



Characterization of potential spermatogonia biomarker genes in the European eel (*Anguilla anguilla*)

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Abstract Identification of specific molecular markers for spermatogonial stem cells in teleost is crucial for enhancing the efficacy of reproductive biotechnologies in aquaculture, such as transplantation and surrogate production in fishes. Since it is not yet possible to distinguish spermatogonial stem cells of European eel (*Anguilla anguilla*) using specific molecular markers, we isolated spermatogonial cells from immature European eels to find these potential

markers. We attempted this by studying three candidate genes: *vasa*, *nanos2*, and *dnd1*. Two *vasa* (*vasa1* and *vasa2*) genes, *nanos2*, and *dnd1* were identified, characterized, and studied in the muscle, testis, and isolated spermatogonia. Our results showed that *vasa1* and *vasa2* had the highest levels of expression when measured by qPCR. In situ hybridization and immunochemistry assays showed that the four genes were localized explicitly in type A spermatogonia.

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However, *vasa1* and *vasa2* exhibited stronger signals in the immature testicular tissue than the other two potential markers. According to this, *vasa1* and *vasa2* were found to be the most effective markers for spermatogonial cells in the European eel.

Keywords Testis · Fish · Gene expression · *Vasa* · *Dnd1* · *Nanos2*

Introduction

The European eel (*Anguilla anguilla*) has a complex catadromous life cycle that includes a long reproductive migration across the Atlantic Ocean to reach their spawning site in the unknown areas of the Sargasso Sea (Schmidt 1923). During the last decades, different factors have significantly impacted eel populations, resulting in a decline in the continental stocks of the eels (Dekker 2002). As a result, the European eel was included in 2007 in the Appendix II of CITES and the Red list of IUCN. Currently, eel production in aquaculture is based on extractive fishing, as their production cycle has not yet been closed. Therefore, to reduce pressure on eel stocks, reproduction in captivity is required. One of the main issues for the reproduction in captivity of the eels is that the reproductive development is blocked in the pre-pubertal stage until their oceanic reproductive migration starts (Dufour et al. 1988). In general, limited development of gonads has been recorded when eels first migrate to oceans (gonadosomatic index = 1–2), although there are slight differences depending on the environmental conditions (Gentile et al. 2022; Palstra et al. 2011). Keeping these eels in captivity does not enhance further gonadal development, indicating the essentiality of environmental triggers, such as photoperiod, salinity, and temperature regimes, for sexual maturation (Burgerhout et al. 2019; van Ginneken and Maes 2005). Achieving sexual maturation in captive conditions requires long hormonal treatments, limited by a great variation in the response of each eel (reviewed by Asturiano 2020). Regarding this, it is crucial to understand the molecular mechanisms involved in germ cell development to make advances in the reproduction of the European eel. For instance, spermatozoa production depends on the subpopulation of spermatogonial stem cells, which in fishes are considered to be undifferentiated type A spermatogonia

(Schulz et al. 2010). Studying molecular markers for spermatogonial stem cells in fish provides a potential application for new reproductive biotechnologies, such as type A spermatogonia (SPGA) transplantation, in vitro spermatogenesis or reprogramming of spermatogonial cell lines into pluripotent cells (Robles et al. 2017; Thoma et al. 2011). Several proteins have been identified in spermatogonial populations in certain fish species (Bosseboeuf et al. 2014; Lacerda et al. 2013; Nagasawa et al. 2012; Ozaki et al. 2011). Some candidates, such as Notch1 (Yano et al. 2009), Ly75 (Nagasawa et al. 2010), Plzf (Ozaki et al. 2011), Oct-4 (Sánchez-Sánchez et al. 2010), and SGSA-1 (Kobayashi et al. 1998), have been proposed, but specific molecular markers for fish spermatogonial stem cells have yet to be discovered (Lacerda et al. 2018).

Vasa, or DDX4 (DEAD-box helicase 4), belongs to the RNA helicase of the DEAD (Asp-Glu-Ala-Asp) family. Vasa is implicated in the translational control of RNA activation (Hay et al. 1988; Lasko and Ashburner 1988). It has been identified in various metazoan species, such as cnidaria (Mochizuki et al. 2001), non-vertebrate Ecdysozoa (Schüpbach and Wieschaus 1986), mammals (Castrillon et al. 2000), or teleost species (Cao et al. 2012), and has been reported to be a germ cell marker in the animal kingdom.

Dead end (Dnd) protein encodes an RNA-binding protein (RBP) essential for primordial germ cell (PGC) migration and gametogenesis in vertebrates. In 2003, *dnd1* was recognized as a component of zebrafish (*Danio rerio*) germplasm (also called germ granules or nuage) that is specifically expressed in PGCs throughout embryogenesis (Weidinger et al. 2003). The Dnd1 protein is localized in perinuclear germ granules within PGCs, helping their polarization, motility, and survival.

Nanos is one of the essential genes for the development of germ cells. *Nanos2* expression was found to be restricted to the gonads of adult mammalian males and has been reported to be a specific marker of the male germinal stem cells of mice. In medaka (*Oryzias latipes*) and zebrafish, ovarian germ stem cells and spermatogonia in adult gonads exhibited expression of *nanos2* (Aoki et al. 2009; Draper 2017; Nakamura et al. 2010).

In our present study, we aimed to determine spermatogonia molecular markers by identifying *vasa*, *nanos2*, and *dnd1* genes in the testicular tissue of immature European eels. In addition, we studied the expression pattern of these genes in the testis, in isolated spermatogonia, and muscles, and have

determined their distribution within the testicular tissue through fluorescent in situ hybridization (FISH), immunohistochemistry, and immunocytochemistry.

Material and methods

Fish handling and sampling

Farmed immature European eels were bought alive at a local supermarket and transported to the facilities in the Laboratory of Fish Reproduction of the Universitat Politècnica de València (Valencia, East coast of Spain). Fish were anaesthetized upon arrival with benzocaine (60 ppm) and euthanized by decapitation. The maturation status of the eels was visually verified by checking the shading of the pectoral fin, which was transparent because of the immature stage of the eels (Okamura et al. 2007; Peñaranda et al. 2018). After dissection, only males were selected. Testes ($n=39$) and muscle ($n=23$) tissue samples were collected and stored in RNAlater (Ambion-Inc., Austin, TX, USA) and kept at $-20\text{ }^{\circ}\text{C}$ until RNA extraction.

Isolation of spermatogonia

Testes from immature male European eels ($n=13$) were dissected and washed with a phosphate-buffered saline (PBS), and the abdominal fat was removed. Testes were cut into small pieces and then transferred into 15-mL tubes containing 10 mL PBS and 0.2% trypsin for enzymatic dissociation. The mixture was incubated for 2 h at $22\text{ }^{\circ}\text{C}$. DNA was eliminated by adding $40\text{ }\mu\text{g/mL}$ DNase I (Pan-Reac AppliChem, Spain). Between 10 and 12 pipetting of the mixture was performed every 30 min to get better tissue dissociation. The obtained suspension was then filtered with a $40\text{-}\mu\text{m}$ meshed filter to separate debris, and the enzymatic reaction was stopped by adding 1% bovine serum albumin (BSA; Sigma-Aldrich).

