



Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part D

journal homepage: www.elsevier.com/locate/cbpd

Molecular evolution of nitric oxide synthases in metazoans

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ARTICLE INFO

Article history:

Received 2 June 2010

Received in revised form 26 August 2010

Accepted 30 August 2010

Available online xxxxx

Keywords:

Nitric oxide synthases

Molecular evolution

Endothelial NOS

ABSTRACT

Nitric oxide synthases (NOS), the enzymes responsible for the NO synthesis, are present in all eukaryotes. Three isoforms (neuronal, inducible and endothelial), encoded by different loci, have been described in vertebrates, although the endothelial isoform seems to be restricted to tetrapods. In invertebrates, a variety of NOS isoforms have been variably annotated as “inducible” or “neuronal”, while others lack precise annotation. We have performed an exhaustive collection of the available NOS amino-acid sequences in order to perform a phylogenetic analysis. We hypothesized that the NOS isoforms reported in vertebrates derive from 1) different invertebrate NOS, 2) a single invertebrate ancestral gene, through an event related to the double whole genomic duplication that occurred at the origin of vertebrates, and 3) the endothelial form of NOS appeared late in the evolution of vertebrates, after the split of tetrapods and fishes. Our molecular evolution analysis strongly supports the second scenario, the three vertebrate NOS isoforms derived from a single ancestral invertebrate gene. Thus, the diverse NOS isoforms in invertebrates can be explained by events of gene duplication, but their characterization as “inducible” or “neuronal” should only be justified by physiological features, since they are evolutionarily unrelated to the homonym isoforms of vertebrates.

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1. Introduction

Nitric oxide synthases (NOSs, EC 1.14.13.39) are ubiquitous in living organisms, including Bacteria (Chen and Rosazza, 1994). Nitric oxide (NO) in mammalian tissues is produced from L-arginine *via* catalysis by at least three different NO synthase (NOS) isoforms, namely neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Alderton et al., 2001; Ghosh and Salerno, 2003). The first two are constitutively expressed mainly in the central and peripheral nervous system and vascular endothelial cells, respectively (Toda and Ayajiki, 2006). iNOS is not constitutive but it is induced by cytokines and polysaccharides in macrophages and other cell types. Evidence has also been accumulated for the role of NO in non-mammalian vertebrates although in some of them, like fishes, the endothelium mainly produces vasodilating prostanoids instead of NO (Toda and Ayajiki, 2006). A phylogenetic appearance of an endothelium-derived, NO-mediated mechanism for vasodilation might be associated with

the air-breathing. According to this idea, the appearance of an endothelial isoform of NOS would be associated to the water–air transition (Olson and Villa, 1991; Toda and Ayajiki, 2006). Regarding NOS from invertebrates, the production and utilization of NO is not well understood, although similar physiological functions have been described for this molecule (Korneev et al., 2005; Trimmer et al., 2001; Elphick et al., 1995, 1996; Kuzin et al., 1996). From a genomic and functional point of view, several NOS isoforms have been described in invertebrates (Palumbo, 2005; Cholazati et al., 2010) including amphioxus, where its constitutive expression has been reported histochemically in several organs (Zhang et al., 2002). However, their evolutionary relationships with the vertebrate isoforms remain unknown.

NOS enzymes usually exist as homodimers, containing independent oxygenase and reductase domains that are connected to each other by a central calmodulin (CaM) binding sequence (Pfeiffer et al., 1999; Roman et al., 2002). Besides the CaM domain, there exist other cofactor-binding sites: flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), NADPH and postsynaptic density 95 (PDZ) domains. These sites are evolutionarily conserved. Nevertheless, the number of them varies according to the isoform and the organism, and even, some of these domains can be absent. For example, the PDZ domain is only present in the N-terminus of the neuronal isoform of vertebrates (Luckhart and Rosenberg, 1999; Stasiv et al., 2001; Scheinker et al., 2005; Matsuo et al., 2008) and also in NOS from some invertebrates, such as *Limax valentianus* and *Nematostella vectensis* (Putnam et al., 2007; Matsuo et al., 2008).

Abbreviations: nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; 2R, Model of two rounds of whole genomic duplication in vertebrates; CaM, calmodulin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; NADPH, nicotinamide-adenine dinucleotide phosphate; PDZ, postsynaptic density 95 domain.

