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

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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*) *nanos*1 gene (*nos*1). 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: *Xcat*2 in *Xenopus laevis* (Mosquera et al., 1993), *nos*1and *nos*2, (Köprunner et al., 2001) in zebrafish (*Danio rerio*) and *nanos*1 (Haraguchi et al., 2003), *nanos*2 and *nanos*3 (Tsuda et al., 2003) in

mouse (*Mus musculus*). It is known that *Xcat*2 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 *nanos*1 is not detected in PGCs (Haraguchi et al., 2003), *nanos*2 and *nanos*3 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 *nos*2 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 *nos*1 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 *nos*1 mutants contain oocytes, but fail to maintain oocyte production (Draper et al., 2007). Moreover, *nos*1 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 *nos*1 (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-*nos*1 3’UTR strand-capped mRNA (Saito et al., 2008; Higaki et al., 2010; Kawakami et al., 2010). Thus visualization of PGCs using GFP-*nos*1 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 *nos*1-3’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 (cc*nanos*) and established a construct including GFP fused to the 3'UTR of cc*nanos*. 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

behavior.

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

Artificial insemination was performed by the dry method. The eggs were stripped on polyvinylidenechloride film (Saran Wrap; Asahi Chemical Industry, Tokyo, Japan and/or Kure Wrap; Kureha Chemical Industry, Tokyo, Japan), inseminated with the sperm, and then fertilized in fertilization solution (tap water containing 0.2% urea and 0.25% NaCl). The combinations of eggs and sperm are described below. Dechorionation of the eggs was carried out before blastodisc formation by a slight modification of the method described by Yamaha and Yamazaki (1993). Fertilized eggs were dechorionated with Ringer's solution (128 mM NaCl, 2.8 mM KCl and 1.8 mM $CaCl_2$) containing 0.1% trypsin (Difco, Detroit, MI, USA) and 0.4% urea for about 10 min, and then washed with culture Ringer's solution containing 1.6% albumen. Operated and control denuded embryos without chorion were cultured in separate wells filled with the culture Ringer's solution for 1 day, and thereafter moved to separate 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

156 cDNA was synthesized and single-stranded overhands were removed, using Takara's
157 cDNA cloning system (Takara).
158 cc*nanos* cDNA fragments were amplified using sense and antisense degenerate
159 primers designed based on a consensus sequence from the aligned deduced amino acid
160 sequences of *nos*1 from several vertebrates species, using DNASIS version 3.5 (Hitachi
161 Software Engineering, Yokohama, Japan) (Table 1). PCR was carried out in a final
162 volume of 50 µl containing 0.5–1 pg cDNA, 400 nM of each primer, 800 µM of each
163 dNTR, and 2.5 U Ex Taq (Takara). PCR was carried out for 35 cycles using a Thermal
164 Cycler Dice Gradient (Takara) under the following conditions: denaturing at 94°C for
165 30 s annealing at 51°C for 30 s, and extension at 72°C for 20 s. PCR products were
166 separated by 1% agarose gel electrophoresis, and selected bands were cut out and
167 purified using QIAprep Spin Miniprep Kit (Qiagen, Venlo, Netherlands). Purified DNA
168 fragments were subcloned into the plasmid vector pGEM-T Easy (Promega, Madison,
169 WI, USA), using a Ligation-Convenience Kit (Nippon Gene, Tokyo, Japan), and
170 positive clones were sequenced using Big Dye Terminator v3.1 Cycle Sequencing Kit
171 (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 3100 Genetic Analyzer
172 (Applied Biosystems).
173

174 *2.3. 5’ and 3’ rapid amplification of cDNA ends (RACE)-PCR*

175

176 Common carp ovary was used for the construction of cDNA for RACE-PCR
177 using a SMART-RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). For
178 both the 3’-RACE and the 5’-RACE, nested primers (NP1, NP2 and NP3, NP4,
179 respectively) were designed from cc*nanos* cDNA fragments (Table 1). Based on the
180 *nanos* cDNA fragments amplified by 5’- and 3’-RACE-PCR, sense (cc*Nanos*-UTR-S)
181 and antisense (cc*Nanos*-UTR-A) primers were designed for the untranslated regions
182 (Table 1). PCR was carried out as described above (denaturation at 94°C for 30 s,
183 annealing at 56°C for 30 s, and extension at 72°C for 30 s).
184 These PCR products were sequenced following the method described above,
185 and cc*nanos* cDNA sequences containing the entire open reading frame were obtained.
186 cc*nanos* cDNA products representing independent, full-length PCR clones were each
187 sequenced five times to detect PCR errors.

