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**Visualization and motility of primordial germ cells using green fluorescent protein
fused to 3'UTR of common carp *nanos*-related gene**

Yutaka Kawakami^{a,*}, Taiju Saito^a, Takafumi Fujimoto^{a,b}, Rie Goto-Kazeto^{a,c}, Eisuke
Takahashi^a, Shinji Adachi^d, Katsutoshi Arai^e and Etsuro Yamaha^a

^aNanae Fresh Water Laboratory, Field Science Center of Northern Biosphere, Hokkaido
University, Nanae 041-1105, Japan

^bCenter for Aquaculture and Environmental Research, Fisheries and Oceans Canada,
West Vancouver, BC V7V 1N6, Canada

^cSupport Office for Female Researchers, Administration Center, Hokkaido University,
Sapporo 060-0808, Japan

^dLaboratory of Aquaculture Biology, Faculty and Graduate School of Fisheries Science,
Hokkaido University, Hakodate 041-8611, Japan

^eLaboratory of Aquaculture Genetics and Genomics, Faculty and Graduate School of
Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

*Corresponding author: Tel: +81-138-65-2344, Fax: +81-138-65-2239

- 19 E-mail address: yutakakjp@hokudai-nanae.jp
- 20
- 21 Yutaka Kawakami: yutakakjp@hokudai-nanae.jp
- 22 Taiju Saito: taiju@hokudai-nanae.jp
- 23 Takafumi Fujimoto: motchan@fish.hokudai.ac.jp
- 24 Rie Goto-Kazeto: goto-kazeto@hokudai-nanae.jp
- 25 Eisuke Takahashi: etakahashi@hokudai-nanae.jp
- 26 Shinji Adachi: s-adachi@fish.hokudai.ac.jp
- 27 Katsutoshi Arai: araikt@fish.hokudai.ac.jp
- 28 Etsuro Yamaha: eyamaha@hokudai-nanae.jp
- 29

Abstract

Primordial germ cells (PGCs) are the only cells in developing embryos with the potential to transmit genetic information to the next generation. We previously visualized the PGCs of several teleostean embryos by injecting RNA synthesized from constructs encoding green fluorescent protein (GFP) fused to the 3'UTR of the zebrafish (*Danio rerio*) *nanos1* gene (*nos1*). However, this technique was not always suitable for visualizing PGCs in embryos from all teleost species. In this study, we compared the visualization of PGCs in common carp (*Cyprinus carpio*) embryos using two artificial constructs containing GFP fused to the 3'UTR of *nanos* from either common carp or zebrafish. Visualization was better using GFP fused to the 3'UTR of the *nanos* gene from common carp, compared with that from zebrafish. The visualized PGCs successfully migrated toward the gonadal ridge after transplantation into goldfish host embryos, suggesting that they maintained normal migratory motility. These techniques could be useful for the production of inter-specific germline chimeras using common carp donor PGCs.

Keywords: *nanos*, primordial germ cell, common carp, goldfish, germline chimera

1. Introduction

Germ cells are the only cells with the potential to transmit genetic information to the next generation. In most organisms, primordial germ cells (PGCs) are set aside from somatic cells in early development. Germplasm is defined as a substance present in the cytoplasm of the eggs and early embryos of many species that is inherited by only some cells of the embryo, and determines the fate of the PGCs (Eddy, 1975). The germplasm includes electron-dense masses of granules and fibrils, rich in RNA and protein (Wylie, 2000), which are known as polar granules in *Drosophila* (Mahowald, 1962). Many genes are required for the localization and formation of these polar granules in *Drosophila* (Nusslein-Volhard et al., 1987). The *nanos* gene, which encodes an RNA-binding zinc-finger protein, was first identified as a polar granule component in *Drosophila* (Wang and Lehmann, 1991). Although the formation of PGCs does not require *nanos* activity, the absence of maternal *nanos* results in abnormal PGC morphology and their failure to migrate to and be incorporated in the gonad (Kobayashi et al., 1996; Forbes and Lehmann, 1998; Deshpande et al., 1999).

nanos-related genes have been reported in vertebrates: *Xcat2* in *Xenopus laevis* (Mosquera et al., 1993), *nos1* and *nos2*, (Köprunner et al., 2001) in zebrafish (*Danio rerio*) and *nanos1* (Haraguchi et al., 2003), *nanos2* and *nanos3* (Tsuda et al., 2003) in

mouse (*Mus musculus*). It is known that *Xcat2* RNA is transported with the germplasm to the vegetal cortex during oogenesis, where it is associated with germinal granules in a process that depends on cis-acting elements in the 3'UTR (Kloc et al., 2000). Although mouse *nanos1* is not detected in PGCs (Haraguchi et al., 2003), *nanos2* and *nanos3* are differentially expressed in PGCs. *nanos2* is predominantly expressed in male germ cells, and the elimination of this gene results in a complete loss of spermatogonia. *nanos3* is found in migrating PGCs, and the elimination of this factor results in the complete loss of germ cells in both sexes (Tsuda et al., 2003).

