for the physiology and development of this structure in the axolotl, and perhaps generally in pre-metamorphic amphibians. Therefore, our data highlighted that the expression of at least one *nos* gene has a functional role in gnathostome gills.

Recently, a single origin of pharyngeal gills predating the divergence of cyclostomes and gnathostomes was suggested [26]. Therefore, we investigated whether either of the two arctic lamprey *nos* paralogues is expressed in developing gills, but found them expressed mainly in the nervous system, mouth and pharynx, similar to the expression pattern previously reported in the cephalochordate amphioxus [43,44]. This led us to speculate that either the expression of *nos* genes in gills was acquired in gnathostomes after the divergence from cyclostomes, or alternatively, gill expression was a feature of their last common ancestor but lost in the lineage of cyclostomes.

In conclusion, our findings pave the way for future studies that aim to investigate the ontogenetic role of NO in gill development of aquatic vertebrates. It would be interesting to understand more about species-specific regulatory mechanisms that drive different *nos* genes expression patterns in gills in different species.

4. Methods

(a) Phylogenetic analysis

Nos sequences used for evolutionary analyses were retrieved from NCBI, Ensembl, Skatebase and DDBJ databases (electronic supplementary material, table S1). We used proteins from *Homo sapiens*, *Anolis carolinensis* and *Xenopus tropicalis* as internal references, and two non-vertebrate chordates as outgroups: the cephalochordate *Branchiostoma lanceolatum* NosA, NosB and NosC, and the tunicate *Ciona robusta* Nos.

For phylogenetic analysis, Nos amino acid sequences were aligned using the MUSCLE algorithm [45] as implemented in MEGAX (v. 10.2.4) [46]. The alignment was trimmed by TRIMAL v. 1.2rev59 [47] and then formatted into a nexus file using READAL (bundled with the TRIMAL package) (electronic supplementary material, File S1). The Bayesian inference tree was constructed using MrBAYES v. 3.2.6 [48], under the assumption of an LG + I + G evolutionary model. Two independent MrBAYES runs of 2 000 000 generations were performed, with four chains each and a temperature parameter value of 0.05. The tree was considered to have reached convergence when the standard deviation stabilized under a value of less than 0.01. A burn-in of 25% of the trees was performed to generate the consensus tree (1 500 000 post-burn-in trees). The maximum-likelihood phylogenetic tree was inferred on the same multi-sequence alignment (electronic supplementary material, file S1) using IQ-TREE v. 2.1.3 [49] with 1000 replicates, using automatic selection of best-fit model with MODELFINDER [50] and branch support assessed with the ultrafast bootstrap approximation [51] (electronic supplementary material, figure S7).

(b) Synteny

With the aim of finding synteny blocks flanking the *nos2* and *nos3* orthologues, we employed the Synteny Database [52,53]. Additional information was retrieved in NCBI, ENSEMBL (v. 102) and GENOMICUS (v. 100.01) [52].

(c) Gene expression analysis by *in situ* hybridization

Whole-mount *in situ* hybridization experiments were performed for all *nos* paralogues following species-specific protocols previously described: spotted gar [54], bichir and sturgeon [55], lamprey [56] and shark [57]. Embryo and tissue collection, and protocol modifications to the *in situ* hybridization are reported in the electronic supplementary material, Extended methods.

Data accessibility. Accession numbers of protein sequences used in the phylogenetic analysis are available in the electronic supplementary material, table S1. Primer sequences used for the synthesis of *in situ* hybridization riboprobes and in qRT-PCR experiments are given in the electronic supplementary material, table S3. Electronic supplementary material is available online [58].

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All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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Supplementary Material

- Extended Methods -

Collection of embryos and tissues

Spotted gar *L. oculatus* adult specimens were collected from the Atchafalaya River basin, Louisiana (USA), and cultured in a 2 m diameter tank containing artificial spawning substrate. Spawning was induced by injection of Ovaprim® (0.5 ml/kg) and embryos were raised in fish water (salinity 1 ppt) at 24°C in a 14/10 h light/dark cycle [1]. The developmental staging was determined following hours or days post fertilization in addition to morphological criteria [2]. Embryos of *L. camtschaticum* were obtained by artificial fertilization, cultured at a temperature ranging between 9 and 12°C, and staged as previously described [3,4]. Embryos of *S. torazame* were obtained, cultured, and staged as previously described [5,6]. Bichir *P. senegalus* embryos were obtained from the breeding colony at the Department of Zoology, Charles University, Prague (Czech Republic) by natural breeding. Embryos were kept at 28°C and staged using Diedhiou and Bartsch guidelines [7]. Sterlet sturgeon *A. ruthenus* embryos were obtained from the hatcheries of the Research Institute of Fish Culture and Hydrobiology in Vodnany, University of South Bohemia (Czech Republic). Embryos were raised in tanks containing E2 Pen/Strep zebrafish medium and incubated at 17°C until the desired stages, according to Dettlaff and collaborators (1993) [8]. Gill tissues from two adult axolotls (RRID:AGSC_110A) were collected under benzocaine anesthesia (University of Kentucky, USA, IACUC protocol 2017-2580). For spotted gar embryos at 7 dpf (Long & Ballard stage 24) and 11 dpf (Long & Ballard stage 28), longer proteinase K (10 µg/mL) digestion times were performed, respectively 25 and 35 minutes at 24°C. Moreover, endogenous melanin pigment was removed using bleaching solution [(3% hydrogen peroxide (H₂O₂) and 1% potassium hydroxide (KOH) in distillate water (ddH₂O)] for a few minutes. For 14 dpf gar embryos (Long & Ballard stage 31), we performed *in situ*

