DEVELOPMENT OF GERM CELL TRANSPLANTATION IN PANGASIID CATFISH

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Animal Production Technology

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นายรังสรรค์ ดวงแก้ว

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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รังสรรค์ ดวงแก้ว : การพัฒนาการปลูกถ่ายเจิร์มเซลล์ในปลากลุ่มแพงกาซิดแคทฟิช (DEVELOPMENT OF GERM CELL TRANSPLANTATION IN PANGASIID CATFISH) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.สุรินทร บุญอนันธนสาร, 157 หน้า.

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อพัฒนาเทคโนโลยีการปลูกถ่ายเซลล์สืบพันธุ์ในปลากลุ่มแพงกาซิด (Pangasiid) โดยการศึกษาวิจัยในครั้งนี้ได้แบ่งเป็น 4 การทดลอง

การทดลองที่ 1 เป็นการศึกษาผลของอายุของปลาผู้ให้ (donor fish) ต่อประสิทธิภาพของการปลูกถ่ายเซลล์สืบพันธุ์ โดยการทดลองในครั้งนี้ได้ศึกษาในปลาชิวข้าวสารญี่ปุ่น (Japanese medaka, *Oryzias latipes*) เนื่องจากเป็นปลาที่มีช่วงชีวิตสั้น การทดลองนี้เริ่มจากการแสดงความสัมพันธ์ของอายุต่อการเปลี่ยนแปลงโครงสร้างของตา และอัณฑะของปลาที่อายุ 1 2 3 4 8 และ 18 เดือน ผลการศึกษาครั้งนี้สามารถสรุปได้ว่า อายุของปลามีผลต่อจำนวน ASG แต่ไม่มีผลต่อประสิทธิภาพการปลูกถ่ายเซลล์สืบพันธุ์

การทดลองที่ 2 ได้ทำการโคลน และศึกษาโครงสร้างของ mRNA ของยีน *vasa* เพื่อใช้เป็นยีนเครื่องหมายเซลล์สืบพันธุ์ในการศึกษาอายุที่เหมาะสมของปลาสวาย (*Pangasianodon hypopthalmus*) ยีน *vasa* ของปลาสวาย (*Phy-vasa)* ประกอบไปด้วยโมทิฟที่สำคัญที่เป็นเอกลักษณ์ของยีน *vasa* ผลการศึกษารีเวิร์สทรานสคริปชันพีซีอาร์ (reverse transcription PCR) พบว่า mRNA ของ *Phy-vasa* แสดงออกเฉพาะในอัณฑะ และรังไข่เท่านั้น และการศึกษาด้วยเทคนิคอินไซตูไฮบริไดเซชัน (in situ hybridization) พบว่า mRNA ของ *Phy-vasa* แสดงออกเฉพาะเซลล์สืบพันธุ์ และสรุปได้ว่า กระบวนการเคลื่อนย้ายของเซลล์ไพรมอร์เดียล (Primordial germ cell; PGC) เกิดขึ้นในปลาวัยอ่อนที่อายุ 2-10 วันนับจากวันหลังผสม (days post fertilization) และในลูกปลาที่อายุ 10-20 วันนับจากวันหลังผสม พบว่า PGCs ในอวัยวะสืบพันธุ์เริ่มมีเซลล์โซมาติก (somatic cell) มาเจริญรอบ นอกจากนี้ในปลาที่มีอายุ 25-30 วันนับจากวันหลังผสม กระบวนการการแบ่งเซลล์เพื่อเพิ่มจำนวนของ PGCs เริ่มเกิดขึ้น

การทดลองที่ 3 เป็นการศึกษาเพื่อหาสภาวะที่เหมาะสมสำหรับการผลิตปลาสวายทริพพลอยด์ (triploid fish) ที่มีโครโมโซม (chromosome) 3 ชุด เพื่อใช้เป็นลูกปลาผู้รับ (recipient fish) ผลการทดลองพบว่า การช็อคไข่ปลาด้วยความเย็นที่อุณหภูมิน้ำ 7.5°C เป็นระยะเวลา 30 นาที เป็นวิธีการที่ทำให้ได้ลูกปลาทริพพลอยด์ 90% โดยมีอัตราการฟักของไข่ปลาเท่ากับ 35.34% และอัตราอดที่ระยะเวลา 7 วันนับจากวันหลังผสม อยู่ที่ 20.00% และพบว่า เซลล์เม็ดเลือดแดงของปลาทริพพลอยมีขนาดนิวเคลียส และปริมาณดีเอ็นเอมากกว่าปลาดิพพลอยด์ที่มีโครโมโซม 2 ชุด (diploid fish) ประมาณ 1.5 เท่า ค่าดัชนีความสมบูรณ์เพศ (gonadosomatic index; GSI) ของปลาดิพพลอยด์มีขนาดใหญ่กว่าปลาทริพพลอยด์ การศึกษานี้พบว่า กระบวนการสร้างเซลล์สืบพันธุ์ (gametogenesis)

ของปลาทริพพลอยด์มีความผิดปกติ อย่างไรก็ตามพบว่า ค่าสมรรถนะการเจริญเติบโต ค่าโลหิตวิทยา และการพัฒนาอวัยวะสืบพันธุ์ในช่วงปลาวัยอ่อนของปลาดิพพลอยด์ และปลาทริพพลอยด์ไม่มีความแตกต่างกัน

การทดลองที่ 4 มีจุดประสงค์ที่จะพัฒนาการปลูกถ่ายเซลล์สืบพันธุ์โดยใช้เซลล์สเปอร์มาโตโกเนีย (spermatogonia; SG) และโอโอโกเนีย (oogonia; OG) จากปลาบึก (*Pangasianodon gigas*) และใช้ปลาสวายเป็นปลาผู้รับ โดยทำการฉีด SG และ OG เข้าสู่ช่องท้องของปลาผู้รับทั้งปลาที่เป็นดิพพลอยด์และทริพพลอยด์พบว่า อัตราการที่ SG และ OG ของปลาบึกเข้าฝังเซลล์ในอวัยวะสืบพันธุ์ (colonization rate) ของปลาสวายดิพพลอยด์ และปลาสวายทริพพลอยด์มีค่าเท่ากับ 80.00±16.33% และ 90.00±20.00% ตามลำดับ นอกจากนี้ได้ใช้เทคนิครีเวิร์สทรานสคริปชันพีซีอาร์อาร์เอฟแอลพี (reverse transcription PCR-RFLP) เพื่อยืนยันผลการฝังตัวของเซลล์สืบพันธุ์ปลาบึกในปลาสวาย ผลการศึกษานี้ชี้ให้เห็นว่า เซลล์ต้นกำเนิดเซลล์สืบพันธุ์จากปลาบึกมีการเคลื่อนย้ายและเข้าไปอาศัยอยู่ในอวัยวะสืบพันธุ์ของปลาสวายที่เป็นปลาผู้รับได้

โดยสรุป การศึกษานี้ได้ข้อมูลทางชีววิทยา เทคนิค และวิธีการสำหรับการปลูกถ่ายเซลล์สืบพันธุ์ และถือเป็นความสำเร็จขั้นต้นในการปลูกถ่ายเซลล์สืบพันธุ์ และปลาที่ได้รับการปลูกถ่ายเซลล์จะนำไปเลี้ยงต่อเพื่อทดสอบว่าเทคนิคที่ใช้ในการศึกษาในครั้งนี้สามารถผลิตพ่อแม่ปลาอุ้มบุญของปลาบึก

สาขาวิชาเทคโนโลยีการผลิตสัตว์ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2560 ลายมือชื่ออาจารย์ที่ปรึกษา _____

RUNGSUN DUANGKAEW : DEVELOPMENT OF GERM CELL TRANSPLANTATION IN PANGASIID CATFISH. THESIS ADVISOR : ASSOC. PROF. SURINTORN BOONANUNTANASARN, Ph.D., 157 PP.

GERM CELL TRANSPLANTATION/PANGASIID FISH/MEDAKA/VASA

This study aimed to develop germ cell transplantation (GCT) technology in Pangasiid fish. In order to accomplish GCT, four experiments were conducted.

Experiment I investigated the aging effects of donor fish on the efficiency of GCT in medaka (*Oryzias latipes*) since it has the short life cycle. The experiment began by determining age-related changes in the eye and testis at 1, 2, 3, 4, 8 and 18 months of age. There were age-related effects on the number of type A spermatogonia (ASG) but not for the efficiency of GCT.

Experiment II cloned and characterized *vasa* mRNA (*Phy-vasa*) for use as a gene maker for determining the suitable age of the recipient larvae of striped catfish (*Pangasianodon hypopthalmus*). *Phy-vasa* contained all of the predicted consensus motifs that are shared within the Vasa family among other fish. By RT-PCR, *Phy-vasa* mRNA was observed only in the gonad. Using in situ hybridization, *Phy-vasa* mRNA was expressed specifically only in germ cells. Migration of primordial germ cells (PGCs) were found most abundantly in larvae, 2-10 days post-fertilization (dpf) and the genital ridges containing PGCs and somatic cells were formed at 10-20 dpf. The proliferation of PGCs began in larvae between 25-30 dpf.

Experiment III determined the optimal condition for production of triploid striped catfish for the use as recipient larvae. The results showed that cold-shock at

7.5°C for 30 min was the optimum process to obtain 90% triploid fish with 35.34% of hatching rate and 20.00% of survival rate. The triploid red blood cell (RBC) has significantly larger nuclear sizes and DNA content (1.5 times) than that of diploid fish. The larger GSI in diploid fish was observed. Gametogenesis of triploid fish appeared to be disorder. However, growth performance, hematological indices and early gonadal development of diploid and triploid fish were similar.

Experiment IV developed GCT using donor germ cell [Spermatogonia (SG) and oogonia (OG)] from the Mekong giant catfish (*Pangasianodon gigas*) and recipient larvae of striped catfish. GCT was performed by microinjecting SG or OG into the peritoneal cavity of either diploid or triploid larvae. The colonization rates of SG and OG in the gonad of diploid and triploid fish were ranged within 80.00±16.33% and 90.00±20.00%, respectively. RT-PCR-RFLP confirmed the incorporation of the donor germ cell in the gonad of the striped catfish. These findings suggest that the transplanted immature germ cell of the Mekong giant catfish migrated toward and incorporated into the genital ridge of the recipient striped catfish larvae.

In conclusion, this study provided biological information, techniques and methods for GCT. The preliminary success of GCT was achieved. Culture of the transplanted fish have been carried out to confirm whether our technology could produce surrogate broodstock of the Mekong giant catfish.

School of Animal Production Technology Student's Signature Rungsun

Academic Year 2017 Advisor's Signature

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Rungsun Duangkaew

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LISTS OF ABBREVIATIONS

ANOVA	=	Analysis of variance
ASG	=	Type A spermatogonia
bp	=	Base pair
BSG	=	Type B spermatogonia
cDNA	=	Complementary DNA
CTM	=	Centigrade temperature minutes
dpf	=	Days post fertilization
dph	=	Days post hatching
dpt	=	Days post transplantation
FBS	=	Fetal bovine serum
GCL	=	Ganglion cell layer
GCT	=	Germ cell transplantation
GFP	=	Green fluorescent protein
GSI	=	Gonadosomatic index
H&E	=	Hematoxylin and eosin
HP	=	Hydrostatic pressure shock
IgG	=	Immunoglobulin G
INL	=	Inner nuclear layer
IPL	=	Inner plexiform layer
IS/OS	=	Inner segment/outer segment of the photoreceptor cells
L-15	=	Leibovitz's L-15 Medium

LISTS OF ABBREVIATIONS (Continued)

$MgCl_2$	=	Magnesium chloride
mRNA	=	Massenger RNA
O	=	Maturing oocytes
OG	=	Oogonia
ONL	=	Outer nuclear layer
OPL	=	Outer plexiform layer
OSC	=	Oogonial stem cells
PCR	=	Polymerase chain reaction
PGC	=	Primordial germ cell
PO	=	Previtellogenic oocyte
PSC	=	Primary spermatocyte
RACE	=	Rapid amplification of cDNA ends
RBC	=	Red blood cell
RPE	=	Retinal pigment epithelium
SSC	=	Secondary spermatocyte
SSCs	=	Spermatogonial stem cells
SZ	=	Spermatozoa
TL	=	Total length
tRNA	=	Transfer RNA
U ml^{-1}	=	Unit per milliliter
UTP	=	Uridine triphosphate
UTR	=	Untranslated region

CHAPTER I

INTRODUCTION

Sustainable fish production commonly includes seed production and rearing of juveniles and grow-out phases. Seed production can be obtained from natural fertilization and/or artificial insemination which is the key step to enable the development of sustainable of full-cycle fish production. To date, Seed production technology in many fish species have been well-established. However, seed production has been limited to some aquacultured-related fish in which larvae propagation cannot be practically induced in captivity. Therefore, development of a technology to produce fish larvae in some species which have been difficult to induce maturation in captivity is needed. Recently, germ cell transplantation (GCT) in fish has been established. GCT is a technique to isolate an immature germ cell from a fish (donor fish) and transplant them into fish larvae (recipient fish). Consequently, the transplanted recipient fish is raised until maturation, and they would become surrogate broodstock for production of donor-derived offspring. GCT would provide as useful tool for several applications including aquaculture practice and conservative aspects.

Germ cell precursor is known as primordial germ cell (PGC) which originates in a particular region of the embryo. During early embryonic development, PGCs locate in extragonadal area. Later, PGCs migrate toward the presumptive gonad site and incorporate into the genital ridge of recipient fish. Subsequently, PGCs proliferate and differentiate via spermatogenesis and oogenesis in male and female,

respectively, for generation of sperm in male or eggs in female. In male vertebrates, spermatogonial stem cells (SSCs) are at the foundation of spermatogenesis. SSCs are defined like all other stem cells, by their ability to balance self-renewing divisions and differentiating divisions. This balance maintains the stem cell pool and meets the proliferative demand of the testis to produce millions of sperm. Studies of SSCs are complicated because these cells are few in number and no unique identifying characteristics have been reported to date (Phillips et al., 2010). Aging-induced deterioration of function of SSCs have been demonstrated in mammals. To date, spermatogonial transplantation assay was demonstrated as an effective tool to study the function and biology of SSC in mice (Brinster and Zimmermann 1994; Kanatsu-Shinohara et al., 2006. Thus, age-related infertility has been intensively studied using animal model particularly in mice (Schmidt et al., 2011; Ogawa et al., 2003; Ryu et al., 2006). Aging process affected both intrinsic function of spermatogonial stem cells (SSCs) and extrinsic microenvironment. For instant, aging affected rate of SSC proliferation in SSC culture. The rate of SSC proliferation in *in vitro* culture of SSC isolated from old mice was lower comparing to that from younger. Decrease in expression of gene that were crucial for SSC self-renewal and elevation in expression of gene that were involved in SSC differentiation was observed in aged SSC culture. Long term culture of SSC also had significantly lower transplantation efficiency (Schmidt et al., 2011). However, aging had negative effects on niche microenvironment which was the major factor to deteriorate testis in mouse (Ogawa et al., 2003; Ryu et al., 2006). Therefore, whether age would affect function of SSC and/or niche regulatory remains to be extensively investigated which would enable to understand aging programing on reproductive system.