Expression of *nos2* mRNA in zebrafish is first detected in the forming somites and in specific domains in the nervous system, but it is not detected in the PGCs during the first 2 days post-fertilization (dpf), while *nos1* is expressed in the germplasm and in the PGCs. Knock-down experiments have revealed that this gene may play roles in the migration and survival of PGCs during embryogenesis (Köprunner et al., 2001). Young female zebrafish *nos1* mutants contain oocytes, but fail to maintain oocyte production (Draper et al., 2007). Moreover, *nos1* 3'UTR-containing RNAs were specifically stabilized in the PGCs, but exhibited rapid degradation in somatic tissues (Köprunner et al., 2001). Using this phenomenon, PGCs in embryos can be visualized by injecting artificial RNA encoding a construct that includes green fluorescent protein (GFP) and/or

DsRed, fused to the 3'UTR of *nos1* (Köprunner et al., 2001; Weidinger et al., 2002; Slanchev et al., 2005).

We have successfully produced intra- and/or inter-specific germline chimeras in teleosts by transplanting GFP-visualized PGCs from embryos injected with GFP-*nos1* 3'UTR strand-capped mRNA (Saito et al., 2008; Higaki et al., 2010; Kawakami et al., 2010). Thus visualization of PGCs using GFP-*nos1* 3'UTR derived from the zebrafish gene could provide a powerful tool for producing inter-specific germline chimeras in teleosts using the single PGC transplantation method (Saito et al., 2008; Kawakami et al., 2010). However, the efficiency of PGC visualization varies among teleost species, and few studies have compared the efficiency of PGC visualization using GFP constructs with zebrafish *nos1*-3'UTR with that using the *nanos*-3'UTR from the species to be visualized.

Common carp (Cypriniformes, *Cyprinus carpio*) is one of the most important commercial species of fish because of its high market value. However, carp take a relatively long time to reach maturity (2 or 3 years), and it may be possible to reduce this period by using host fish with shorter pubertal periods, such as zebrafish and/or goldfish. It is therefore important to establish a suitable technique for visualizing common carp PGCs. In this study, we therefore cloned the common carp *nanos*-related

gene (*ccnanos*) and established a construct including GFP fused to the 3'UTR of *ccnanos*. We then confirmed the GFP-visualized PGCs of embryos injected with mRNA synthesized from the construct. We also analyzed the motility of the visualized PGCs after transplantation into goldfish hosts (*Carassius auratus*).

2. Materials and methods

2.1. Fishes

Immature 1-year-old female common carp, mature common carp, goldfish, and zebrafish were maintained in the Nanae Fresh Water Laboratory, Hokkaido University. Ovary samples from immature common carp were frozen in liquid nitrogen and kept at -80°C prior to the preparation of total RNA or poly(A) + RNA.

Fertilized common carp eggs and sperm were obtained in June–July 2010. Stock mature fish maintained at 13–16°C in a plastic tank were moved to a 1,000-l spawning tank, containing water at a depth of 30–50 cm and at a temperature of 20–24°C with a spawning bed, to induce ovulation by thermal treatment (males: 5–10 fish per tank, females: 2–3 fish per tank). Mature eggs and sperm were artificially stripped from the parent fish during the early morning, after checking spawning

120 behavior.

121 Mature goldfish eggs were obtained after artificial induction by hormonal
122 injection of 10 IU/g body weight of human chorionic gonadotropin (Asuka Seiyaku,
123 Tokyo, Japan). Sperm was collected from anesthetized male goldfish using
124 microcapillary tubes.

125 Artificial insemination was performed by the dry method. The eggs were
126 stripped on polyvinylidenechloride film (Saran Wrap; Asahi Chemical Industry, Tokyo,
127 Japan and/or Kure Wrap; Kureha Chemical Industry, Tokyo, Japan), inseminated with
128 the sperm, and then fertilized in fertilization solution (tap water containing 0.2% urea
129 and 0.25% NaCl). The combinations of eggs and sperm are described below.

130 Dechoriation of the eggs was carried out before blastodisc formation by a slight
131 modification of the method described by Yamaha and Yamazaki (1993). Fertilized eggs
132 were dechorionated with Ringer's solution (128 mM NaCl, 2.8 mM KCl and 1.8 mM
133 CaCl_2) containing 0.1% trypsin (Difco, Detroit, MI, USA) and 0.4% urea for about 10
134 min, and then washed with culture Ringer's solution containing 1.6% albumen.

135 Operated and control denuded embryos without chorion were cultured in separate wells
136 filled with the culture Ringer's solution for 1 day, and thereafter moved to separate
137 wells filled with second culture solution (1.8 mM CaCl_2 and 1.8 mM MgCl_2) until 4

dpf.

The developmental schedule for goldfish was staged from fertilization to the end of epiboly at 20°C based on Yamaha et al. (1999). Hatching occurred at 4 dpf. After hatching, fry were fed with *Artemia nauplii* for about 1 month, and thereafter with a commercial fish feed.