hybridizations on cryosections, as previously described [9], including modifications reported in [10].

Mining of sequences not available in databases

Nos sequences from bowfin (*A. calva*) were obtained from a draft genome assembly [11]. Lamprey *nosA* and *nosB* genes were obtained by TBLASTN v2.2.31+ searches [12] from the v1.0 draft genome of arctic lamprey *L. camtschaticum* [13] and the germ line draft genome of sea lamprey *P. marinus* [14]. Initial predictions were extended, corrected, and confirmed by RACE PCRs in the case of *L. camtschaticum*. Both *P. marinus nosA* and *nosB* were manually curated using Wise2 [15]. The single *nos* gene sequence from the inshore hagfish *E. burgeri* was obtained from a *de novo* transcriptome assembly [16]. Sequences of *nos1*, *nos2*, and *nos3* genes from the cloudy catshark *S. torazame* were obtained from a *de novo* transcriptome assembly [16]. A partial *nos1* sequence (g15096.t1) was found in the European eel *A. anguilla* transcriptome database (EeelBase 2.0) but it was deliberately excluded because of alignment ambiguities.

Selection Analysis

We assessed signals of selection footprints in the *nos* genes by estimating the non-synonymous to synonymous substitution rate ratio ($\omega = dN/dS$). For this, we inferred a phylogenetic tree including the available sequences from vertebrate species clade (as previously described; electronic supplementary material, Table S1 and File S1). Under the assumption of neutral evolution, ω is expected to have a value of 1. Positive and purifying (negative) selection are indicated when $\omega > 1$ and $\omega < 1$, respectively [17]. We used the PAML wrapper EasyCodeML 1.31 [18] using the codon-aware filtered gene alignments to fit substitution models to the data for ω estimation. We used a two-ratio branch model assuming that specific branches have an ω that differs from that throughout the rest of the tree [19–21]. We did 3 distinct analyses and in each analysis we tested if all the branches

of each clade (*nos1*, *nos2*, *nos3*) had a different ω from that throughout the rest of the tree. Pairwise comparisons of these models were performed using likelihood-ratio tests [22]. If the p-value of the LRTs (likelihood-ratio tests) was significant, then we accepted that the value of ω is different in our clade (gene) with respect to the rest of the tree against to the assumption of an equal ω for all the tree (for all the three genes).

Gene expression analysis by *in situ* hybridization

For spotted gar embryos at 7 dpf (Long & Ballard stage 24) and 11 dpf (Long & Ballard stage 28), longer proteinase K (10 $\mu\text{g/mL}$) digestion times were performed, respectively 25 and 35 minutes at 24°C. Moreover, endogenous melanin pigment was removed using bleaching solution [(3% hydrogen peroxide (H_2O_2) and 1% potassium hydroxide (KOH) in distillate water (ddH_2O)] for a few minutes. For 14 dpf gar embryos [2](Long & Ballard stage 31), we performed *in situ* hybridizations on cryosections, as previously described [9], including modifications reported in [10].

Transversal vibratome sections of bichir and sturgeon embryos (thickness 50 μm) were made on whole-mount hybridized embryos upon embedding in gelatin/albumin/glutaraldehyde [23]. Shark embryos were embedded in paraffin after whole-mount *in situ* hybridization assays, and frontal sections (10 μm) were obtained with a microtome.