188

189 *2.4. Sequence analysis*

190 The GenBank accession numbers of the sequences compared with our common
191 carp *nanos* sequence were: cc*nanos* (AB576134), zebrafish *nos*1 and *nos*2 (AY052376

192 and AI585000), Japanese medaka (*Oryzias latipes*) *nanos*1 and *nanos*2 (BJ074258 and
193 EU074259), Atlantic salmon (*Salmo salar*) *nanos*1 (BT050235), *Xenopus laevis Xcat*2
194 (X72340), mouse (*M. musculus*) *nanos*1, *nanos*2 and *nanos*3 (AB095029, AB095972
195 and AB095973).

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

200

201 *2.5. Construction of GFP-cc*nanos-*3'UTR vector*

202 A GFP-containing vector specifically-expressed in PGCs, designated
203 pCS-GFP-cc*nanos*-3'UTR, was constructed as follows: GFP with the SP6 promoter at a
204 5' upstream region, introducing a *Hind* III site at the 5' end (*Hind*III-SP6-GFP-S) and a
205 *Xho*I site at the 3' end (*Xho*I-GFP-A), and a 343-bp fragment of the *nanos* 3'UTR with
206 a reduced poly(A) tail introducing a *Xho*I site at the 5' end (*Xho*I-cc*Nanos*-3'UTR-S)
207 and an *Xba*I site at the 3' end (*Xba*I-cc*Nanos*-3'UTR-A), were inserted at the *Hind*III
208 and *Xba*I sites of the pCS2+ vector (Table 1).

209

210 *2.6. Microinjection of mRNA*

211 Common carp, goldfish, and zebrafish embryos were injected at the 1–4-cell
212 stage with 80 pg of GFP-cc*nanos*-3'UTR or GFP-zebrafish *nos*1-3'UTR strand-capped
213 mRNA, synthesized by SP6 transcription from a *Not*I-linearized plasmid using the
214 mMESSAGE mMACHINE system (Ambion, Austin, TX). Common carp and goldfish
215 embryos injected with GFP-cc*nanos* 3'UTR were cultured at 15, 18 or 20°C in 96-well
216 plates, homogenized at the appropriate stage corresponding to 10–15 or 15–20 somites,
217 and GFP-labeled cells were then obtained. Zebrafish embryos were then cultured at
218 26°C in 96-well plates, and somatic embryos with GFP-labeled PGCs were counted
219 after 24 h.

220

221 *2.7. Whole-mount* in situ *hybridization (WISH) using* vasa *probe*

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

228 described by Otani et al. (2002) and Saito et al. (2006). GFP-labeled embryos were
229 fixed overnight in 4% paraformaldehyde at 4°C. Common carp *vasa* mRNA in embryos
230 was analyzed by amplifying a 0.5-kbp cDNA fragment including the 3'-UTR by PCR,
231 using primers that introduced an SP6-promoter site at the 3' end (*Vasa*-Probe-S and
232 SP6-*Vasa*-Probe-A in Table 1). The cDNA fragment was purified by
233 phenol/chloroform/isoamyl alcohol and ethanol precipitation. Antisense digoxigenin
234 (DIG)-labeled RNA *vasa* probes were applied using 100–200 ng *vasa* cDNA fragment
235 and a DIG RNA labeling kit (Roche, Mannheim, Germany).
236
237 *2.8. PGC transplantation*
238 Donor embryos injected with GFP-cc*nanos*-3'UTR or GFP-zebrafish
239 *nos*1-3'UTR strand-capped mRNA were incubated at 15, 18 or 20°C. Donors with
240 visualizable PGCs (Fig. 1A) were dissociated into single cells using 0.25% sodium
241 citrate in Ringer's solution. PGCs from dissociated cells were transplanted into host
242 goldfish blastulae, according to the method of Kawakami et al. (2010). The
243 transplantation procedure took around 2 h, during which one isolated PGC was picked
244 up with a glass micro-needle under a stereomicroscope (Fig. 1B) and transplanted into
245 the marginal region of the blastodisc of each goldfish blastula (Fig. 1C). Chimeric