We used zebrafish from a golden-strain that had been maintained in our laboratory in a closed colony since 2006. Parental brood stock was maintained at 26–28°C under a 16-h light:8-h dark photoperiod. Embryos were dechorionated with 0.1% trypsin (Difco) in Ringer’s solution. Dechorionated embryos were then cultured at 26°C in 96-well plates individually filled with Ringer’s solution for 24 h, followed by culture in different wells filled with a second culture solution.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR) and cDNA cloning

Total RNA was extracted from common carp ovaries using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Poly(A)+ RNAs were subsequently isolated from total RNA using Oligotex-dT-30 (Takara, Otsu, Japan). Isolated RNA was denatured at 70°C for 10 min, placed on ice, and reverse transcribed with M-MLV. Second-strand

cDNA was synthesized and single-stranded overhangs were removed, using Takara's cDNA cloning system (Takara).

ccnanos cDNA fragments were amplified using sense and antisense degenerate primers designed based on a consensus sequence from the aligned deduced amino acid sequences of *nos1* from several vertebrates species, using DNASIS version 3.5 (Hitachi Software Engineering, Yokohama, Japan) (Table 1). PCR was carried out in a final volume of 50 µl containing 0.5–1 pg cDNA, 400 nM of each primer, 800 µM of each dNTP, and 2.5 U Ex Taq (Takara). PCR was carried out for 35 cycles using a Thermal Cycler Dice Gradient (Takara) under the following conditions: denaturing at 94°C for 30 s annealing at 51°C for 30 s, and extension at 72°C for 20 s. PCR products were separated by 1% agarose gel electrophoresis, and selected bands were cut out and purified using QIAprep Spin Miniprep Kit (Qiagen, Venlo, Netherlands). Purified DNA fragments were subcloned into the plasmid vector pGEM-T Easy (Promega, Madison, WI, USA), using a Ligation-Convenience Kit (Nippon Gene, Tokyo, Japan), and positive clones were sequenced using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

2.3. 5' and 3' rapid amplification of cDNA ends (RACE)-PCR

Common carp ovary was used for the construction of cDNA for RACE-PCR using a SMART-RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). For both the 3'-RACE and the 5'-RACE, nested primers (NP1, NP2 and NP3, NP4, respectively) were designed from *ccnanos* cDNA fragments (Table 1). Based on the *nanos* cDNA fragments amplified by 5'- and 3'-RACE-PCR, sense (*ccNanos*-UTR-S) and antisense (*ccNanos*-UTR-A) primers were designed for the untranslated regions (Table 1). PCR was carried out as described above (denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s).

These PCR products were sequenced following the method described above, and *ccnanos* cDNA sequences containing the entire open reading frame were obtained. *ccnanos* cDNA products representing independent, full-length PCR clones were each sequenced five times to detect PCR errors.

2.4. Sequence analysis

The GenBank accession numbers of the sequences compared with our common carp *nanos* sequence were: *ccnanos* (AB576134), zebrafish *nos1* and *nos2* (AY052376

and AI585000), Japanese medaka (*Oryzias latipes*) *nanos1* and *nanos2* (BJ074258 and EU074259), Atlantic salmon (*Salmo salar*) *nanos1* (BT050235), *Xenopus laevis* *Xcat2* (X72340), mouse (*M. musculus*) *nanos1*, *nanos2* and *nanos3* (AB095029, AB095972 and AB095973).

A phylogenetic tree was constructed using the neighbor-joining method (Saitoh and Nei, 1987). For this analysis, 1,000 bootstrap replicates were carried out using ClustalW version 1.83 (DNA Data Bank of Japan, <http://www.ddbj.nig.ac.jp/index-j.html>).

2.5. Construction of GFP-ccnanos-3'UTR vector

A GFP-containing vector specifically-expressed in PGCs, designated pCS-GFP-ccnanos-3'UTR, was constructed as follows: GFP with the SP6 promoter at a 5' upstream region, introducing a *Hind* III site at the 5' end (*Hind*III-SP6-GFP-S) and a *Xho*I site at the 3' end (*Xho*I-GFP-A), and a 343-bp fragment of the *nanos* 3'UTR with a reduced poly(A) tail introducing a *Xho*I site at the 5' end (*Xho*I-ccNanos-3'UTR-S) and an *Xba*I site at the 3' end (*Xba*I-ccNanos-3'UTR-A), were inserted at the *Hind*III and *Xba*I sites of the pCS2+ vector (Table 1).

2.6. Microinjection of mRNA

Common carp, goldfish, and zebrafish embryos were injected at the 1–4-cell stage with 80 pg of GFP-*ccnanos*-3'UTR or GFP-zebrafish *nos1*-3'UTR strand-capped mRNA, synthesized by SP6 transcription from a *NotI*-linearized plasmid using the mMACHINE mMACHINE system (Ambion, Austin, TX). Common carp and goldfish embryos injected with GFP-*ccnanos* 3'UTR were cultured at 15, 18 or 20°C in 96-well plates, homogenized at the appropriate stage corresponding to 10–15 or 15–20 somites, and GFP-labeled cells were then obtained. Zebrafish embryos were then cultured at 26°C in 96-well plates, and somatic embryos with GFP-labeled PGCs were counted after 24 h.

2.7. Whole-mount in situ hybridization (WISH) using vasa probe

Dechorionated embryos were fixed with 4% paraformaldehyde in phosphate-buffered saline overnight at 4°C at each stage during development. Fixed embryos were stored in 100% methanol at -20°C. The identity of the GFP-labeled cells in common carp embryos as PGCs was confirmed by comparing the locations of the GFP-labeled cells with *vasa* (GenBank accession number: AF479820) mRNA-expressing cells, using WISH, according to a modification of the method

described by Otani et al. (2002) and Saito et al. (2006). GFP-labeled embryos were fixed overnight in 4% paraformaldehyde at 4°C. Common carp *vasa* mRNA in embryos was analyzed by amplifying a 0.5-kbp cDNA fragment including the 3'-UTR by PCR, using primers that introduced an SP6-promoter site at the 3' end (*Vasa-Probe-S* and *SP6-Vasa-Probe-A* in Table 1). The cDNA fragment was purified by phenol/chloroform/isoamyl alcohol and ethanol precipitation. Antisense digoxigenin (DIG)-labeled RNA *vasa* probes were applied using 100–200 ng *vasa* cDNA fragment and a DIG RNA labeling kit (Roche, Mannheim, Germany).