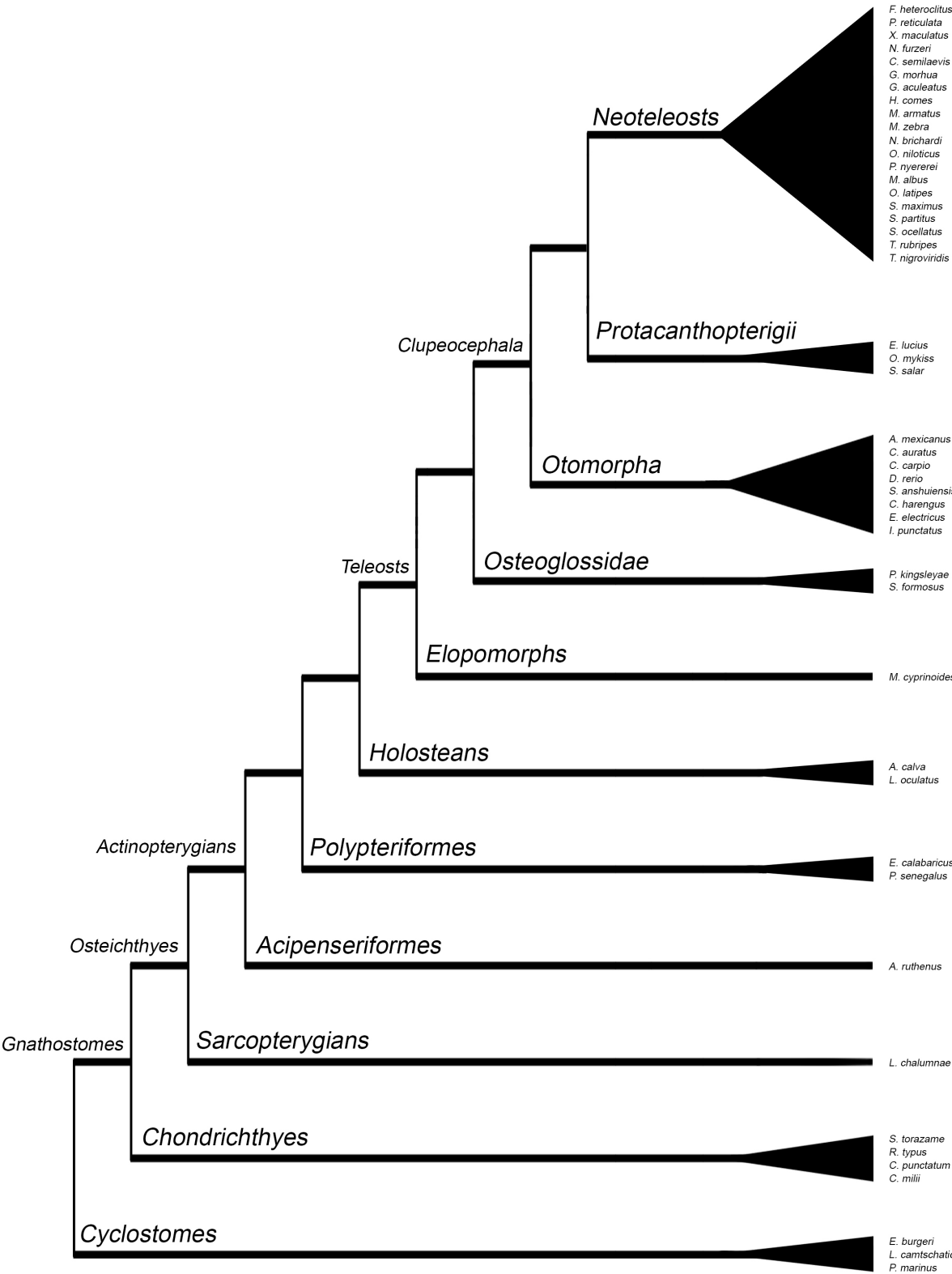
Real-time PCR

Expression levels of *nos* genes in axolotl *A. mexicanum* gills were analysed by RT-qPCR using specific primers reported in electronic supplementary material, Table S3. The *atpf51* gene was used as a reference and data were analysed using the $\Delta\Delta\text{CT}$ method.

