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Migration of Primordial Germ Cells During Late Embryogenesis of Pikeperch *Sander lucioperca* Relative to Blastomere Transplantation

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ABSTRACT

Güralp H., Pocherniaieva K., Blecha M., Policar T., Pšenička M., Saito T. (2017): **Migration of primordial germ cells during late embryogenesis of pikeperch *Sander lucioperca* relative to blastomere transplantation**. Czech J. Anim. Sci, 62, 121–129.

Pikeperch *Sander lucioperca* is a valuable fish in Europe, and basic information about its embryonic development, especially primordial germ cell (PGC) migration, is important for use in biotechnology. We categorized pikeperch embryonic development into six stages as in other fish species: zygote, cleavage, blastula, gastrula, segmentation, and hatching and described PGC migration. PGCs were visualized by injection of synthesized green fluorescent protein (GFP) within the 3'untranslated region (UTR) mRNA of *nanos3.* GFP-positive PGCs appeared in all embryos at approximately 100% epiboly. Time-lapse imaging revealed the PGC migration pattern from their initial appearance to location at the gonadal ridge. We conducted blastomere transplantation (BT) at the blastula stage. Donor embryos were labelled with GFP-*nos3* 3'UTR mRNA and tetramethylrhodamine dextran to label PGCs and somatic cells, respectively. Twelve BT chimeras were produced, with eight surviving to hatching. All exhibited donor-derived somatic cells in the developing body. The PGCs from donor embryos were observed to migrate towards the gonad region of the host embryos. Our results indicated that BT can be successfully applied in pikeperch, and these findings may be useful to produce germline chimeras in percids.

Keywords: germ cell transplantation; germ cell migration; germ-line chimera; *nanos*; primordial germ cell visualization

Pikeperch *Sander lucioperca* is a valuable recreational and commercially important freshwater fish for inland European aquaculture (Hilge and Steffens 1996). It belongs to the order Perciformes, which also includes numerous commercially and ecologically important marine species (Nelson 2006). The scientific community has recently shown interest in surrogate reproduction of fish using

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germ cell transplantation technology (GCTT) (Takeuchi et al. 2001; Yamaha et al. 2003; Okutsu et al. 2007; Saito et al. 2008, 2011). The gametes of a donor fish can be obtained via a germ-line chimera after germline stem cell transplantation. Germ cell transplantation provides benefits, including reducing the time for fish to reach sexual maturity and the space required for aquaculture (Psenicka et al. 2015), banking the genetic resource by cryopreservation of germ cells (Linhartova et al. 2014; Psenicka et al. 2016), producing fish by *in vitro* cultivation of germ cells without maintaining broodfish (Shikina et al. 2013), and the possibility of producing marine fish on land. Keeping marine fish in captivity generally requires costly equipment, such as large cages and nets, not used for freshwater species. Culture of marine fish on land is expensive, since maintaining relatively small volumes of seawater in a stable condition, with the salt concentration affected by evaporation, is a challenge. It may be of value to reproduce marine fish species in freshwater using surrogate breeding. However, the only report on successful production of a germ-line chimera in fish of different habitats is GCT between the catadromous Japanese eel *Anguilla japonica* and the freshwater zebrafish *Danio rerio* (Saito et al. 2011).

A germline chimera can be generated through spermatogonia transplantation into larvae (Lacerda et al. 2013). However, pikeperch larvae are sensitive and cannot survive transplantation or even mild anesthesia (Guralp, unpublished data). Transplantation of blastomeres (BT) containing PGCs is probably the only method for the generation of a germ-line chimera that can be successfully applied in pikeperch as our previous work has demonstrated (Guralp et al. 2016). However, the contribution of donor PGCs to the host embryo is an issue that needs to be addressed, as the donor-derived cells must be incorporated into the developing embryo. Knowledge of embryonic development is crucial to the assessment of PGC contribution to the recipient embryo. Lack of basic information on germ cell development during embryogenesis in pikeperch impedes the application of such surrogate production techniques. The present study focused on characteristics of late embryogenesis.

