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Cite this article: Annona G *et al*. 2022 Evolution of the nitric oxide synthase family in vertebrates and novel insights in gill development. *Proc. R. Soc. B* **289**: 20220667. https://doi.org/10.1098/rspb.2022.0667

Received: 2 June 2022
Accepted: 19 July 2022

Subject Category:
Evolution

Subject Areas:
developmental biology, evolution

Keywords:
vertebrate evolution, genome duplication, gene duplication and loss, *nos*, phylogenomics, synteny

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.6136963.

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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 coelacanths, 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 1*a* blue shading; electronic supplementary material, figure S2-a). Most fish lineages retained Nos2, including chondrichthyans (*Callorhinchus 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 1*a*, yellow shading), although a *nos2* gene loss event occurred at the stem of Neoteleostei (figure 1*b*), 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*, 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 1*a*, 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 1*a*, red shading). The absence of *nos3* in clupeocephalans indicates a gene loss event at the stem of this group (figure 1*c*). 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 ($\omega1 = 0.035$), Nos2 ($\omega1 = 0.092$) and Nos3 ($\omega1 = 0.082$) (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.

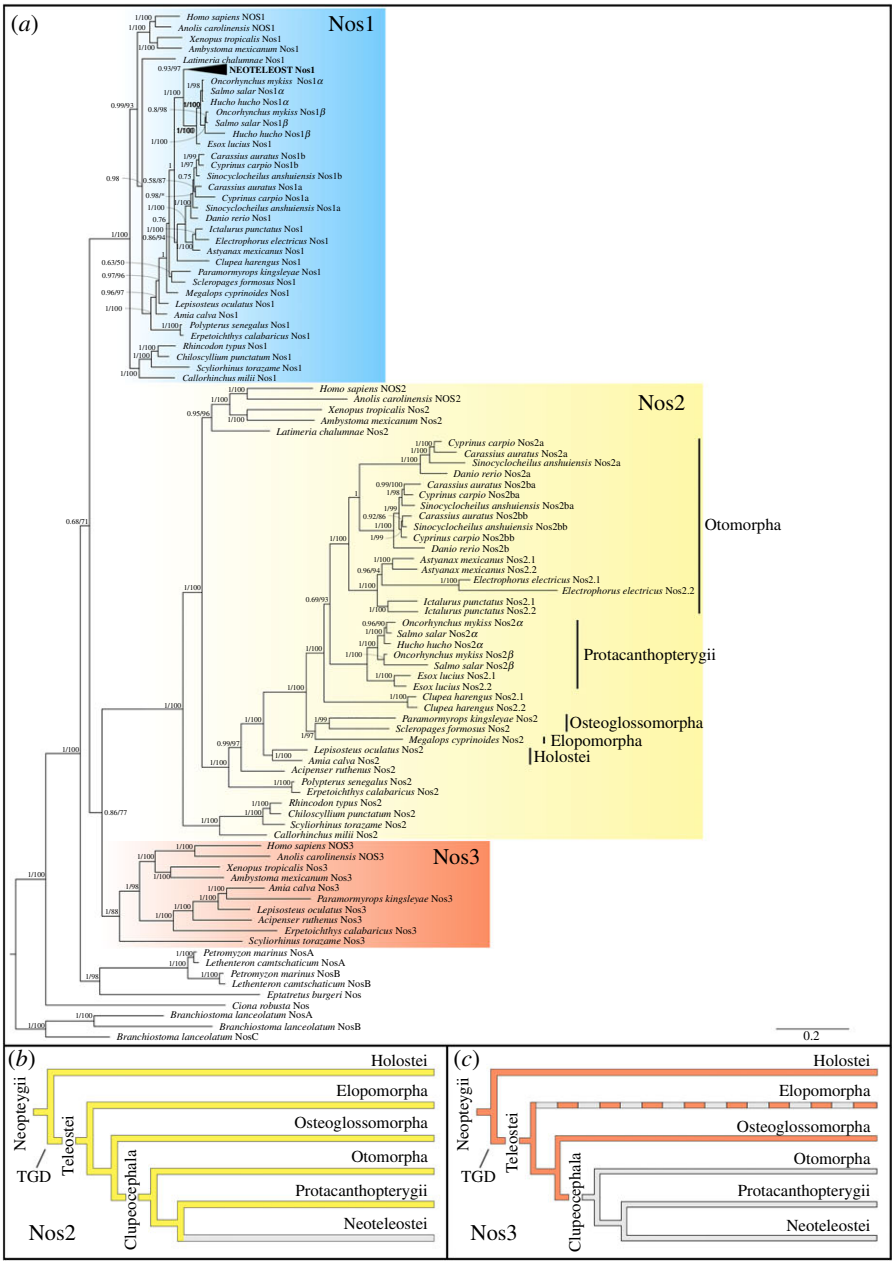