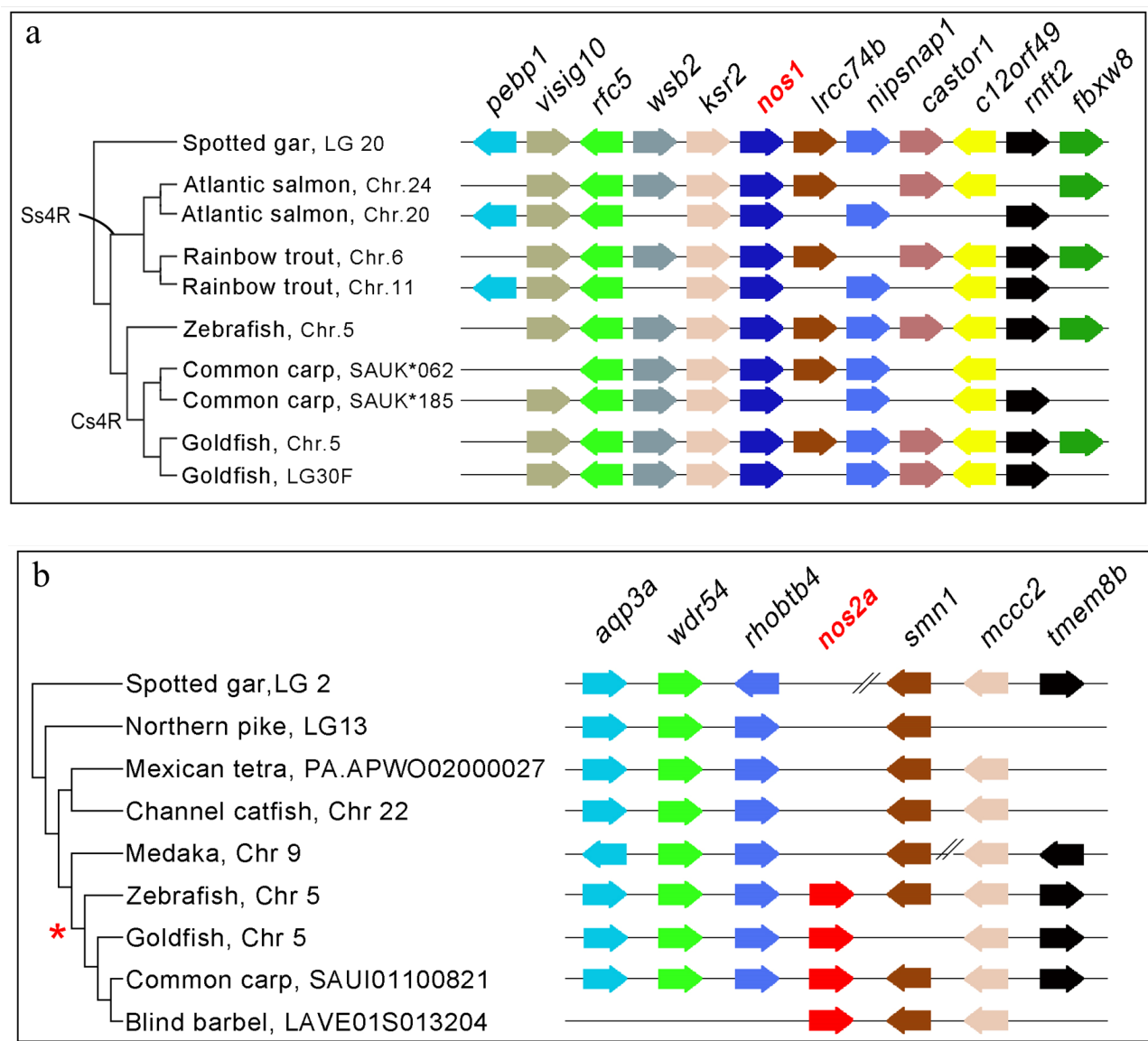
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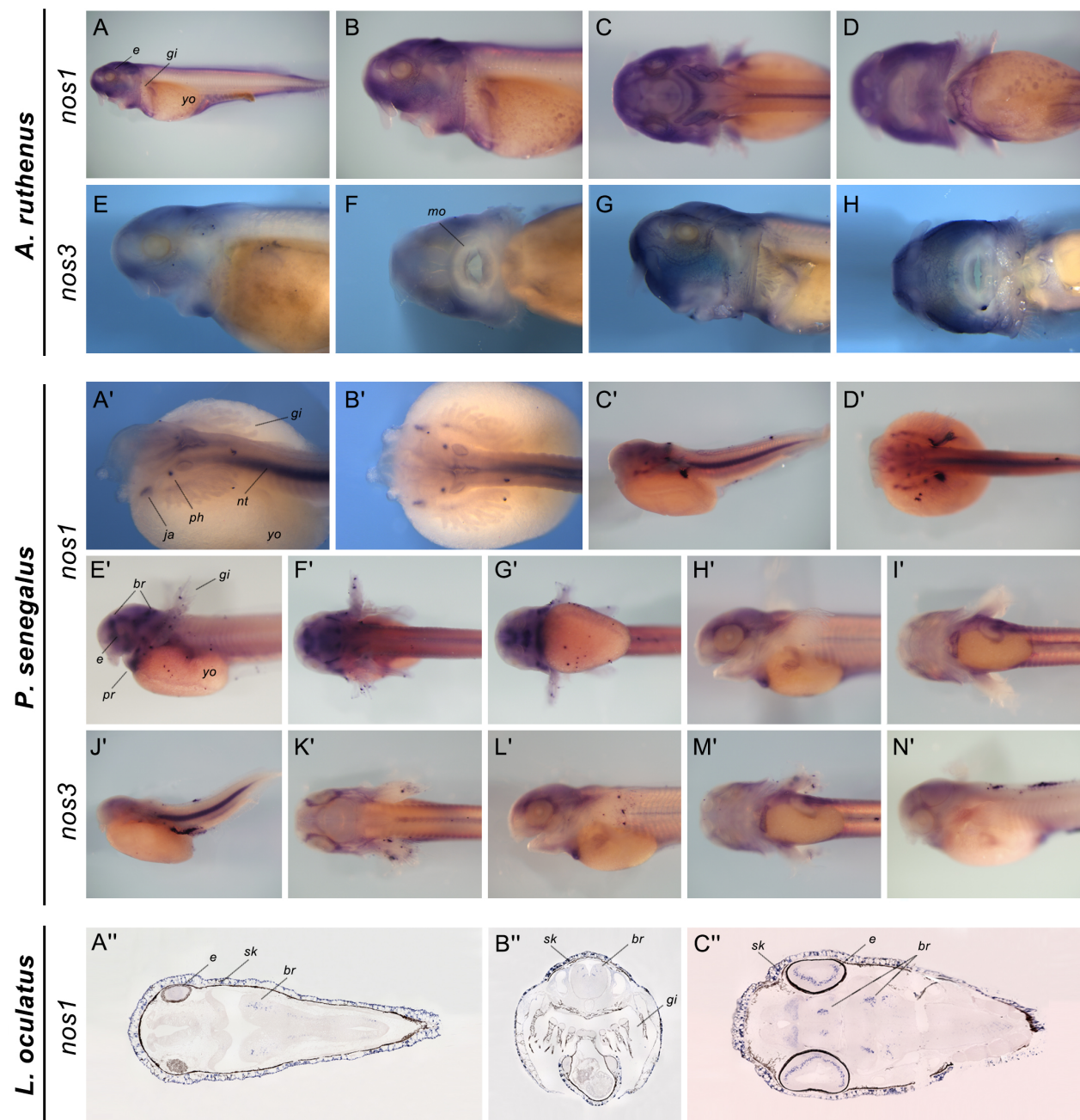
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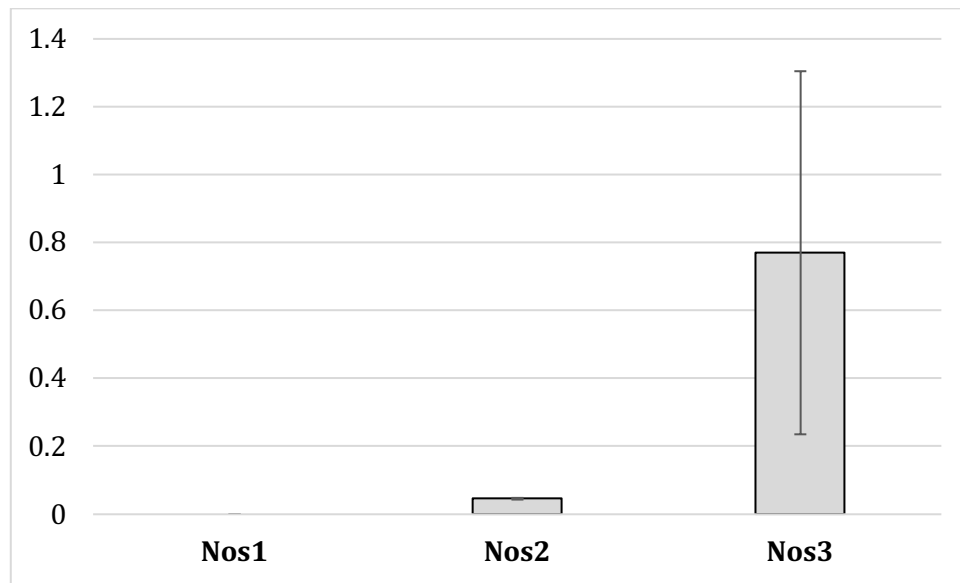
Supplementary Figure 1. Taxonomic classification of fish clades included in the present study.