The origin and migratory pathways of PGCs vary among animals (Richardson and Lehmann 2010; Saito et al. 2014) and are not identical even in teleosts (Saito et al. 2006). The origin and migration pathway of PGCs have been studied in fish species including zebrafish, loach *Misgurnus anguillicaudatus*, goldfish *Carassius auratus*, medaka *Oryzias latepis*, Pacific herring *Clupea pallasii*, ice goby *Leucopsarion petersii*, tench *Tinca tinca* (Weidinger et al. 1999; Fujimoto et al. 2006; Saito et al. 2006; Linhartova et al. 2014), sturgeon *Acipenser* spp. (Saito et al. 2014), and flounder *Paralichthys olivaceus* (Li et al. 2015; Wang et al. 2015). The PGC migration pattern can vary, even in species within a family, as Saito et al. (2008, 2010) reported for Cyprinidae. Nevertheless, it has recently been revealed that germline chimeras can be produced between distantly related family members occupying different habitats (catadromous and freshwater). Japanese eel PGCs migrate and localize to the genital ridge of the zebrafish embryo after transplantation, although donor-derived PGCs disappear during the development of the chimeras (Saito et al. 2011). The pattern of PGC migration during late embryonic development of percids is unclear.

The PGCs can be visualized beginning at the mid-gastrula stage of embryogenesis by injecting synthetic mRNA constructed with a green fluorescent protein (GFP) sequence within the 3' untranslated region (UTR) of RNA of *vasa* or *nanos3* into 1–2 cell stage embryos (Koprunner et al. 2001; Yoshizaki et al. 2005; Saito et al. 2006; Linhartova et al. 2014). Saito et al. (2006) labelled PGCs in six species by injecting GFP-*nos3* 3'UTR zebrafish mRNA and found its function to be conserved.

The goal of this study was to use GFP labelling to describe the migration of PGCs from gastrula through hatching, and to evaluate the potential of BT in pikeperch. The results will provide needed information on generating germline chimeras in pikeperch.

MATERIAL AND METHODS

Ethics. The maintenance of fish and experimental procedures were conducted according to the criteria of the EU Harmonized Animal Welfare Act of the Czech Republic, Act No. 246/1992 Coll.

Fish and preparation of embryos. Pikeperch were obtained from the recirculating aquaculture system at the University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of

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Waters during the spawning season, March through April, 2013 and 2015. The fish were anesthetized with clove oil at 0.03 ml/l water (Dr Kulich Pharma, s.r.o., Czech Republic). Ovulation and spermiation were induced by the intramuscular injection of human chorionic gonadotropin (Chorulon; Merck, USA) (Kristan et al. 2013). Eggs and sperm were collected from three females and three males ~ 83 d° after the hormone treatment. Eggs were inseminated with the sperm in filtered and aerated tap water at 15°C. Fertilized eggs were treated for 10 min with 0.2% trypsin and 0.4% urea in Ringer's solution (128 mM NaCl, 2.8 mM KCl, 1.8 mM $CaCl_2 \cdot 2H_2O$) buffered by 10 mM TAPS to pH 8.5 to remove stickiness and soften the chorion. After enzyme treatment, embryos were individually dechorionated using fine forceps. The dechorionated embryos were divided into ten 1% agar-coated Petri dishes (diameter 95 mm) with each dish containing up to 50 embryos and incubated in Ringer's solution buffered by 10 mM HEPES to pH 7.5 containing 1.6% albumin until the completion of epiboly (Guralp et al. 2016). Ringer's solution was replaced every 2 h. After completion of epiboly, embryos were transferred to a second culture solution (1.8 mM $MgCl_2$ and 1.8 mM $CaCl_2$) containing 0.01% penicillin and 0.01% streptomycin and incubated at 15°C. A control group of approximately 80 embryos with chorion was incubated in filtered and aerated tap water in 120 mm plastic Petri dishes with water replaced every 4 h.

Definition of stages. The developmental stages were defined morphologically by stereomicroscopy (Leica M165FC; Leica, Germany) and photographed (Leica DFC425C). The embryos were observed during the somitogenesis period every 2 h.

Observation of PGCs. Zebrafish *nos3* 3'UTR mRNA containing green fluorescent protein (GFP) was synthesized using the mMESSAGE mMACHINE Kit (Life Technologies Corp., USA) according to manufacturer's instructions, and 300 ng/µl in 0.2M KCl was injected into the blastodisc of 2-cell pikeperch embryos to visualize the PGCs (Saito et al. 2006) (Figure 1A). PGCs were observed in darkness from the 100% epiboly until hatching using fluorescence stereomicroscopy (Leica M165FC), and photographed (Leica DFC425C).