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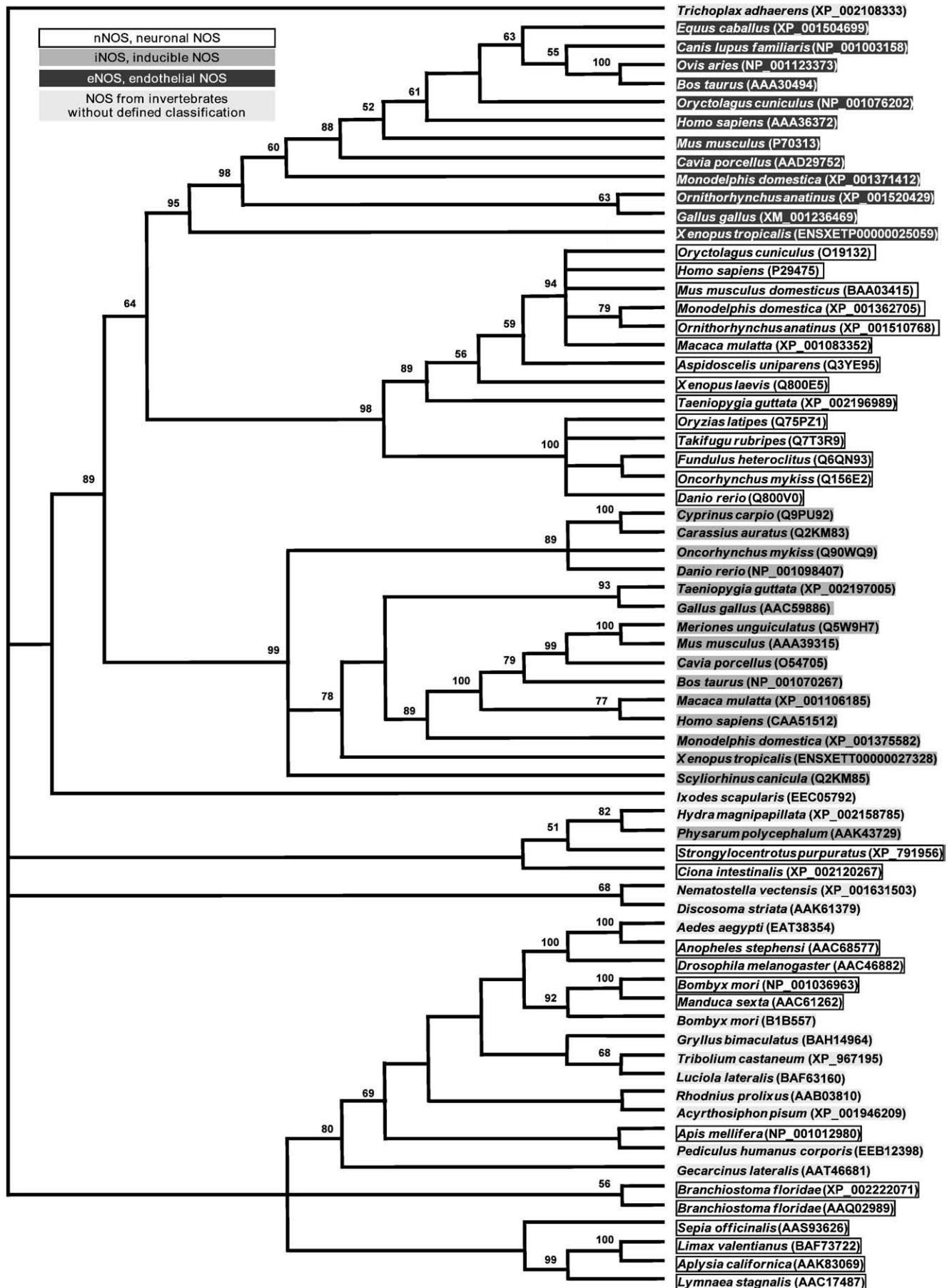
Table 1
Amino-acid sequences retrieved from Genbank and EMBL (release of July of 2009). Sources, references and accession numbers are specified. **nNOS**, neuronal NOS; **iNOS**, inducible NOS; **eNOS**, endothelial NOS; , NOS from invertebrates, functionally and structurally different from known NOS, so they have not classification defined; ps, partial sequence; v, vertebrate; i, invertebrate.