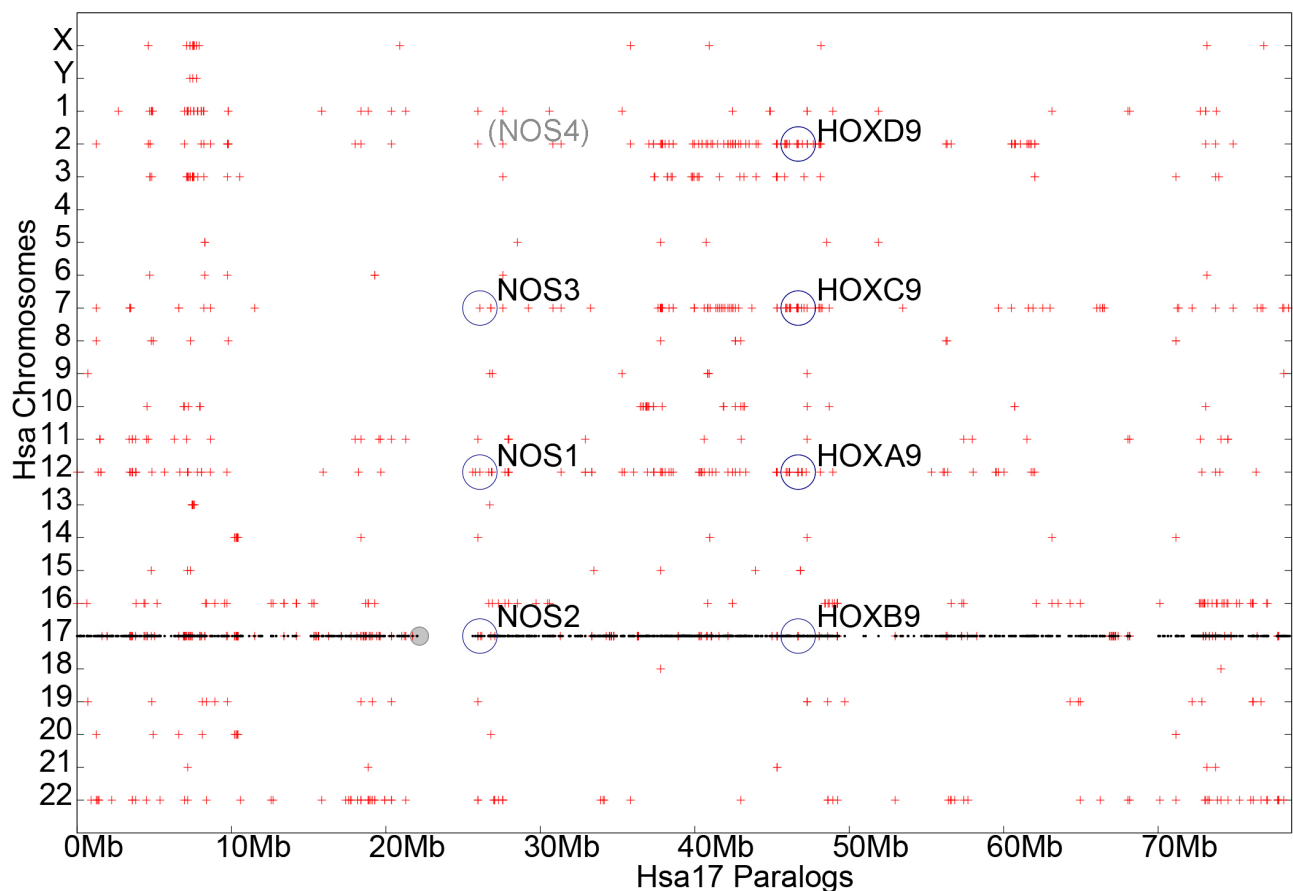
Supplementary Figure 2. Synteny conservation analysis of *nos1* (a) and *nos2a* genes (b). Orthologs from different species are represented with the same colour code. The direction of arrows indicates chromosomal gene orientation. The symbol // indicates long distance on the chromosome. The red asterisk indicates that *nos2a* has been gained exclusively in cyprinids.