246 embryos were observed and photographed using a fluorescence stereomicroscope,
247 model MZ16F (Leica Microsystems, Inc., Bannockburn, IL, USA).
248
249 **3. Results and discussion**
250 The nucleotide sequence of the cloned cDNA in this study is shown in Fig. 2A.
251 The common carp cDNA contains an open reading frame of 462 bp, encoding 154
252 amino acid residues. All the cysteines in both proteins are found within the homology
253 region and in the same positions. The C-terminal region of this gene contains two
254 unusual Cys-Cys-His-Cys (CCHC) motifs (underlined in Fig. 2A), which each bind one
255 equivalent of zinc with high affinity in *Drosophila* (Curtis et al., 1997). The CCHC
256 motifs characteristic of the *nanos* (*nos*) gene can be represented as:
257 Cys-X2-Cys-X12-His-X10-Cys-X7-Cys-X2-Cys-X7-His-X4-Cys (Fig. 2B) (Köprunner
258 et al., 2001; Mosquera et al., 1993). When the amino acid sequence corresponding to the
259 common carp gene was compared with that of other known *nanos* (*nos*) genes, the
260 protein showed the highest homology with zebrafish *nos*1 (Fig. 3). Partial sequences for
261 the zebrafish *nos*2 gene have been reported, and include a zinc-finger domain with high
262 homologies to medaka *nanos*1 and Atlantic salmon *nanos*1 (see Fig. 2B). Thus the
263 common carp gene cloned in this study appeared to be a *nanos* gene, most closely

related to zebrafish *nos*1.

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

282 gibel carp (Xu et al., 2005) embryogenesis. WISH demonstrated that common carp cells
283 labeled with GFP-cc*nanos*-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 *nos*1 expression
286 have been reported to involve the interaction of the microRNA, miR-430 and the
287 *nos*1-3'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 *nos*1-3'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 *nos*1-3'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-cc*nanos*-3'UTR, suggesting that this 3'UTR has the same function as the zebrafish
298 *nos*1-3'UTR. However, injection of the artificial GFP mRNA fused with the
299 cc*nanos*-3'UTR visualized both common carp and goldfish PGCs more effectively than

injection of the zebrafish *nos*1-3'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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425 **Figure Legends**

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

435

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

443 CCHC zinc finger domain are labeled in gray. 3'UTR sequences of 504–846 bps were
444 used for constructs in this study.
445
446 **Fig. 3.** Phylogenetic tree of eight vertebrate *nanos* (*nos*). Branch lengths are
447 proportional to genetic distance. One thousand boot-strap replicates were performed,
448 and values supporting nodes are shown next to inner branches. Carp; common carp
449 *nanos* (AB576134), Zebrafish; zebrafish *nos*1 (AY052376), Medaka1; medaka *nanos*1
450 (BJ074258), Medaka2; medaka *nanos*2 (EU074259), Atl.salmon; Atlantic salmon
451 *nanos*1 (BT050235), *Xenopus*; *Xenopus laevis Xcat*2 (X72340), Mouse1; mouse *nanos*1
452 (AB095029), Mouse2; mouse *nanos*2 (AB095972) and Mouse3; mouse *nanos*3
453 (AB095973).
454
455

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

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

DPs (degenerate primers), primers for amplification of cc*nos*1fragments; cc*nanos*-UTRs (untranslated regions), sense and antisense primers for the sequencing of cc*nanos* containing the open reading frame.