The optimal stage of recipients is essential for germ cell transplantation technique. Newly hatched larvae were chosen to be used as recipients as they did not possess a functional immune system, as indicated by the lack of differentiation in both their thymus and their T-cells. Lack of a functional immune system allowed the immunorejection of exogenous (donor-derived) germ cells to be avoided (Lacerda et al., 2013). Like in other vertebrates, fish PGCs are generally large in size, have a low nucleocytoplasmic ratio and have a distinct nuclear border and granular nuclear chromatin van Winkoop et al., 1992; Patino and Takashima, 1995). Identification of PGCs, particularly at the developmental stages before complete migration toward genital ridge is difficult and need a molecular marker to confirm PGC characterization. Morphological characterization together with the aid of a molecular marker would enable the investigation of early reproductive development in fish larvae. There are several genes that are specifically expressed in germ cells including *vasa*, *nanos*, *dazl*, *deadend*, and *cxcr4b* in living organisms that could be potential candidates as a marker (review in Raz, 2003). Among them, *vasa* gene products have been extensively used as a molecular marker for identification of germ cells. For instance, the ubiquity of *vasa* allowed it to be used to identify PGCs in larvae, as well as in spermatogonia and oogonia throughout the reproductive cycle (Braat et al., 2000; Úbeda-Manzanaro et al., 2014; Yoshizaki et al., 2002).

In GCT, diploid recipients have ability to proliferate and differentiate both donor-derived cells and endogenous PGCs. Therefore, the efficiency of GCT or the percentage of donor-derived offspring was variable. In order to improve the efficiency of production of donor-derived offspring, sterile fish or fish that cannot produce their own gamete but could support the development of the transplanted germ cells is

needed. Triploidization is alternative method to provide sterile recipients. Triploid fish with three complete sets of chromosomes instead of two sets in diploid individuals can be generated by preventing the extrusion of the second polar body during the second meiotic division in embryonic development (Benfey, 1999). Triploidization techniques were developed in several aquaculture-related fish species. It was demonstrated that gonad development in triploid undergoes normally for mitotic division. However, gametogenesis was arrested because of inducing a meiotic disorder. Therefore, triploid fish could be a recipient fish for GCT since it could contribute normal gonad development but disorder in functional gamete production. Therefore, the triploid fish could be effectively used as surrogate recipients in GCT.

Germ cell transplantation has potential applications for the preservation of endangered species and seed production for commercially important large body species. Currently, the family Pangasiidae comprises of 21 recognized species, some of Pangasiid catfish are economically important and/or endangered species. Among Pangasiid catfish, the Mekong giant catfish, *Pangasianodon gigas*, has been attracted globally because it is the world's largest freshwater fish. The maturation of the Mekong giant catfish species was reported at more than 15 years old and at 37 kg of body weight (Phayao, 2000). The Mekong giant catfish has been considered to be Critically Endangered (IUCN) and is already listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). The striped catfish, *Pangasianodon hypophthalmus*, occasionally becomes maturity at 900 g of body weight. The striped catfish at 2 years old can generally be broodstock and able to produce functional gamete. In addition, its full cycle culture is well-developed. Moreover, the striped catfish is belonged to the same genus and therefore is

considered to be evolutionarily related with the Mekong giant catfish. Germ cell transplantation technology enables a practical tool to generate the offspring from fish that are limited for seed production. For example, a number of fish which have large body size and long generation time, thus need extensive farm space to maintain the broodstock. In addition, seed production of some fish species which is near extinction will be accomplished by using germ cell transplantation technique. Therefore, the striped catfish could be a good candidate to be a recipient for GCT of the Mekong catfish.

This study aims to develop GCT in the Pangasiid catfishes (family Pangasiidae). In order to establish GCT, characterization of immature germ cell which were used for donor cells were carried out in experimental medaka fish. Therefore, aging effect of spermatogonia on transplantation efficiency was investigated in medaka. In addition, since a molecular marker of germ cell is needed to characterize PGC of the recipient striped catfish, cloning and characterization of *vasa* cDNA was performed and used to identify the suitable age of recipient larvae. Moreover, in order to develop surrogate broodstock which would be able to provide only donor-derived offspring, this study investigated the suitable method to produce triploid striped catfish. Consequently, GCT was developed by using the Mekong giant catfish as donor fish and Striped catfish, *Pangasianodon hypophthalmus* as a recipient.

1.1 Research objectives

The aim of this study is to develop GCT technology in *Pangasiid* fish. In order to accomplish GCT, four objectives were investigated as the following:

1.1.1 To investigate age-related spermatogonail stem cell (SSC) function of spermatogonia in medaka.

1.1.2 To clone and characterize *vasa* mRNA which was used as a gene maker for determining the suitable age of recipient larvae of striped catfish (*Pangasianodon hypopthalmus*) and study the early reproductive development in striped catfish larvae.

1.1.3 To determine the optimal condition of production of triploid striped catfish, *P. hypopthalmus* for the use as recipient larvae.

1.1.4 To develop GCT using donor germ cell from the Mekong giant catfish (*P. gigas*) and recipient larvae of striped catfish (*P. hypopthalmus*).

1.2 Research hypothesis

1.2.1 Aging related SSC in spermatogonia might has effect on GCT efficiency.

1.2.2 The *vasa* could be used as gene marker for germ cells to study early gonadogenesis.

1.2.3 Triploid fish would grow normally, and its gonadal development would not be able to produce functional gamete.

1.2.4 For GCT by transplanting immature germ cell from the Mekong giant catfish into recipient striped catfish, the gonad of the striped catfish would be able to support colonization of immature germ cell from the Mekong catfish.

1.3 Scope and limitation of the study

This research project aims to develop GCT in Pangasiids catfish. In order to study the aging effect of SSC in spermatogonia, spermatogonia transplantation using

donor cell from various ages of donor fish was conducted in experimental madaka. Cloning and characterization of *vasa* mRNA was conducted to use as gene marker to study early development of gonad in recipient larvae. Triploidization in the striped catfish was carried out in the striped catfish, and its gonad development was demonstrated. Finally, GCT in Pangasiids catfish was developed by using the Mekong giant catfish as donor and the striped catfish as recipient fish.

1.4 Expected results

1.4.1 Information about age-related SSC function in medaka was achieved.

1.4.2 Gene maker for reproductive systems in recipient fish (striped catfish; *P. hypopthalmus*) and biological information of the early reproductive development in striped catfish larvae

1.4.3 Information of the optimal condition of production of triploid striped catfish, *P. hypopthalmus* which suitable to use as recipient larvae

1.4.4 The methodology of xenogenic GCT in Pangasiid fish using the striped catfish as the recipient fish

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CHAPTER II

LITERATURE REVIEW

2.1 Germ cell transplantation

2.1.1 Cell type of vertebrate

Cells are the primary units of life in humans and in other living things. As we all know, cells were discovered by Mr. Hooke in the 1600's. From then on, it was known to be the functional, primary, or smallest unit of life. They are also called the building blocks of life. There are two primary types of cells. These are the prokaryotic and eukaryotic cells. Prokaryotic cells are usually found in microorganisms such as bacteria and cyanobacteria while eukaryotic cells are found in multi-level or multi-cellular living organisms. All sexual organisms are composed of two fundamental cell types: germ cells and somatic cells.

Precursor of germ cells is known as primordial germ cell (PGC) which is originate in a particular region of the embryo. In fish, during early embryonic development, PGCs are extragonadal area. Later, PGCs migrate toward the presumptive gonad site. While the differentiating gonads, PGCs are developed to gonocytes. Male gonocytes are precursors of the spermatogonial stem cells (SSCs). However, female gonocytes are precursors of the oogonial stem cells (OSCs). Both SSCs and OSCs becomes a life-long reservoir of germline stem cells in males and females, respectively (Lacerda et al., 2013).

2.1.2 Stemness of immature germ cell

In male vertebrates, spermatogonial stem cells (SSCs) are the foundation of spermatogenesis. SSCs are undifferentiated spermatogonia which are responsible for preservation of spermatogenesis. Consequently, SSCs continuously self-renew as well as produce progenitor spermatogonia that are committed to differentiate and transform to spermatozoa. The balance of these processes was crucial to regulate the quantity and quality of SSCs to sustain spermatogenesis which was controlled by intrinsic function of cellular gene networks and/or extrinsic stem cell niche (Oatley and Brinster, 2008; Griswold and Oatley, 2013). This balance maintains the stem cell pool and meets the proliferative demand of the testis to produce millions of sperm each day. In mammalian ovaries, it is well known that mitotic division of germ cells completes before birth. Neo-oogenesis does not occur afterwards. Therefore, the number of oocytes to be ovulated is limited to the number of primordial follicles that are generated during embryogenesis. In contrast, in many vertebrates with high fecundity such as teleost, the number of oocytes is thought to be infinite. In the adult ovary of these species actually, mitotic germ cells are histologically observed as oogonia and thus the proliferation of oogonia can supply mature eggs continuously throughout the life (Nakamura et al., 2011). Therefore, progenitor cells of gametogenesis including type A spermatogonia and oogonia are possible to apply for GCT technique.

Aging-induced deterioration of function of SSCs have been demonstrated in mammals. To date, spermatogonial transplantation assay was demonstrated as an effective tool to study the function and biology of SSC in mice (Brinster and Zimmermann 1994; Kanatsu-Shinohara et al., 2006). Thus, age-related

infertility has been intensively studied using animal model particularly in mice (Schmidt et al., 2011; Ogawa et al., 2003; Ryu et al., 2006). Aging process affected both intrinsic function of spermatogonial stem cells (SSCs) and extrinsic microenvironment. For instant, aging affected rate of SSC proliferation in SSC culture. The rate of SSC proliferation in *in vitro* culture of SSC isolated from old mice was lower comparing to that from younger. Decrease in expression of gene that were crucial for SSC self-renewal and elevation in expression of gene that were involved in SSC differentiation was observed in aged SSC culture. Long term culture of SSC also had significantly lower transplantation efficiency (Schmidt et al., 2011). In addition, aging had negative effects on niche microenvironment which was the major factor to deteriorate testis in mouse (Ogawa et al., 2003; Ryu et al., 2006). Therefore, whether age would affect function of SSC and/or niche regulatory remains to be extensively investigated which would enable to understand aging programing on reproductive system in fish.

2.1.3 Principle of germ cell transplantation

The technique for GCT in fish simply mimics the principle of gonadal development in vertebrates. During the early stage of development, genital ridge is formed with somatic cells, and PGCs are developed outside of the gonad. Later, PGCs migrate toward the genital ridge with pseudopodia by chemotaxis (Raz, 2004). After PGCs have settled in genital ridge, PGCs are surrounded by somatic cells and start proliferation (Yoshizaki et al., 2002). Later, sex differentiated-mechanism occurs. GCT is the technique to introduce the PGCs or the progenitor of gamete into peritoneal cavity of recipient larvae. The donor PGCs or germ cell-like PGCs subsequently migrate and incorporate toward the genital ridge of recipient fish. Then,

recipient somatic cells support the donor cell proliferation and differentiation. In general, more closely related species between donor and recipient fish will enable more successful of transplantation efficiency. As a result, recipient fish produces donor-derived offspring, enabling surrogate broodstock technology (Fig. 2.1). This technique is considered to be a powerful tool for applying to a number of fish species in which artificial seed production is a major constraint such as fish with large body size and endangered species.

GCT have been developed in a number of animals. For example, PGCs were transferred to the blood stream of developing chicken embryos. After the chicken was reached to sexual maturity, PGCs were able to produce donor-derived offspring (Tajima et al., 1993). In addition, GCT was established in rodents. This method utilized a cell suspension obtained from donor testis, which was injected into the seminiferous tubules of infertile recipient mice. SSCs present in the injected cell suspension were colonized to the recipient seminiferous tubules. After maturation, donor spermatogenesis was observed in the injected mices (Brinster and Zimmermann, 1994). Moreover, GCT in non-rodents mammals such as goats (Honaramooz et al., 2003), sheep (Rodriguez-Sosa et al., 2006), cattle (Herrid et al., 2006), pigs (Mikkola et al., 2006), monkeys (Hermann et al., 2007) and felines (Silva et al., 2012) have also been reported. Therefore, GCT provides a useful tool for not only understanding of germ cells physiology, but also contributing to the fields of experimental animal research and zootechnical science.

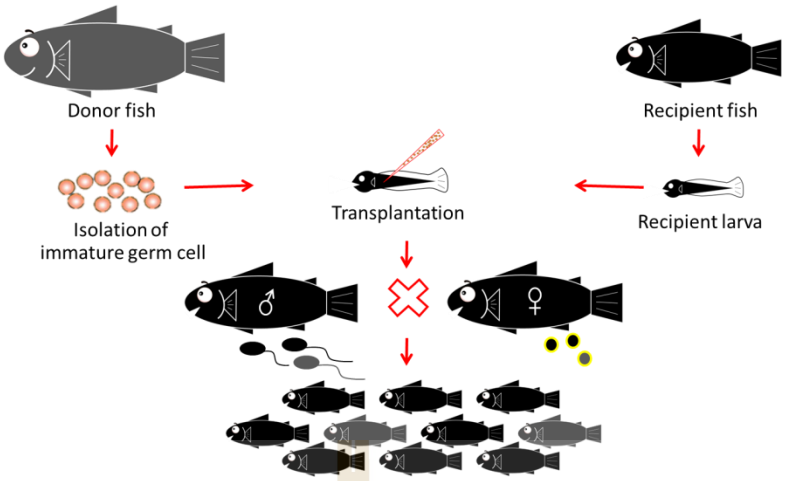

Figure 2.1 Scheme of germ cell transplantation in fish

In fish, GCT has been established in salmonid, medaka and some marine fish by using different method of cell dissociation (Table 2.1) (Takeuchi et al., 2004; Okutsu et al., 2006; Nakamura et al., 2011; Takeuchi et al., 2009; Yazawa et al., 2010). For example, primordial germ cell from donor rainbow trout was introduced into the peritoneal cavity of masu salmon recipient larvae (Takeuchi et al., 2004). Since the donor fish was transgenic rainbow trout which carried green fluorescent protein (gfp) driven by the vasa promoter (Yoshizaki et al., 2000), the migration and incorporation of introduced PGCs could be easily followed. The donor PGCs proliferated and differentiated under the normal condition. Furthermore, the recipient salmon contained trout sperms, and they could produce donor-derived rainbow trout using the masu salmon as the surrogate parents. Although the efficiency of donor-derived offspring was low (0.4%), this was the first report demonstrating interspecific germ cell transplantation in fish (Takeuchi et al., 2003; 2004). In general, PGCs are found in young fish or fish larvae which contain only small number of PGCs. It also

needs the biological skills to isolate PGCs from fish larvae. As a result, using PGC for transplantation would be a promising technique for basic study but not for aquaculture industries. Spermatogonia have a potential to become stem cell including differentiation ability (Yoshida et al., 2007). A high number of spermatogonial cells are found in testes through life cycle of male fish. Therefore, isolation of spermatogonial cells is much more practical than that of PGC. Transplantation of spermatogonial cell of rainbow trout with *gfp*-labeled was conducted in masu salmon larvae (Okutsu et al., 2006). The migration and incorporation of donor spermatogonia from rainbow trout could be achieved in masu salmon larvae. Further, the incorporated spermatogonia was be able to proliferate, generate functional sperm and produce donor-derived offspring (18.9%) (Okutsu et al., 2006). When spermatogonia were transplanted into female larvae, they could produce functional eggs, demonstrating sexual plasticity property of spermatogonia (Okutsu et al., 2006). Oogonial cells from rainbow trout were also transplanted into masu salmon larvae. Subsequently, donor oogonia was reported to incorporate and differentiate in recipient salmon. Oogonia cell was also able to produce oogonia-derived sperm in male recipient and generate rainbow trout offspring, demonstrating sexual plasticity property of oogonia (Yoshizaki et al., 2010). Combined together, both spermatogonia and oogonia behave characteristics of PGC-like cell and sexual plasticity to produce donor-derived gametes.