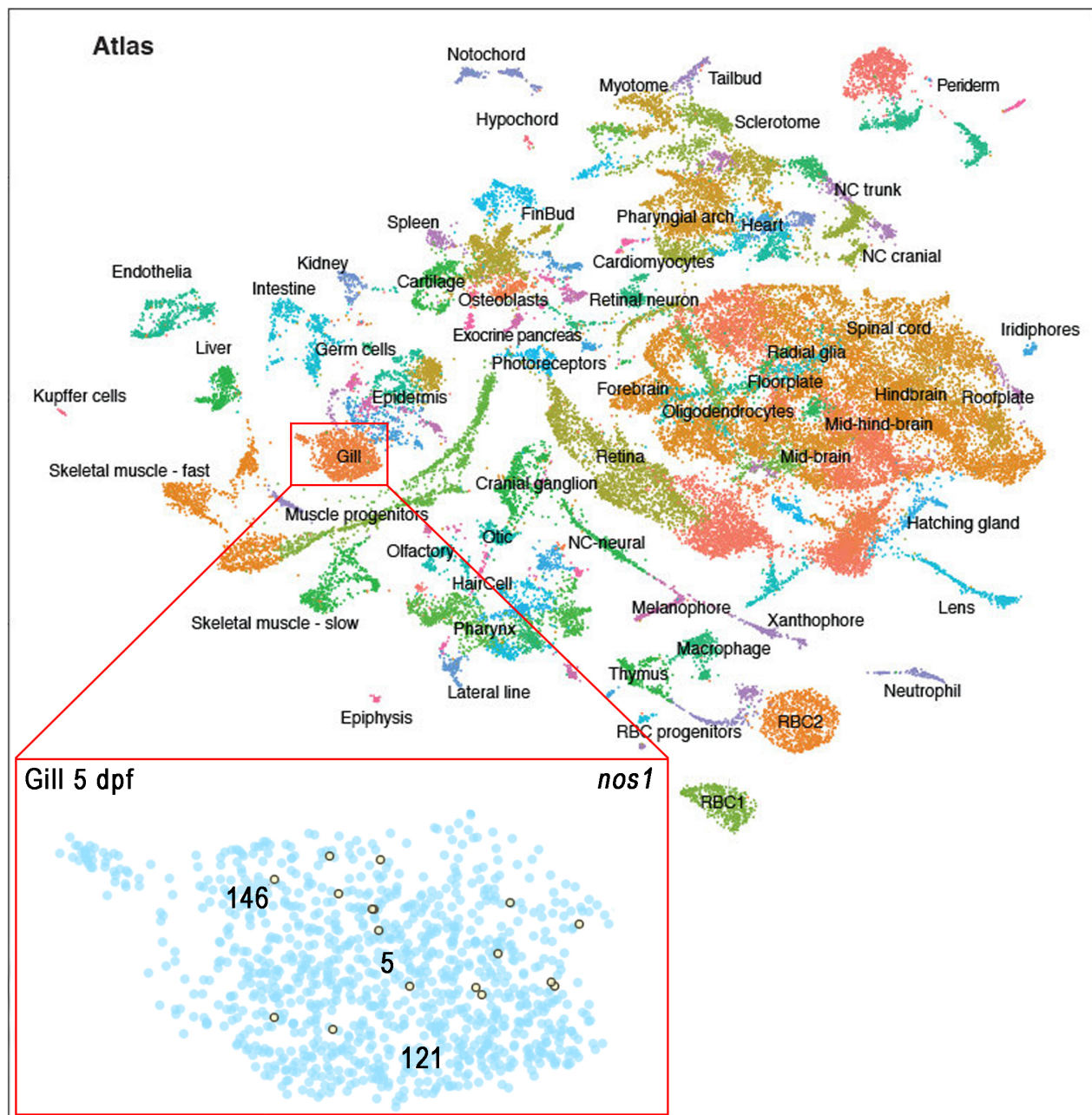
Supplementary Figure 3. Expression patterns of remaining *nos* genes in non-teleost fishes. *Acipenser ruthenus* *nos1* is expressed mainly in the head region, with intense staining in the brain (B-C), eye (C), and skin (A-D); *nos3* is diffusely expressed in the head skin (E-H). *Polypterus senegalus*, both *nos1* and *nos3* are expressed in the notochord at early developmental stages (A'-D', J'), and in the pericardial region (G'-I', L'-M'). Early in the development *nos1* is also expressed in the jaw joint area and pharynx (A'-B'), while later in the brain (E'-G'); *nos3* is expressed in the eye (H'). *Lepisosteus oculatus* (histological sections): *nos1* is expressed in several areas of the developing brain (A''-C''), eye (C''), and skin (A'', B'', C''). No expression was obtained for *nos2* during development. Abbreviations: br, brain; e, eye; gi, gill; ja, jaw joint area; mo, mouth; nt, notochord; ph, pharynx area; pr, pericardial region; sk, skin; yo, yolk.