2.8. PGC transplantation

Donor embryos injected with GFP-*ccnanos*-3'UTR or GFP-zebrafish *nos1*-3'UTR strand-capped mRNA were incubated at 15, 18 or 20°C. Donors with visualizable PGCs (Fig. 1A) were dissociated into single cells using 0.25% sodium citrate in Ringer's solution. PGCs from dissociated cells were transplanted into host goldfish blastulae, according to the method of Kawakami et al. (2010). The transplantation procedure took around 2 h, during which one isolated PGC was picked up with a glass micro-needle under a stereomicroscope (Fig. 1B) and transplanted into the marginal region of the blastodisc of each goldfish blastula (Fig. 1C). Chimeric

embryos were observed and photographed using a fluorescence stereomicroscope,
model MZ16F (Leica Microsystems, Inc., Bannockburn, IL, USA).

3. Results and discussion

The nucleotide sequence of the cloned cDNA in this study is shown in Fig. 2A.

The common carp cDNA contains an open reading frame of 462 bp, encoding 154 amino acid residues. All the cysteines in both proteins are found within the homology region and in the same positions. The C-terminal region of this gene contains two unusual Cys-Cys-His-Cys (CCHC) motifs (underlined in Fig. 2A), which each bind one equivalent of zinc with high affinity in *Drosophila* (Curtis et al., 1997). The CCHC motifs characteristic of the *nanos* (*nos*) gene can be represented as:

Cys-X2-Cys-X12-His-X10-Cys-X7-Cys-X2-Cys-X7-His-X4-Cys (Fig. 2B) (Köprunner et al., 2001; Mosquera et al., 1993). When the amino acid sequence corresponding to the common carp gene was compared with that of other known *nanos* (*nos*) genes, the protein showed the highest homology with zebrafish *nos1* (Fig. 3). Partial sequences for the zebrafish *nos2* gene have been reported, and include a zinc-finger domain with high homologies to medaka *nanos1* and Atlantic salmon *nanos1* (see Fig. 2B). Thus the common carp gene cloned in this study appeared to be a *nanos* gene, most closely

264 related to zebrafish *nos1*.

265 Köprunner et al. (2001) developed a PGC-labeling technique using RNA
266 synthesized from a construct including GFP fused to the 3'UTR of zebrafish *nos1*. This
267 technique was able to produce germline chimeras using donor GFP-visualized PGCs as
268 follows: 50–100 GFP-expressing cells from the marginal region of a mid-blastula stage
269 zebrafish injected with GFP-zebrafish *nos1*-3'UTR mRNA were picked up and
270 transplanted into host embryos. The GFP-zebrafish *nos1*-3UTR mRNA was rapidly
271 degraded in the somatic cells, but was stably maintained in PGCs, leading to GFP
272 protein expression specifically in PGCs. Moreover, the GFP-visualized PGCs migrated
273 to the gonadal ridge of the host, and donor PGC-derived gametes were induced. This
274 technique has been utilized for labeling zebrafish PGCs (Weidinger et al., 2002;
275 Slanchev et al., 2005; Higaki et al., 2010; Kawakami et al., 2010). In the current study,
276 GFP-visualized cells were confirmed at the somite stage in common carp embryos
277 injected with mRNA synthesized from a GFP-*ccnanos*-3'UTR construct (Fig. 1A). *vasa*
278 RNA has been demonstrated to be a germ cell marker in teleosts, including zebrafish
279 (Raz, 2003), gibel carp (*Carassius auratus gibelio*) (Xu et al., 2005), Nile tilapia
280 (*Oreochromis niloticus*) (Kobayashi et al., 2000), and medaka (Shinomiya et al., 2002).
281 *vasa* protein expression has been analyzed during zebrafish (Baat et al., 2000) and

282 gibel carp (Xu et al., 2005) embryogenesis. WISH demonstrated that common carp cells
283 labeled with GFP-ccnanos-3'UTR mRNA colocalized with cells expressing *vasa*
284 mRNA (Fig. 1D and 1E), confirming the identity of the GFP-labeled cells as PGCs.