Figure 1. Evolution of the Nos gene family. (*a*) Phylogenetic analysis of Nos proteins in chordates. The tree topology was inferred by Bayesian inference and maximum-likelihood methods, with the exact topology obtained from the former shown here (see the electronic supplementary material, figure S7 for the maximum-likelihood tree). Numbers at nodes represent posterior probability values (left) and maximum-likelihood bootstrap support for 1000 replicates (right). (*b*,*c*), Evolutionary scenarios indicating the loss of Nos2 event in Neoteleostei (*b*) and Nos3 in Clupeocephala (*c*) as grey lines. Nos3 in Elopomorpha is absent, although parsimony suggests it was present in stem elopomorphs, and it is indicated with a dashed line. TGD stands for teleost-specific genome duplication. (Online version in colour.)

To better understand the gene loss and expansion events highlighted by our phylogenetic analysis, we next analysed the microsynteny (genes linked in proximity) of *nos* genes in different species. This revealed a complex evolutionary scenario for *nos2* compared to *nos1* and *nos3*. Specific *nos2* duplications in different lineages are explained by distinct evolutionary events in teleosts. First, the lack of synteny conservation between *nos2a* and *nos2b* in cyprinids, and the lack of *nos2a* in the expected location in non-cyprinid fishes (electronic supplementary material, figure S2-b) indicates that these paralogues originated in a specific gene duplication event in a common ancestor of the lineage, independently from the teleost-specific genome duplication (TGD) (the alternative explanation would require numerous *nos2a* losses in several fish lineages), in which while *nos2b* has remained in the ancestral genomic location, *nos2a* has been translocated to a different position in the genome (figure 2*a*; 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 1*a* and 2*a*). In the case of salmonids, we name