Species	Common name	Phylum	Protein	Source	Reference	Accession number
<i>Homo sapiens</i>	Human (v)	Chordata	nNOS	Brain	Nakane et al., 1993	P29475
<i>Takifugu rubripes</i>	Japanese pufferfish (v)	Chordata	nNOS	Undetermined	Wilson et al.(direct submission)	Q773R9
<i>Oryzias latipes</i>	Japanese ricefish (v)	Chordata	nNOS	Undetermined	Yamamoto T and Suzuki N.(direct submission)	Q75PZ1
<i>Oryctolagus cuniculus</i>	Rabbit (v)	Chordata	nNOS	Brain	Jeong and Yim(direct submission)	O19132
<i>Aspidoscelis uniparens</i> (also <i>Cnemidophorus uniparens</i>)	Desert grassland whiptail lizard (v)	Chordata	nNOS	Brain	Sanderson et al., 2008	Q3YE95
<i>Oncorhynchus mykiss</i>	Rainbow trout (v)	Chordata	nNOS	Undetermined	McNeill and Perry, 2006	Q156E2
<i>Xenopus laevis</i>	Claw-footed toad (v)	Chordata	nNOS	Brain	Scheinker et al. (direct submission)	Q800E5
<i>Fundulus heteroclitus</i>	Mummichog (v)	Chordata	nNOS	Undetermined	Havird et al. (direct submission)	Q6QN93
<i>Danio rerio</i>	Zebrafish (v)	Chordata	nNOS	Embryonic nervous system	Poon et al., 2003	Q800V0
<i>Mus musculus domesticus</i>	House mouse (v)	Chordata	nNOS	Brain	Ogura et al., 1993	BAA03415
<i>Drosophila melanogaster</i>	Fruit fly (i)	Arthropoda	nNOS	Whole organism (including brain)	Regulski and Tully, 1995	AAAC4682
<i>Manduca sexta</i>	Tobacco hornworm (i)	Arthropoda	nNOS	Olfactory system	Nighorn et al., 1998	AAAC61262
<i>Apis mellifera</i>	Honey bee (i)	Arthropoda	nNOS	Antenna and the antennal lobes	The Honeybee Genome Sequencing Consortium, 2006	NP_001012980
<i>Anopheles stephensi</i>	Asian malaria mosquito (i)	Arthropoda	nNOS	Midgut and carcass	Luckhart and Rosenberg, 1999	AAAC68577
<i>Lymnaea stagnalis</i>	Pond snail (i)	Mollusca	nNOS(1a)	Neuronal cells	Korneev et al., 1998	AAAC17487
<i>Bombyx mori</i>	Silkworm (i)	Arthropoda	nNOS	Various organs and functions (including immune defense system)	Imamura et al., 2002	NP_001036963
<i>Aplysia californica</i>	California sea hare (i)	Mollusca	nNOS(1a)	Neurons	Sadreyev et al., unpublished	AAK83069
<i>Ciona intestinalis</i>	Sea squirt (i)	Chordata	nNOS	Undetermined	Undetermined	XP_002120267
<i>Ornithorhynchus anatinus</i>	Platypus (v)	Chordata	nNOS(1b)	Liver, lung, brain, spleen	Undetermined	XP_001510768
<i>Lehmanna valentiana</i> (<i>Limax valentianus</i>)	Terrestrial slug (i)	Mollusca	nNOS	Brain	Matsuo et al., 2008	BAF73722
<i>Sepia officinalis</i>	Common cuttlefish (i)	Mollusca	nNOS(1a)	Optic lobes and ink glands	Scheinker et al. 2005	AAAS93626
<i>Monodelphis domestica</i>	Gray short-tailed opossum (v)	Chordata	nNOS <i>in silico</i>	Undetermined	Undetermined	XP_001362705
<i>Taeniopygia guttata</i>	Zebra finch (bird) (v)	Chordata	nNOS <i>in silico</i>	Undetermined	Undetermined	XP_002196989
<i>Macaca mulatta</i>	Rhesus monkey (v)	Chordata	nNOS <i>in silico</i>	Undetermined	Undetermined	XP_001083352
<i>Strongylocentrotus purpuratus</i>	Sea urchin (i)	Echinodermata	nNOS <i>in silico</i>	Undetermined	Undetermined	XP_791956
<i>Branchiostoma floridae</i>	Florida lancelet or amphioxus (i)	Chordata	nNOS <i>in silico</i>	Testes	Putnam et al., 2008	XP_002222071
<i>Branchiostoma floridae</i>	Florida lancelet or amphioxus (i)	Chordata	NOS <i>in silico</i>	Testes	Panchin et al., unpublished	AAQ02989
<i>Xenopus tropicalis</i>	Western clawed frog (v)	Chordata	nNOS <i>in silico</i>	Undetermined	Hubbard et al., 2002	ENSXETT0000027328
<i>Taeniopygia guttata</i>	Zebra finch (bird) (v)	Chordata	NOS(2a) <i>in silico</i>	Undetermined	Undetermined	XP_002197005
<i>Gallus gallus</i>	Chicken (v)	Chordata	NOS(2a)	Macrophage	Lin et al., 1996	AAAC59886
<i>Meriones unguiculatus</i>	Mongolian gerbil (v)	Chordata	NOS	Glandular Stomach	Matsubara et al., 2004	Q5W9H7
<i>Cyprinus carpio</i>	Common carp (v)	Chordata	iNOS	Phagocyte	Saeji et al., 2000	Q9PU92
<i>Mus musculus</i>	House mouse (v)	Chordata	iNOS	Macrophage	Xie et al., 1992	AAA39315
<i>Homo sapiens</i>	Human (v)	Chordata	iNOS	Chondrocyte	Charles et al., 1993	CAA51512