Table 2.1 Gonadal dissociation for germ cell isolation

Species	Gonadal type	Dissociation enzyme	Incubation	Reference
Nibe croaker	Testis	0.25% trypsin	3 h at 25°C	Takeuchi et al. (2009)
Nile tilapia	Testis	0.5% trypsin	3 h at 25°C	Farlora et al. (2014)
Rainbow trout	Genital ridges from hatched embryos	0.5% trypsin	2 h at 20°C	Takeuchi et al. (2003)
	Postspermiation testis	0.5% trypsin	2 h at 20°C	Okutsu et al. (2006)
	Testis	0.5% trypsin	2 h at 20°C	Sato et al. (2014)
Siberian sturgeon	Testis or ovary	0.3% trypsin	2 h at 23°C	Psenicka et al. (2015)
Yellowtail	Testis	0.25% trypsin	3 h at 20°C	Higuchi et al. (2011)
	Testis	0.4% collagenase H with 0.03% dispase II	2 h at 25°C	Morita et al. (2012)

Germ cell transplantation was also carried out in marine fish. In order to develop germ cell transplantation methodology, microinjection of spermatogonia into allogenic larvae was first conducted in Nibe croaker (*Nibea mitsukurii*) (Takeuchi et al., 2009). The maintenance of Nibe croaker broodstock is well-developed which enable the establishment of surrogate broodstock in marine fish. Later, xenogenic germ cell transplantation was demonstrated by introduction of spermatogonia of chub mackerel (*Scomber japonicus*) and yellow tail (*Seriola quinqueradiata*) into larvae of Nibe croaker (Yazawa et a., 2010; Higuchi et al., 2011). In addition, the study of oogonia transplantation was also reported that ovarian germ cells transplantation from transgenic Pearl danio (*Danio albolineatus*) into sterile *Danio* hybrid recipients was successfully (Wong et al., 2011). This technique provides application for conventional breeding to increase a number of broodstock carrying germ cells with desirable genetic traits (Morita et al., 2012).

Another approach was to confirm the surrogate broodstock that could produce the donor-derived offspring. Albino-strain which is easily classified by color property was used as recipient fish in zebrafish (Lin et al., 1992). As a result, when the recipient fish were maturation, they could produce the donor-offspring which possessed different color from parents. Furthermore, the albino-strain was also used as donor fish in germ cell transplantation of rainbow trout (Okutsu et al., 2006).

2.1.4 *vasa* gene

There are several genes that are specifically expressed in germ cells including *vasa*, *nanos*, *dazl*, *deadend*, and *cxcr4b* in living organisms that could be potential candidates as a marker (review in Raz, 2003). Among them, *vasa* gene products have been extensively used as a molecular marker for identification of germ

cells. The *vasa* gene is widely demonstrated as an essential germline marker. It was originally identified in *Drosophila* as an essential gene for germline development, and since then it has been studied in the germline of many animals (Raz, 2000). *vasa* expression allowed be used to identify PGCs in larvae, as well as spermatogonia and oogonia throughout the reproductive cycle (Braat et al., 2000; Yoshizaki et al., 2002; Úbeda-Manzanaro et al., 2014).

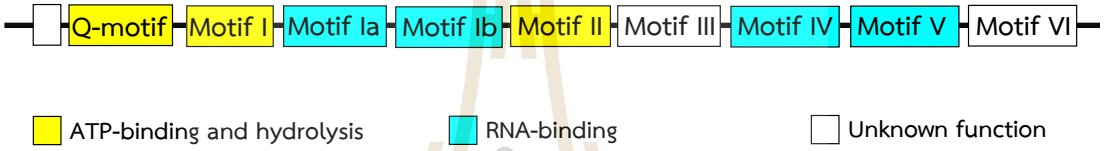

Figure 2.2 ATP-dependent RNA helicase of the DEAD-box family

Source : Cordin et al. (2006)

Vasa is a founding member of the family of DEAD-box proteins, and its consensus sequence L-**D**-**E**-**A**-**D**-X-(M/L)-L-X-X-G-F shared with other DEAD-box members (e.g. eIF4A, p68, and PL10) reflects a unique version of the B-motif of ATP-binding proteins (Fig. 2.2) (Linder et al., 1989). Along with its RNA helicase domain and its highly conserved carboxy-terminal regions, Vasa appears to be a part of a larger translational complex (Johnstone and Lasko, 2004). The *vasa* RNA is expressed in the germ cells of many organisms**.** For example, *vasa* RNA is uniformly distributed in early *Drosophila* embryos, but consistent with its function, the protein is found localized to the posterior pole, where it is associated with the polar granules in the germ plasm**.** Similar discrepancies between RNA and protein expression, in

which the vasa protein is found in a restricted number of cells relative to the RNA and is localized to specific subcellular structures, have also been described in *Xenopus* and the nematode (Raz, 2000). The localization of *vasa* RNA to the germ plasm allowed them to follow precisely the distribution of the germ plasm to the cells of the early embryo (Knaut et al., 2000). Until late blastula stages, the four cells that contain germ plasm divide asymmetrically, so that only one of the blastomeres resulting from each division inherits the germ plasm labeled by *vasa* RNA which will become the future PGCs. The germline stem cells of *Drosophila* are abundant with Vasa, and it now appears to be essential for mitotic activity through an interaction with chromatin (Pek and Kai, 2011). Vasa was also found to be essential for cell cycle progression in early embryos of the sea urchin (Yajima and Wessel, 2011b). The function of the *vasa* gene can be inferred from its expression pattern in different organisms**.** With the exception of the mouse (and probably other mammals), the *vasa* gene product is expressed in or localized to the PGCs at early gonad development (Yajima and Wessel, 2011a).

The application of *vasa* gene has been done in many investigations. Due to the localization of vasa expression, in situ hybridization approach can be done with *vasa* antisense probe. For example, *vasa* probe used to detect the allogeneic germ cell in yellowtail (Morita et al., 2012) or transplanted xenogenic germ cells in recipient chub mackerel (Yazawa et al., 2010) and Nibe croaker (Higuchi et al., 2011).

Using *vasa* gene expression as marker gene to characterize PGC at early development of fish larvae, the optimal stage of recipient fish larvae were identified for GCT. Newly hatched larvae were chosen to be used as recipients as they did not possess a functional immune system, as indicated by the lack of differentiation in

both their thymus and their T-cells. Lack of a functional immune system allowed the immunorejection of exogenous (donor-derived) germ cells to be avoided (Lacerda et al., 2013). Like in other vertebrates, fish PGCs are generally large in size, have a low nucleocytoplasmic ratio and have a distinct nuclear border and granular nuclear chromatin (van Winkoop et al., 1992; Patino and Takashima, 1995). Despite these hallmarks, identification of PGCs, particularly at the developmental stages before complete migration toward genital ridge, is difficult and demonstrates a need for a molecular marker to confirm PGC identification. Morphological characterization together with the aid of a molecular marker would enable the investigation of early reproductive development in fish larvae.

2.2 Mekong giant catfish (*Pangasianodon gigas*)

Figure 2.3 Mekong giant catfish

Mekong giant catfish (Fig. 2.3) is one of the world's largest freshwater fish, measuring up to three meters in length and weighing in excess of 300 kg. Mekong giant catfish is endemic to the Mekong basin. It is known from the Tonle Sap Lake,

Tonle Sap River, and the Mekong River. The local Thai name “Pla Beuk” has been known to Thai and Lao people, and the common name, “Mekong giant catfish” has been known worldwide. Historical reports indicated that the species was abundant in the early 1900s. However, in the 1970s, local fisheries began to report the disappearance of this fish. Current population size is unknown, but a decline of more than 80% over the last 28 years can be estimated from past annual catch records, qualifying the species for critically endangered under criterion A (Mattson et al., 2002).

Fishing effort in the Mekong basin in general is increasing. Fishing effort specifically for this species in the Mekong River remains constant, although it may be increasing in some areas, such as in the Tonle Sap Lake. Habitat loss and degradation are also serious threats to this fish. There has been increasing siltation or water pollution of the Mekong mainstream through past deforestation practices in the northern parts of the Mekong River area. It is a migratory species. From October to December each year, the species moves out of the lower Mekong to migrate upstream into northeastern Cambodia and possibly Lao PDR, or Thailand to spawn (Mattson et al., 2002). The loss of migratory routes through the construction of dams may pose a serious threat to the species' spawning habitat and also have a negative impact on fish abundance in the river (Mattson et al., 2002).

Artificial breeding of this species was achieved for the first time in 2001. Individuals artificially spawned from wild-caught parents have been released into the Mekong since 1985, however this practice is now thought to have stopped, and fish are now only introduced into reservoirs and not into the Mekong river. The fish almost certainly spawns upstream of Chiang Khong, Thailand. Possible spawning site

includes the Kok River near Chiang Saen, Thailand. Previously known spawning sites in the Mekong River are between Loei and Nong Khai Provinces and in Ubon Ratchathani Province before the river fully enters Lao. First maturation is 15-17 years, from artificial breeding recorded of the first offspring from wild spawners in the Thai Department of Fishery's ponds. Generation length for captive fish is possibly 35 years, but this is probably not representative of the wild fish. For wild individuals, generation length has been reported as less than ten years, however this is difficult to verify. The best estimate of generation length is between 10 and 15 years, but this is a very uncertain estimate and further research on the life history of this species is needed to confirm this (Mattson et al., 2002).

2.2.1 Conservation of Mekong giant catfish

This species has been listed on CITES Appendix I since 1975. The species occurs in a Biosphere Reserve in the Tonle Sap Lake, and a Ramsar site in northeastern Cambodia, although neither of these sites offers real protection for the species. In Thailand, fishing for this species is regulated based on a quota license of less than 20 catches annually. The Department of Fisheries in Thailand began releasing captive-bred individuals in 1985. Between 2000 and 2003, approximately 10,000 captive-bred fish were released into the Mekong. Captive-bred individuals are no longer released into the Mekong. However, they are released into reservoirs in Thailand. Large fish are now caught regularly in some Thai reservoirs but there is no evidence of self-sustaining populations. However, the fish have also been artificial hybridized with *P. hypophthalmus* for aquaculture purposes (Mattson et al., 2002).

2.3 Striped catfish (*Pangasianodon hypophthalmus*)

Figure 2.4 Striped catfish

Striped catfish (Fig. 2.4), the local Thai name “Pla Sa Wai”, is the scaleless freshwater fish. The biggest was 1.5 m. in length (Mattson et al., 2002). The striped catfish is relatively fast-growing catfish. It is an omnivore and inhabits in main channels and floodplains of large rivers and seasonally moves up to floodplains and wetlands for feeding and nursing (Vidthayanon and Hogan, 2013). This fish grows approximately one kilogram per year. The fish reaches full maturity between two to four years. Spawning adults migrate upstream each year in at the beginning of the flood season. Eggs are sticky and apparently deposited on submerged vegetation. Maximum life span is estimated at approximately 20 years (Pangasiids at the Steinhart Aquarium in San Francisco, U.S.A.) (Vidthayanon and Hogan, 2013). It has been an economically important fish. Full-cycle of the striped catfish has been well-know. In order to raise the striped catfish as broodstock, fish have been grown at the stocking density of 800 fish per Rai (1,600 M^2). This species has been bred in captivity; therefore, it is widely cultured throughout Thailand and Vietnam (Vidthayanon and Hogan, 2013).

2.4 Triploid fish

Most vertebrates are diploid, meaning that they possess two complete chromosome sets in their somatic cells. Polyploid individuals possess one or more additional chromosome sets, bringing the total to three in triploids, four in tetraploids, and so on. Polyploidy has played a major role in economically important groups of fishes, including many cyprinids and all salmonids and catostomids (Schultz, 1980; Allendorf and Thorgaard, 1984; Ferris, 1984). There are a few extant triploid species, all of which are unisexual (female) and exhibit atypical modes of reproduction (Schultz, 1980; Purdom, 1984).

The methods used to induce triploidy and the applications of these biotechnologies to aquaculture and fisheries management are well described. Artificially produced triploids differ from conspecific diploids in three fundamental ways: they are generally more heterozygous (Allendorf and Leary, 1984; Leary et al., 1985), they have larger but fewer cells in a variety of tissues, and their gonadal development is disrupted to some extent. Triploid cell nuclei contain, by definition, 50% more DNA than diploid cell nuclei. Nuclear volume is increased in triploids to accommodate this extra genetic material. A corresponding increase in cellular volume typically results due to the approximate maintenance of the diploid ratio of nuclear to cytoplasmic volume. Despite increased cell size, triploid individuals are not, as a rule, larger than diploids. This appears to be due to a reduction in cell numbers in those tissues and organs containing larger cells (Benfey et al., 1999). Gonadal development in triploids is typically disrupted early in gametogenesis, when cells enter meiosis. This is presumably due to mechanical problems associated with the pairing of homologous chromosomes in the presence of a third set of homologues. Although

poorly studied in fish, there was some histological evidence to support this presumption (Gui et al., 1992). Impaired gametogenesis typically has much greater physiological effects in females than in males.

2.4.1 Triploid hematology

Many studies have demonstrated that erythrocyte cellular and nuclear dimensions are increased in triploids (Fig. 2.5). This fact is so well accepted that some measure of erythrocyte size is frequently used as the sole criterion for determining ploidy level in fish. Increased size is apparently not uniform in all dimensions: many of the studies have demonstrated a greater proportionate increase in cellular and/or nuclear length than in width (Sezaki et al., 1991).

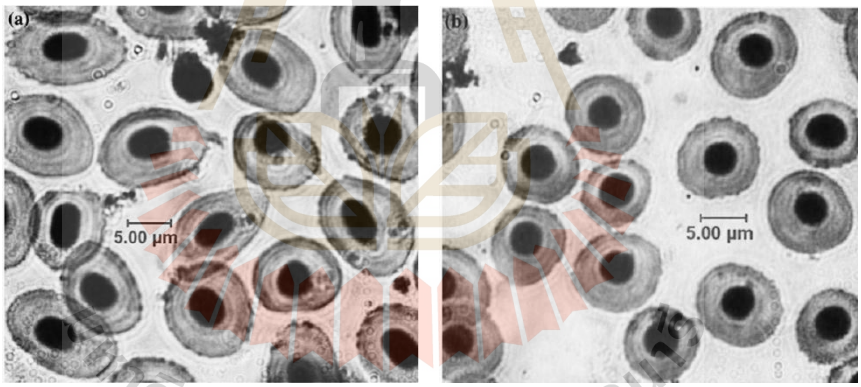

Figure 2.5 Erythrocytes of triploid (a) and diploid (b) African catfish (×1,000) showing more elongated shape and larger size of triploid erythrocytes

Source : Karami et al. (2010)

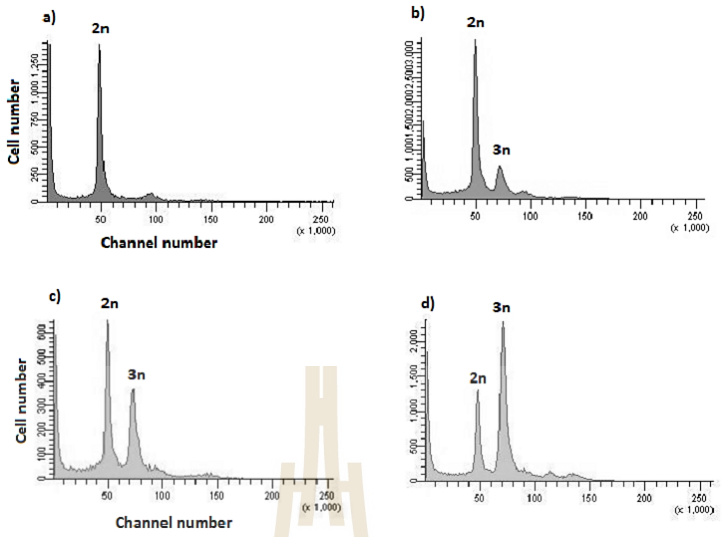

Figure 2.6 Flow cytometric histograms for the relative DNA content of somatic cells when somatic cells of normal diploid catfish were used as standard of normal diploidy. Diploid (2n) larva of control (a), triploid (3n) and diploid larva of treated groups (5 mM, 10 mM and 15 mM as b,c,d respectively)

Source : Turan and Guragac (2014)

Recently, many of the studies have demonstrated DNA content of triploids by flow-cytometry. Turan and Guragac (2014) had reported that induction of triploidy with caffeine treatment in the African catfish (*Clarias gariepinus*) to suppress the second meiotic division (Fig. 2.6). Preston et al. (2013) conducted to determine the optimal timing of application post-fertilization, define optimal pressure intensity and duration of the shock and study the effect of temperature (6-12°C) on triploid yield in brown trout (*Salmo trutta* L.). Examination of triploid fish were carried out by blood smear and flow cytometry since these techniques were simple

and accurate. The results showed the high triploid yields (82.5-100%) when treated by hydrostatic pressure shock (HP) 10,000 psi applied at 300 centigrade temperature minutes (CTM) for at least 5 minutes duration or shocked in HP 10,000 psi applied using water temperatures of 6, 8, 10 and 12°C. The HP shocks all started at 300 CTM (at 50, 37.5, 30 and 25 minutes post fertilization, respectively) for durations of 8 minutes 20 s, 6 minutes 15 s, 5 minutes and 4 minutes 10 s respectively. The high deformability of fish erythrocytes may also allow for increased erythrocyte volume without any effect on capillary blood flow (Parsons, 1993).