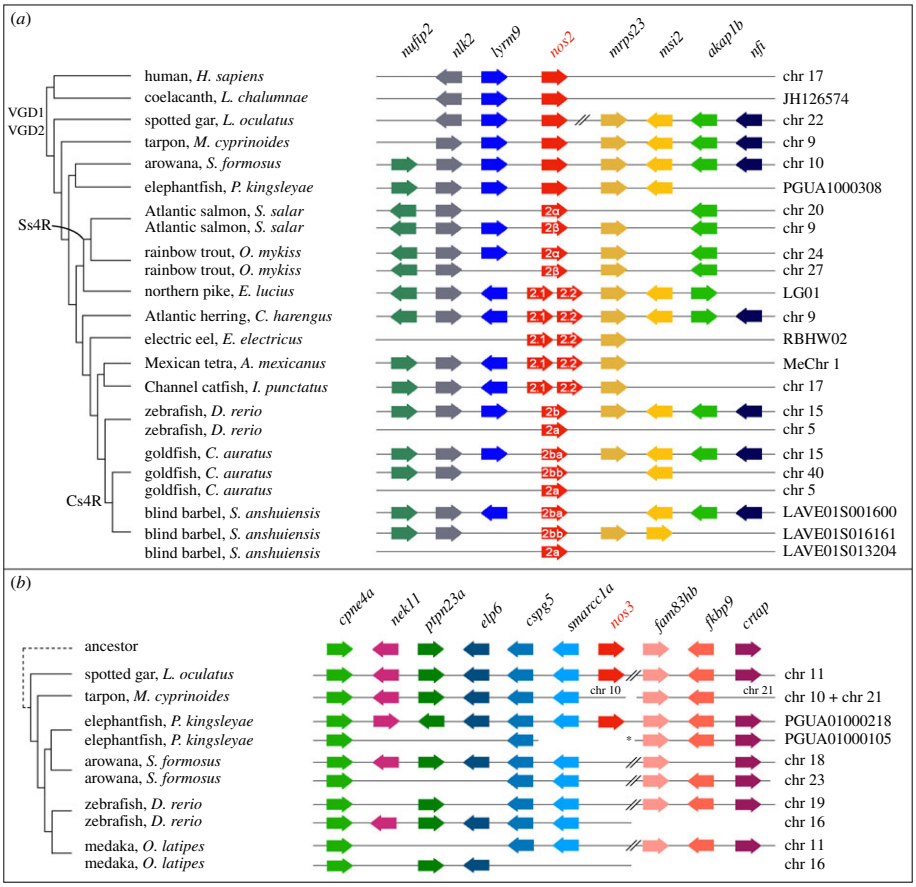

Figure 2. Conserved microsynteny of *nos2* and *nos3*. (*a*) The *nos2* paralogues derived from different duplication modalities: carp-specific genome duplication (Cs4R) (*nos2ba* and *nos2bb* in the goldfish and blind barbel); salmonid-specific genome duplication (Ss4R) (*nos2α* and *nos2β* in the Atlantic 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 2*a*). 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 2*a*).

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 2*b*). While these appear to correspond to one of the TGD ohnologons (figure 2*b*), there are two other *Pa. kingsleyae* scaffold segments (from scaffolds 72 and 104) that together seem to represent the second TGD ohnologon, but lacking the expected *nos3* TGD ohnologue (figure 2*b*). Zebrafish chromosomes 16 and 19 and medaka chromosomes 11 and 16 contain orthologous regions to the two *Pa. kingsleyae* and *Le. oculatus* TGD ohnologons, 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 2*b*). Regardless, the most parsimonious explanation for the *nos3* repertoire in ray-finned fishes is that, one of the two *nos3* TGD ohnologues 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 1*c* and 2*b*).

(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 3*a*,*b*) and increased at 6 dpf (figure 3*c*,*d*). At 7 dpf, embryos showed clear *nos3*