Tracking migrating PGCs. The migrating PGCs were recorded by a stereomicroscope equipped with a camera (Leica DFC425C) for 2-hour periods with 30 s between frames. The position of each PGC was marked, and photos were merged into a single image in order to track PGCs.

Blastomere transplantation. The donor embryos were labelled with GFP-*nos3* 3'UTR mRNA and tetramethylrhodamine (TMR)-dextran (molecular weight (MW) = 10 000) at the 2–4 cell stage. The BT from labelled into non-labelled embryos was performed as described by Saito et al. (2010), so that transplanted PGCs and somatic cells could be visualized in the chimeric embryos. We aspirated the labelled blastomeres into a glass microneedle connected to a microinjector (Eppendorf, Germany) from the marginal region of the donor embryo blastoderm at the blastula stage and transplanted them into the same region of the host blastoderm at the same stage (Figure 3A). The chimeric embryos were examined under a fluorescence stereomicroscope (Leica M165FC). Images of the embryos were obtained using the appropriate filters for GFP and rhodamine fluorescence and were merged into a single image using ImageJ software.

RESULTS

Late embryonic development in pikeperch. The embryonic development in pikeperch was separated into cleavage (1.5–7.5 hpf), blastula (9–18.75 hpf), gastrula (21–39 hpf), segmentation (45–105 hpf), and hatching (125 hpf) periods based on morphological features. Three groups of embryos from different females were observed, and the embryonic periods were completed by hatching period at 125 hpf.

PGC migration in pikeperch. PGCs were observed in all embryos injected with synthesized GFP-*nos3* 3'UTR mRNA from 100% epiboly at 39 hpf. The mean number of PGCs in each embryo at the early segmentation period was 12.6 (standard deviation 8.52, range = 2–35). The PGCs showed migration to, and localization at, the gonad area during development and were confirmed as PGCs.

Time-lapse imaging and analysis revealed the migration pattern of PGCs from the time and place of their appearance to localization at the gonadal ridge (Figure 1). The PGC migration patterns and localization were similar among embryos although in some the PGCs appeared only, or in lower numbers, on one side of the body. At the beginning of the segmentation period (the 1–12

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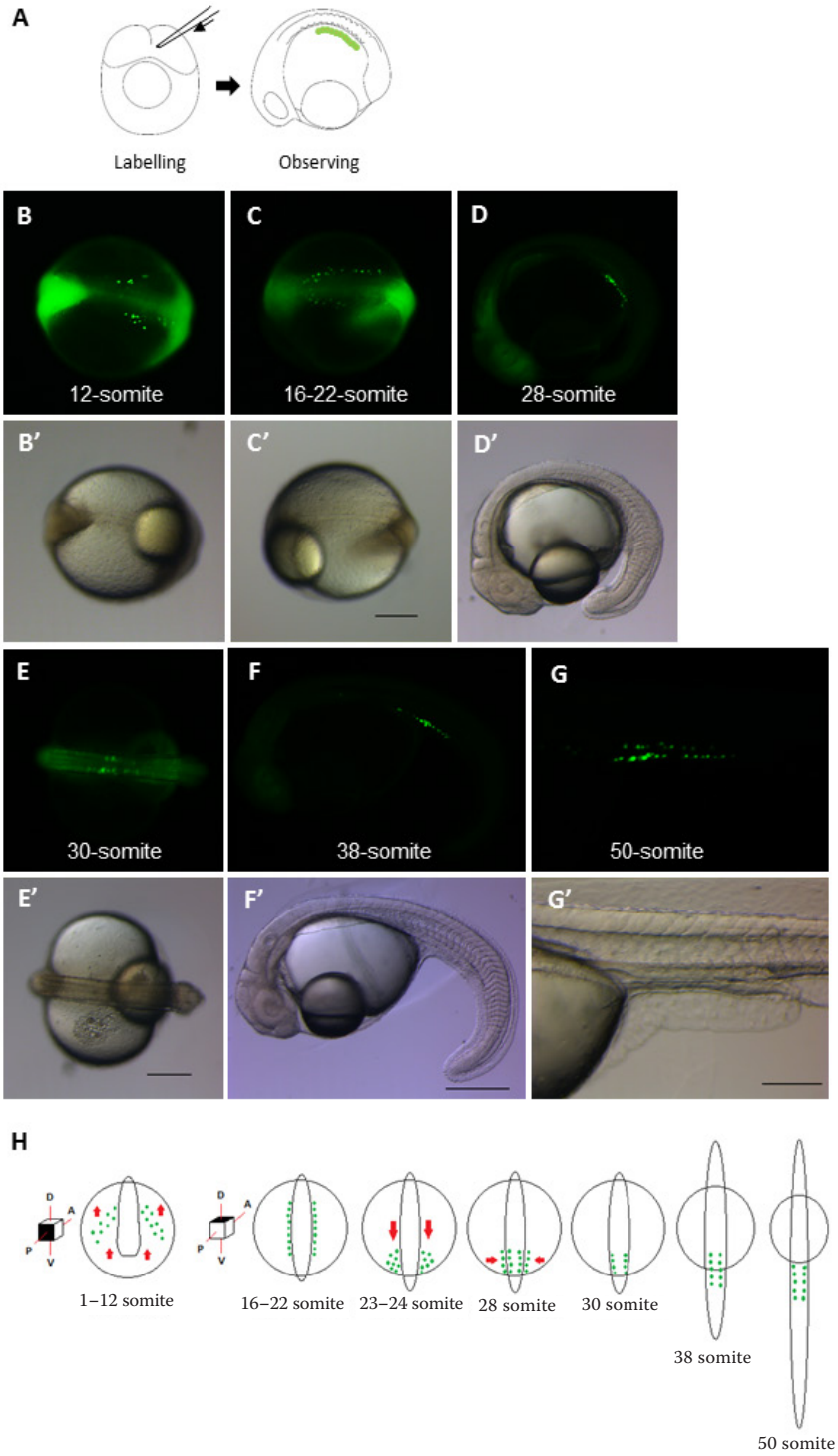