<i>Danio rerio</i>	Zebrafish (v)	Chordata	NOS(2a)	Macrophage	Poon et al., 2008	NP_001098407
<i>Carassius auratus</i>	Goldfish (v)	Chordata	NOS(2b)	Macrophage	Laing et al., 1996	Q2KM83
<i>Cavia porcellus</i>	Domestic guinea pig	Chordata	iNOS	Lung	Shirato et al., 1998	O54705
<i>Scyliorhinus canicula</i>	Small-spotted catshark (v)	Chordata	iNOS	Spleen	Reddick et al. (direct submission)	Q2KM85
<i>Oncorhynchus mykiss</i>	Rainbow trout (v)	Chordata	iNOS	Macrophage from liver	Laing et al., 1996	Q90WQ9
<i>Bos taurus</i>	Bovine (v) cattle	Chordata	NOS(2a)	Hepatocyte	Widdison et al., 2007	NP_001070267
<i>Macaca mulatta</i>	Rhesus monkey (v)	Chordata	NOS(2a) <i>in silico</i> (isoform3)	Hepatocyte	Undetermined	XP_001106185
<i>Physarum polycephalum</i>	Plasmodial slime molds	Amoebozoa	NOS(2a)	Undetermined	Golderer et al., 2001	AAK43729
<i>Monodelphis domestica</i>	Gray short-tailed opossum (v)	Chordata	NOS <i>in silico</i>	Undetermined	Undetermined	XP_001375582
<i>Homo sapiens</i>	Human (v)	Chordata	eNOS	Endothelial cells	Marsden et al., 1992	AAA36372
<i>Bos taurus</i>	Bovine (v) cattle	Chordata	eNOS	Aortic endothelial cells	Lamas et al., 1992	AAA30494
<i>Ovis aries</i>	Caprine (v) sheep	Chordata	eNOS	Endothelial cell	Cale et al., 2005	NP_001123373
<i>Equus caballus</i>	Horse (v)	Chordata	eNOS <i>in silico</i> (3a)	Undetermined	Undetermined	XP_001504699
<i>Mus musculus</i>	House mouse (v)	Chordata	eNOS	Endothelial cell from fetal heart and liver	Gnanapandithen et al., 1996	P70313
<i>Oryctolagus cuniculus</i>	Rabbit (v)	Chordata	eNOS <i>in silico</i>	Myocardial cell	Wan et al., 2009	NP_001076202
<i>Cavia porcellus</i>	Domestic guinea pig (v)	Chordata	eNOS <i>in silico</i>	Undetermined	Derst et al., unpublished	AAQ29752
<i>Canis lupus familiaris</i>	Dog (v)	Chordata	eNOS	Endothelial cells	Schwemmer and Bassenge, 1999	NP_001003158
<i>Ornithorhynchus anatinus</i>	Platypus (v)	Chordata	eNOS	Liver, lung, brain, spleen	Undetermined	XP_001520429
<i>Xenopus tropicalis</i>	Western clawed frog, Amphibia (v)	Chordata	eNOS <i>in silico</i>	Undetermined	Hubbard et al., 2002	ENSXETG00000011979
<i>Gallus gallus</i>	Chicken (v)	Chordata	eNOS <i>in silico</i> (ps)	Undetermined	Undetermined	XM_001236469
<i>Monodelphis domestica</i>	Gray short-tailed opossum (v)	Chordata	eNOS <i>in silico</i>	Undetermined	Undetermined	XP_001371412
<i>Pediculus humanus corporis</i>	Human body louse (i)	Arthropoda	NOS <i>in silico</i>	Salivary gland	Kirkness et al., unpublished	EEB12398
<i>Ixodes scapularis</i>	Black-legged tick (i)	Arthropoda	NOS <i>in silico</i>	Embryonated eggs	Caler et al., unpublished	EEC05792
<i>Gryllus bimaculatus</i>	Two-spotted cricket (i)	Arthropoda	NOS	Brain	Takahashi et al., 2009	BAH14964
<i>Luciola lateralis</i>	Japanese firefly (i)	Arthropoda	NOS	Whole body	Ohtsuki et al., 2008	BAF63160
<i>Rhodnius prolixus</i>	Blood sucking bug	Arthropoda	NOS	Salivary gland	Yuda et al., 1996	AAAB03810
<i>Bombyx mori</i>	Silkworm (i)	Arthropoda	NOS	Wing disc	Sato et al., 2008	B1B557
<i>Geocarcinus lateralis</i>	Blackback land crab (i)	Arthropoda	NOS <i>in silico</i>	Nerve ganglia, Y-organ, gill, gonad	Kim et al., 2004	AAT46681
<i>Nematostella vectensis</i>	Starlet sea anemone (i)	Cnidaria	NOS <i>in silico</i>	Undetermined	Putnam et al., 2007	XP_001631503
<i>Discosoma striata</i>	Striped mushroom (i)	Cnidaria	NOS <i>in silico</i>	Undetermined	Panchin et al., unpublished	AAK61379
<i>Acyrtosiphon pisum</i>	Pea aphid (i)	Arthropoda	NOS <i>in silico</i>	Undetermined	Undetermined	XP_001946209
<i>Trichoplax adhaerens</i>	Not assigned (i)	Placozoa	NOS <i>in silico</i> (ps)	Undetermined	Srivastava et al., 2008	XP_002108333
<i>Aedes aegypti</i>	Yellow fever mosquito (i)	Arthropoda	NOS <i>in silico</i>	Undetermined	Nene et al., 2007	EAT38354
<i>Hydra magnipapillata</i>	Hydra (i)	Cnidaria	NOS <i>in silico</i>	Undetermined	Undetermined	XP_002158785
<i>Tribolium castaneum</i>	Red flour beetle (i)	Arthropoda	NOS <i>in silico</i>	Undetermined	Undetermined	XP_967195