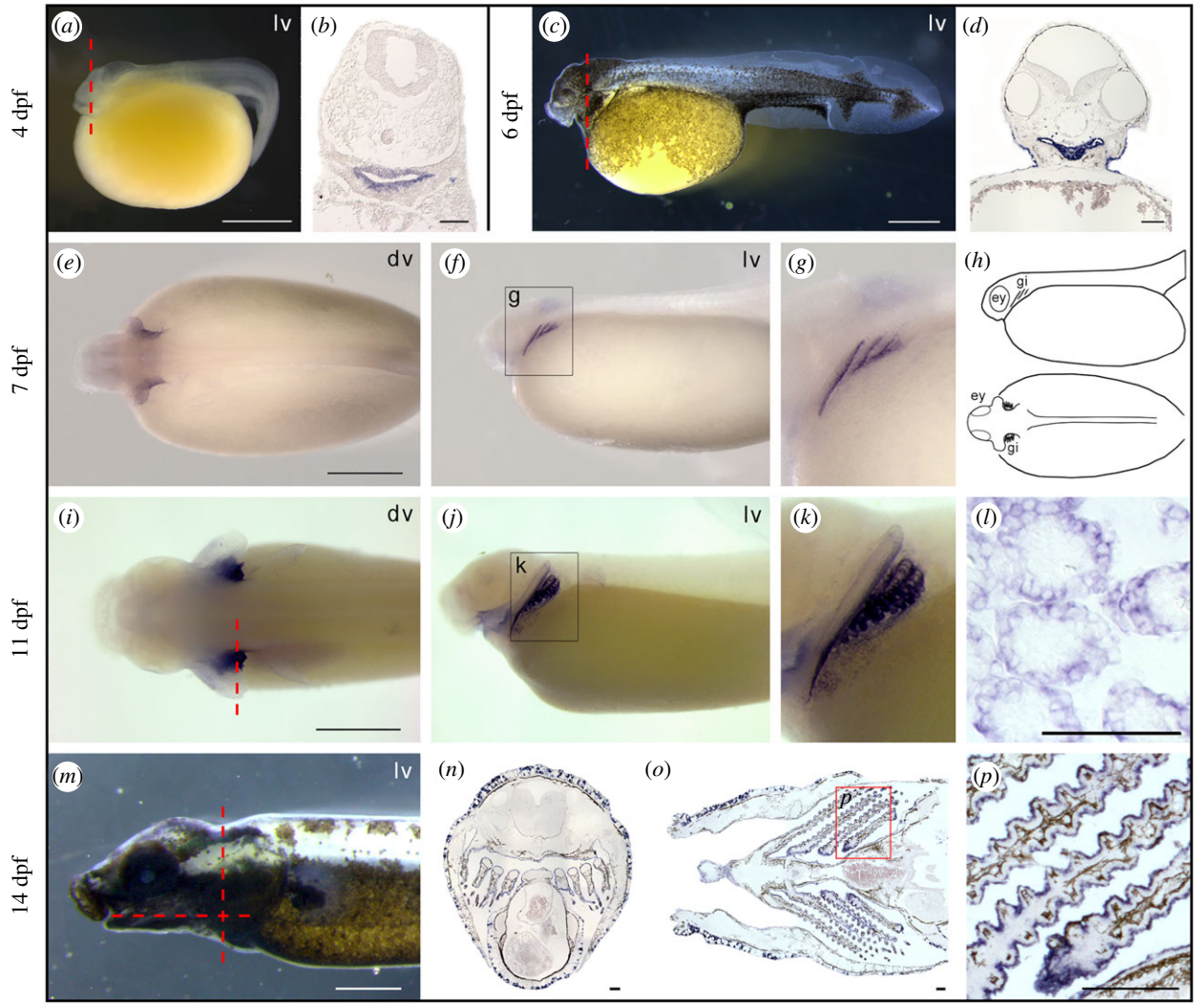

Figure 3. Spotted gar *nos3* localization during development. Expression of *nos3* is localized in the pharyngeal area in 4 dpf (*a*,*b*) and 6 dpf (*c*,*d*) embryos, in pharyngeal arches in 7 dpf larvae (*e*–*g*) schematized in (*h*), in developing gills in 11 dpf late larvae (*i*–*l*), and in gill 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*,*l*,*n*,*o*,*p*). (Online version in colour.)

expression in developing arches III, IV, and V (figure 3*e*–*g*). Later, at 11 dpf, the positive signal is localized in gill filaments (figure 3*i*–*k*). Histological sections highlighted the presence of *nos3* in the epithelium of branchial lamellae (figure 3*l*), also confirmed by the signal in gill structures in an advanced stage of maturation in 14 dpf juveniles (figure 3*m*–*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 4*a*–*c*) and bichir embryos (figure 4*d*–*f*), while *nos1* is expressed in gills of catshark embryos (figure 4*g*–*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