Figure 1. Migration pathway of primordial gem cells (PGCs) in pikeperch embryos at 15°C
(**A**) schematized experimental design of PGCs observation, PGCs were labelled with green fluorescent protein (GFP)-*nos*3 3'UTR mRNA; (**B–B'**) PGCs migrated from posterior to anterior part of embryonic body at 12 somite stage (54 hpf); (**C–C'**) PGCs localized at ventral side of embryonic body at 16–22 somite stage (64 hpf); (**D–D'**) PGCs migrated to posterior at 24–28 somites (70 hpf); (**E–E'**) PGCs localized on both sides of germinal line on ventral side of embryonic body at 30 somites (78 hpf); (**F–F'**) PGCs extended through yolk to germinal line (90 hpf); (**G–G'**) PGCs localized on both sides of germinal line (125 hpf); (**H**) scheme of PGC migration, posterior view at 1–12 somites and dorsal view at 16–50 somites. Scale = 200 μm

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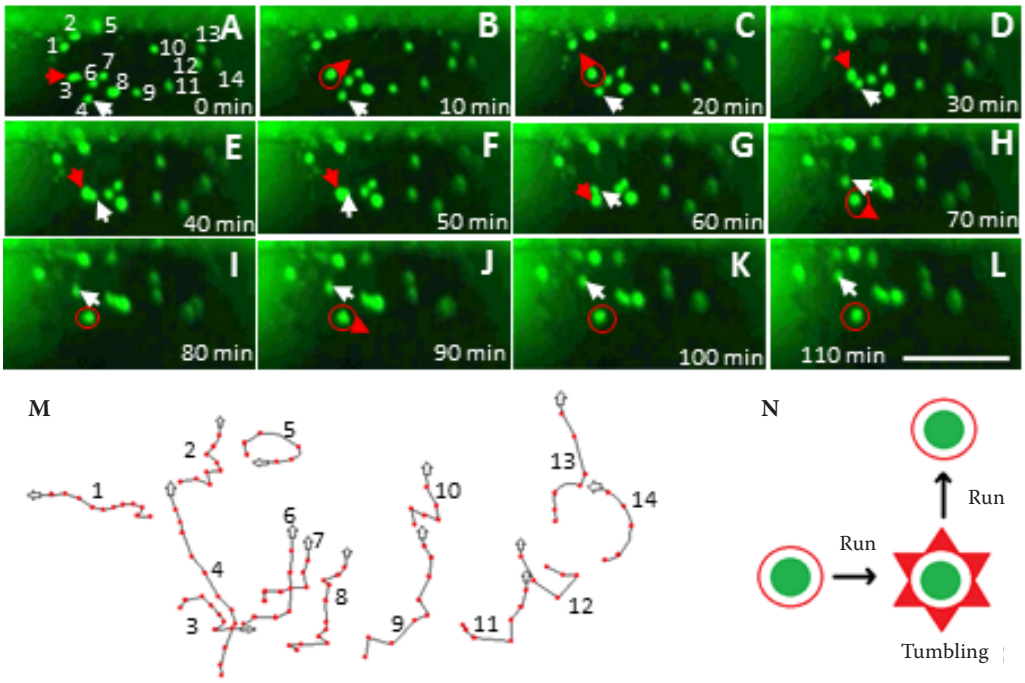