To enrich the percentage of spermatogonial stem cells, a Percoll density gradient was employed. The Percoll solution at 33% (v/v) was prepared and transferred into a 50-mL plastic tube. The cell suspension was slowly transferred to tubes avoiding mixing layers. After that, the suspension was centrifuged at $400\times g$ for 20 min at $20\text{ }^{\circ}\text{C}$ using a progressive acceleration and deceleration centrifuge program. When completed, the middle layer containing spermatogonia was extracted from the mixture and transferred to

a 15-mL plastic tube. It was then diluted $3\times$ in PBS and centrifuged again at $400\times g$, $20\text{ }^{\circ}\text{C}$ for 20 min. After this second centrifugation, the supernatant was carefully removed, and the pellet containing the testicular cells was resuspended in PBS. The obtained testicular cells were observed under a light optical microscope, and due to their spherical appearance, large size ($10\text{--}20\text{ }\mu\text{m}$), and large nuclei ($6\text{--}10\text{ }\mu\text{m}$) (Robles et al. 2017), the germ cells at the early developmental stage were distinguished from other cell types, and their number was counted using a Neubauer cell hemocytometer at $\times 40$ magnification. Cell viability was evaluated by adding 0.4% trypan blue.

RNA extraction and reverse transcription

RNA from the enriched spermatogonia fraction was extracted immediately after their isolation, while testis and muscle RNA were extracted from samples kept in RNAlater. Total RNA was isolated using phenol/chloroform extraction in the Trizol reagent (Life Technologies, Inc., Carlsbad, CA) according to Peñaranda et al. (2010). RNA concentration and 280/260 and 280/230 ratios were determined using NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain). DNase I treatment and first-strand complementary DNA (cDNA) synthesis were performed from 500 ng of total RNA using a QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Identification of *vasa1*, *vasa2*, *nanos2*, and *dnd1* sequences from European and Japanese eel genome datasets

The *vasa1*, *vasa2*, *nanos2*, and *dnd1* sequences in vertebrate species were retrieved from NCBI (<http://www.ncbi.nlm.nih.gov/>) and Ensembl (<https://www.ensembl.org/index.html>) databases. All the genomic sequences of *vasa1*, *vasa2*, *nanos2*, and *dnd1* were retrieved from European (*Anguilla anguilla*; for accession/ID number GCF_013347855.1) (Henkel et al. 2012a) and Japanese eel (*Anguilla japonica*) genomes by performing the TBLASTN algorithm of the CLC DNA Workbench software (CLC bio, Aarhus, Denmark) (Henkel et al. 2012b). European eel *vasa1*, *vasa2*, *nanos2*, and *dnd1* sequences were then used to retrieve the genomic sequence coding for the respective genes in the Japanese eel genome (Henkel et al. 2012b). The exons and

splicing junctions were predicted using the empirical nucleotidic splicing signatures, that is, intron begins with “GT” and ends with “AG.” The percentage of European and Japanese eel *Vasa* (DEAD-box helicase 4) and DDX3X (DEAD-box helicase 3X-linked) identity was calculated with the Sequences Identities And Similarities (SIAS) server (imed.med.ucm.es/Tools/sias.html).

Phylogenetic analysis of *vasa1*, *vasa2*, *nanos2*, and *dnd1*

Phylogenetic analyses were conducted to retrace the evolutionary scenarios of *vasa*, *nanos2*, and *dnd1* families, as well as to determine and classify the number of paralogs, gene duplications, and gene losses on osteichthyans of key-phylogenetical positions: a representative of an early sarcopterygian, the coelacanth (*Latimeria chalumnae*); mammalians; sauropsids (squamates, chelonians, and archosaurians); the non-teleost actinopterygian spotted gar; and the European and Japanese eels, as members of an early group of teleosts (elopomorphs), and other teleosts. Three phylogenetic trees were constructed with amino acid sequences of known or predicted sequences of *Vasa* and its closest paralogous gene called DDX3X, *Nanos2* (nanos C2HC-type zinc finger 2), and *Dnd1* (dead end 1) (for accession/ID number, see Supplementary Table A).

The sequences were retrieved from NCBI or ENSEMBL, first aligned using Clustal Omega (Sievers et al. 2011) with Seaview 4.5.4 software (<http://doua.prabi.fr/software/seaview>), and later manually adjusted. The JTT (Jones, Taylor, and Thornton) protein substitution matrix of the resulting alignment was determined using ProTest software (Abascal et al. 2005). The alignment of peptide sequences was performed with amino acid sequences of *Vasa* retrieved

from NCBI for European eel (accession numbers XP_035234983.1 and XP_35248267.1), Japanese eel (accession numbers ASV71763.1 and BAO21641.1), and rice field eel (*Monopterus albus*, accession number ABA54551.1) to provide further details of *vasa* genes in eels (for alignment, see Supplementary Fig. A).

The three phylogenetic trees were constructed based on the sequence alignments using the maximum likelihood method (PhyML software, (Stamatakis and Ott 2008)) with 1000 bootstrap replicates and subsequently visualized using treedyn (<http://phylogeny.lirmm.fr/phylo.cgi/>). Human and fugu (*Takifugu rubripes*) DDX10 (DEAD-box helicase 10), human RBM47 (RNA-binding motif protein 47), and human NANOS1 (nanos C2HC-type zinc finger 1) were used as out-group for the *Vasa*, *Dnd1*, and *Nanos2* phylogenetic analyses, respectively (for accession/ID number, see Supplementary Table A).

Gene expression analysis by quantitative real-time PCR

Quantitative real-time polymerase chain reactions (qPCR) were carried out using specific qPCR primers for European eel *vasa1*, *vasa2*, *nanos2*, and *dnd1* (Table 1). Acidic ribosomal phosphoprotein P0 (*arp*) was used as a reference gene as previously used for eel (Morini et al. 2015).

Primers

European eel *vasa1*, *vasa2*, *nanos2*, and *dnd1*-specific qPCR primers were designed based on European eel coding sequences using Primer3 Software (Whitehead Institute/Massachusetts Institute of Technology,

Table 1 Specific primers sets used for qPCR

Name of gene	5'-3'	Efficiency	Length
<i>vasa1</i>	F TTTGGAGGGAGAGGTAGAGG R CTCATTTCTGATGCGTTCC	105	69
<i>vasa2</i>	F GTGTATGAGGTCACCCAGTA R CTCTTGGTCTCTACAAACAC	98	98
<i>dnd1</i>	F CGGGACATCTACGAGGACAA R TTCATCATCAGGCGGAACTC	106	77
<i>nanos2</i>	F GAGCCAGCAGAGCAGAAA R CCGTCCTTCGCCTTCA	97	194
<i>arp</i>	F GTGCCAGCTCAGAACACTG R ACATCGCTCAAGACTTCAATGG	105	107

Boston, MA, USA). All primers were designed on two different exons to avoid amplifying potential genomic contamination. Primers were purchased from Integrate DNA Technology Inc. (IDT, Coralville, IA).

SYBR Green assay

Expression of *vasa1*, *vasa2*, *nanos2*, and *dnd1* genes in spermatogonia, gonads, and muscles (negative control) of immature European eels was measured by performing qPCR assays using a model 7500 unit (Applied Biosystems; Foster City, CA, USA) with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas Corp. Glen Burnie, MD, USA). qPCR program was performed as an initial step of 95 °C for 10 min and 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. To evaluate assay specificity, the machine performed a melting curve analysis directly after PCR by slowly (0.3 °C/s) increasing the temperature from 60 to 95 °C, with continuous registration of any changes in fluorescent emission intensity. The total volume for each qPCR reaction was 20 µL, with 5 µL of diluted cDNA (1:20) template, forward and reverse primers (250 nM each), and SYBR Green/ROX Master Mix (12 µL). Serial dilutions of the cDNA pool of gonad tissues were run in duplicate and used for standard curve to measure *vasa1*, *vasa2*, *nanos2*, and *dnd1* in the spermatogonia, testis, and muscle. As a calibrator, a 1/32 standard curve dilution was included in each run of the corresponding gene. Target and reference genes in samples were run in duplicate PCR reactions. A non-template control (cDNA replaced by water) for each primer pair was replicated in all plates. Data for all genes were normalized to eel reference gene *arp*. qPCR calculations were performed according to Roche Applied Science protocol, Technical Note No. LC 13/2001, part 4 “Calibrator normalized relative quantification.”