We have performed an exhaustive collection of the available NOS amino-acid sequences in the gene databases, and we have selected a representative group of them in order to perform an evolutionary analysis. We hypothesized about three alternative evolutionary scenarios for eumetazoan NOS: 1) The three isoforms reported in

vertebrates derive from different NOS of invertebrates, 2) The three NOS isoforms of vertebrates derive from a double duplication of an ancestral gene related to the 2R event (Ohno, 1970; Hall et al., 1994; Graur and Li, 2000), or 3) The endothelial form of NOS appeared late in the evolution of vertebrates, after the split of tetrapods and fishes, a

Fig. 1. Strict consensus tree based on amino-acid sequence of nitric oxide synthase catalytic domain from selected species in this study. The tree was obtained from 153 trees computed by the maximum-parsimony criterion, with PROTPARS weighting. GenBank/EMBL/DBJ accession numbers are given in parenthesis. Bootstrap values greater than 50% are shown. There is a clear separation of vertebrate NOS sequences from the invertebrate ones.



hypothesis supported by the lack of evidence of an eNOS ortholog in fishes. The aim of our study has been to elucidate which of these hypotheses fits better with the origin of the vertebrate NOS, and to clarify the evolutionary relationships between vertebrate and invertebrate NOS isoforms performing similar functions.

2. Material and methods

2.1. Computational search and collection of sequences

We carried out the sequence search at two levels. First, we performed the simplest search retrieving all the available protein sequences stored as NOS in GenBank and UniProtKB/TrEMBL databases, belonging to both, invertebrates and vertebrates. We sorted the sequences by groups (nNOS, iNOS and eNOS) based on their annotations. For each subgroup, we selected conserved regions using the program MEME (http://meme.sdsc.edu/meme4_1/cgi-bin/meme.cgi) (Bailey and Elkan, 1994). We entered the unaligned sequences, keeping all the default values except that for “maximum number of motifs to find”, which we set at 10. The motifs obtained were submitted to MAST (http://meme.ncbr.net/meme4_1/cgi-bin/mast.cgi) (Bailey and Gribskov, 1998) for searching further NOS in the databases. The searches were repeated until no novel sequences were detected at the *e*-value threshold established.

2.2. Identification and annotation of functional domains

For this purpose, we used the interface SMART (http://smart.embl-heidelberg.de/help/smart_about.shtml). SMART (Simple Modular Architecture Research Tool) allows the identification and annotation of genetically mobile domains and the analysis of domain architectures. These domains are extensively annotated with respect to phyletic distributions, functional class, tertiary structures and functionally important residues (Schultz et al., 1998; Letunic et al., 2002).

2.3. Sequence alignment and preliminary phylogenetic reconstruction

The alignments of all amino-acid sequences retrieved with an informative length and functional domains were carried out using the CLUSTALX program (Thompson et al., 1997) and then refined both manually and automatically by using the Gblocks software (Castresana, 2000), which eliminated the poorly aligned positions and divergent regions of the alignments. The Gblocks program was run for the alignments of each individual gene with stringent parameters (setting at default values except for “allowed gap positions” that was set at “with half”), in order to preserve only the common informative sites.

Preliminary phylogenetic analysis was carried out under the maximum-parsimony (MP) criterion using the PAUP (Phylogenetic Analysis Using Parsimony) software version 4.0b10 (David L. Swofford, Laboratory of Molecular Systematics, Museum Support Center, Smithsonian Institution, Washington, DC 20560-0534, USA). We employed the command *contree* to compute a strict consensus tree when needed. The trees were always rooted with *Trichoplax adhaerens*.

2.4. Selection of organisms and phylogenetic analysis of NOS

Starting with the information available, length of sequences and functional domains exhibited, we chose a cluster of sequences that were also representative of the different clades obtained in the

preliminary screening. This group of sequences was used in the phylogenetic analysis.