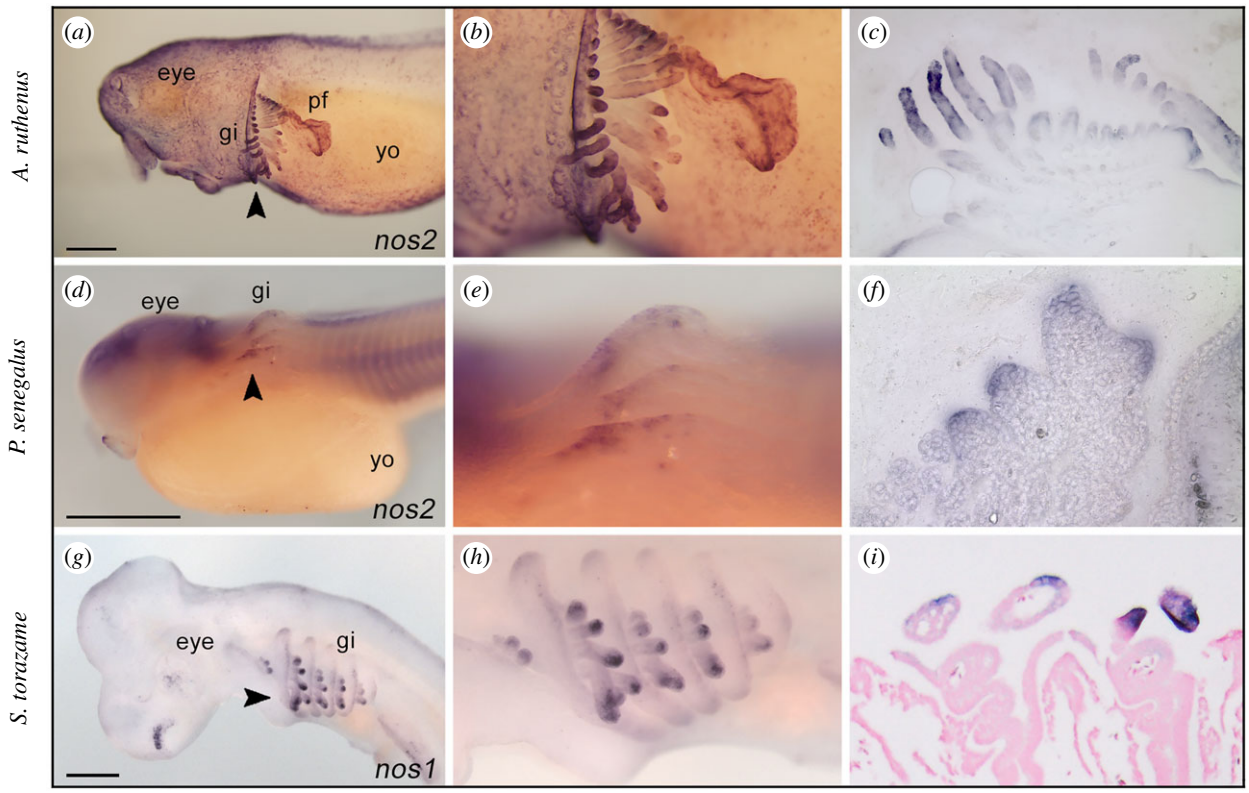

Figure 4. Expression of *nos* genes in developing gills of sturgeon, bichir, and shark embryos. The expression of *nos2* in the gills of sterlet sturgeon *Acipenser ruthenus* (14 mm stage, *a–c*) and bichir *Polypterus senegalus* (stage 31, *d,e*); *nos1* in the shark *Scyliorhinus torazame* (stage 27, *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 µm) and frontal section (*I*, 10 µm). gi, gill; yo, yolk; pf, pectoral fin. Scale bar in (*a,d,g*) is 0.5 mm. (Online version in colour.)