Figure 2. Primordial gem cells (PGCs) migration at 28 somites for 110 min (**A**–**L**) in the left cluster of the PGCs, 14 migrating PGCs are clearly observable. Two migrating PGCs (3 and 4) are indicated by white and red arrows intercepting. During migration, the PGCs showed tumbling behaviour. The tumbling is indicated by red circles with arrowheads showing the direction of movement. A single arrow indicates the migration orientation and position of PGC. A single circle shows position of PGC; (**M**) = migration pathway of each PGC (1 to 14); (**N**) = illustration of migrating PGCs behaviour. PGCs are in green and the direction of movement is shown by red arrows. Tumbling movement is illustrated in all possible orientations by red arrows on the circle. Scale = 100 µm

somite stage), the PGCs were widely distributed on both sides of the trunk in the posterior half of the embryonic body; some were located ventrally, especially in the posterior near the developing tail bud (Figure 1B). As the trunk and tail bud became more prominent around the 16–22 somite stage (Figure 1C), the PGCs began to migrate and were loosely aligned along both sides of the developing somites. The PGCs on the posterior ventral surface of the body migrated laterally and joined to form a loose PGC alignment. The PGCs in the anterior region migrated posteriorly and narrowed their distribution area. During this shift, the width of the PGC distribution increased. The aligned posterior PGCs continued to migrate but they were confined to target areas. At the 23–24 somite stage, PGCs continued to migrate towards the axis of the body and a more confined area, to form two PGC clusters. The subsequent step, at the 28 somite stage, represented completion of active migration, positioned on the ventral side of the embryonic body (Figure 1D). The PGCs displayed slower movement for a short distance on the ventral side of the body during the late segmentation period until located at the origin of the germinal line at the end of the yolk extension. The final step of migration, at the 30–50 somite stage, was the localization of PGCs in alignment on both sides of the germinal region of the embryo (Figure 1E–H).

During the migration process, PGCs exhibited active behaviour as reported in zebrafish PGC migration, characterized by the formation of protrusions and "run and tumble" behaviour (Figure 2). Each PGC moved through the pathway and, after tumbling, either continued to move in the same direction or in other directions for varying distances depending on the its original position.

Blastomere transplantation in pikeperch. Twelve BT chimeras were produced, and eight survived to hatching. All showed donor-derived cells

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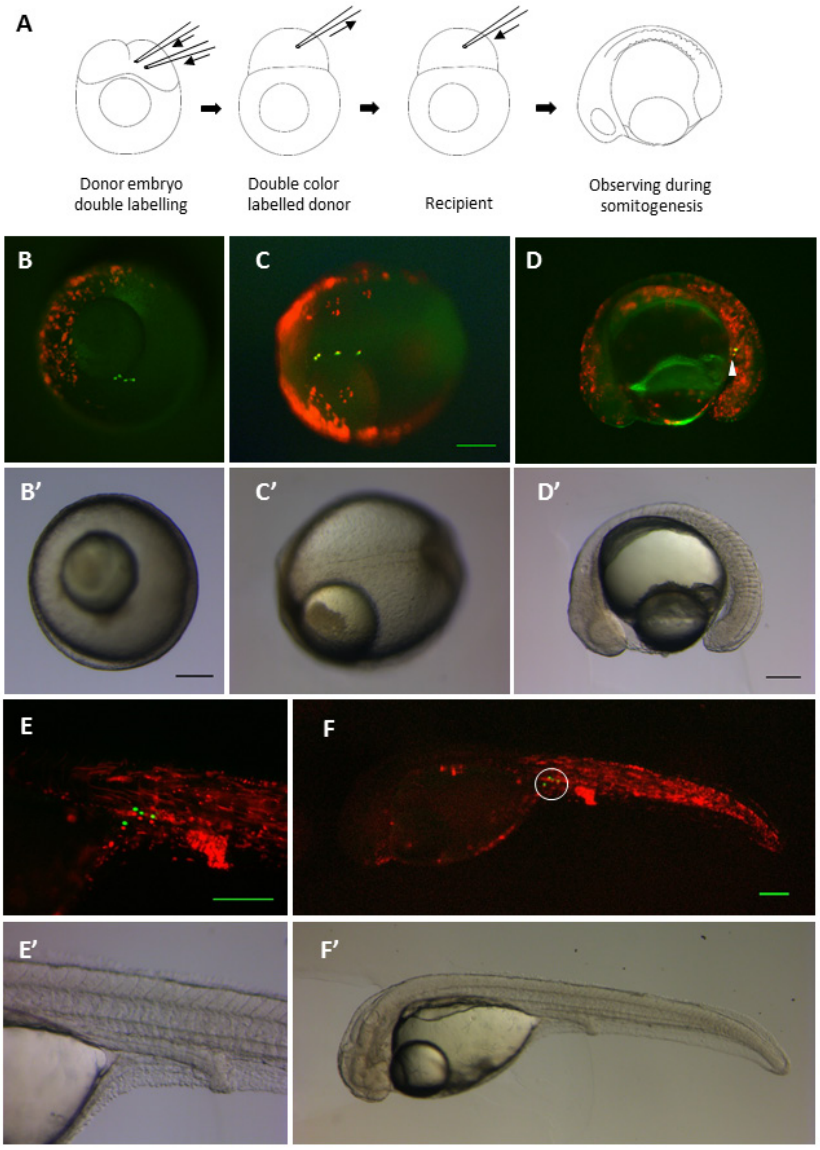