A new alignment of the selected sequences was performed with the CLUSTALX program (Thompson et al., 1997). We aligned separately the whole sequence of the NOS protein and the amino-acid sequence of the conserved NO synthase catalytic domain. Then, we automatically refined the alignments with the above mentioned Gblocks software (Castresana, 2000) but using relaxed conditions in order to preserve as much information as possible: “the minimum number of sequences for a conserved position” and “the minimum number of sequences for a flank position” were both set at a half the number of sequences, “the maximum number of contiguous non-conserved positions” was set at 10, “the minimum length of a block” was set at 5 and “the allowed gap positions” was set at “with half”. Finally, the phylogenetic trees were reconstructed by neighbor-joining (NJ) and maximum-parsimony (MP) methods using the already mentioned PAUP. Confidence levels for the phylogenetic trees were assessed using bootstrap with 100 replicates. Additionally, we also weighted the amino-acid substitutions using the PROTPARS matrix (Felsenstein, 1993) that gives the minimum number of codon changes needed to account for the amino-acid substitutions.

3. Results and discussion

3.1. Retrieval of NOS sequences and preliminary phylogenetic analysis

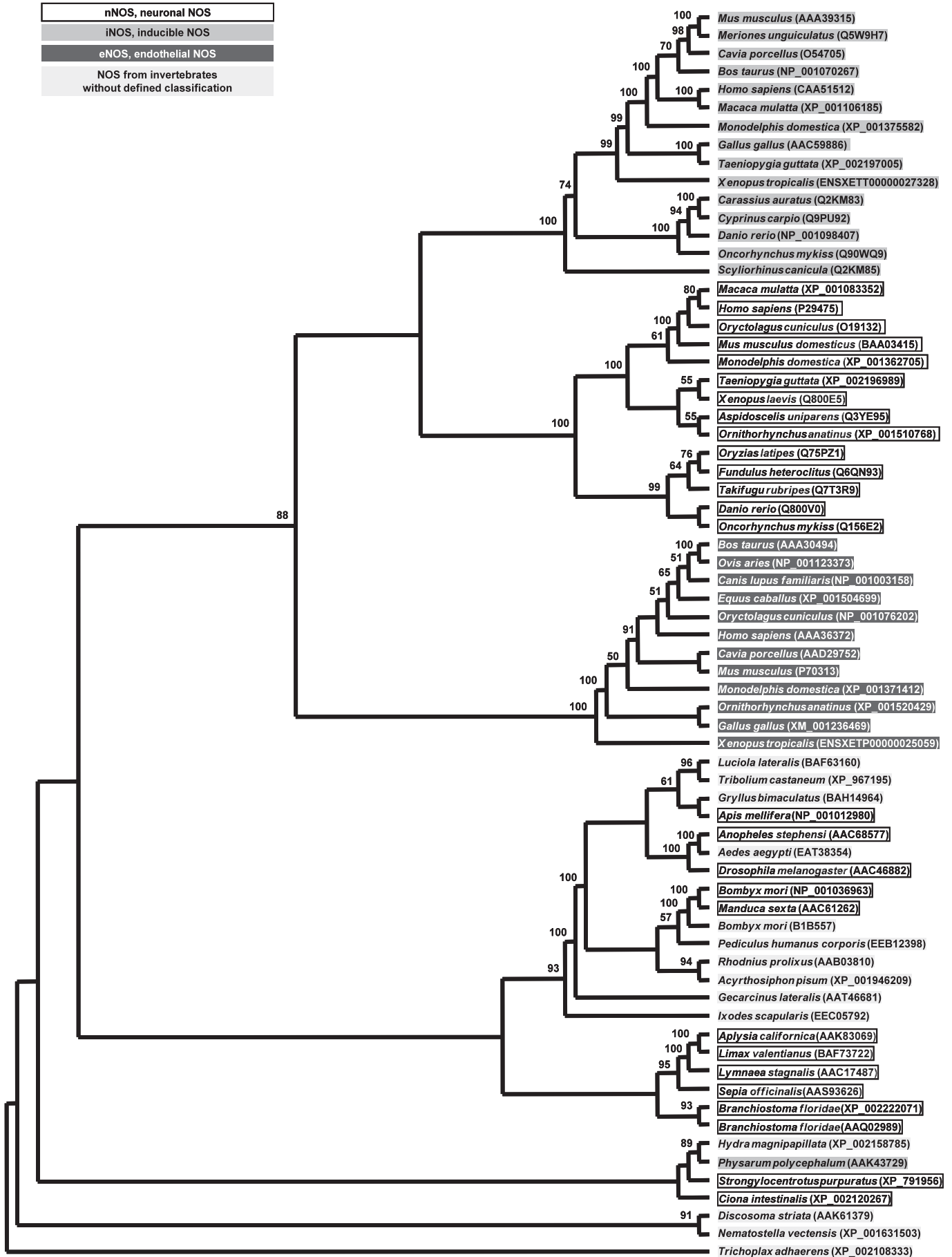
All the confirmed, putative and *in silico* deduced NOS sequences, retrieved from the databases, are summarized in Table S1. Among them, we discarded for further analysis most of the partial sequences, with a length shorter than 300–400 nucleotides (100–150 amino-acids), due to the lack of informative sites and the difficulty to align them with the other ones. The eNOS sequence from *Gallus gallus* (XM_001236469) has only 138 amino-acids. For this reason it was discarded in a first alignment. However, we later decided to include it, since the evolutionary information provided by this sequence revealed to be significant, as we will see below.