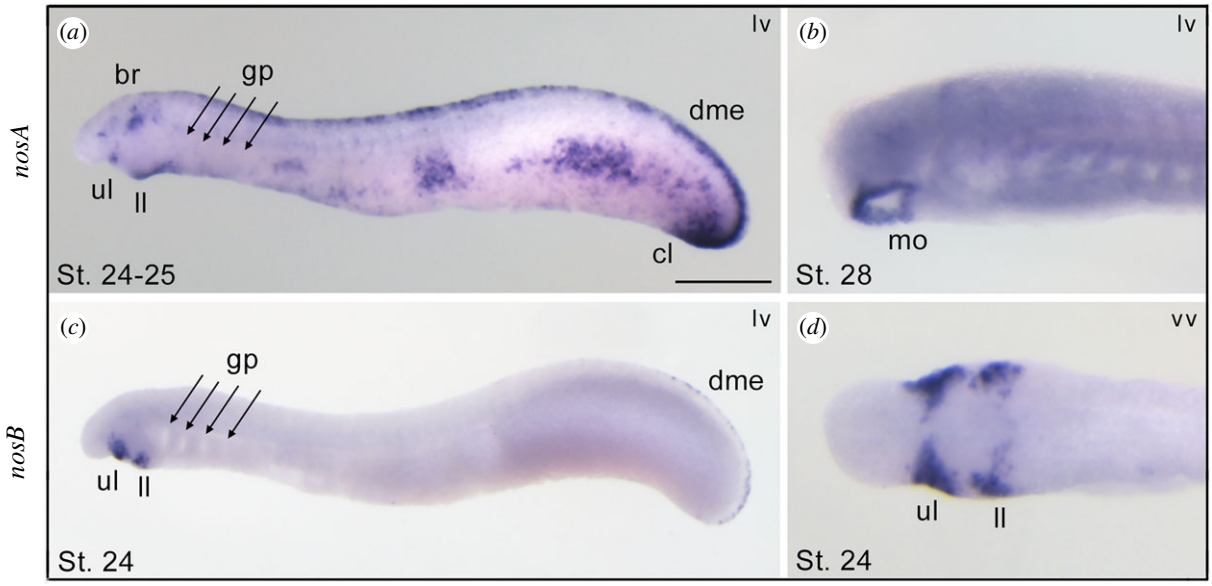

Figure 5. Expression patterns of *nosA* and *nosB* in larvae of the arctic lamprey. At stages 24–25 the *nosA* is expressed in the brain, mouth, upper and lower lip, dorsal midline epidermis, and cloaca (*a*). At stage 28, *nosA* expression is restricted to the mouth (*b*). The *nosB* is exclusively 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