2.4.2 Sexual maturation of triploids

Differences in gonadal growth between females and males in both the number and size of gametes were observed. The majority of germ cells do not progress through first meiotic prophase in triploids of either sex, thus resulting in small ovaries with a small number of previtellogenic oogonia and primary oocytes, but large testes with a large number of spermatogonia and primary spermatocytes (Takeuchi et al., 2017). Many of the studies have reported the production of viable spermatozoa by triploids, at greatly reduced numbers, resulting in very dilute milt. Abnormalities in spermatozoan morphology have also been described (Kawamura et al., 1995; Nakamura et al., 1993; Teong, 1991). When milt from triploid males is used to fertilize normal haploid eggs, the resultant progeny typically begin development but die at embryonic and larval stages. It was revealed that both the brain and the pituitary gland of triploid females remain responsive to steroid signals (Benfey et al., 1989; Breton and Sambroni, 1996). Steroidogenic cells are present and active in triploid males, resulting in the normal pattern of endocrine changes associated with sexual maturation in diploids (Benfey, 1999).

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CHAPTER III

AGING-RELATED ACTIVITY OF SPERMATOGONIAL STEM CELL IN MEDAKA: A VERTEBRATE MODEL FOR INVESTIGATION OF SPERMATOGONIAL STEM CELL PROPERTY

3.1 Abstract

Aging process in deterioration of stem cell function has been attracted to investigate to explore aging programing on reproductive system. The medaka could serve as an excellent model for studying aging-related biological characteristics of spermatogonial stem cells (SSCs) which exhibit self-renewal and producing progenitors committed to differentiation into spermatozoa. In this study, using the medaka, aging effect of SSC activity was investigated in type A spermatogonia (ASG) which behave as SSCs in teleost. The experiment began by determining age-related changes in the eye at 1, 2, 3, 4, 8 and 18 months of age. Morphological changes of testis in the medaka at these ages were demonstrated. The gonadosomatic index (GSI) and number of ASG were significantly different among fish at 4-months (the onset maturation), 8- and 18 months (senescent age) of age. In addition, low water temperature appeared to retard testis development, and therefore, had effects on GSI and ASG number. Aging-related activity of SSCs in ASG was performed using transgenic medaka expressing green fluorescent specifically in germ cells. In order to

investigate aging effect of ASG, GFP-expressed ASG were isolated from fish at 4 (the onset of maturation) and 18 (senescence) months of age and transplanted to hybrid recipient larvae, which provide as young niche microenvironment. The result showed that there were no significant differences in colonization rate of ASG isolated from both ages. In addition, similar germ-line transmission rates were observed. Moreover, water temperature had no significant effects on SSCs function. Combined together, these findings suggested that there was aging and temperature effects on ASG number. However, when different age of ASG were transplanted into young niche microenvironment gonad, aging-related activity of SSCs in ASG was not observed.

3.2 Introduction

Global life expectancy in human has increased, therefore, age-related infertility has been attracted to investigate. Age-related decline in fertility has been demonstrated in mammals, and a number of mechanisms and/or factors were involved such as alteration of endocrine system, oxidative stress, microenvironment and DNA mutation which, therefore, caused male infertility (Gunes et al., 2016; Yamada et al., 2016). SSCs are undifferentiated spermatogonia which are responsible for preservation of spermatogenesis. Consequently, SSCs continuously self-renew as well as produce progenitor spermatogonia that are committed to differentiate and transform to spermatozoa. The balance of these processes was crucial to regulate the quantity and quality of SSCs to sustain spermatogenesis which was controlled by intrinsic function of cellular gene networks and/or extrinsic stem cell niche (Oatley and Brinster, 2008; Griswold and Oatley, 2013).

Aging-induced deterioration of function of (SSCs) have been demonstrated in mammals. To date, spermatogonial transplantation assay was demonstrated as an effective tool to study the function and biology of SSC in mice (Brinster and Zimmermann 1994; Kanatsu-Shinohara et al., 2006). Thus, age-related infertility has been intensively studied using animal model particularly in mice (Schmidt et al., 2011; Ogawa et al., 2003; Ryu et al., 2006). Aging process affected both intrinsic function of spermatogonial stem cells (SSCs) and extrinsic microenvironment. For instant, aging affected rate of SSC proliferation in SSC culture. The rate of SSC proliferation in *in vitro* culture of SSC isolated from old mice was lower comparing to that from younger. Decrease in expression of gene that were crucial for SSD self-renewal and elevation in expression of gene that were involved in SSC differentiation was observed in aged SSC culture. Long term culture of SSC also had significantly lower transplantation efficiency (Schmidt et al., 2011). However, aging had negative effects on niche microenvironment which was the major factor to deteriorate testis in mouse (Ogawa et al., 2003; Ryu et al., 2006). Therefore, whether age would affect function of SSC and/or niche regulatory remains to be extensively investigated which would enable to understand aging programing on reproductive system.

Vertebrates display diverse reproductive strategies, particularly, fish exhibit the greatest diversity of reproductive models. For example, some fish are transgender which change their sex according to social status and/or during certain age (Todd et al., 2016). Extraordinarily, salmon are semelparous which die following mating (Kindsvater et al., 2016). A number of fish are seasonal reproduction which could produce functional gamete only specific period yearly while the others are nonseasonal reproduction which has ability to produce functional gamete throughout

the year. In teleost, type A spermatogonia (ASG) behaved as SSCs. In addition, ASG transplantation technology has been well-developed in a number of fish. The transplanted spermatogonial cells were be able to incorporate into recipient gonad, resume gametogenesis to produce donor-derived gamete and generate donor derived offspring (Takeuchi et al., 2003; review in Yoshizaki et al., 2000). Thus, fish could be useful models to investigate age-associated SSC properties among vertebrates which could partly provide information about aging-related evolution process to extend theories of biological aging in animals. Using rainbow trout (*Oncorhynchus mykiss*) which is seasonal breeder, during spermiation, residual spermatogonia are suggested as SSCs which had capability to support spermatogenesis in the next spawning season. Together with ASG transplantation technique, seasonal associated SSC characteristics were demonstrated (Sato et al., 2017).

In nonseasonal reproductive fish, the balance of self-renewal and producing of progenitors committed to differentiate into active gamete would be a main factor affecting heterogeneity of SSCs, and how age-associated function of SSC in nonseasonal fish remains to be clarify. The medaka which are native to East Asia has been widely used as an experimental fish for vertebrate model. Medaka have short life cycle, and the mating system has been well-developed under laboratory condition. Germ cell transplantation technology and cryopreservation of testicular tissue were demonstrated which employ the medaka to use for investigate SSC properties (Seki et al., 2017). In addition, transgenic medaka expressing GFP specifically in germ cells was established (Tanaka et al., 2001) which employed to use as donor fish to monitor SSC function. Moreover, interspecific hybrid medaka, particularly, Japanese medaka (*Oryzias latipes*) and Chinese Hainan medaka (*O. curvinotus*) was sterile because of

aberration of gametogenesis (Hamaguchi and Sakaizumi, 1992). These hybird offspring would be benefit to use as recipient fish to produce only donor-derived offspring. Taken together, medaka would be an excellent fish model to study age-related SSC function which would provide applications for further preserving animal genetic resources and reproductive management. In this study, as the model of nonseasonal fish, age-associated function of SSC were investigated in the medaka. Age-associated change in retinal morphology was used to validate medaka aging. Age-related development of testis was displayed. In addition, using ASG transplantation technique, age-related SSC function was demonstrated in their efficiency of transplantation efficiency and germline transmission.

3.3 Materials and methods

3.3.1 Recipient fish

Both Japanese medaka (*Oryzias latipes*) and Chinese Hainan medaka (*O. curvinotus*) used in this study were maintained at the Tokyo University of Marine Science and Technology (Tokyo, Japan) under laboratory condition (Kinoshita et al, 2009). Fish were kept in plastic tanks (15 L) with aeration. Fish were fed commercial diet (Otohime, Aquatic Enterprise Co., Malaysia) *ad libitum* for 4 times daily, 10.00, 13.00, 16.00 and 19.00.

Hybrid embryos were obtained by natural mating. Ten pairs of female Japanese medaka and Chinese Hainan medaka or their reciprocal were cultured in tank (15 L) with aeration. Every morning, fertilized eggs were observed at the genital pore of female. The fertilized eggs were transferred into Petri disc and incubated in

26°C with replacing dechlorinated water daily until hatching. The larvae at 11-13 days post fertilization (dpf) were used for transplantation.

3.3.2 Donor fish

In this study, we used transgenic orange medaka carrying *vasa-GFP* in which the germ cells exhibit green fluorescence, predominantly in ASG. Fish were cultured under laboratory condition as described as the above.

In order to investigate the effects of water temperature on testis development, fish at 4 months of age (the onset of maturation) and 14 months of age (pre-senescence) were transfered to maintain at 10°C until they became 8 and 18 months of age, respectively.

3.3.3 Fish sampling and histological study

In order to investigate age-related changes in the eye and testis development, transgenic male at 1-, 2-, 3-, 4-, 8-, and 18-months of age were sampled. In addition, Donor fish at 8 and 18 months of age which were cultured at 10°C for 4 months were performed histological study of testicular tissues. Experimental fish were anesthetized with 2-phenoxyethanol (Wako Pure Chemical Industries, Ltd, Japan) at 2 ppm. Subsequently, fish and isolated testis were weighed to calculate the gonadal somatic index (GSI=gonad weight × 100 × body weight^{-1}). The GSI of the experimental fish at 1, 2, 3, 4, 8, and 18 months of age were 1.74±0.44, 0.63±0.25, 0.55±0.23, 0.77±0.05, 0.63±0.04, and 0.62±0.03 g, respectively. Eyes and testis were collected and fixed in Bouin's fixative solution at 4°C for 24 h. After washing with 70% ethanol, the fixed specimens were dehydrated using an ethanol series and then embedded in paraffin, sectioned to a thickness of 5 µm, and stained with hematoxylin and eosin (H&E).

In order to determine the localization of ASG in testicular lobules, paraffin sections were subjected to immunofluorescent staining. Paraffin sections were immune-stained using mouse anti-GFP antibody (11 814 460 001; Roche, Basel, Switzerland). The secondary goat anti-mouse IgG conjugated to Alexa 488 (Life Technology, Driverockville, US) was used. The primary antibody against GFP and the secondary antibody were diluted to 1:500 and 1:200, respectively.

3.3.4 Isolation of ASG from testicular cells

In order to determine the number of ASG, fish at 4-, 8-, and 18-months of age were collected testes. In addition, testes were isolated from fish at 8-, and 18-months of age that were maintained at 10°C. The isolated testes were dissociated using the method described previously (Seki et al., 2017). Briefly, testes (2 mg) were minced and incubated with the mixture of 0.2% collagenase H (Roche Diagnostics, Mannheim, Germany), 0.17% dispase II (Sanko Junyaku Co., Ltd., Tokyo, Japan), 10% FBS (Gibco Invitrogen Co.) and 900 U ml^{-1} DNase I (Roche Diagnostics) in L-15 medium (pH 7.8 with Hepes, Gibco Invitrogen Co., Grand Island NY, USA) for 90 minutes at 26°C. The dissociated cell suspension was rinsed with L-15 medium containing 10% FBS to eliminate enzymatic activity. In order to discard non-dissociated cell clumps, the cell suspension was filtrated through a nylon mesh (42-μm) (Tokyo Screen Co., Ltd.). After centrifugation at 200 × g for 10 min at 4°C, the resulting pellets were suspended in the L-15 medium containing 10% FBS. The number of ASG per mg testis were determined.

3.3.5 Spermatogonial transplantation and determination of colonization

ASG isolated from donor medaka carrying *vasa-GFP* of 4- and 18-months of age were used for spermatogonial transplantation. The hybrid recipients

were anesthetized with 2-phenoxyethanol at 0.3 ppm before transplantation. Approximately 10,000 ASG were microinjected into the peritoneal cavity of hybrid recipient larvae. A variety number (12-39 recipient hatchlings) of hybrid recipient larvae were obtained each time. Triplicate transplantation experiment were conducted to determine the colonization rate. At 30 days post-transplantation (dpt), the recipients were dissected, and incorporation of donor ASG was assessed using a fluorescent microscope (model BX-51N-34FL; Olympus) equipped with a GFP filter (U-MWIB; Olympus). Colonization rate was determined as the number of recipients carrying donor ASG in their gonads divided by the total number of analyzed recipients.

3.3.6 Germline transmission

In order to determine germline transmission, each transplanted recipient male were mated with Japanese female medaka (1:1 sex ratio) in breeding tank (1 L) for 1 week. The donor-derived embryos (6 dpf) had GFP primordial germ cell at the presumptive gonad. The germline transmission rate of recipient male was conducted as triplicate (n=10/donor replicate). In addition, the germline transmission rate of recipient female was conducted as triplicate (n=10/donor replicate). In breeding tank (1L), each transplanted recipient female were mated with Japanese male medaka (1:1 sex ratio) for 1 week. GFP was observed at the germinal disc of the donor-derived eggs (1 dpf).

3.3.7 Statistical Analysis

Data are presented as means ± SE unless otherwise stated. All data were analyzed one-way analysis of variance (ANOVA) using SPSS for Windows (Release 14) (SPSS Inc. Chicago, IL, USA). When significant differences were

observed among the groups, Tukey's multiple comparison test was performed to rank the groups. Values were considered statistically significant when the calculated *P* values were less than 0.05 ($P<0.05$).

3.4 Results

In order to investigate the aging-effects of ASG on transplantation efficiency, we used transgenic medaka carrying *vasa-GFP* which expressed GFP specifically in germ cells particularly in immature germ cells. In this study, to validate fish age, age-related changes in the eye were investigated in fish at age of 1, 2, 3, 4, 8 and 18 months (Fig. 3.1). The results showed clear alteration in retinal pigment epithelium (RPE) according to fish age. With increasing age, the thickness of RPE layer altered, and their morphological character appeared pleomorphic. In addition, the basement membrane of RPE changed. Moreover, the thickness of inner/outer segments (IS/OS) where the photoreceptors such as rod and cone cells are located decreased with increasing age. Also, pleomorphism appearance was observed in the IS/OS, particularly in the eye of fish at 18-month of age.