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	Total	Survive	PGSs positive	PGSs negative
RNA-injected	42	41	41	0
BT-chimera	12	8	2	6

Figure 3. Blastomere transplantation (BT)
(**A**) schematic illustration of BT experimental design. The donor embryos were co-labelled with green fluorescent protein (GFP)-*nos3* 3'UTR mRNA and tetramethylrhodamine-dextran at 1–2 cell stage. Labelled donor blastomeres were aspirated into a glass needle and injected into the recipient at the blastula stage. Transplanted donor cells were observed during somitogenesis; (**B–F**) chimeras under light and fluorescence microscopy – red-labelled cells are somatic cells, and GFP-labelled cells are primordial gem cells (PGCs) (**B–B'**) at 4 somite stage; (**C–C'**) at 16 somite stage; (**D–D'**) at 22 somite stage, two PGCs indicated by arrowhead; (**E–E'**, **F–F'**) at 44 somite stage embryos, PGCs indicated by circle; (**G**) = efficacy of BT. Scale = 200 μm

in the developing body. As the chimeric embryos developed, donor cells mingled with host cells and were distributed throughout the body, including somites, brain, and intestine (Figure 3B–F). Two of the eight embryos exhibited GFP-labelled PGCs at the gonadal ridge (Figure 3B–F).

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DISCUSSION

PGC migration in pikeperch. The migration route of PGCs was identified by GFP visualization for the first time in pikeperch. The efficacy of microinjection has been evaluated by the presence of PGCs in embryos of 100%, as reported in ice goby, zebrafish, and Pacific herring (Saito et al. 2006). The appearance of PGCs on only one side of the body in some embryos was similar to findings in these studied model fish species. We observed 2–35 labelled PGCs in pikeperch embryos, similar to reports in other species (Saito et al. 2006). The number of visualized PGCs in the embryos can depend on the efficacy of microinjection, and may not show the actual quantity of PGCs. Saito et al. (2011) reported that the number of GFP-positive PGCs varied among eel embryos, due to the difficulty of microinjection. PGCs have been previously visualized from 100% epiboly in tench; from 50% epiboly in zebrafish, medaka, loach, and eel; and from 90% epiboly in ice goby (Saito et al. 2006, 2011). Our results were consistent with studies regarding the conserved function of GFP-*nos3* 3'UTR mRNA in teleosts (Saito et al. 2006).