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

Construct	Injected embryo	Experiments	No. of injected embryos	No. of normal embryos	No. of embryos with with GFP-labeled PGCs (%)
GFP-cc*nanos*-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 *nos*1-3'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-cc*nanos*-3’UTR mRNA

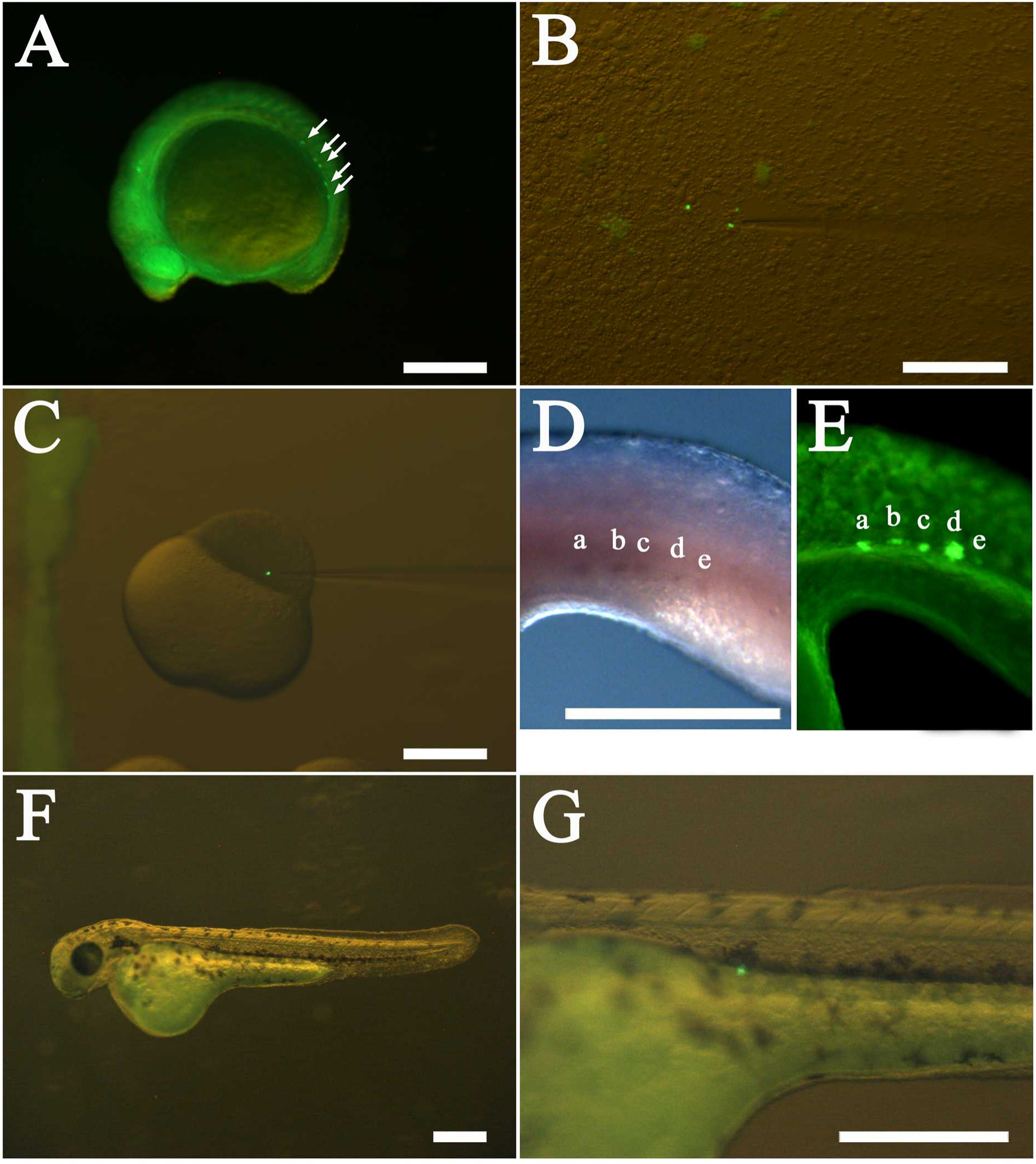

Fig.1

A

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                                                                TCAGCGCGTGCTTCACGGTTCTGTCGCACCTGGCGGATGTC   41