285 In zebrafish, the mechanisms underlying germline-specific *nos1* expression
286 have been reported to involve the interaction of the microRNA, miR-430 and the
287 *nos1*-3'UTR. Although the miR-430 target mRNAs are equally susceptible to repression
288 in somatic cells and PGCs (Giraldez et al., 2005, 2006), the deadenylation element in
289 the *nos1*-3'UTR compensates for miR-430-mediated repression in PGCs (Mishima et al.,
290 2006). Mechanisms such as this are likely to be highly conserved among teleost fish,
291 and PGCs in several teleost embryos have been visualized by injection of
292 GFP-zebrafish *nos1*-3'UTR, e.g., in Clupeiformes (herring, *Clupea pallasii*),
293 Cypriniformes (pearl danio, *Danio albolineatus*; goldfish, *C. auratus*; loach, *Misgurnus*
294 *anguillicaudatus*), Beloniformes (medaka) and Perciformes (ice goby, *Leucopsarion*
295 *petersii*) (Saito et al., 2006; Yamaha et al., 2010). The results of this study demonstrated
296 that PGCs in common carp embryos could also be visualized using
297 GFP-ccnanos-3'UTR, suggesting that this 3'UTR has the same function as the zebrafish
298 *nos1*-3'UTR. However, injection of the artificial GFP mRNA fused with the
299 ccnanos-3'UTR visualized both common carp and goldfish PGCs more effectively than

injection of the zebrafish *nos1*-3'UTR (see Table 2). The reason for this difference is currently unclear, though the new method represents a powerful tool, with potential applications related to the visualization of common carp and/or goldfish PGCs.

When these GFP-visualized cells were isolated and transplanted into goldfish blastulae, they migrated to the gonadal ridge at 4 dpf (Fig. 1F and 1G). Migration to the gonadal ridge is one the characteristics of PGCs (Saito et al., 2008; Kawakami et al., 2010), thus supporting the identification of the GFP-visualized cells as PGCs. PGCs from earlier-stage embryos were more likely to migrate to the host gonadal ridge than those from more advanced developmental stages (Table 3). Thus PGCs isolated from earlier developmental stages are likely to be more successful in producing germline chimeras, as in zebrafish (Kawakami et al. 2010; Saito et al., 2010). The replacement of germ cells requires removal of the host endogenous PGCs. PGC differentiation was blocked by injection of a *dead end* (*dnd*) antisense morpholino oligonucleotide (MO) (Ciruna et al., 2002; Weidinger et al., 2003), and germline chimeras using zebrafish hosts have successfully been achieved by injection of a *dnd* antisense (Saito et al., 2008; Higaki et al., 2010; Kawakami et al. 2010). Further studies are needed to develop techniques for asexualizing goldfish to use as hosts.

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Figure Legends

Fig. 1. The visualization and migration of green fluorescent protein (GFP)-labeled cells (primordial germ cells: PGCs) in common carp embryos using GFP-*ccnanos*-3'UTR mRNA. (A) Common carp 10–15-somite embryo. Arrows indicate GFP-labeled cells. (B) GFP-labeled cells isolated from common carp 15–20-somite embryo. (C) Transplantation of GFP-labeled cells from common carp 15–20-somite embryo to goldfish host. (D) GFP-labeled PGCs. (E) *vasa in situ* hybridization. Note that the same cells are identified by GFP-labeling and *vasa in situ* hybridization. (F, G) Migration of donor-derived PGCs in host. Host at 4 days post-fertilization. a, b, c, d and e were estimated to represent the same signals, respectively. Bars, 500 μ m.

Fig. 2. The common carp *nanos* gene encodes a *nanos*-like zinc-finger protein. (A) Nucleotide sequence of common carp *nanos* gene, with the predicted amino acid sequence indicated in single-letter code below the open reading frame. The characteristic C-terminal CCHC CCHC zinc-finger domain with its conserved residues is underlined. (B) Comparison of the amino acid sequence of the zinc-finger domain among *nanos*-like proteins from common carp (*nanos*), zebrafish (1: *nos1*, 2: *nos2*), and medaka (1: *nanos1*, 2: *nanos2*). The amino acid residues comprising the conserved

CCHC zinc finger domain are labeled in gray. 3'UTR sequences of 504–846 bps were used for constructs in this study.

Fig. 3. Phylogenetic tree of eight vertebrate *nanos* (*nos*). Branch lengths are proportional to genetic distance. One thousand boot-strap replicates were performed, and values supporting nodes are shown next to inner branches. Carp; common carp *nanos* (AB576134), Zebrafish; zebrafish *nos1* (AY052376), Medaka1; medaka *nanos1* (BJ074258), Medaka2; medaka *nanos2* (EU074259), Atl.salmon; Atlantic salmon *nanos1* (BT050235), *Xenopus*; *Xenopus laevis* *Xcat2* (X72340), Mouse1; mouse *nanos1* (AB095029), Mouse2; mouse *nanos2* (AB095972) and Mouse3; mouse *nanos3* (AB095973).

Table 1 Primers used for cloning, PCR, vector construction and whole mount *in situ* hybridization analysis of common carp *nanos* (*ccnanos*).