In order to study development of testis of medaka during pubertal through senescence ages, histological studies of testis at various ages of medaka including 1, 2, 3, 4, 8 and 18 months of age were performed. Fig. 3.2 showed that the testis of medaka is typical lobular-type with a lobular cavity at the center which contain various stages of testicular germ cells such as ASG, type B spermatogonia, primary spermatocyte, secondary spermatocyte and spermatozoa. Most ASGs were located at germinal epithelium (Fig. 3.2). At 1-month age, testis was small (body weight=0.60±0.01 g; GSI=1.74±0.44%) which contained ASG, type B

spermatogonia, primary spermatocyte and secondary spermatocyte throughout testis, and rare spermatozoa were observed (Fig. 3.2B). The size of testis of 2-, 3- ,4-, 8-months of age fish increased with increasing age; however, the size of testis of fish at 18-month decreased (Fig. 3.2C, E, G, I, K). Indeed, the GSI of fish 2-, 3- ,4-, 8-, 18-months of age were 0.63±0.25, 0.55±0.23, 0.77±0.05, 0.63±0.04, and 0.62±0.03 %, respectively. During these ages, all stages of testicular cells including ASG, type B spermatogonia, primary spermatocyte, secondary spermatocyte and spermatozoa were observed (Fig. 3.2D, F, H, J, L). In addition, spermatozoa were found at the center of the lobule's lumen in fish at 3- ,4-, 8-, 18- months of age (Fig. 3.2F, H, J, L), indicating the maturity stage of medaka. During maturity ages, the number of spermatozoa increased with increasing age of fish, and the majority of spermatozoa was observed in testis of fish at 8-months (Fig. 3.2J).

In order to investigate the effect of temperature on testis development and number of ASG, we incubated the medaka at 10°C for 4 months comparing with that raised under laboratory condition at 26°C. Lower temperature was observed to affect testis development. The medaka at 8 months of age that were incubated at 10°C, had smaller testis comparing with that of the same age (Fig. 3.2K-L). The population of spermatozoa were lower, and cavities inside the cyst of spermatozoa were observed. Similarly, lower in spermatozoa population appeared in the testis of fish at 18 months of age that were incubated at 10°C (Fig. 3.2O-P) These findings suggested that low temperature led to lower the spermatozoa differentiation process. Low temperature appeared to decrease ASG number in the testis of the medaka at 8 months of age (maturation stage) However, increase in ASG number was observed in the testis of the medaka at 18 months of age (senescent stage) that were incubated at 10°C. These

observations suggested that temperature had significant effect on ASG number, and the effects were different depending on age.

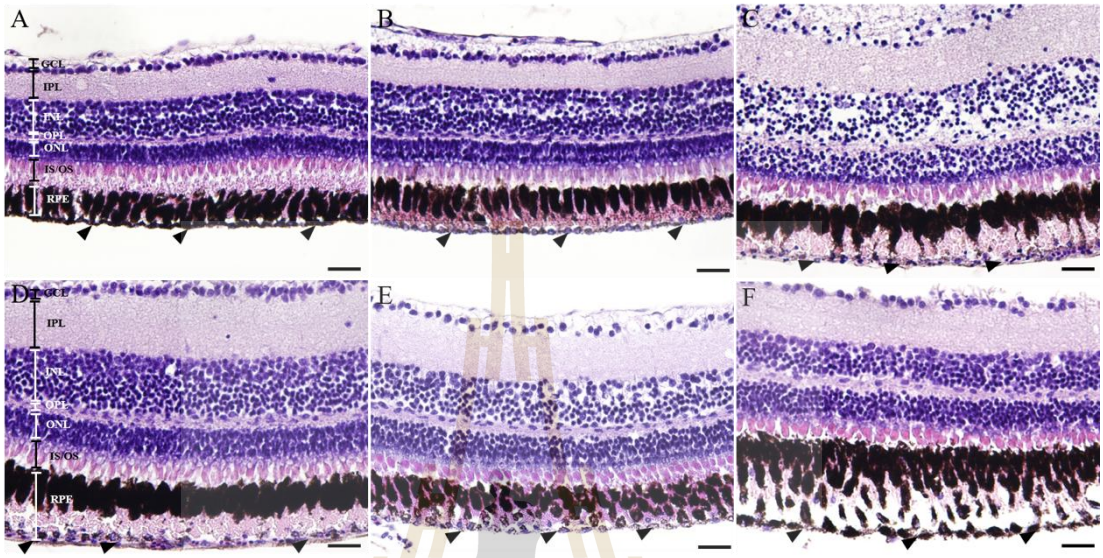

Figure 3.1 Aging-related changes in retina. Transverse section stained with hematoxylin and eosin was performed for retina collected from medaka at ages of 1 (**A**), 2 (**B**), 3 (**C**), 4 (**D**), 8 (**E**) and 18 (**F**) months. Cell layers are indicated: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS/OS, inner segment/outer segment of the photoreceptor cells, RPE, retinal pigment epithelium. Arrow heads indicated the basement membrane of RPE. Scale bar = 20 µm. Note that aging-effect on retina morphology was observed in medaka through 18 months of age.

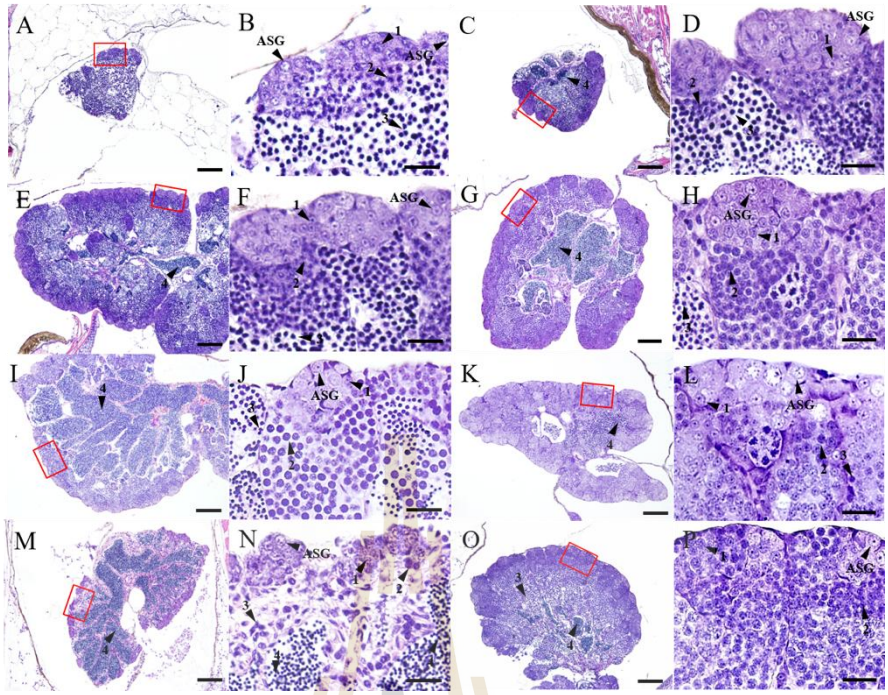

Figure 3.2 Histological study of donor testis of the transgenic medaka. Testis were collected from medaka at ages of 1 (**A-B**), 2 (**C-D**), 3 (**E-F**), 4 (**G-H**), 8 (**I-J**) and 18 (**M-N**) months. In addition, testis of medaka at 8 (K-L) and 18 (O-P) months of age that was incubated at 10°C. Overview of testicular structure (**A, C, E, G, I, K, M, O**). Rectangles show the area rich in ASG. Higher magnification images of each rectangle in **A, C, E, G, I, K, M, O** are showed in **B, D, F, H, J, L, N, P,** respectively. Germ cells were found at various differentiation stages. Type A spermatogonia (ASG) is categorized by the largest cells germ cells (>10μm) with a prominent dense bodies and clear cytoplasm. Type B spermatogonia (BSG) is categorized by the germ cells which are smaller size than ASG and form clusters with fragmented dense bodies. Primary spermatocyte (PSC), secondary spermatocyte (SSC) and spermatozoa (SZ) were indicated. Scale bars 100 μm (**A, C, E, G, I, K, M, O**) and 100 μm (**B, D, F, H, J, L, N, P**)

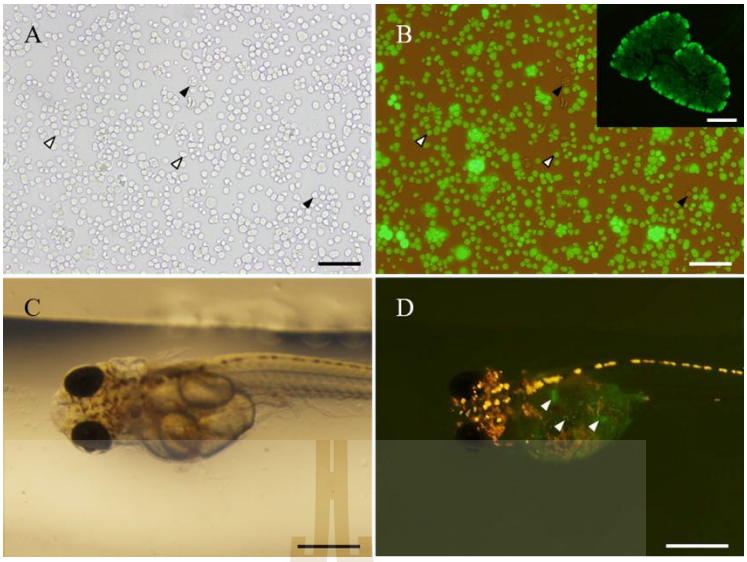

Figure 3.3 Donor ASG expressing GFP obtained from *vasa-Gfp* transgenic medaka for transplantation. Dissociated testicular cells in the brightfield view (**A**) and in the fluorescent view (**B**). ASG were scattered along the testicular walls which showed strong green fluorescent (**B**). When approximately 10,000 ASG expressing GFP were transplanted into the body cavity of medaka, GFP-expressing donor cells inside the body cavity were observed in the brightfield view (**C**) and in the fluorescent view (**D**). Scale bars 50 µm (**A** and **B**) and 500 µm (**C** and **D**)

It was demonstrated that only testicular cells capable of incorporating recipient gonads were ASG (Yano et al., 2009). Viable ASG showed green fluorescence with diameter of 8-10 µm (Fig. 3.3A-B). The fish at 1, 2, and 3 months of age contain small testis (less than 1 mg); therefore, they are not practical to use as donor fish because a number of fish were needed to sacrifice in order to obtain enough cell number of ASG for transplantation. In this study, we thus use fish at 4 (the onset of maturation), 8 (maturation) and 18 (senescence) months of age for preparing

testicular cell suspension. Table 3.1 showed that the size of fish increased with age of fish increased; however, GSIs were not significantly different. The number of isolated ASG were counted only GFP-expressed cells (diameter of 8-10 μm) (Fig. 3.3A-B). Comparing to fish at 4 months of age, fish at 8 months of age had higher number of viable ASG. However, when fish became 18 months old, their viable ASG significantly decreased.

Table 3.1 Body weight, GSI and number of ASG at various ages of medaka maintained at different temperature (mean ± standard error, n = 3).

Age (months)	**Incubation temperature**	**Body weight (mg)**	**GSI (%)**	**No. of ASG ($\times 10^5$ cells/mg gonad)**
4	26°C	344.33 ± 24.66^{b}	0.77 ± 0.05	6.47 ± 0.23^{b}
8	26°C	479.67 ± 31.63^{aX}	0.63 ± 0.04	8.78 ± 0.62^{aX}
	10°C	279.00 ± 22.11^{Y}	0.72 ± 0.06	5.92 ± 0.51^{Y}
18	26°C	538.00 ± 34.24^{a}	0.62 ± 0.03	2.69 ± 0.22^{cY}
	10°C	451.67 ± 26.87	0.67 ± 0.04	6.17 ± 0.17^{X}

Means with different small superscripts in each column differ significantly from each other among age (P<0.05).

Means with different capital superscripts differ significantly between incubation temperature within same age (P<0.05).

Table 3.1 showed that fish were incubated at different water temperature for 4 months had effects on number of viable ASG. At both 8 and 18 months of age, fish which were incubated at 10°C had significantly lower ASG number. Note that low

water temperature led to decrease body weight of fish; however, significant difference was observed only the fish at 8 months of age.

In order to investigate the effect of donor aging on efficiency of germ cell transplantation, donor fish at age of 4 (the onset of maturation) and 18 (senescence) months were used for preparing the testicular cell suspension. Approximately 10,000 GFP-expressing ASGs were transplanted into the body cavity of recipient fish (Fig. 3.3C-D). Because of variable fecundity in ovulation, 12-39 larvae per replication (3 replicates) were performed transplantation, and only healthy fish were culture for colonization checking. GFP donor cells were observed in the genital ridge of transplanted fish (Fig. 3.4C-D) but not in the gonads of non-transplanted fish (Fig. 3.4A-B) by fluorescence microscopy. These observations indicated the incorporation of the transplanted ASG in the recipient gonad. Table 3.2 showed that there were no significant differences in the colonization rate of ASG which were isolated from donor fish at age of 4 and 18 months. These findings could suggest no aging effect of donor fish for the efficient colonization of germ cell transplantation. In addition, Table 1 showed that there were no significant of colonization rate of ASG obtained from fish at 18 months of age at different water temperature, demonstrating that rearing temperature had no effects on ASG activity.

In this study, the recipient fish was hybrid medaka. This hybrid fish was sterile; therefore, both male and female could become surrogate broodstock to produce only donor-derived offspring. Fig. 3.4E-F showed donor-derived GFP oocytes inside the ovary obtained from transplanted female. Also, the male recipient produced GFP donor-derived GFP testicular cells (Fig. 3.4G-H). These findings demonstrated that the colonized donor cells could developed in the gonad of sterile recipient fish.

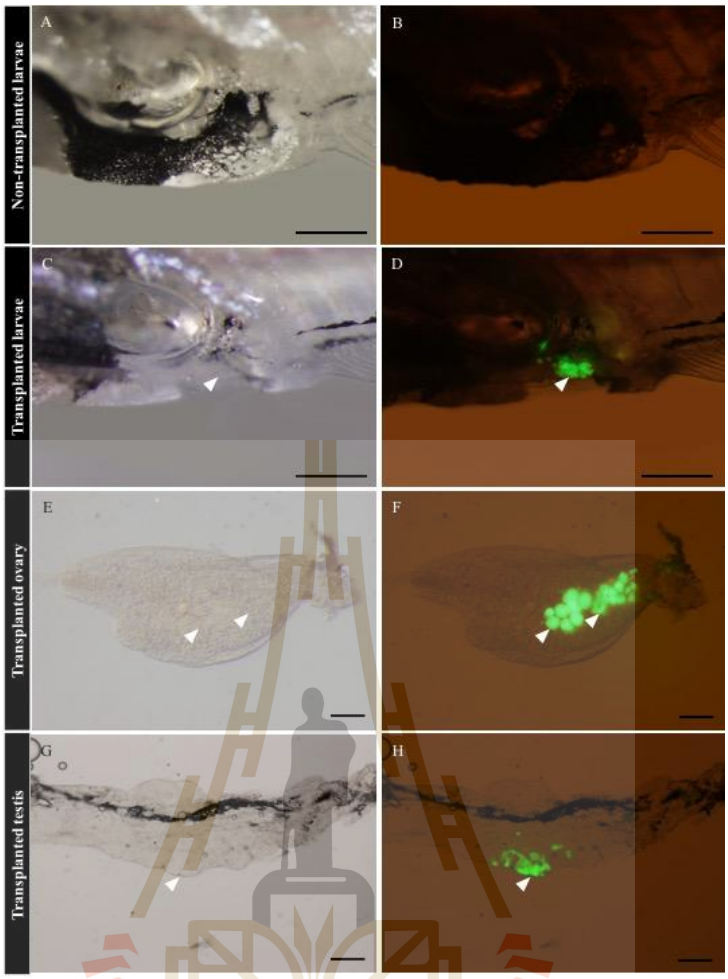

Figure 3.4 Colonization of transplanted GFP-expressing donor cells in recipient gonad. Wild-type non-transplanted fish showing no GFP-expressing donor cells in the gonad (**A-B**). GFP-expressing donor cells incorporated in the transplanted recipient fish (**C-D**). Note that hybrid recipient is sterile. When hybrid recipients were transplanted with GFP-expressing donor ASG, GFP-expressing eggs were observed in the ovary collected from the transplanted recipient female (**E-F**). GFP-expressing testicular cells were observed in the testis collected from the transplanted recipient male (**F-H**). Scale bars 1 mm (**A, B, C, D**) and 200 µm (**E, F, G, H**)