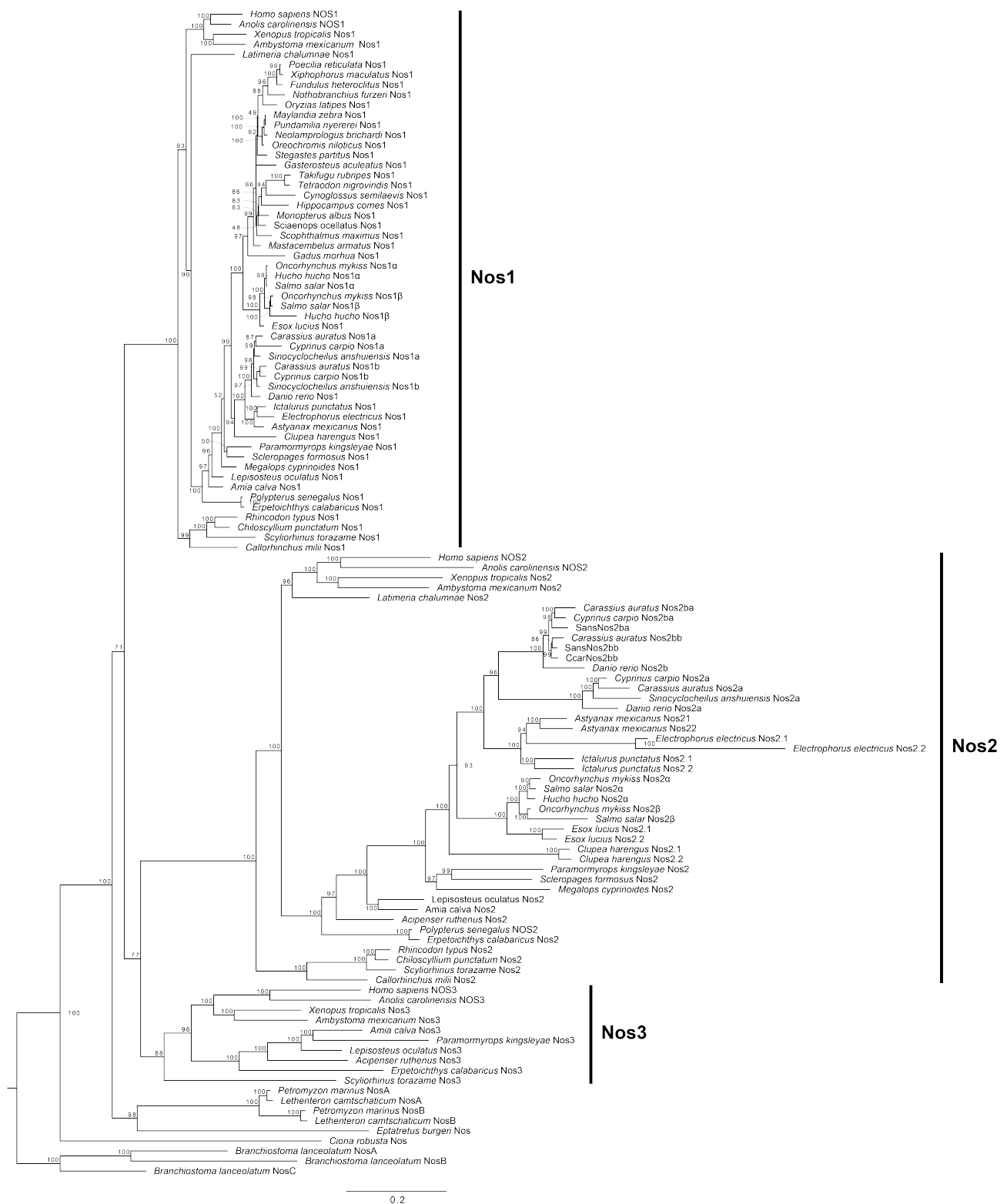
Supplementary Figure 4. Quantitative real-time PCR analysis of *nos* genes expression of axolotl *Ambystoma mexicanum* gills. Target gene expression was normalized to *atpf51* as a reference gene. Relative expression values were calculated using the $\Delta\Delta CT$ method. Error bars show the standard deviation of biological replicates. Expression estimates for the two *nos3* biological replicates were approximately 5x and 28x higher than the highest *nos2* estimate.