Localization analyses

Fluorescent in situ hybridization (FISH)

To detect the distribution of the potential spermatogonia markers (*vasa1*, *vasa2*, *nanos2*, and *dnd1*), testes from four immature European eels were dissected. For FISH, RNAscope Multiplex

Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics Inc. [ACD], CA, USA) was used following the manufacturer instructions. ACD created probes as *vasa1* (Gene ID 118213421), *vasa2* (Gene ID 118206405), *nanos2* (Gene ID manually characterized using CLC software; see the “RNA extraction and reverse transcription” section), and *dnd1* (Gene ID 118224086) of European eel. β -actin probe, targeting LOC118216518 of European eel β -actin-1 (Gene ID DQ493907.1), was used as the positive control. Probe diluent was used as the negative control. Testes samples were fixed in 10% neutral buffered formalin for 24 h at room temperature (RT). Then, samples were dehydrated in an increasing percentage of ethanol series (70, 80, 90, 95, 100%) followed by xylene and embedded in resin (Technovit 7100) according to the manufacturer’s instructions. Sections of 5 µm thickness were cut with a Microtome HM325 and were mounted on Superfrost® plus slides (Thermo Fisher Scientific, MA, USA). Two slides were stained with hematoxylin–eosin (H&E) to determine the testis maturation stage (Peñaranda et al. 2010).

After dewaxing and rehydration, antigen retrieval was done by boiling the tissue sections in RNAscope® Target Retrieval Reagents at 98–102 °C for 15 min. Tissue sections were incubated with the appropriate probe at 40 °C for 2 h for probe hybridization. Localization of *vasa1*, *vasa2*, *nanos2*, and *dnd1* RNA transcripts was done by labelling them with Opal 520 fluorophore (Akota Biosciences, MA, USA) by incubating the slides at 40 °C for 30 min. Preliminary testing of optimal fluorophore concentration showed that the 1:750 dilution of the Opal fluorophore displayed a more intense signal than the 1:1000 dilution. Therefore, 1:750 was used in further analyses (see Supplementary Fig. B). Slides were counterstained with DAPI at RT for 30 s, mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific, MA, USA) and covered with a coverslip. The slides were stored in the dark at 4 °C. Between 5 and 15 photographs were taken after at least 8 h and until the 2 following weeks using a camera (Moticam 1080, MoticEurope S.L.U, Barcelona, Spain) mounted on a phase-contrast microscope (Nikon Eclipse 80i). Positive signal was visible as fluorescent dots within a cell, correlated with the number of RNA copies. A quantitative analysis according to the manufacturer’s instruction was

applied to evaluate results using the image-based software QuPath version 0.4.1 (Bankhead et al. 2017). The software estimated the number of spots per cell counting the number of single spots and clusters. An approximate number of 1500 cells were counted in each photograph.

More detailed photographs were taken using an AxioObserver 780 confocal microscope (Zeiss, Jena, Germany) to diagnose the cell type in which the signal was present.

Immunohistochemistry

Three immature European eel males were sacrificed as described above, and their testes were dissected and fixed in modified Davidson's fixative [mDF; 30% of 37–40% formaldehyde, 15% EtOH, 5% glacial acetic acid in ddH₂O; (Howroyd et al. 2005)] for 24 h at RT. Tissues were subsequently dehydrated in increasing EtOH series, cleared in xylol and embedded into paraffin. Sections of 5 µm thickness were cut and mounted on Superfrost Ultra Plus® slides (Thermo Fisher Scientific, Massachusetts, USA). One slide was first stained with standard H&E staining to verify the sample's testicular morphology and identify spermatogonia.

Immunohistochemistry localization of *Vasa*, *Nanos2*, and *Dnd* proteins was done using the 3,3'-diaminobenzidine (DAB) immunoperoxidase visualization method. After dewaxing and rehydration, antigen retrieval was done by boiling the sections in the HistoVT One antigen retrieval solution (Nacalai Tesque Inc, Kyoto, Japan) in a TintoRetriever pressure cooker (Bio SB, Santa Barbara, CA, USA) under the 80–86 °C low-pressure setting for 20 min. Endogenous peroxidases were inhibited by treating the sections with 3% H₂O₂ in PBS for 30 min, after which non-specific binding was blocked with a blocking solution composed of 10% FBS and 10% goat serum in PBS for 1 h at RT. After the preliminary testing of optimal antibody concentrations, sections were labelled with anti-*vasa* (1:200; Abcam, ab13840), anti-*nanos2* (1:10; Abcam, ab76568), or anti-*dnd1* (1:10; Sigma-Aldrich, AV41198) antibodies diluted in the blocking solution, for 1 h at RT. Subsequently, the sections were labelled with a goat anti-rabbit (1:250; Abcam, ab6721) antibody conjugated to horseradish peroxidase for 30 min at RT. The signal was then visualized by applying a solution of 0.05% DAB and 0.015% H₂O₂ for approximately

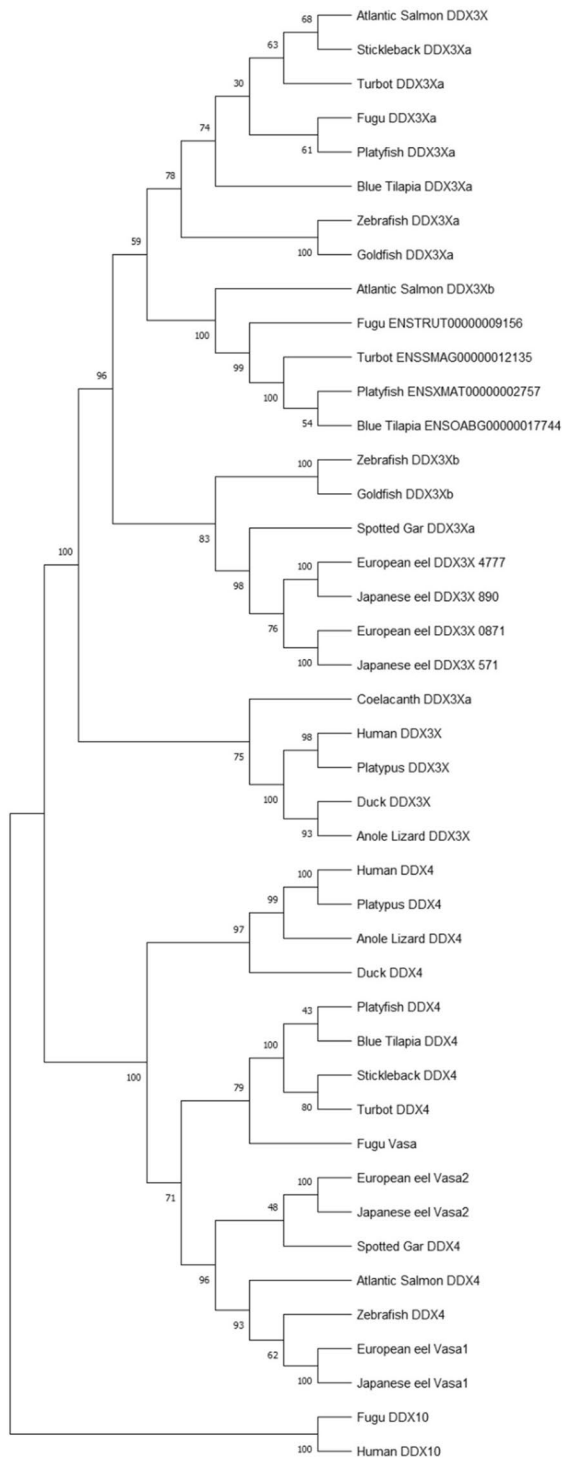
5 min. After washing, the slides were counterstained with hematoxylin for 3 min, dehydrated in increasing EtOH series, cleared in xylol, and mounted with DPX. Sections labelled only with the secondary antibody were considered secondary-antibody control sections, while sections that were not labelled with antibodies but stained with DAB were termed DAB controls. Samples were visualized under the Nikon 6100 Epifluorescent microscope.