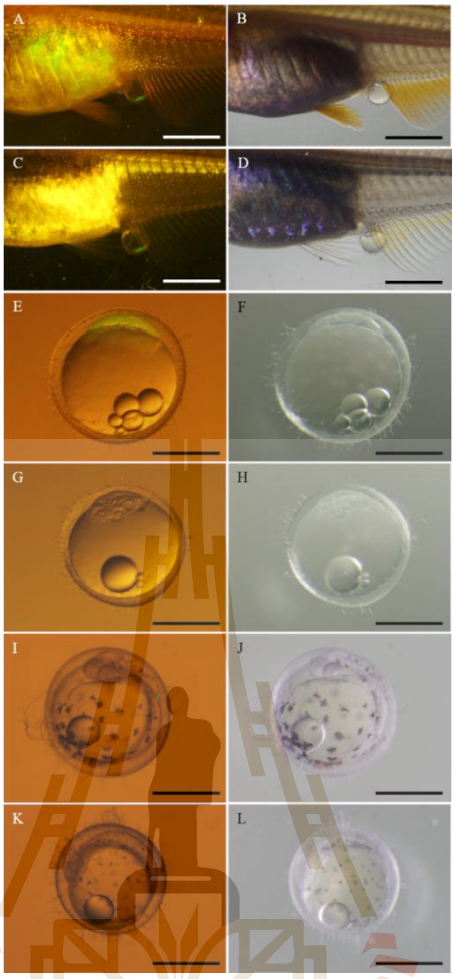

Figure 3.5 Donor derived embryos obtained from progeny tests. **A**: A number of GFP-expressing eggs were observed in transgenic medaka carrying vasa-GFP. **C**: Transplanted recipient female produced GFP-expressing egg. **E**: donor-derived embryo showing GFP in the blastodisc. **G**: Embryo obtained from wild-type nontransplanted female. **I**: Recipient male produced donor-derived embryo showing GFP at the presumptive genital. **K**: Embryo obtained from wild-type nontransplanted male. **B, D, F, H, J, L**: The same field as in **A, C, E, G, I, K**, respectively, viewed in the bright light. Scale bars 2 mm (**A, B, C, D**) and 500 µm (**E, F, G, H, I, J, K, L**)

Table 3.2 Colonization rate and germ-line transmission rate of donor ASG obtained from medaka at age of 4 and 18 months maintained at different temperature (mean ± standard error, *n* = 3).

Replication	Colonization			Transmission		
	4 months	18 months	18 months	4 months	18 months	18 months
	26°C	26°C	10°C	26°C	26°C	10°C
1	8/33	8/36	10/32	3/20	4/20	3/20
2	7/28	10/39	8/34	2/20	2/20	2/20
3	3/12	4/17	3/14	1/20	6/20	1/20
Means±SE (%)	24.75 ± 0.25	23.80 ± 1.00	25.40 ± 2.99	10.00 ± 2.89	20.00 ± 5.77	10.00 ± 2.89

No significant differences were observed among experimental age and temperature

In order to determine aging-effect on germ-line transmission rate, donor ASG obtained from donor fish at 4 and 8 months of age were used for transplantation. Approximately 10,000 GFP-expressing ASGs were microinjected into the body cavity of recipient larvae (3 replicates with 30 larvae per replication). Among 30 transplanted fish of each replicate, 10 recipient females or males were mated with wild-type nontransplanted male or female, respectively. In transgenic medaka, GFP-expressing ovulated eggs were observed inside body cavity (Fig. 3.5A-B). GFP-expressing donor-derived eggs were also observed in recipient female (although less number of ovulated eggs), indicating that female could produce functional gametes (Fig. 3.5C-D). Fig. 5E-F showed that the female could produce donor-derived embryo showing GFP comparing with wild-type embryo showing no GFP (Fig. 3.5G-H). In addition, crossing with wild-type nontransplanted female, recipient male produced donor-derived embryo showing GFP at the presumptive genital ridge (Fig. 3.5I-J) which were not observed in the wild-type embryo (Fig. 3.5K-L). There were no significant differences in the total germ-line transmission rate of both recipient male and female (20 fish) transplanted with 4- and 18-months donor ASGs ($P>0.05$), although the transmission rate of broodstock which were transplanted with ASG isolated from 4 months of age was lower than that of 8 months of age (Table 3.2). These observations suggested that there was no aging-effect of donor fish for germ-line transmission. In addition, we investigated whether different rearing temperature of donor fish at 18 months of age would affect the germline transmission rate. Table 3.2 showed that donor fish at 18 months of age which were maintained at 10°C had lower transmission rate but no significant difference ($P>0.05$) when compared that of the normal condition (26°C). Note that the transmission rate of ASG obtained from fish

at 18 months of age at 10°C appeared to be similar to that isolated from fish at 4 months of age.

3.5 Discussion

In mammal**,** aging-associated changes in eyes were well-demonstrated by explanation of several theories (Salvi et al., 2006; Samuel et al., 2011). Similar ageing-associated changes in eyes were also revealed in zebrafish and medaka (Kishi et al., 2009; Ding et al., 2010). Therefore, morphological status of eye could be used as a biomarker to trace process of tissue aging. It was reported that medaka lifespan in laboratory condition (water temperature at 27°C) was about 1 year (Shima and Mitani, 2004). Under laboratory condition (water temperature at 27°C) the median lifespan of medaka was approximately 22 months. In this study, therefore, we performed histological study on eye of medaka at age of 1-3 months (pubertal stages), 4-8 months (adolescent stages) and 18 months (senescent stage). Our results showed clear morphological aging-related alteration in eye which were consistent to the previous studies (Kishi et al., 2009; Ding et al., 2010).

Asynchronous gonad in medaka was previously demonstrated in both male and female (Mita et al., 2000; Iwamatsu, 2004). In this study, the observation of a range of developmental stages of testicular cells from ASG to spermatozoa at age of 1 month through 18 months indicated an asynchronous-type testis in medaka. The scattered ASG were found along the walls of the testicular lobules. These ASG were suggested to be SSCs which are capable for self-renewal and to provide spermatogenesis throughout reproductive life. In this study, from ages of 4 months (the onset of maturation) to 8 months (maturation), which is the ages of regular

spermatogenesis, spermatozoa lobules increased with increasing age. Also, the number of ASG increased, demonstrating high rate of self-renewal of committed progenitors during adolescent ages. The testis of medaka at age of 18 months was decrease in size, and decline in spermatozoa lobules were observed. In addition, a few scattered of spermatogonial cells including type A and B were observed along the wall of lobule. Consequently, the relative number of ASG were decreased. Our findings demonstrated aging negatively influenced fertility performance, indicating the signs of reproductive senescence. Taken together, aging-relating effect on number of SSCs were clearly observed in medaka which might be partly affected by imbalance of SSCs self-renewal and differentiation. Similarly, the aging effects on SSCs self-renewal and differentiation was described in mice which were resulted from deterioration of niche microenvironment (Ryu et al., 2006).

In general, extrinsic factor such as water temperature tightly influenced reproductive function in a poikilothermic animal. For example, water temperature influenced gonad maturity in *Brachyhypopomus pinnicaudatus* (Quintana et al., 2004). Variation in water temperature affected GSI and sperm production in Atlantic cod (*Gadus morhua*) (Yoneda and Wright, 2005). Reduction of spermatogenesis at high temperature was demonstrated in damselfish (*Acanthochromis polyacanthus*) (Donelson et al., 2010). Under the laboratory condition (26°C), medaka is nonseasonal breeder which is able to reproduce all year round. In this study, low temperature seemed to lower spermatozoa population at both experimental ages. Similarly, low temperature was suggested to affect medaka reproduction. For instant, it was revealed that spermatogenesis processes in medaka at 15°C was less than that at 25°C (Egami and Hyodo-Taguchi, 1967). Low temperature (15°C) decreased

fertility but did not arrest (Koger et al., 1999).

In this study, the effect of temperature was studied by incubation the medaka at low temperature at sub-optimal at 10°C for 4 months. Temperature stress influenced ASG number, and the effect depended on age. The fish at age of 8 months were incubated at 10°C (since they became 4 months) for 4 months. Consequently, they have similar ASG number when compared to the fish at age of 4 months but lower number of ASG comparing with the fish at the same age of 8 months. This observation might suggest that low temperature might retard self-renewal process of ASG. However, at age of 18 months, lower temperature led to increase ASG number, suggesting that low temperature might slow down the decline in reproductive performance in senescent age. Taken together, these results suggested that water temperature influenced the balance of self-renewal and differentiation rates. The interactions of intrinsic (age) and extrinsic (temperature) factors influenced the balance of self-renewal and differentiation of ASG. Indeed, the effects of temperature on testicular development was revealed in tilapia. Low temperature (20°C), when compared to high temperature (30-35°C), seemed to induce renewal of ASG and proliferation of Sertoli and Leydig cells (Alvarenga and Franca, 2009).

Hybrid sterility was demonstrated in several fish (reviewed in Golpour et al., 2016). Interspecific hybrid in several fish were reported to be sterile including medaka (Hamaguchi and Sakaizumi, 1992; Takahashi et al., 2005). In this study, the offspring of interspecific hybrid of Japanese medaka (*O. latipes*) x Chinese Hainan medaka (*O. Curvinotus*) was used as recipient fish. It was reported that while female offspring of these hybrid could lay eggs, the males were sterile (Hamaguchi and Sakaizumi, 1992). Nevertheless, in this study, under our experimental condition, we

could not obtain eggs from the female hybrid. Sterility pattern of this hybrid was demonstrated as gametic sterility since gonads in these hybrids were be able to sexually differentiate, but normal oogenesis and spermatogenesis were severely impaired. Consequently, the hybrid offspring provide reproductive advantage to use as recipient because the gonads in the transplanted hybrid recipient would be sexually differentiation and able to generate only donor-derived offspring.

In order to determine whether there was an aging-related stem cell activity of ASG in the nonseasonal medaka reproduction, we performed spermatgonial transplantation to evaluate stem cell activity among ASG cell subpopulation isolated from medaka expressing *vasa-gfp* comparing between 4 months (the onset of maturation) and 18 months (the senescent age) of age. Our results showed that ASG isolated from testis of fish at 4 and 18 months of age had similar incorporation rate into the genital ridge of recipient larvae. In addition, with donor cells isolated from both ages, no significant differences in the germ-line transmission rate were observed. These findings indicated that the ASG isolated from fish between at the onset of maturation and the senescent age exhibited similar SSCs activity *in vivo* for migration, incorporation into the recipient larvae gonad and differentiation to produce functional gamete. Moreover, water temperature which affected the number of ASG had no significant effects on transplantation efficiency for both colonization rate and germline transmission rate, suggesting that water temperature influenced the quantity of SSCs but not quanlity of SSCs activity. In general, since the efficiency of germ cell transplantation would depend on both SSCs function per se and niche/ microenvironment in gonad of recipient larvae. Therefore, the very young gonadal environment in fish larvae would exert strong niche regulatory function for SSCs

migration, colonization, self-renewal and differentiation irrespective of SSCs aging. It was demonstrated that the transplantation of SSCs from old mice could continue to produce spermatogenesis in young males, suggesting that, rather than SSCs per se, the niche factors exerted important effects on deteriorates in the testis of old males (Ryu et al., 2006). The stem cell niche such as sertoli cells is essential for determining SSCs activity (Oatley et al., 2011). Nevertheless, it was demonstrated that aging influenced the decline in both SSC numbers and activity when different age of SSCs was transplanted into mice at 1 year of age. Therefore, both SSCs and testicular somatic environment affected aging process (Zhang et al., 2006). Aging negatively influenced the function of SSCs in mice both *in vivo* and *in vitro*. It was revealed that *in vitro* culture had more negative effect on SSC function because of a reduction in the expression of gene involved in SSC self-renewal and an increase in the expression of gene related to germ cell differentiation (Schmidt et al., 2011). Taken together, there was aging-related effect of niche microenvironment on transplantation efficiency. However, whether SSC aging or its combination effect with niche regulatory factor would influence the transplantation efficiency remained to be intensively clarified.

In conclusion, this study demonstrated the aging effects on stem cell activity of ASG in medaka, a nonseasonal breeding fish. Age-related changes in development of testicular cells were obviously demonstrated which significantly influenced the number of ASG. Water temperature also affected ASG number. Using transplantation of ASG into young niche microenvironment larvae, neither aging nor water temperature influenced stem cell activity of ASG.

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CHAPTER IV

A *VASA* HOMOLOG IN THE STRIPED CATFISH (*PANGASIANODON HYPOPHTHALMUS*): CHARACTERIZATION AND EXPRESSION IN GONAD AND PRIMORDIAL GERM CELLS DURING LARVAL DEVELOPMENT

4.1 Abstract

Tracking reproductive system development during larval development requires a known molecular gene marker that is specifically expressed in germ cells in order to confirm histological characterization. The *vasa* gene has been shown to be specifically expressed in the germline, thus acting as a useful molecular marker in germ cells. In this study, cloning and characterization of vasa in the striped catfish, *Pangasianodon hypophthalmus*, was carried out and designated as Phy-vasa. *Phy-vasa* contained all of the predicted consensus motifs that are shared within the vasa family in other fish, including RG and RGG repeats, ATPase motifs, and a DEAD box. Phylogenetic-tree construction using various DEAD-box family proteins demonstrated that, as expected, *Phy-vasa* clustered within the Vasa family. By reverse transcription PCR, *Phy-vasa* mRNA was observed only in the testis and ovary. Using in situ hybridization, we showed that it was expressed specifically only in germ cells, with strong expression detectable in spermatogonia and oogonia.

histological characterization, together with *in situ* hybridization using a *Phy-vasa* probe, was performed to investigate reproductive development in catfish larvae. Our results showed that migration of primordial germ cells (PGCs) were found most abundantly in larvae, 2-10 days post-fertilization (dpf) and the genital ridges containing PGCs and somatic cells were formed at 10-20 dpf. The proliferation of PGCs began in larvae between 25-30 dpf. Our findings provide a valuable insight into early gonadal development in the striped catfish.