We observed that the PGCs in pikeperch migrated to the region of gonad development and were localized there, consistent with the results of studies in other fish species (Saito et al. 2004, 2006; Li et al. 2015; Wang et al. 2015). Studies have revealed differing migratory patterns of PGCs during embryonic development among fish species. The migration path of PGCs in pikeperch showed similarities with goldfish; however, unlike goldfish and similar to herring, the PGCs in pikeperch clustered in the posterior region of the body. A comparison of our results with other reports shows wide variations in PGC migratory paths among species. The source of the differing migration patterns among fish may be the combination and balance of chemo-attractant signals, which have been described in detail for zebrafish and medaka (Doitsidou et al. 2002; Boldajipour et al. 2008; Saito et al. 2015). In olive flounder, *sdf-1* mRNA encoding a protein that guides PGC migration is expressed in the ventral region of the embryonic body at the 15 somite stage, and PGCs are localized in this region at that stage. In zebrafish, *sdf-1* is not expressed in this region, and PGCs do not migrate toward the ventral side of the body at the 15 somite stage. These species use the same attractant system, but PGC migration shows different patterns.

Blastomere transplantation. We successfully established a technique to produce germ-line chimeras in pikeperch with the rate of efficacy of PGC transfer in chimeras higher than reported in zebrafish (Saito et al. 2010). While donor-derived PGCs were observed in the gonad region in 8.97% of zebrafish chimeras, 25% of pikeperch chimeras exhibited donor PGCs at the gonad region in our study. The donor cells were well distributed throughout the blastoderm, and the distribution of the donor somatic cells in pikeperch BT chimeras was similar to that of zebrafish (Saito et al. 2010) and rainbow trout *Oncorhynchus mykiss* (Takeuchi et al. 2001). Transplanted somatic cells were evenly distributed throughout the embryonic body including somites, brain, and intestine, while PGCs were located in the gonad region.

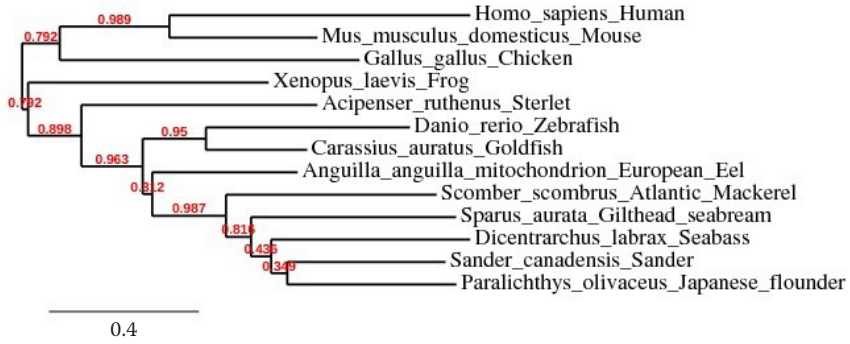

Figure 4. Phylogenetic relationships of the *Sander* species to other eukaryotes
Phylogenetic tree with branch length is proportional to the number of substitutions per site. It shows the closest marine fish species, which can potentially be donor species for blastomere transplantation in percids. Cytochrome b sequence of species was used to construct the tree using Phylogeny.fr software after searching in NCBI

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It has been reported that the phylogenetic relationship does not affect the migration of donor PGCs into the gonad region of the host in interspecies transplantation (Saito et al. 2010). On the other hand, donor-derived PGCs did not develop in the chimeras generated by a single PGC transplantation between distantly related family members, although the donor-derived germ cells did migrate and localize to the genital ridge of recipient embryo (Saito et al. 2011). Therefore, BT might be effective in generation of inter-species germ-line chimeras between phylogenetically close family members, considering the phylogenetic position of pikeperch and its developmental pattern, which is similar to marine fish such as sea bass *Dicentrarchus labrax* and Japanese flounder *Paralichthys olivaceus* (Figure 4). Pikeperch can be a potential host to generate inter-species germ-line chimeras between freshwater and marine species. Micromanipulation will allow surrogate production of endangered and commercially valuable percids, for instance, asprete *Romanichthys valsanicola*, and, potentially, reproduction of brackish water species such as European perch *Perca fluviatilis* and marine fish such as sea bass and Japanese flounder in freshwater.

CONCLUSION

In this study, we described the migration of PGC, based on the knowledge of the late embryonic development of pikeperch from gastrula to hatching. We established a reliable technique to transplant PGCs in pikeperch embryos. Further study is needed to determine whether donor germ cells in the chimera will differentiate into functional gametes. Deletion of endogenous PGCs must be established to conduct exclusive production of donor gametes in pikeperch. More importantly, although it is often overlooked, a method of culturing a small number of manipulated larvae and juveniles is needed for practical surrogate production. Based on the described techniques and results of the present study, GCTT using a pikeperch model can be realized.

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