Name	Primer sequence	Nucleotide numbers corresponding to the annealing site (Fig. 2)
<i>ccNanos</i> -DP-S	5'-TGGAVSGACTACHTGGGTCT-3'	
<i>ccNanos</i> -DP-A	5'-CCNGTGGCNCRCAYAGNGGACA -3'	
NP1	5'-GGACCCTGACGACGGAGAAAAGAGA-3'	223–247 bp
NP2	5'-TTCTGCAGCTTCTGCAAACACAACG-3'	294–318 bp
NP3	5'-GCGCGGTCTTTTAAGTAGTGCGAGGT-3'	338–364 bp
NP4	5'-TGCAGAACTTCTTCTCGGATGGGTG-3'	275–300 bp
<i>ccNanos</i> -UTR-S	5'-TCAGCGCGTGCTTCACGGTTC-3'	1–21 bp
<i>ccNanos</i> -UTR-A	5'-AGCATTAAAAGCGTTTATATATTTACCAC-3'	817–846 bp
<i>Hind</i> III-SP6-GFP-S	5'-AAGCTTATGGTGAGCAAGGGCGAGGA -3'	
<i>Xho</i> I-GFP-A	5'-CTCGAGTTACTTGTACAGCTCGTCCA-3'	
<i>Xho</i> I- <i>ccNanos</i> -3'UTR-S	5'-CTCGAGACCGGACGTTTCTGAACCG-3'	
<i>Xba</i> I- <i>ccNanos</i> -3'UTR-A	5'-TCTAGAAGCATTAAAAGCGTTTATATATTT-3'	
<i>Vasa</i> -Probe-S	5'-AGTGTCCAGTGCTGGTGGCCACG -3'	
SP6- <i>Vasa</i> -Probe-A	5'-ATTTAGGTGACACTATAGAACATTTTAACAAACATGCTG -3'	

DPs (degenerate primers), primers for amplification of *ccnos1* fragments; *ccnanos*-UTRs (untranslated regions), sense and antisense primers for the sequencing of *ccnanos* containing the open reading frame.

Table 2 Appearance of somatic embryos with GFP-labeled PGCs injected with GFP-*ccnanos*-3'UTR or GFP-zebrafish *nos1*-3'UTR mRNA at 1–4 cells.

Construct	Injected embryo	Experiments	No. of injected embryos	No. of normal embryos	No. of embryos with GFP-labeled PGCs (%)
GFP- <i>ccnanos</i> -3'UTR	Common carp	1	101	72	53 (73.6)
		2	95	80	46 (57.5)
		3	91	78	38 (50.7)
	Zebrafish	1	40	36	33 (91.7)
		2	35	31	31 (100.0)
		3	61	58	56 (96.6)
	Goldfish	1	38	37	23 (62.2)
		2	42	37	25 (67.6)
		3	54	53	31 (58.5)
GFP-zebrafish <i>nos1</i> -3'UTR	Common carp	1	48	37	2 (5.4)
		2	50	40	4 (10.0)
	Goldfish	1	44	43	10 (23.3)
		2	47	46	11 (23.9)
		3	53	50	8 (16.0)

Table 3 Localization of transplanted PGCs from common carp and/or goldfish embryos in host goldfish at 4 days post-transplantation at 20°C

Trial no.	Donor	Stage (donor)	No. of manipulated embryos	No. of normal embryos (%)	No. of embryos with PGCs at		
					Total (%)	Gonadal ridge (%)	Ectopic (%)
1	Common Carp	10–15 somite	158	148 (93.7)	140 (88.6)	44 (27.8)	96 (60.8)
2	Common Carp	15–20 somite	33	21 (63.6)	17 (51.5)	3 (9.1)	14 (42.4)
3	Common Carp	15–20 somite	75	34 (45.3)	19 (25.3)	8 (10.7)	11 (14.7)
4	Common Carp	15–20 somite	56	48 (85.7)	46 (82.1)	13 (23.2)	33 (58.9)
5	Common Carp	15–20 somite	50	22 (44.0)	22 (44.0)	5 (10.0)	17 (34.0)
Control							
6	Goldfish	10-15 somite	24	19 (79.2)	15 (62.5)	7 (29.2)	8 (33.3)
7	Goldfish	10-15 somite	10	10 (100.0)	10 (100.0)	4 (40.0)	6 (60.0)
8	Goldfish	15-20 somite	20	9 (45.0)	8 (40.0)	3 (15.0)	5 (25.0)