A preliminary maximum-parsimony analysis, including the 110 sequences retrieved, is available as supplementary material (Fig. S1).

3.2. Identification of functional motifs

Employing the web-based program SMART, we analyzed all the amino-acid sequences and we localized their functional domains. In vertebrates, we found that all the neuronal isoforms of NOS always had one PDZ domain at their N-terminus, as expected (Luckhart and Rosenberg, 1999; Stasiv et al., 2001; Scheinker et al., 2005; Matsuo et al., 2008). The sequences of nNOS for the vertebrate organisms *Aspidoscelis uniparens* and *Ornithorhynchus anatinus* (Q3YE95 and XP_001510768, respectively), although possessing the catalytic domain, were incomplete, so this could be the reason of lacking the PDZ motif. In contrast, most of the invertebrate NOS proteins lack of PDZ domain in their N-termini. Striking exceptions for this rule have been described in NOS from *L. valentianus* and *N. vectensis* (Putnam et al., 2007; Matsuo et al., 2008). This latter organism has a fragment of a PDZ domain juxtaposed to a putative NOS gene in the same direction in its genome (Matsuo et al., 2008). We have found three new exceptions: Two sequences from *Branchiostoma floridae* (AAQ02989 and XP_002222071), bearing one and two PDZ regions, respectively. The third exception is the invertebrate chordate *Ciona intestinalis* (XP_002120267), whose NOS sequence has a complete PDZ domain. This PDZ region shares a 45.3% of similarity with that from *L. valentianus* and a high-scoring BLASTp result

Fig. 2. Strict consensus tree based on amino-acid sequence of nitric oxide synthase protein from selected species in this study. The consensus tree was obtained from 24 trees computed by the maximum-parsimony criterion, with PROTPARS weighting. GenBank/EMBL/DDJB accession numbers are given in parenthesis. Bootstrap values greater than 50% are shown. The NOS sequences of vertebrates are clustered by isoforms, differently from invertebrates which show a taxonomic clustering. The main groups are supported by high bootstrap values.



(above 50% of identities) with the PDZ domain of the nNOS of vertebrates. Dehal et al. (2002) described whole vertebrate gene families in *C. intestinalis*, suggesting that ascidians contained the basic ancestral complement of the vertebrate genome. The existence of a PDZ domain-containing NOS in *C. intestinalis* also provides consistency to the hypothesis of Matsuo et al. (2008) about the origin of nNOS in vertebrates. They proposed that the PDZ domain-containing type NOS was a prototype of Eumetazoan NOS, and the PDZ domain was lost from NOS during the evolution of insects. Furthermore, as reported by some authors, the existence of a PDZ motif in invertebrates does not imply neural functions; for instance, it is suggested an immunological role for one of the NOS from *B. floridae* (AAQ02989) (Godoy et al., 2006) whereas the NOS from *N. vectensis* physiologically resembles iNOS from vertebrates (Ancil et al., 2005; Ancil, 2009).

3.3. Selection of amino-acid sequences for phylogenetic analyses

According to the preliminary phylogenetic analysis performed (Fig. S1) we selected 69 sequences, which are summarized in Table 1. Trees were inferred by NJ and MP criteria, using alignments of both, the amino-acid sequences spanning the NO synthase catalytic domain and the whole NOS protein sequences. The trees obtained under both criteria and for both types of sequences (whole and catalytic domain) are shown in Supplementary Figs. S2 to S5. We also obtained MP trees using the PROTPARS matrix for amino-acid substitution weighting. The strict consensus trees obtained by this method are shown in Figs. 1 and 2. In all these cases, the trees showed a similar topology, irrespectively of the criterion used or if whole protein or the NO synthase domain were considered. In all the cases a complete separation between vertebrate and invertebrate NOS was obtained, being supported the vertebrate clade by bootstrap values of 88–89% when the PROTPARS weighting was used. This result does not support the first of the hypotheses considered in the Introduction, that the vertebrate isoforms arise from different invertebrate NOS. Thus, our analysis does not support the existence of a single nNOS ortholog in eumetazoans.

Within vertebrates, the three NOS isoforms appear clearly clustered, with each isoform cluster being supported by high bootstrap values (between 95 and 100% in the PROTPARS weighted, MP analysis). However, clustering between the three isoforms was variable and not supported by bootstrapping. If we consider the double duplication of an ancestral gene as the origin of the different NOS isoforms (a hypothesis already forwarded by Hall et al., 1994 and Godoy et al., 2006), this event would have not allowed for accumulation of molecular synapomorphies in the common ancestor of a given couple of isoforms.