Immunocytochemistry

Spermatogonia were isolated as described above (see the "Isolation of spermatogonia" section), and the cells were fixed in 10% NBF for 15 min at RT. The cells were centrifuged at 300×g for 10 min and washed 3 times in PBS. After the last wash, the cells were resuspended in PBS containing 10% polyethylene glycol (PEG) to avoid crystallization of the salts contained in the PBS after evaporation and left overnight at 4 °C. Approximately 200,000 cells were transferred per well of a chamber slide (Nunc Lab-Tek, NY, USA) and were centrifuged at 1000×g for 5 min. The supernatant was aspirated, and the cells were left to dry at RT for 2 h. The cells were then permeated by using 0.25% Triton X-100 in PBS for 10 min, and no antigen retrieval was done. As both FISH and immunohistochemistry displayed that *vasa* was more optimal in labelling spermatogonia, cells were labelled only with the anti-*vasa* (1:200; Abcam, ab13840) antibody for 1 h at RT. Subsequently, the sections were labelled with a goat anti-rabbit (1:500; Abcam, ab150078) antibody conjugated to Alexa 555 for 30 min at RT, counterstained with DAPI, and mounted in 90% glycerol in PBS. Samples were visualized under the Nikon 6100 Epifluorescent microscope.

Statistics

Results are shown as mean ± standard error of the mean (SEM). Statistical analyses were performed to compare the transcript levels in the muscle, testes, and spermatogonia and the difference in the number of spots estimated between markers. Shapiro–Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. Due to the heteroscedasticity of variance, means were compared by the non-parametric Kruskal–Wallis test. Differences



were considered significant when $P < 0.05$. All statistical procedures were performed using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA).

Fig. 1 DEAD-box helicase consensus phylogenetic tree. The phylogenetic tree was constructed based on the amino acid sequences of DDX3X (DEAD-box helicase 3X-linked) and Vasa (DEAD-box helicase 4, DDX4) (for the references of each sequence, see Table 1) using the maximum likelihood method with 1000 bootstrap replicates. The number shown at each branch node indicates the bootstrap value (%). The DEAD-box helicase 10 (DDX10) was used as the out-group

Results

Characterization and phylogenetic analyses of potential spermatogonial biomarkers in eel

Vasa and its closest paralog gene *DDX3X*

The search in European and Japanese eel genomes (Henkel et al. 2012a, b) revealed two eel *vasa* paralogous genes, called *vasa1* and *vasa2*, and two *ddx3x* paralogous genes, called *ddx3x1* and *ddx3x2*, orthologous to the other vertebrate *vasa* and *ddx3x*, respectively. Only partial *vasa2* and *ddx3x2* sequences were achieved from the European and Japanese eel draft genomes. The missing part corresponds to the 3' start. Two more exons are missing in the middle of the European eel *vasa2* sequence. European eel *vasa1* and *vasa2* display 98.24 and 90% (due to the missing exon in European eel *vasa2*) identity compared to their respective Japanese eel *vasa1* and *vasa2*. European eel *ddx3x1* and *ddx3x2* display 98.31 and 99.33% identity compared to their respective Japanese *ddx3x1* and *ddx3x2*.

A single *vasa* gene has been retrieved in a non-teleost actinopterygian, the spotted gar, as in sarcopterygians and other teleost species. The coelacanth *vasa* is missing in databases. A single *ddx3x* gene has been retrieved in the spotted gar and in the coelacanth, a basal sarcopterygian, as in humans, tetrapods, and some other teleost species. Other teleosts such as fugu (*T. rubripes*), platyfish (*Xiphophorus maculatus*), tilapia (*Oreochromis aureus*), turbot (*Scophthalmus maximus*), salmon (*Salmo salar*), goldfish (*Carassius auratus*), or zebrafish exhibit two *ddx3x* paralogous genes.

In the phylogenetic analyses, *Vasa* and *DDX3X*, both members of the DEAD-box helicase family, clustered in two monophyletic groups. In each *Vasa* and *DDX3X* monophyletic groups, actinopterygian and sarcopterygian sequences clustered in two well-supported clades (Fig. 1).

Fig. 2 Nanos2 (nanos C2HC-type zinc finger 2) consensus phylogenetic tree. The phylogenetic tree was constructed based on the amino acid sequences of Nanos2 (for the references of each sequence, see Table A) using the maximum likelihood method with 1000 bootstrap replicates. The number shown at each branch node indicates the bootstrap value (%). The Nanos1 (nanos C2HC-type zinc finger 1) was used as the out-group