ATG GCA TTT TCT CTT CTC CAT TAC ATC TTG TCG GCT CAT GGA TCT ATG GAG TCT ACA   98
 M   A   F   S   L   L   H   Y   I   L   S   A   H   G   S   M   E   S   T      19

AAT CAG TAC TTT CAG CCC TGG AAG GAT TAC ATG GGT CTG GCA GAC ATG ATC AGA GGC  155
 N   Q   Y   F   Q   P   W   K   D   Y   M   G   L   A   D   M   I   R   G      38

ATG CAG CGG CCA GCA GAG CAG CCA GAC GCG CTC CTG GAG TCT CCA AGC GGC CCG ACG  212
 M   Q   R   P   A   E   Q   P   D   A   L   L   E   S   P   S   G   P   T      57

CGA GCG CAC GGG ACC CTG ACG ACG GAG AAA AGA GAC CCG GAA CGC GGC AAG AGC ACC  269
 R   A   H   G   T   L   T   T   E   K   R   D   P   E   R   G   K   S   T      76

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      95

GAA GCC GTG TTC ACC TCG CAC TAC TTA AAA GAC CGC GCT GGA GAC GTG ACG TGC CCG  383
 E   A   V   F   T   S   H   Y   L   K   D   R   A   G   D   V   T   C   P     114

TAC TTG AGC CAG TAC GTG TGT CCC CTG TGC GGA GCC ACC GGG GCC AAA GCG CAC ACC  440
 Y   L   S   Q   Y   V   C   P   L   C   G   A   T   G   A   K   A   H   T     133

AAG AGA TTC TGC CCG CTC GTG GAC AAA ACC TAC AGC TCC GTG TAC GCC AAA TCA ACA  497
 K   R   F   C   P   L   V   D   K   T   Y   S   S   V   Y   A   K   S   T     152

TGG TGA ACCGGACGTTTCTGAACCGAAGAAGATCTGGCCACGGTATACAAGAAGGACGTTTTTACGAGTAGTT  570
 W   *                                                                         153

TTAATATTCCAGTTTTAATTGTTCAATCCATAATGGCTTGTGTGTAAGTTTGCATGCATGTGTGCTTTTTTTTTT  645

TTGGTGTTTGATTTTTGCACGGTTTTTTGTCTTCCTCTTGTGTGCAGTGGTGTTGTTTTTCACTCTAACAAACTT  720

GTACACAAGCCAGTTGGCTTGCTACAGGTGCAACCACGTGTGAACTAGCGCTTTCTTGTTAATTTTACTAAAAAA  795

AAAGTATCTTGTGATTAATCTGTGGTGAAATATATAAACGCTTTTAATGCT                           846
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B