4.2 Introduction

The *vasa* gene, also known as *Ddx4*, belongs to the DEAD-box (Asp–Glu–Ala–Asp) protein family. Vasa expression is ubiquitous in germ cells in a wide evolutionary range of organisms (Schupbach and Wieschaus, 1986; Hay et al., 1988; Olsen et al., 1997; Castrillon et al., 2000). In addition, *vasa* was revealed to be a maternal expression gene, as its mRNA was found during embryonic development (Yoshizaki et al., 2000a). The *vasa* gene is also a conserved ATP-dependent RNA helicase that has crucial functions in primordial germ cell (PGC) formation and germ cell differentiation (Hay et al., 1988; Liu et al., 2009). In mice, using gene-targeting analysis, the *vasa* homolog was revealed to be involved in the proliferation and differentiation of male germ cells (Tanaka et al., 2000). However, *vasa* was shown not to be crucial in PGC proliferation in Japanese rice fish. It was, however, suggested to be essential in PGC migration (Li et al., 2009). Thus, although the precise function of vasa does not appear to be conserved among animal taxa, the expression of *vasa* is specific in the germline lineage. PGCs are the progenitor cells that lead to production of a gamete. Like in other vertebrates, fish PGCs are generally

large in size, have a low nucleocytoplasmic ratio and have a distinct nuclear border and granular nuclear chromatin (Van Winkoop et al., 1992; Patino and Takashima, 1995). Despite these hallmarks, identification of PGCs, particularly at the developmental stages before complete migration toward genital ridge, is difficult and demonstrates a need for a molecular marker to confirm PGC identification. Morphological characterization together with the aid of a molecular marker would enable the investigation of early reproductive development in fish larvae. There are several genes that are specifically expressed in germ cells including *vasa*, *nanos*, *dazl*, *deadend*, and *cxcr4b* in living organisms that could be potential candidates as a marker (review in Raz, 2003). Among them, *vasa* gene products have been extensively used as a molecular marker for identification of germ cells. For instance, the ubiquity of *vasa* allowed it to be used to identify PGCs in larvae, as well as in spermatogonia and oogonia throughout the reproductive cycle (Braat et al., 2000; Yoshizaki et al., 20002; Ubeda-Manzanaro et al., 2014). For example, the localization of *vasa* mRNA was used as a useful tool to characterize and trace the migration of PGCs in Drosophila, zebrafish, medaka, rainbow trout and brown-marbled grouper (Fujiwara et al., 19954; Braat et al., 2000; Shinomiya et al., 2000; Yoshizaki et al., 2000a; Ubed-Manzanaro et al., 2014 Boonanuntanasarn et al., 2016). Moreover, transgenic animals carrying a green fluorescent protein whose expression is driven by the *vasa* promoter were established and used for tracking the development of germ cell transplantation, which contributes to the tracking of spermatogonial stem cells in mice and fish (Yoshizaki et al., 20020b; Takeuchi et al., 2003; Okutsu et al., 2006; Niikura et al., 2009). Therefore, characterization of *vasa* has been an attractive probe to develop in a number of fish species since it can provide valuable information and

has applications in research involving in reproductive technology such as germ cell transplantation (Nagasawa et al., 2009; Boonanuntanasarn et al., 2016).

Germ cell transplantation technology in fish has been developed by using spermatogonia from donor fish and transplanting them into recipient larvae during the period of germ cell migration (Takeuchi et al., 2003; Okutsu et al., 2006; Morita et al., 2012). Indeed, establishment of a reliable germ cell transplantation technique would offer a number of practical applications for both basic and applied research and innovation in fish studies .For example, germ cell transplantation could be a useful technology for restoring endangered species, cloning of animals carrying desired phenotypes and producing offspring of large fish using small fish as a surrogate brood stock .To develop this technique, precise identification of germ cells including PGCs and spermatogonia cells is required. Particularly, identification of early reproductive development in the desired recipient fish would be an important prerequisite. To achieve high efficiency of germ cell transplantation, a species with a well-established culture procedure is desired as recipient for donor fish that are evolutionarily related (Yazawa et al., 2010).

The family Pangasiidae has been found across southern Asia in both freshwater and brackish water. Among them, many species have been categorized as endangered species, such as the Mekong giant catfish (*Pangasianodon gigas*) and the Chao Phraya giant catfish (*Pangasius sanitwongsei*). In addition, several pangasiid catfish, particularly, the striped catfish (*Pangasianodon hypophthalmus*) have been extensively cultured and are economically important as they are a major source of whitefish products on the world market. Full-cycle cultivation of the striped catfish is well-established at all stages, including hatchery, seed production and grow-out

farming. Therefore, the striped catfish has potential as a recipient fish for development of germ cell transplantation in Pangasiid catfish, which would be applicable for conservation and aquaculture practices. Therefore, in this study we cloned and characterized *vasa* cDNA in the striped catfish. In order to determine the specificity of gene expression. The restricted analysis expression of *vasa* mRNA in both testis and ovaries. Various tissues were demonstrated performed. In addition, using *in situ* hybridization, localization of *vasa* expression in both immature and mature gonads was demonstrated. Furthermore, we showed that *vasa* expression was could be used as a marker for identification of PGCs during early gonadal development. The localized expression of *vasa* in both immature and mature gonads was presented.

4.3 Materials and methods

4.3.1 Fish

Striped catfish used in this study were cultured at a farm at the Suranaree University of Technology, Nakhon Ratchasima. Immature fish (0.12-0.85 kg) were maintained in an earthen pond (10 × 40 × 1.5 m^3). They were fed with commercial feed (36% crude protein, 4% crude fat) at 2% body weight at 10:00 and 16.00 h every day. For mature fish and for larvae production, brood stock (females 1.5-2.5 kg, males 1.0-2.5 kg) were maintained in an earthen pond (10 × 40 × 1.5 m^3). The broodstocks were fed with commercial feed (25% crude protein, 3% crude fat) at 5% body weight at 10:00 and 16.00 h every day. Throughout the experimental period, the water temperature was maintained at 26-30°C, pH 7.4-8.5, dissolved oxygen 3.0-5.1 mg/l.

For seed production, brood stock (1:1, male/female) were transferred to a canvas pond (2 m^3) for hormonal injection. Female fish were injected twice. During the first injection, fish were injected with one dose of fish pituitary extract (PE) and 300 IU kg^{-1} of human chorionic gonadotropin. After 8-12 h, the second injection was performed by injecting the female with 4 doses of fish PE and 900 IU kg^{-1} of human chorionic gonadotropin. After 10-12 h, artificial fertilization was conducted by mixing eggs and milt before activating fertilization with 0.85% NaCl. After washing three times to remove excess sperm, the fertilized eggs were transferred into hatching tanks (0.5 m^3). After 36 h, the hatched larvae were then transferred into a new tank (0.5 m^3) at a density of 100 fish per l with aeration. The larvae were fed with newly hatched brine shrimp (5-15 individuals ml^{-1}) from 3-14 days post-hatching (dph). Subsequently, the fry were fed with commercial powdered diets (36% crude protein, 4% crude fat).

4.3.2 Molecular cloning of *vasa* and *β-actin* cDNAs

Two fish, including one male (800 g body weight, gonad weight 0.31 g, gonadosomatic index (GSI) 0.04%) and one female (840 g body weight, gonad weight 2.46 g, GSI 0.29%) were sampled and dissected to collect tissues for total RNA extraction. Total RNA was extracted from sampled tissues (~100 mg) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and digested with RNase-free DNase I (Promega, Madison, WI, USA), according to the manufacturer's instructions. To clone the full-length cDNA of the *P. hypophtalmus vasa* gene (*Phy-vasa*), 3′ and 5′ rapid amplification of cDNA ends (3′ and 5′ RACE) was performed using a SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. The 3′ and 5′ ends of *Phy-vasa* cDNA were

cloned with nested polymerase chain reaction (PCR). For the 3′ end, two gene-specific primers (forward), vasa-F1 and vasa-F2 (Table 4.1), were generated based on the highly conserved regions of other *vasa* genes. For the 5′ end, two gene-specific primers (reverse) were designed, vasa-R1 and vasa-R2 (Table 4.1), according to the DNA sequences of the 3’ RACE product. Reverse and forward primers for 3′ and 5′ end amplification (Table 4.1), respectively, were provided by the SMART^{TM} RACE Kit. PCR was carried out in a total volume of 25 µl, consisting of 2.5 µl of cDNA, 400 µM of each dNTP, 2.5 pmol of each primer, 2.5 mM $MgCl_2$, 1X LA Taq^{TM} buffer, and 1.25 U LA Taq (Takara Shuzo, Shiga, Japan). PCR was carried out at 95°C for 3 min, followed by 35 reaction cycles of 45 s at 95°C, 45 s at 62°C, and 90 s at 72°C. The final elongation step was carried out at 72°C for 5 min.

The partial cDNA of *β-actin* was cloned using a SMART^{TM} RACE cDNA Amplification Kit (Clontech) and designated as *Phy-actin*. Two gene-specific primers (β-actin-F and β-actin-R) (Table 4.1) were designed according to the highly conserved regions of other teleost -actin genes. PCR was conducted as described earlier. The PCR was performed at 94°C for 3 min, followed by 35 reaction cycles including 45 s at 95°C, 30 s at 62°C, and 30 s at 72°C. The final elongation step was carried out at 72°C for 5 min.

PCR-amplified DNA fragments of the expected size were isolated, purified, and ligated into the pGEM T-Easy plasmid (Promega). All experimental plasmid DNA (at least five sequenced clones per cDNA in both directions) was sent for sequencing at Macrogen, Inc. (Seoul, Korea). The plasmids containing the 3′ RACE amplicon (p*Phy-vasa*) and -actin fragment (p*β-actin*) were kept for further use.

Multiple sequence alignment of Vasa proteins was performed using CLC Main Workbench 7.9.1. A phylogenetic tree was accomplished with MEGA version 7.0 using the Neighbor-Joining (NJ) method (Kumar et al., 2016), based on the Poisson model. The phylogenetic tree was constructed with Vasa proteins and PL10 family proteins from different teleost species.

4.3.3 Reverse transcription PCR

The expression of *Phy-vasa* in the heart (H), liver (L), spleen (Sp), stomach (St), intestine (I), kidney (K), muscle (M), testis (T) and ovary (O) of immature *P. hypophthalmus* were examined using reverse transcription (RT) PCR. Two fish, including one male (800 g body weight, gonad weight 0.31 g, GSI 0.04%) and one female (840 g body weight, gonad weight 2.46 g, GSI 0.29%) were sampled and dissected to collect tissues for total RNA extraction. Total RNA was extracted from sampled tissues (~100 mg) using TRIzol reagent (Invitrogen) and treated with RNase-free DNase I (Promega), according to the manufacturer's instructions.

First-strand cDNA was synthesized from 2 µg of total RNA using the ImProm- II™ Reverse Transcription System Kit (Promega). A pair of primers was used to amplify *Phy-vasa* (vasa-RT-F and vasa-RT-R). As an internal control, the primers -Actin-F and -actin-R were used to amplify *Phy-actin*. Duplicate PCR of each tissue was performed in a total volume of 10 l consisting of 1 l of cDNA template, 250 M of each dNTP, 1 pmol of each primer, 2.5 mM $MgCl_2$, 1X Green GoTaq Flexi buffer, and 0.25 U GoTaq DNA polymerase (Promega). The PCR was performed at 95°C for 3 min, followed by 35 reaction cycles of 45 s at 95°C, 30 s at 62°C, and 30 s at 72°C. The final elongation step was carried out at 72°C for 5 min. The PCR products of *Phy-actin* and *Phy-actin* were verified with agarose gel electrophoresis and ethidium bromide staining.

Table 4.1 Sequences of primers used in this study.

Primer	Sequence (5′-3′)	PCR
vasa-f1	GAYGABATMHTKGTVGA GTBAGYGG	3′ RACE PCR
vasa-f2	AAGCCBACYCCDGTVCAGAARYAYGG	3′ RACE PCR
vasa-r1	CCABKWVGGMACYTCCTGYTG RGC	5′ RACE PCR
vasa-r2	TTHCCRCAKCGDCCDGTKCKBCCRA	5′ RACE PCR
UPM-L	CTAATACGACTCACTATAGGGCAAGCA GTGGTATCAACGCAGAGT	3′ and 5′ RACE PCR
UPM-S	CTAATACGACTCACTATAGGGC	3′ and 5′ RACE PCR
vasa-RT-F	CGGCAAACCCTTATGTTCAG	RT-PCR
vasa-RT-R	CATTGTTCTCTGCGTACCTG	RT-PCR
β-actin-F	ACTACCTCATCAAGATCCTG	RT-PCR
β-actin-R	TTGCTGATCCACATCTGCTG	RT-PCR

4.3.4 Histological study and *in situ* hybridization

4.3.4.1 Fish sampling

Ovary tissue was collected from mature female fish (1.5 kg body weight; gonad weight 52.08 g; GSI 3.47%) and one immature female fish (160 g body weight, gonad weight 2.46 g, GSI 1.54%). Testis was taken from one mature male fish (1.0 kg body weight; gonad weight 7.84 g; GSI 0.78%) and one immature male fish (140 g body weight, gonad weight 0.11 g, GSI 0.08%). All gonads were fixed in Bouin's solution for 18 h at 4°C The solution was subsequently replaced with 80% ethanol and kept at 4°C until use. Fish larvae at 3, 5, 10, 15, 20 and 30 days post

fertilization (dpf) were also fixed with Bouin's solution for 18 h at 4°C, which was replaced with 80% ethanol and kept at 4°C until use.

Gonad tissue was embedded in paraffin wax and serial transverse slices were cut at 5 m intervals. The sections were subsequently dewaxed, dehydrated and stained with hematoxylin–eosin (H&E) or subjected to *in situ* hybridization with sense or antisense probes to investigate *Phy-vasa* expression levels. The larvae were also embedded in paraffin wax, and serial transverse slices were cut at 5-m intervals. Like the mature gonad slices, transverse sections were subsequently dewaxed, dehydrated, and stained with H&E or subjected to *in situ* hybridization with antisense probes to characterize PGCs.

Templates for antisense and sense RNA probes were prepared by digesting the p*Phy-vasa* plasmid with *Apa*I and *Sal*I. Probes were synthesized by in vitro transcription using Digoxigenin- labeled uridine triphosphate (UTP) (Roche Diagnostics, Mannheim, Germany) with Sp6 or T7 RNA polymerase (Promega) for antisense and sense probes, respectively, according to the manufacturer's instructions. The sections were then subjected to *in situ* hybridization as described by Jangprai et al. (2011), with slight modifications. Briefly, after permeabilization and acetylation, sections were incubated in hybridization solution including 1.5 g/ml antisense or sense probe, 50% formamide, 2X saline solution, citrate buffer pH 4.5, 50 g/ml yeast tRNA, 50 g/ml heparin, 1% sodium dodecyl sulfate, and 10% dextran sulfate. Hybridization was conducted at 65°C for 6 h. The sections were then washed and incubated with an anti- DIG-alkaline phosphatase-conjugated antibody (Roche Diagnostics). The colorimetric reaction was then developed using nitroblue tetrazolium and 5-bromo-4- chloro-3-indolyl phosphate (Roche Diagnostics),

according to the manufacturer's instructions. The sections were counterstained using Nuclear Fast Red (Vector Laboratories, CA, USA).

4.3.4.2 Histological analysis of PGC numbers

Five fish larvae at each stage (1, 2, 3, 4, 5, 6, 8, 9, 13 and 15 dpf) were fixed with Bouin's solution for 18 h at 4°C. The solution was subsequently replaced with 80% ethanol and embedded with paraffin wax. Serial sagittal slices were cut at 5-m intervals, dewaxed, dehydrated, and stained with H&E to determine PGC numbers. One-way analysis of variance following Tukey's honest significant difference test was conducted to rank the numbers of PGCs in each larval stage using SPSS for Windows (Release 10) (IBM Corp., Armonk, NY, USA). Regression analysis of the number of PGCs (Y) or total length (mm) (Y) with larval age (dpf) (X) and goodness of fit (R^2) was also performed.

4.4 Results

The 2,621 bp full-length cDNA of *vasa* was cloned from the gonads of *P. hypophthalmus* and was designated as *Phy-vasa*. *Phy-vasa* contained ~119 bp of 5′ untranslated region (UTR), an open reading frame of 1989 bp, encoding 662 putative amino acids (aa), followed by 513 bp of a 3′-UTR with a poly(A) tail. The predicted *vas*a protein has a calculated molecular weight of 71.564 kDa, a theoretical isoelectric point of 5.24 and the predicted protein shared many conserved motifs with other typical Vasa proteins. For instance, the deduced amino acid sequence contained nine conserved domains of the DEAD-box protein family, including AQTGSGKT (motif I), PTRELI (motif Ia), TPGR (motif Ib), DEAD (motif II), SAT (motif III), MVFVETKR (motif IV), ARGLD (motif V), HRIGRTGR (motif VI), and a GG

doublet. In addition, a GYTKPTPVQ sequence (Q-motif) was present 16 aa upstream of motif I. Moreover, the region between N-terminus and aa 155 was rich in glycine residues (29%). The Phy-Vasa protein also contained four repeating sequences of arginine–glycine (RG) and six repeating sequences of arginine-glycine-glycine (RGG). Conserved tryptophan (W), glutamic acid (E) and aspartic acid (D) residues near the start and stop codons were also present (Fig. 4.1). Phy-Vasa shared 73.5%-86% identity with Vasa from other fish species in the NCBI database (data not shown). The greatest identity of Phy-Vasa with another fish Vasa homolog protein was shown in Fig. 4.2. The highest degree of similarity was observed in the known conserved sequences characteristic of DEAD-box helicase proteins including the RG and RGG repeats at the N-terminus, conserved tryptophan and acidic residues near the start and stop codons, the Q-motif, and motifs I, Ia, Ib, II, III, IV, V and VI. Phylogenetic-tree analysis showed that the *Phy-vasa* was distinctive from the *PL10* gene (Fig. 4.3). In addition, fish Vasa could be separated into several distinct clades, and Phy-Vasa clustered most closely in the Siluriformes clade.