PGCs of donor embryos were labeled by injecting GFP-*ccnanos*-3'UTR mRNA

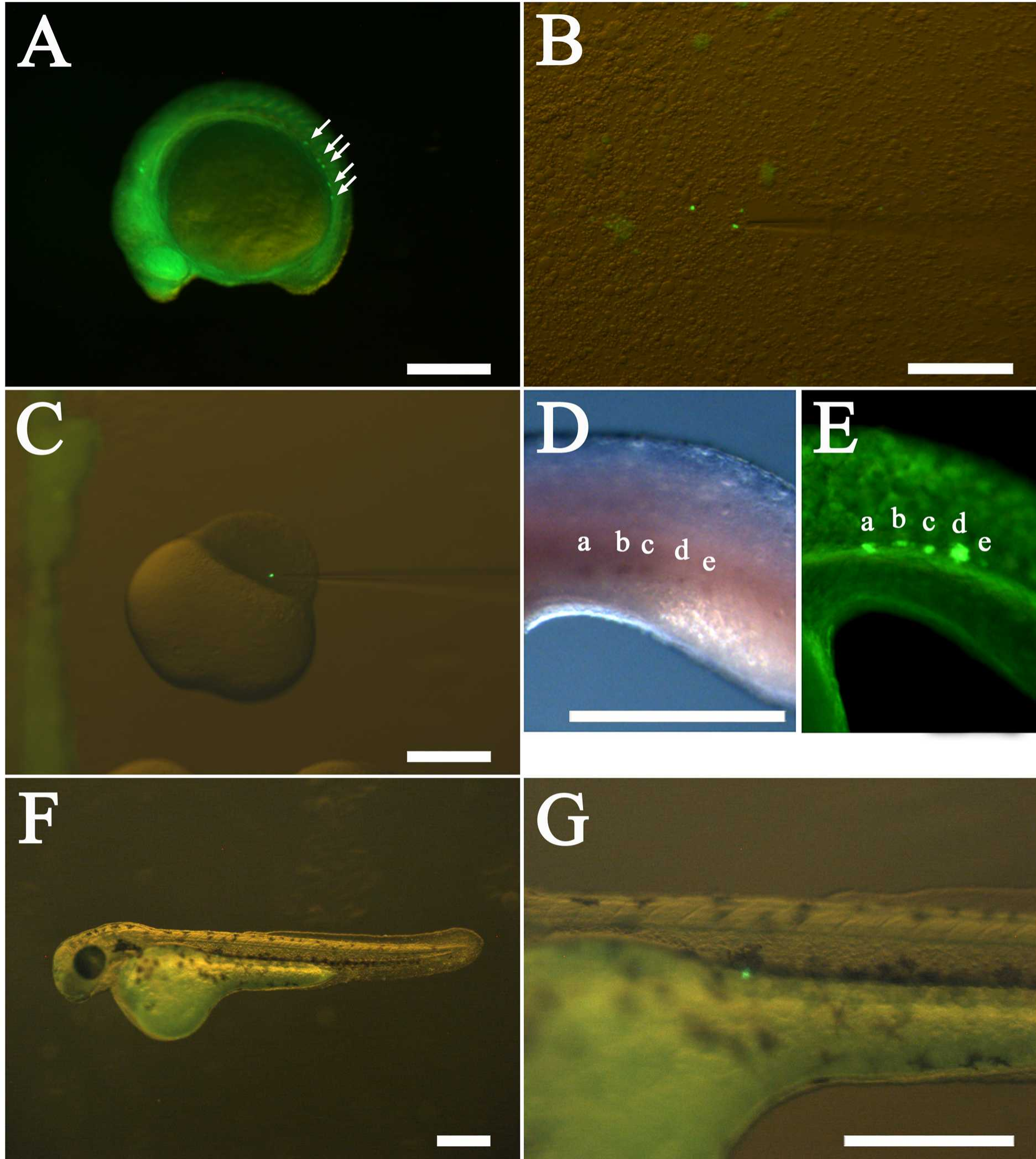


Fig.1

A	TCAGCGCGTGCTTCACGGTTCTGTCGCACCTGGCGGATGTC																		41					
	ATG	GCA	TTT	TCT	CTT	CTC	CAT	TAC	ATC	TTG	TCG	GCT	CAT	GGA	TCT	ATG	GAG	TCT	ACA	98	19			
	M	A	F	S	L	L	H	Y	I	L	S	A	H	G	S	M	E	S	T					
	AAT	CAG	TAC	TTT	CAG	CCC	TGG	AAG	GAT	TAC	ATG	GGT	CTG	GCA	GAC	ATG	ATC	AGA	GGC	155		38		
	N	Q	Y	F	Q	P	W	K	D	Y	M	G	L	A	D	M	I	R	G					
	ATG	CAG	CGG	CCA	GCA	GAG	CAG	CCA	GAC	GCG	CTC	CTG	GAG	TCT	CCA	AGC	GGC	CCG	ACG	212			57	
	M	Q	R	P	A	E	Q	P	D	A	L	L	E	S	P	S	G	P	T					
	CGA	GCG	CAC	GGG	ACC	CTG	ACG	ACG	GAG	AAA	AGA	GAC	CCG	GAA	CGC	GGC	AAG	AGC	ACC	269				76
	R	A	H	G	T	L	T	T	E	K	R	D	P	E	R	G	K	S	T					
	CGC	AGC	AAC	CCA	TCC	GAG	AAG	AAG	TTC	TGC	AGC	TTC	TGC	AAA	CAC	AAC	GGA	GAG	ACC	326				
R	S	N	P	S	E	K	K	F	C	S	F	C	K	H	N	G	E	T						
GAA	GCC	GTG	TTC	ACC	TCG	CAC	TAC	TTA	AAA	GAC	CGC	GCT	GGA	GAC	GTG	ACG	TGC	CCG	383	114				
E	A	V	F	T	S	H	Y	L	K	D	R	A	G	D	V	T	C	P						
TAC	TTG	AGC	CAG	TAC	GTG	TGT	CCC	CTG	TGC	GGA	GCC	ACC	GGG	GCC	AAA	GCG	CAC	ACC	440		133			
Y	L	S	Q	Y	V	C	P	L	C	G	A	T	G	A	K	A	H	T						
AAG	AGA	TTC	TGC	CCG	CTC	GTG	GAC	AAA	ACC	TAC	AGC	TCC	GTG	TAC	GCC	AAA	TCA	ACA	497			152		
K	R	F	C	P	L	V	D	K	T	Y	S	S	V	Y	A	K	S	T						
TGG	TGA	ACCGGACGTTTCTGAACCGAAGAAGATCTGGCCACGGTATAACAAGAAGGACGTTTTTTACGAGTAGTT																570	153					
W	*																							
TTAATATTCCAGTTTTTAATTGTTCAATCCATAATGGCTTGTGTGTAAGTTTGCATGCATGTGTGCTTTTTTTTTTTT																							645	
TTGGTGTTTGATTTTTTGCACGGTTTTTTTGTCTTCCTCTTGTGTGCAGTGGTGTGTTTTTCACTCTAACAAACTT																							720	
GTACACAAGCCAGTTGGCTTGCTACAGGTGCAACCACGTGTGAAGTAGCGCTTTCTTGTTAATTTTACTAAAAAA																				795				
AAAGTATCTTGTGATTAATCTGTGGTGAAATATATAAACGCTTTTAAATGCT																				846				