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Common Carp     CSFCKHNGETEAVFTSHYLKDRAGDVTCPYLSQYVCPLCGATGAKAHTKRFC
Zebrafish 1     CSFCKHNGETEAVYTSHYLKNRDGDVMCPYLRQYKCPLCGATGAKAHTKRFC
Zebrafish 2     CVFCRNNGAPEEVYGSHVLKTPDGRVVCPILRAYTCXLCSANGDNAHTIXYC
Medaka 1        CVFCRNNGAPEEVYGTHILKTGEGRVLCPILRAYTCPLCSANGDNAHTIKYC
Medaka 2        CSFCRHNGESEMVYRSHWLKNQKGDVLCPYLRQYVCPLCGATGAKAHTKRFC
Atl.Salmon1     CVFCRNNGAPEEVYGSHVLKTPDGGVVCPILRAYTCPLCSANGDNAHTIKYC
```

Fig.2

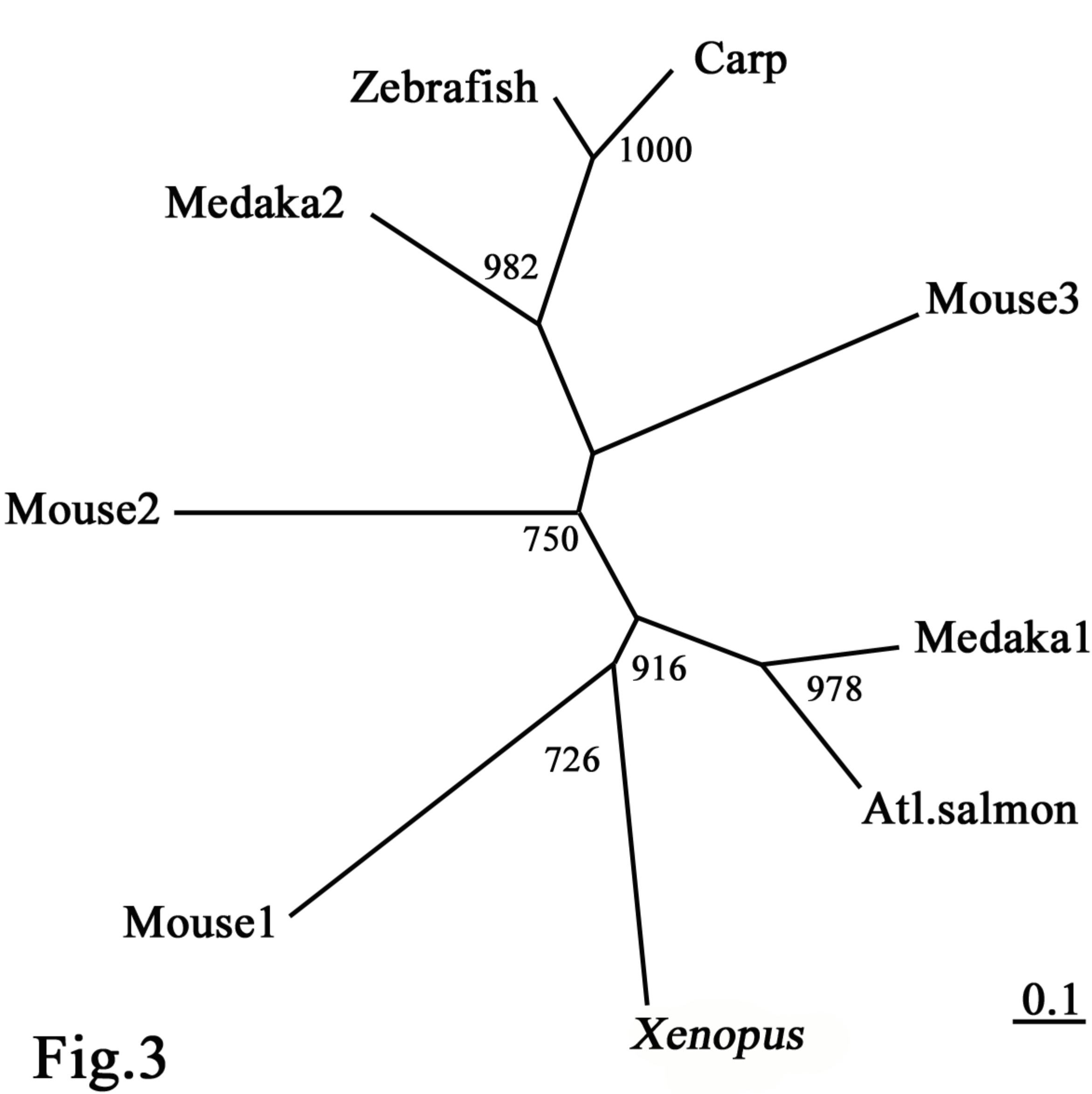
Carp
Zebrafish
1000
Medaka2
982
Mouse3
Mouse2
750
Medaka1
916
978
726
Atl.salmon
Mouse1
0.1
Xenopus

Fig.3