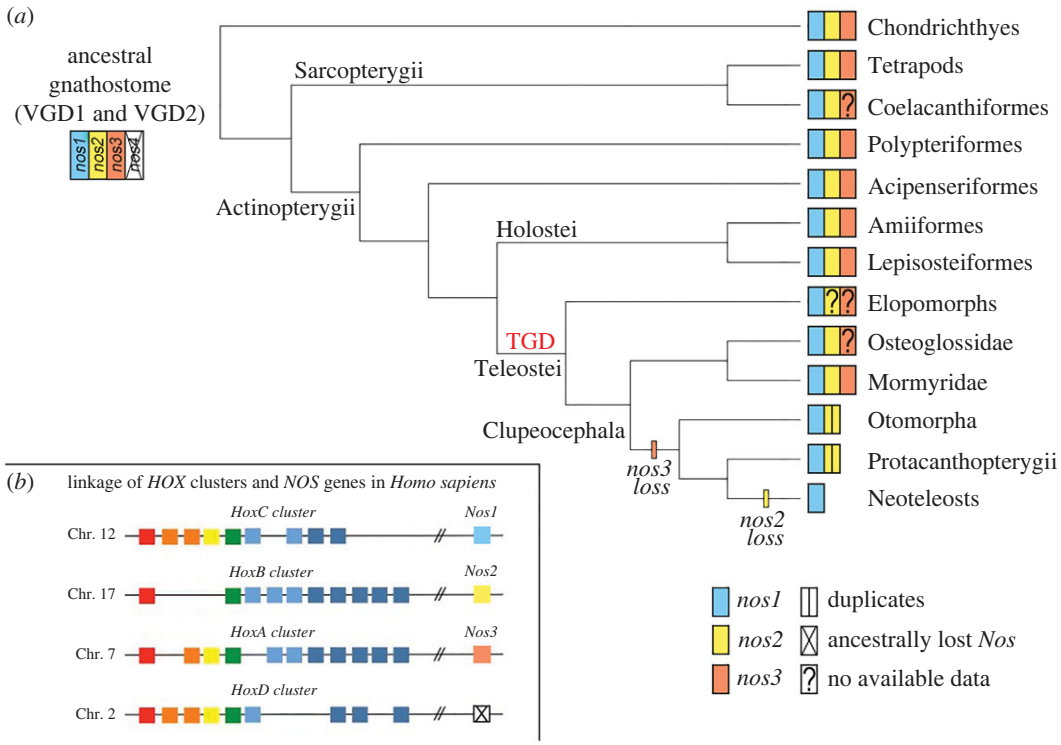

Figure 6. *Nos* evolution in light of recent gene findings in vertebrates. The proposed evolution of *nos* genes in gnathostomes (*a*) supposes an ancestral loss of a predicted fourth *nos* gene, based on the linkage of human *Nos* and *Hox* clusters (*b*). Loss of *nos3* occurred in stem Clupeocephala 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 syntenic 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 1*a* and 2*a*). On the other hand, the cyprinid *nos2b* paralogue independently duplicated in carps after the Cs4R [17], as the conserved synteny suggests (figure 2*a*). In salmonids, synteny analysis also indicates that the two Nos2 paralogues originated secondarily after the Ss4R (figure 2*a*) [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 2*a*). 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 2*b*), 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 6*b*; 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 6*a*).

The apparent lack of *nos*3 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 acidic 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-burnt-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, Ensemble (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].

Authors' contributions. G.A.: conceptualization, data curation, formal analysis, investigation, methodology, writing—original draft, writing—review and editing; I.S.: investigation, methodology; J.P.-A.: conceptualization, data curation, formal analysis, investigation, methodology, validation, writing—original draft, writing—review and editing; D.O.: data curation, formal analysis, writing—review and editing; I.B.: writing—review and editing; R.V.: data curation, investigation, writing—review and editing; J.S.: data curation, investigation, writing—review and editing; V.S.: conceptualization, data curation, investigation, methodology, writing—review and editing; A.F.: data curation, formal analysis, investigation, writing—review and editing; Q.F.: data curation, formal analysis, investigation, writing—review and editing; S.K.: supervision; J.H.P.: conceptualization, data curation, investigation, methodology, supervision, writing—review and editing; S.D.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, supervision, validation, writing—original draft, writing—review and editing.

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

Conflict of interest declaration. The authors declare no competing interests.

Funding. G.A. was supported by the Research grant POR Campania FSE 2014/2020 (IT) and by the EMBO Short Term Fellowship (no. 6936) to visit the Postlethwait laboratory in Oregon (USA) and for the field trip in Louisiana (USA). J.S. is supported by a Marie Skłodowska-Curie grant agreement no. 897949. V.S. is supported by the Charles University Research Centre program no. 204069 and grant no. SVV260571/2020. R.V. and the Ambystoma Genetic Stock Center are supported by US NIH (P40OD019794). J.H.P. and I.B. are supported by the R01 OD011116 grant from the US NIH. I.B. is supported by the US NSF EDGE grant no. 2029216. S.D. is supported by the NOEVO grant from the SZN.

Acknowledgements. We thank Fumiaki Sugahara for the interpretation of arctic lamprey results, Anna Pospisilova for technical assistance with bichir and sturgeon *in situ* hybridizations, and Martin Psenicka, Roman Franek, Michaela Fucikova, Marek Rodina, David Gela, Martin Kahanec for sterlet sturgeon spawns. A special thanks to Robert Cerny for the establishment of the African bichir colony at the Charles University in Prague. The sturgeon work was supported by the Ministry of Education, Youth and Sports of the Czech Republic, project Cenakva (LM2018099) and project Biodiversity (CZ.02.1.01/0.0/0.0/16_025/007370). We also thank Jordi Paps and Giacinto De Vivo for their help on phylogenetic inferences, and to Silvia Perea and Iker Irisarri for their help in the selection analyses.

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