B	Common Carp	CSFCKHNGETEAVFTSHY	LKDRAGDVTCPYLSQYVCPLCGATGAKAHTKRFC
	Zebrafish 1	CSFCKHNGETEAVYTSHY	LKNRDGDVMCPYLRQYKCPLCGATGAKAHTKRFC
	Zebrafish 2	CVFCRNNGAPEEEVYGSH	VLKTPDGRVVCPILRAYTCXLCSANGDNAHTIXYC
	Medaka 1	CVFCRNNGAPEEEVYGTH	ILKTGEGRVLCPILRAYTCPLCSANGDNAHTIKYC
	Medaka 2	CSFCRHNGESEMVYRSH	WLKNQKGDVLCPYLRQYVCPLCGATGAKAHTKRFC
	Atl.Salmon1	CVFCRNNGAPEEEVYGSH	VLKTPDGGVVCPILRAYTCPLCSANGDNAHTIKYC

Fig.2

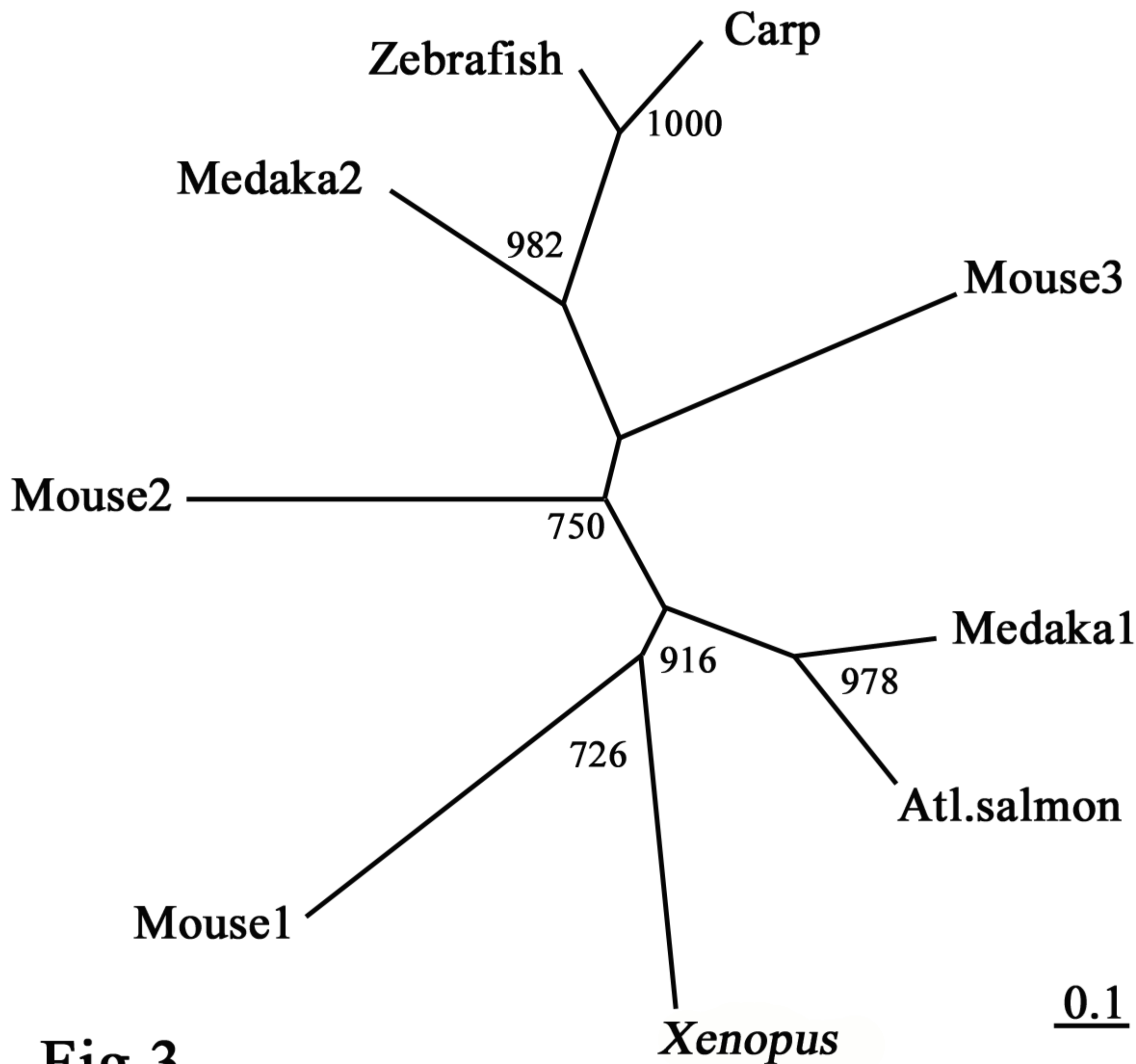


Fig.3