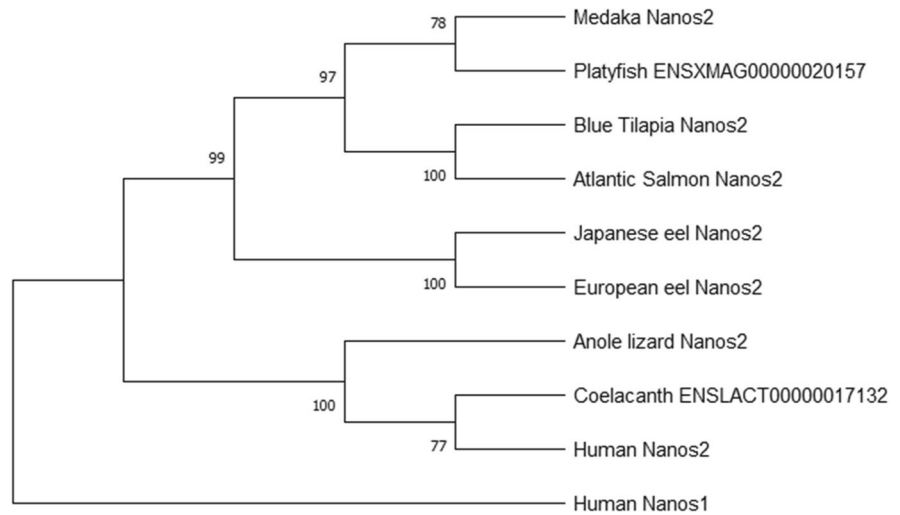
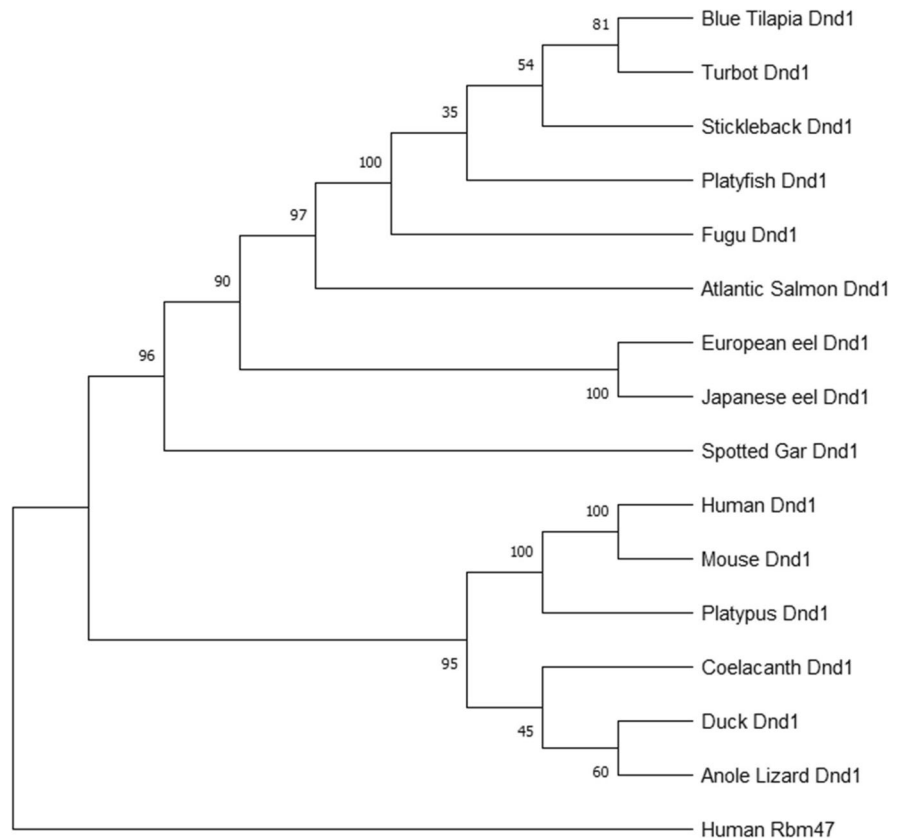


Fig. 3 Dnd1 (dead end 1) consensus phylogenetic tree. The phylogenetic tree was constructed based on the amino acid sequences of dnd1 (for the references of each sequence, see Table A) using the maximum likelihood method with 1000 bootstrap replicates. The number shown at each branch node indicates the bootstrap value (%). The RBM47 (RNA binding motif protein 47) was used as the out-group



Nanos2 and *dnd1*

The search in European and Japanese eel genomes (Henkel et al. 2012a, b) revealed only one eel *nanos2* and one *dnd1* gene, orthologous to the other vertebrate *nanos2* and *dnd1*, respectively. A

single *nanos2* and *dnd1* gene has been retrieved in teleost species, as in sarcopterygians. The spotted gar *nanos2* is missing in the database. In both phylogenetic analyses, actinopterygians and sarcopterygians sequences clustered in two monophyletic groups (Figs. 2 and 3).

Fig. 4 Spermatogonial cells observed under light optical microscope. Spermatogonia are large spherical cells with large nuclei, distinguished from lipids and debris, identified with arrowheads. Scale bar, 25 μ m

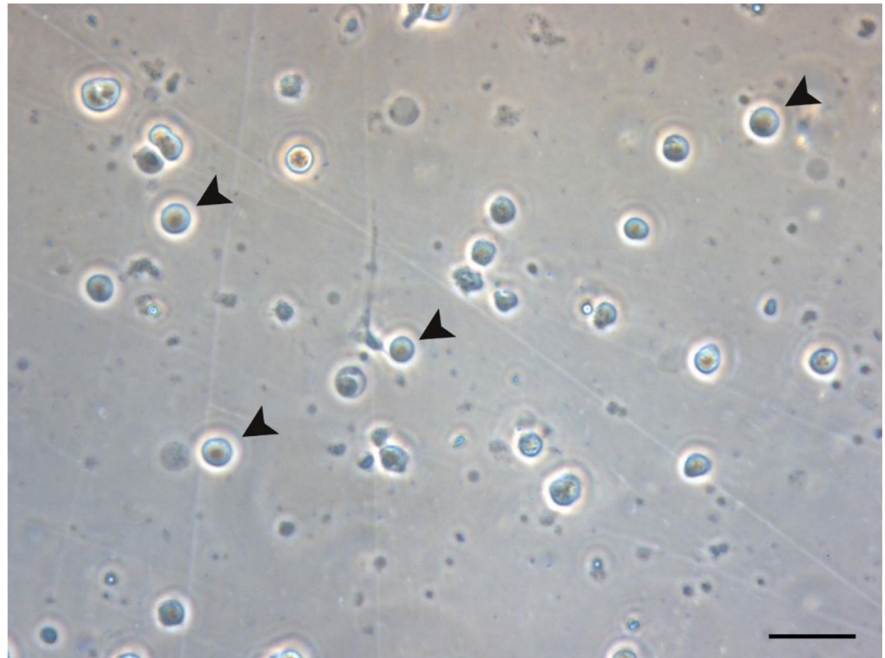
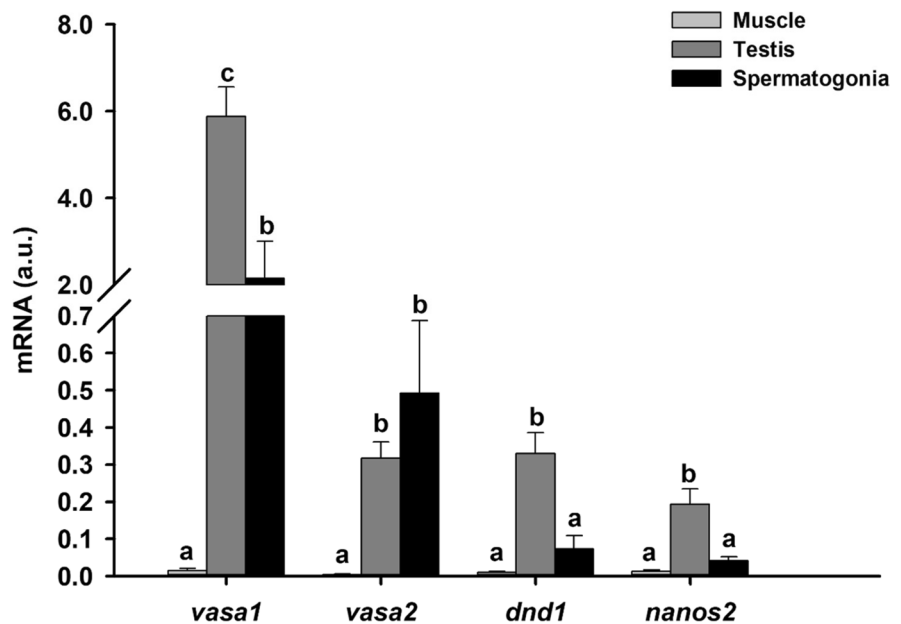
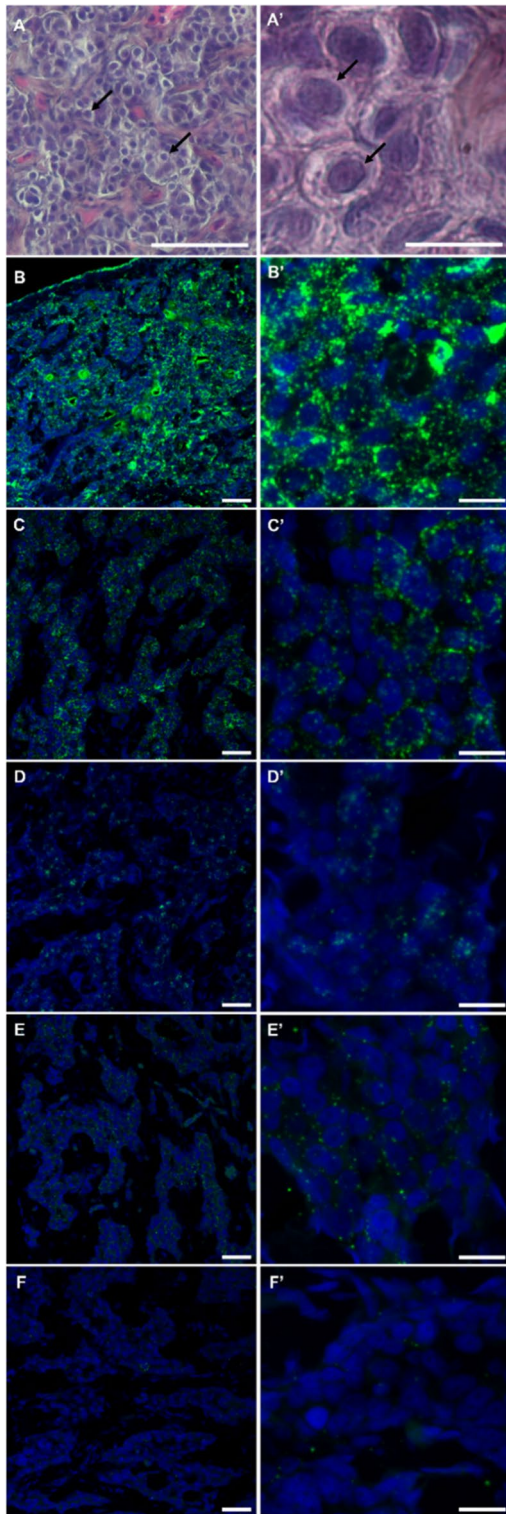