Supplementary Figure 5. Dotplot analysis showing syntenic conservation between human *NOS* genes and *HOX* clusters. Paralogs of human genes are plotted throughout the 23 chromosomes on Y-axis, within a 10 Mb-window, and are represented with red crosses. Dotplot shows syntenic conservation between four chromosomes harbouring *NOS* and *HOX* cluster genes. *NOS1* and *HOXA* cluster are on chromosome 12, *NOS2* and *HOXB* cluster are on chromosome 17, *NOS3* and *HOXC* cluster are on chromosome 7. According to the 2R hypothesis, a fourth *NOS* gene lost during evolution (*NOS4*) should have been positioned on chromosome 2 as the *HOXD* cluster. ENSG00000170689 (*HOXB9*) on Hsa17; ENSG00000128709 (*HOXD9*) on Hsa2; ENSG00000180806 (*HOXC9*) on Hsa12; ENSG00000078399 (*HOXA9*) on Hsa7; ENSG00000007171 (*NOS2*) on Hsa17; ENSG00000089250 (*NOS1*) on Hsa12; ENSG00000164867 (*NOS3*) on Hsa7.



Supplementary Figure 6. Cell types clustering from single cell RNA-seq (scRNA-seq) atlas of zebrafish embryos during organogenesis (Miller lab, University of Oregon, Institute of Neuroscience, Eugene, Oregon). Colours correspond to annotated cell clusters. In the square are highlighted the cell clusters 5, 121 and 146 representing gill at 5 dpf and the specific *nos1*-expressing cells are shown in yellow. Data are obtained from the UCSC Cell Browser (<http://zebrafish-dev.cells.ucsc.edu>). Image modified from Farnsworth *et al.* (2020) [24]



Supplementary Figure 7. Maximum-likelihood phylogenetic tree of chordate Nos proteins. Bootstrap support values for 1000 replicates are indicated at the nodes (1000 bootstrap replicates). Topology of the tree was inferred using IQ-TREE and plotted in FigTree.

Supplementary File 1

Alignment in FASTA format of the Nos protein sequences used to generate the phylogenetic trees of Figure 1 and Supplementary Figure 2.