In addition, the genome sequence projects of *Gallus gallus* (<http://www.ncbi.nlm.nih.gov/genome/guide/chicken/>) and *Xenopus tropicalis* (<http://genome.jgi-psf.org/Xentr4/Xentr4.home.html>) allowed us to identify for the first time putative ortholog sequences of eNOS in these species. If confirmed, the presence of eNOS in amphibians (ENSXETP00000025059) and birds (XM_001236469), suggests that the origin of eNOS predates at least the origin of the mammals. Furthermore, the independent clustering of the three NOS isoforms in vertebrates does not support an origin of eNOS after the origin of the amphibians. If this would be the case, we should expect a separate clustering of the eNOS isoform with its ancestral form, either nNOS or iNOS, in a tetrapod clade, contrarily to that observed in our analysis. Therefore, we should expect the presence of an eNOS gene or pseudogene in fishes. However, neither a simple search by BLAST in the databases nor the exhaustive scanning of the genomes of *Danio rerio* and *Takifugu rubripes* reported any result. This is surprising, since a putative eNOS protein has been detected in fishes by immunohistochemistry and western blot, using antibodies that presumably do not cross-react with other isoforms. This putative eNOS protein has been localized not only in endothelial cells, but also in myocardio-

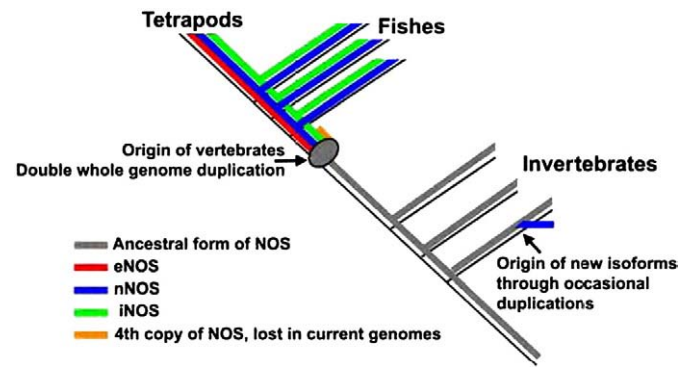


Fig. 3. Evolutionary hypothetical scenario for evolution of nitric oxide synthase genes. Within vertebrates, the three isoforms of NOS would have arisen from an ancestral gene by means of a double duplication occurring simultaneously. The branches leading to the fishes and the invertebrates just represent the paraphyletic nature of these groups. The diversity of NOS isoforms in invertebrates could be explained by occasional events of gene duplication, being the resulting genes evolutionarily unrelated to the homonym isoforms of vertebrates.

cytes, epicardium, podocytes, oocytes and other cells from the ovary (Wang et al., 2007; Amelio et al., 2008; Tripathi and Krishna, 2008). Thus, the issue of a functional eNOS gene in fish remains open.

With regard to invertebrates, we have found a mix of NOS isoforms spread along different Phyla. Basically two isoforms, neuronal and inducible, have been described in invertebrates (Fig. S1). Choi et al. (1995) suggested that different *Bombyx mori* NOS were similar to vertebrate isoforms, but existing a divergence of the enzyme features acquired along evolution. Furthermore, the existence of similar transcripts in mammalian nNOS and dNOS of *Drosophila* would suggest a conserved function for both enzymes, but Regulski and Tully (1995) confirmed the need of more biochemical studies in order to determine whether dNOS was really a nNOS. Our results confirm the independent evolution of isoforms in vertebrates and invertebrates, discarding the first of hypotheses exposed, as we have mentioned before. The grouping of the invertebrate sequences has a taxonomical base: arthropod and mollusc sequences constitute clusters supported by high bootstrap values, particularly when whole sequence and PROTPARS weighting were used (Fig. 2). In this case, even the chelicerate–mandibulate split is significantly observed in the tree. Therefore, the arthropod sequences showing functional similarity with nNOS from vertebrates do not show an evolutionary relationship with the vertebrate nNOS.

In conclusion, the exhaustive collection of all the available NOS gene sequences in the databases, allowed us to perform a thorough phylogenetic analysis in vertebrates and the most important taxa of invertebrates. According to our findings, we can suggest a hypothetical scenario for the evolution of the NOS genes (Fig. 3). Despite the lack of eNOS isoforms in fishes, the vertebrate NOS isoforms probably originated in the 2R event of whole genomic duplication. On the other hand, the diversity of the NOS isoforms in invertebrates could be explained by occasional events of gene duplication. Thus, the annotation of invertebrate NOS as “inducible” or “neuronal” should only be justified by physiological features, but always taking in account that they are evolutionarily unrelated to the homonym isoforms of vertebrates.

Acknowledgment

This work was supported by grants BFU2008-02384 and P08-CTS-03618 (Junta de Andalucía).

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.cbd.2010.08.004.

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