Fig. 5 Expression of *vasa1*, *vasa2*, *dnd1*, and *nanos2* mRNA in the muscle, testis, and spermatogonia of immature male European eels, measured by quantitative PCR (qPCR). Data are normalized to eel acidic ribosomal phosphoprotein P0 (ARP), and results are shown as means \pm SEM ($n = 23$ for muscle; $n = 39$ for testis; $n = 13$ for spermatogonia). Different letters indicate significant differences (Kruskal–Wallis test; $P < 0.05$)



The two eel *Nanos2* sequences form a group branching at the base of the teleost clade, in agreement with the phylogenetical basal position among teleost species (Fig. 2). Similarly, the *Dnd1* sequence of the non-teleost actinopterygian, a holostean, the

spotted gar, branched at the basis of the actinopterygian clade. Both Japanese and European eel *Dnd1* sequences diverged at the base of the other teleost sequences, in agreement with the phylogenetical basal position of elopomorpha among teleosts (Fig. 3).



◀ **Fig. 6** **A, A'** Photographs taken with a phase-contrast microscope of histological sections of immature European eel testis, stained with hematoxylin and eosin. Spermatogonia are identified with arrows. Photographs taken with confocal microscope of immature European eel testis for detection of potential spermatogonia markers by FISH, labelled with Opal 250 (dilution 1:750) and DAPI fluorophores. **B, B'** β -actin (positive control); **C, C'** *vasa1*; **D, D'** *vasa2*; **E, E'** *dnd1*; **F, F'** *nanos2*. Scale bars **A, B, C, D, E, and F**—25 μ m; **A', B', C', D', E', and F'**—10 μ m

Gene expression of potential biomarkers in spermatogonia, testis, and muscle

Isolation of spermatogonia

To measure by qPCR the mRNA expression of 4 different genes, *vasa1*, *vasa2*, *nanos2*, and *dnd1*, in spermatogonia cell population, we previously isolated the spermatogonia cells from the whole testis (Fig. 4). The eels did not receive any treatment that enhances sexual maturation and were therefore in a very immature stage of development. Enzymatic digestion of the testicular tissue resulted in a heterogeneous cell suspension composed of germ cells at early developmental stage and somatic cells (mainly blood cells), but the use of Percoll gradient enriched the spermatogonia cell population. In this sense, the Percoll gradient centrifugation positioned the spermatogonial cells in the middle layer, whereas lipids were in the upper layer and somatic cells pelleted down at the bottom of the tube. Many somatic cells were removed from the spermatogonial cell suspension, but the complete removal could not be achieved. The viability test revealed that $93.5 \pm 1.7\%$ of isolated spermatogonia survived the process.

mRNA expression

Intra-assay coefficient of variation (CV, %) for each gene (*vasa1*, *vasa2*, *nanos2*, *dnd1*) and tissue (muscle, testis, and spermatogonia fraction) was calculated. The mean % CV for each gene was 0.98% (*vasa1*), 2.99% (*vasa2*), 0.70% (*dnd1*), and 1.80% (*nanos2*).

The expression of *vasa1*, *vasa2*, *dnd1*, and *nanos2* genes was detected in testes, enriched spermatogonia fraction, and muscle, with a significantly lower gene

expression in the muscle (Fig. 5). *Vasa1*, *nanos2*, and *dnd1* showed the same expression pattern, with a higher gene expression in the gonad than in the isolated spermatogonia (2.7, 4.5, and 4.8-fold higher, respectively). In contrast, the *vasa2* mRNA transcript did not show significant differences between the gonad and the isolated spermatogonia.

Gene location of potential biomarkers in testis by FISH

A preliminary examination of the testis sections was conducted to determine the maturation stage (Fig. 6A, A'), confirming that most contained cells were SPGA. β -actin signal (positive control) was distributed evenly throughout the tissue without cell-specificity (Fig. 6B, B'). Moreover, the signals of *vasa1* (Fig. 6C, C') and *vasa2* (Fig. 6D, D') were detected only in SPGA, characterized by their large round shape and pronounced nucleus. Both *vasa1* and *vasa2* signal spots were primarily accumulated in clusters, but *vasa1* signaling spots were more widely distributed than *vasa2* ones. The distribution of *dnd1* (Fig. 6E, E') and *nanos2* (Fig. 6F, F') transcripts was also limited to SPGA, but the signal was more dispersed and weaker than those of both *vasa* genes.

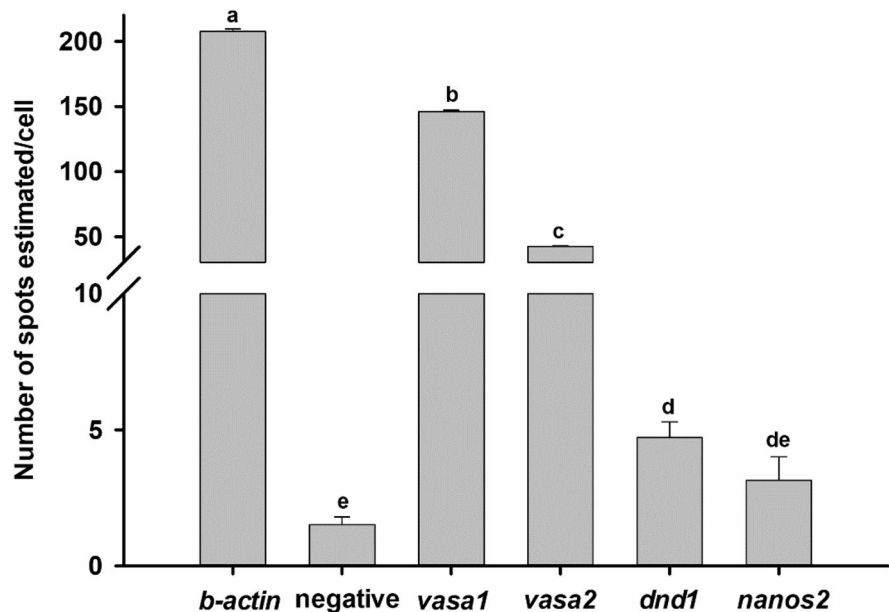
The highest number of spots was found for β -actin (positive control, Fig. 6B, B') which is distributed in the cytoplasm of all cell types, while the negative control did not show fluorescent dots (not shown).

The mean number of estimated spots per cell showed significant differences among the potential spermatogonial markers, where the *vasa1*, as well as *vasa2* transcripts displayed a significantly higher number of estimated spots than *dnd1* and *nanos2* (Fig. 7).

Localization of the potential markers by immunohistochemistry

Immunohistochemistry localization of Vasa, Nanos2, and Dnd1 proteins in the immature European eel testicular tissue conducted through the immunoperoxidase visualization method displayed that all three investigated markers were localized specifically in the germline cells (Fig. 8). As the only germline cells in the immature European eel testicular tissue are SPGA, the three markers were localized within these cells. However, the intensity varied significantly; while the signals for Vasa and Nanos2 were strong, the signal for Dnd, even at a 1:10 dilution ratio, was very weak. As the dilution ratio for the anti-vasa antibody was the highest but still resulted in a strong signal, this protein was the most favorable among the tested ones. Even though the antibodies used in this study were developed against mammalian antigens, their labelling pattern was in accordance with FISH results thus further confirming their specificity to European eel antigens as well. Background DAB staining was noticed in the connective tissue of all

Fig. 7 Expression quantification by QuPath software for β -actin (positive control), negative control, *vasa1*, *vasa2*, *dnd1*, and *nanos2*, showing the average number of spots estimated per cell. Results are shown as means \pm SEM. Different letters indicate significant differences (Kruskal–Wallis test, $P < 0.05$)



samples, including the controls; however, this is a regular occurrence in DAB staining and is not representative of an actual localization signal.

As Vasa was identified as the most suitable SPGA marker in immature European eel testicular tissues by FISH and immunohistochemistry, we tested the efficiency of this marker to identify isolated SPGA within the testicular cell suspension. By immunocytochemistry, we could observe that the signal was detected only in cells with large round nuclei corresponding to the SPGA (Fig. 9), and not in other somatic cells.

Discussion

Duplicated vasa genes in the eel

Few studies reported phylogenetic analyses of potential spermatogonial biomarkers in teleosts, including the DEAD-box helicase family (Vasconcelos et al. 2019; Xu et al. 2005; Ye et al. 2007). To better understand the evolutionary history of the DEAD-box helicase family, we performed phylogenetic analyses on vertebrate amino acid sequences of two members of this family, Vasa and DDX3X. Duplicated *vasa* and *ddx3x* genes have been retrieved from eel genomes. Two *ddx3x* paralogs were also present in other teleost species, while only one *vasa* was retrieved in the other teleost non-elopomorph species. Two events of whole-genome duplication (WGD) (“1R” and “2R”) occurred in ancestral vertebrates (Nakatani et al. 2007), and a third WGD (“3R”) occurred in the teleost lineage (Henkel et al. 2012a; Meyer and Van de Peer 2005; Morini et al. 2022). So, two hypotheses could be considered: duplicated eel DDX3X and Vasa may originate either from teleost 3R or from a specific gene duplication that could have occurred in Elopomorphs or Anguillids. In the first hypothesis, one *vasa* paralog gene would have been lost early after the 3R in the teleost lineage.

Phylogenetic analyses were also performed to characterize eel Dnd1 and Nanos2 among vertebrates. Our in silico analysis revealed the presence of a single *dnd1* gene in European and Japanese eels and in other vertebrates. The presence of a single gene in the eels, in teleosts, in non-teleost actinopterygian, and sarcopterygian species reveals no impact of the TWGD on teleost *dnd1* gene number. This suggests that one of the two *dnd1* paralogs would have been lost early after the

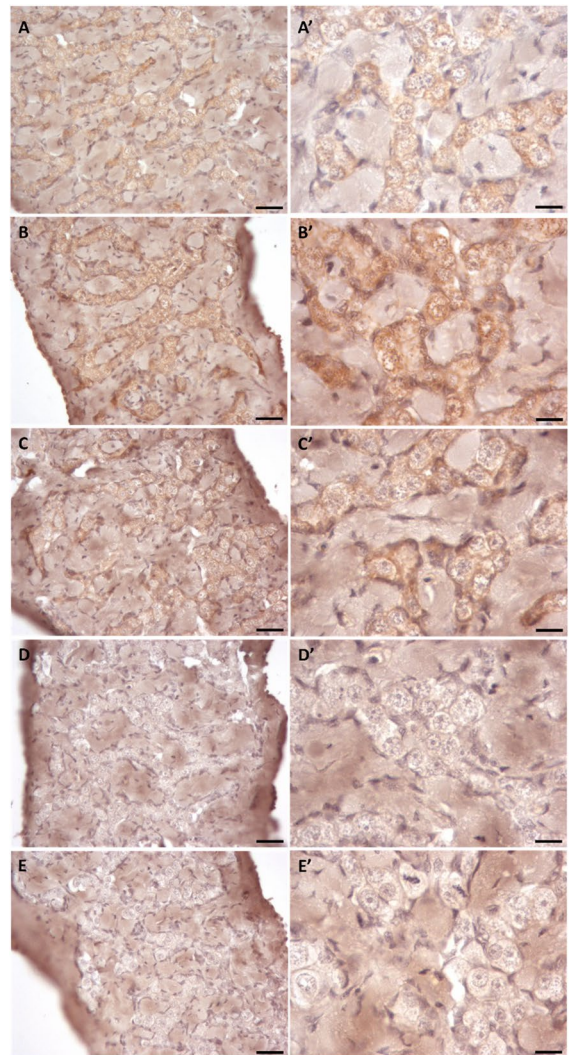


Fig. 8 Immunohistochemical detection of Vasa (A–A’), Nanos2 (B–B’), and Dnd1 (C–C’) antigens in the immature testicular tissue of European eel, as well as the secondary antibody control (D–D’) and the DAB control (E–E’). Each panel under the same letter represents a different magnification. Scale bars A, B, C, D, and E—25 μ m; A’, B’, C’, D’, and E’—10 μ m

TWGD in the teleost lineage. The same hypothesis can arise for the *nanos2* evolutive history.

European eel *vasa*, *dnd1*, and *nanos2* are expressed in spermatogonial cells

Vasa is considered one of the essential markers for identifying germ cells within the animal kingdom (Begum et al. 2022). A species-specific expression

pattern of *vasa* mRNA and protein has been observed in PGCs and germ cells of the ovary and testis in diverse fish species (Cao et al. 2012; Duangkaew et al. 2019; Xu et al. 2005; Yuan et al. 2014). In Japanese eel, *vasa* expression occurs mainly in males during testis differentiation but also in the developing ovary of E2-induced feminizing eels (Jeng et al. 2018). However, in the rice field eel, *vasa* was expressed in oocytes at all stages of oogenesis, including degenerating oocytes of ovotestis and in spermatogonia and primary spermatocytes (Ye et al. 2007). This observation highlights the significance of *vasa* and its potential for a better understanding of the reproductive biology in these organisms.

In present study, we characterized two paralogs of *vasa* in the European eel, while only one *vasa* gene has been described in vertebrates until now. European eel *vasa1* and *vasa2* showed a higher expression in the testis than in muscle. According to the bibliography, *vasa* expression is mainly restricted to the testis and ovary in adult teleosts, but it can also be expressed in other tissues, although at virtually undetectable levels (Blázquez et al. 2011; Nagasawa et al. 2013). Nevertheless, *vasa1* showed a higher expression in the testis than in spermatogonia while no differences were found in *vasa2*. Moreover, FISH results showed that both *vasa1* and *vasa2* are found in SPGA, while immunohistochemistry and immunocytochemistry confirmed that Vasa protein is also expressed in the SPGA. These results suggested that both *vasa* genes may be involved in the reproductive process (Wang et al. 2022; Xu et al. 2014), and the conservation of duplicated eel *vasa* may reflect evolutionary processes such as neo- or sub-functionalization, as showed in other duplicated genes in eels

(Jolly et al. 2016; Nakamura et al. 2017; Maugars and Dufour 2015). Further syntenic studies are needed to clarify the origin of *vasa* genes in teleosts. Together with our qPCR results, both genes could be reliable markers for SPGA in the European eel.

Nanos genes are expressed in germ cell lineage in metazoans. Three *nanos* gene subtypes (*nanos1*, *nanos2*, and *nanos3*) have been described in vertebrates. In mice, *nanos2* was mainly expressed in the male germ cells, and knock-out of *nanos2* led to the lack of spermatogonia (Tsuda et al. 2003, 2006). In fish, depending on the species, specifically the expression of *nanos2* and *nanos3* mRNA and protein has been observed in testicular and ovary germ cells (Aoki et al. 2009; Beer and Draper 2013; Bellaiche et al. 2014; Han et al. 2018; Nakamura et al. 2010; Tsuda et al. 2003). In the European eel, our qPCR analyses revealed that *nanos2* was more expressed in the whole testis compared to the enriched spermatogonia fraction, and low signal was found by the FISH technique.

While *nanos2* is recognized as a marker for SPGA in teleosts, the role of *nanos3* as a specific spermatogonia marker remains unclear (Bellaiche et al. 2014; Han et al. 2018; Lacerda et al. 2014). Despite that, the present study showed that the *nanos2* expression in European eel spermatogonia is low. As a result, the characterization of European eel *nanos3* in testis may be helpful to find a potential germ cell marker inside the *nanos* gene family.

Dead end (*dnd*) gene has been identified as a specific spermatogonial marker in some teleosts (Baloch et al. 2019; Duan et al. 2015; Lin et al. 2013; Yang et al. 2015; Yazawa et al. 2013; Yoshizaki et al. 2016) which suggests that the *dnd* gene may play an important role in spermatogenesis. In our study, eel *dnd1* was identified

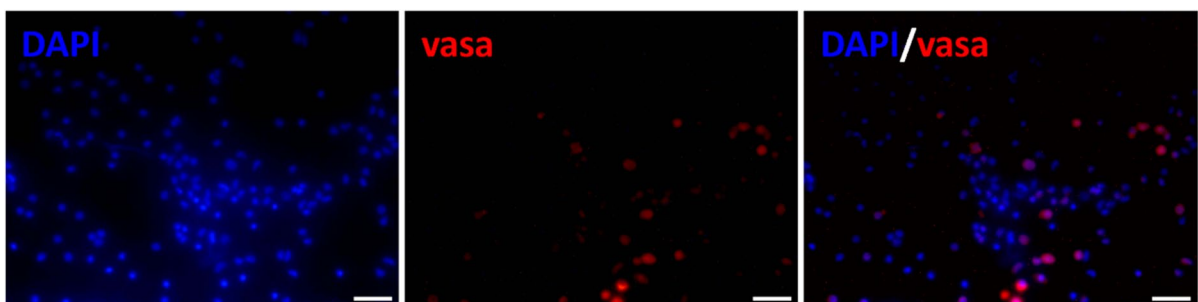


Fig. 9 Immunocytochemical detection of the Vasa-positive cells (displaying red fluorescence) in the testicular suspensions of the immature European eel using DAPI staining (blue fluorescence). Scale bars, 25 μ m

as orthologous to the other teleost *dnd1*, suggesting a similar function than in other species (Booncherd et al. 2024; Wargelius et al. 2016; Zhu et al. 2018). In this sense, a higher *dnd1* expression level has been revealed in the immature testis of the eels than in the isolated spermatogonia. According to our FISH results, *nanos2* and *dnd1* may not be reliable molecular markers for identifying SPGA in the European eel, as they present a weak signal compared to *vasa1* and *vasa2*. Previous studies in mammals have shown that the dissociation of human spermatogonial stem cells from their niche may alter their gene expression profile to a considerable degree, compared with spermatogonia in the in vivo situation (von Kopylow et al. 2010). Considering this, it is difficult to compare the gene expression between whole testis tissue, even it is mostly composed of spermatogonia, and an enriched population of spermatogonia. In the present study, we hypothesize that a higher expression of germ markers (*vasa1*, *nanos2*, and *dnd1*) in the whole testis compared to the enriched spermatogonia fraction is due to the alteration that the dissociation protocol could induce on the spermatogonia expression.

Conclusions

The expression pattern of spermatogonial markers studied so far appears to be dependent on the species, the sex, and the maturing stage in teleosts. In the European eel, two *vasa* (*vasa1* and *vasa2*), one *nanos2*, and one *dnd1* genes were identified and characterized. All three markers were targeted at the testicular tissue and the enriched spermatogonia fraction. In addition, it was detected some differences in the expression among the markers, concluding that European eel *vasa1* and *vasa2* appeared to be the best early-stage germ cell markers of those tested in immature testes.

Author contribution All authors made distinct contributions to the research project. M.B.G. and J.G.H.J. performed investigation, analyses, data curation, methodology validation, animal handling, samplings, and writing of the original draft. Z.M. performed immunochemistry analyses and helped writing the original draft. X.X. and L.F. participated in ISH analyses. A.R.B. participated in qPCR analyses. V.G. and L.P. participated in animal handling and samplings. A.H., M.P., and J.F.A. performed the conceptualization, supervision, project administration, and funding acquisition. M.M. performed the supervision, investigation, analyses, animal handling and samplings, writing, reviewing, and editing the manuscript.

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Data availability Not applicable.

Declarations

Competing interests The authors declare no competing interests.

Ethical approval Experimental animals were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC) and with the recommendations given in the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 regarding the protection of animals used for scientific purposes (BOE 2013). The applied protocols were approved by the Experimental Animal Ethics Committee from the Universitat Politècnica de València and final permission was given by the local government (Generalitat Valenciana, Permit Number: 2019/VSC/PEA/0073). All efforts were made to minimize fish suffering.

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