Tissue distribution of *Phy-vasa* mRNA was determined using RT-PCR (Fig. 4.4). As an internal control, *Phy-actin* was detectable in the heart, liver, spleen, stomach, intestine, kidney, muscle, testis and ovary. However, *Phy-vasa* expression was restricted to the testis and ovary, demonstrating the specificity of *Phy-vasa* expression.

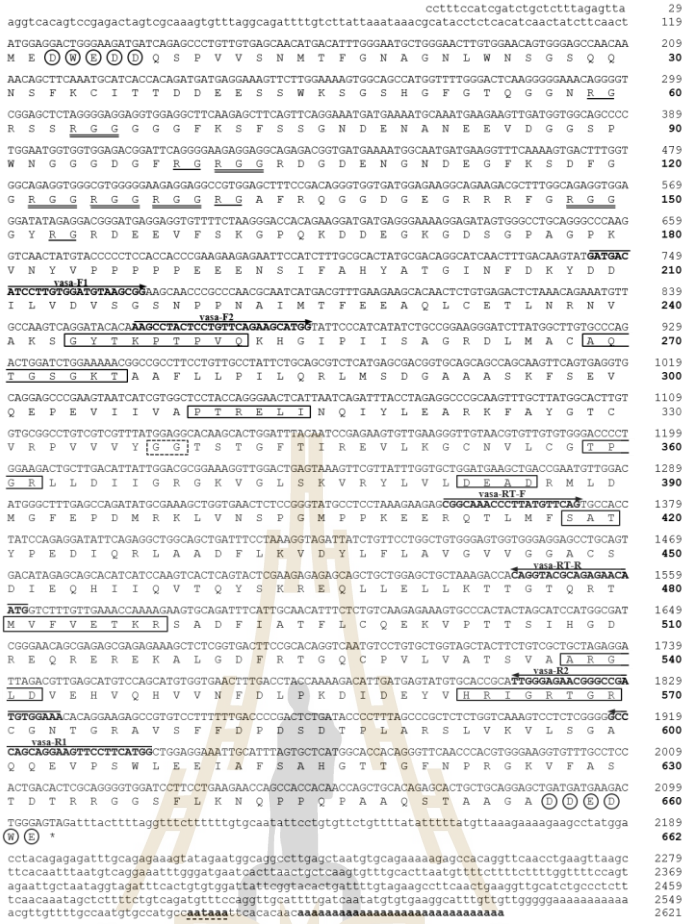

Figure 4.1 Nucleotide sequence of *Phy-vasa* cDNA and translated amino acid sequence of the encoded protein. Open circles indicate the consensus aspartic acid (D), glutamic acid (E), and tryptophan (W) residues next to the start and stop codons. Arginine-glycine (RG) and arginine-glycine-glycine (RGG) repeats in the N-terminal region are underlined and double-underlined, respectively. Open boxes indicate nine consensus sequence motifs of DEAD-box proteins. Open box with dotted line indicates glycine-glycine (GG) consensus doublet. The predicted polyadenylation signal is dotted underlined. Locations of primers used for cloning and RT-PCR are indicated by bold letters with arrow lines. See Table 4.1 for sequences of primers.

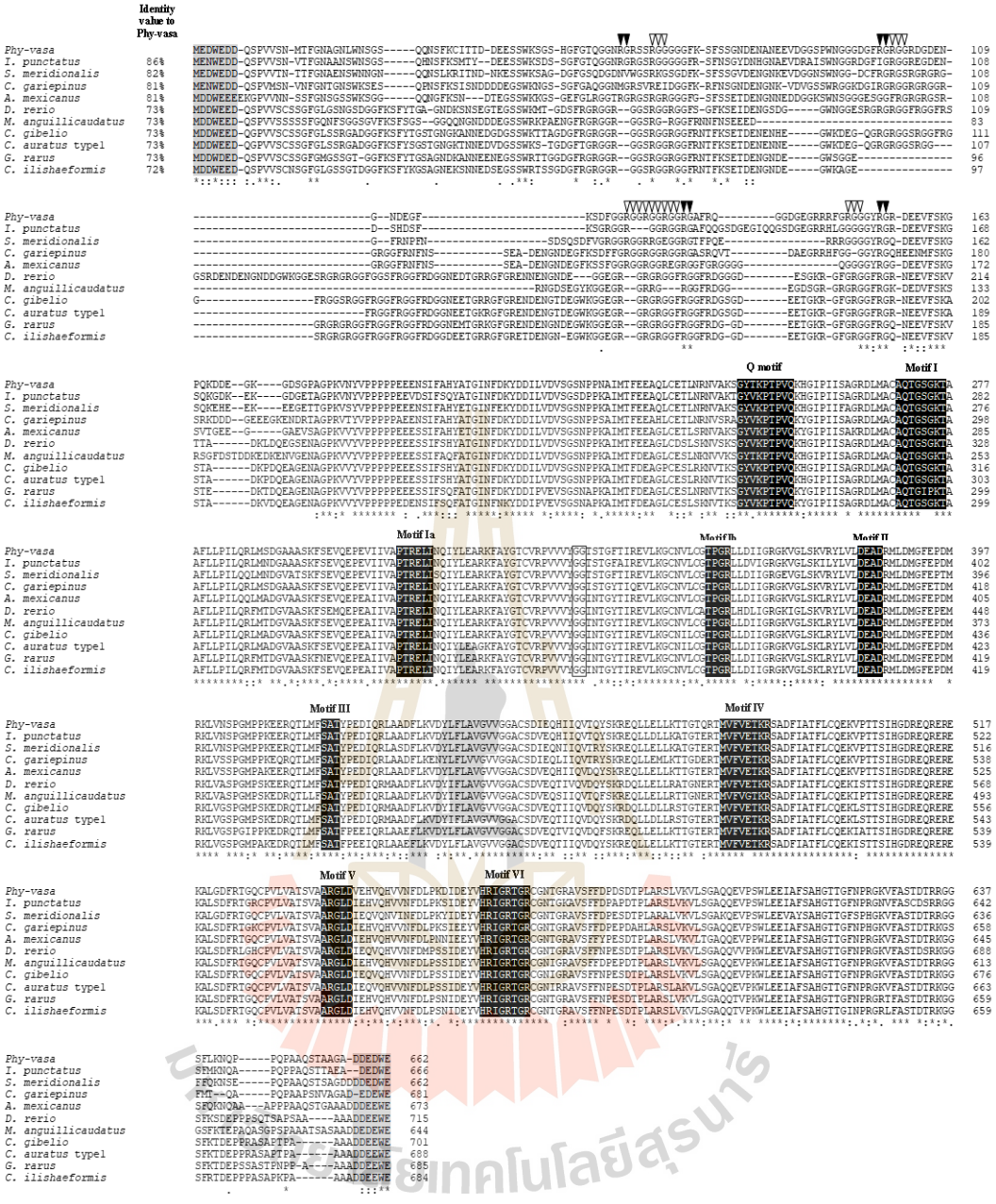

Figure 4.2 Multiple alignment of Phy-Vasa amino acid with known piscine Vasa protein homologs .The black box shows nine consensus sequence motifs of DEAD-box protein . Open box represents glycine-glycine (GC) consensus doublet . Fully-, highly-, and less-conserved amino acid residues are indicated by (*), (:) and (.), respectively .

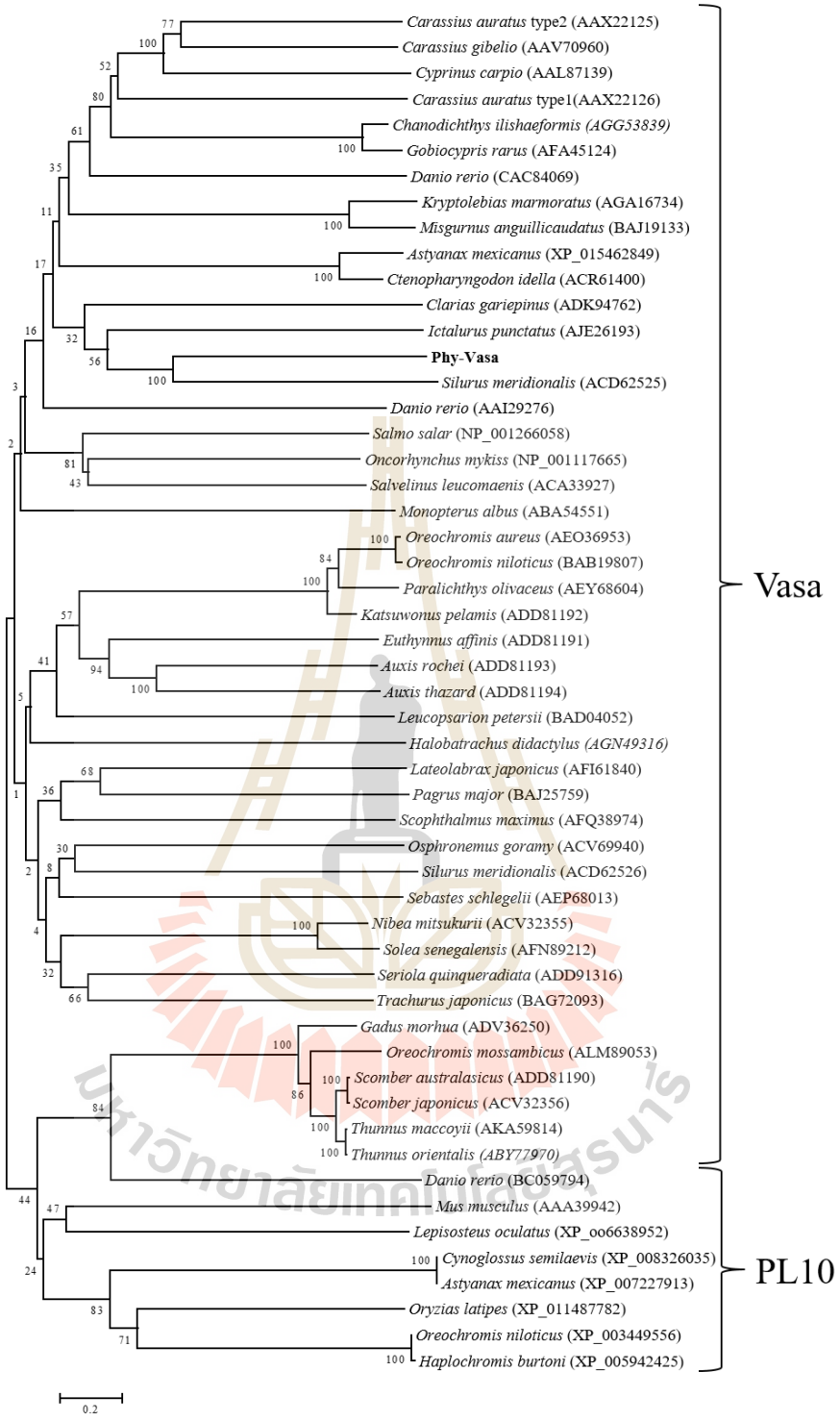

Figure 4.3 Phylogenetic tree of Vasa proteins and PL10 family proteins from different teleost species. GenBank accession numbers of Vasa and PL10 family proteins are indicated in brackets.

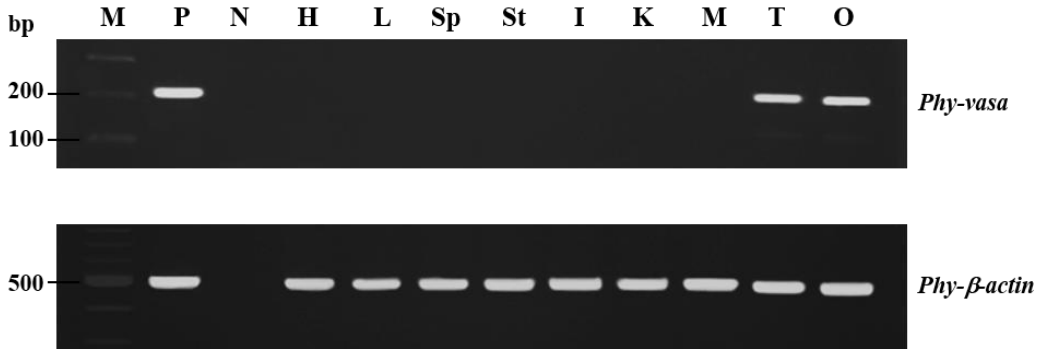

Figure 4.4 RT-PCR of *Phy-vasa* and *Phy-actin* mRNAs in tissues taken from the striped catfish. cDNAs were synthesized using total RNA isolated from heart (H), liver (L), spleen (Sp), stomach (St), intestine (I), kidney (K), muscle (M), testis (T) and ovary (O). Two pairs of primers were used to amplify *Phy-vasa* and β-*actin*, which generated amplicons of 210 (Vasa-RT-F and Vasa-RT-R) and 500 (β-*actin*-F and β-*actin* -R), respectively. Distilled water was used as a negative control (N) and plasmid DNA p*Phy-vasa vasa* or p*β-actin* were used as positive controls (P) for *Phy-vasa* and *Phy-actin*, respectively. M represents DNA marker.

Histological analysis and *in situ* hybridization were carried out in both immature and mature gonads, including testis and ovary (Fig. 4.5). Hybridization of sense *Phy-vasa*-cRNA probes did not display specific signals in the gonad samples taken from striped catfish (Fig. 4.5C, F, I, L), showing the specificity of probe. In immature testis, positive signals were detected with antisense *Phy-vasa*-cRNA probes, with the strongest signals present in spermatogonial cells throughout the testis (Fig. 4.5A, B). The *Phy-vasa*-expressing cells were in various germ cell growth stages, excluding spermatozoa, in the mature testis (Fig. 4.5D, E). Strong signals were observed in spermatogonia (SG), and

reduced expression of *Phy-vasa* was observed in primary spermatocytes and secondary spermatocytes. However, no detectable signal was found in spermatozoa. Strong positive signals for *Phy-vasa* were observed in oogonia and primary oocytes in the immature (Fig. 4.5G, H) and mature ovary (Fig. 4.5J, K) while weaker signals in maturing oocytes were detectable in the mature ovary (Fig. 4.5K). To investigate early gonad development in the striped catfish larvae, serial transverse sections of the whole body were obtained and *in situ* hybridization was performed (Fig. 4.6). Histological characterization demonstrated that PGCs were large in size and had large, rounded nuclei. During the migration process, PGCs were observed under the peritoneal wall in the dorsal part of the peritoneal cavity, where the genital ridge would form during development (Fig. 4.6A,C,E,G,I,K). By *in situ* hybridization, a positive *Phy-vasa* signal was detected in morphologically-characterized PGCs at the dorsocaudal position in the peritoneal cavity (Fig. 4.6B, D, F, H, J, L). In addition, serial sagittal sections of whole fish larvae were obtained to count the number of PGCs per individual larvae (Fig. 4.7). In 1 dpf larvae, no PGCs were observed (data not shown). In larvae 2-9 dpf, only very few PGCs were observed to have migrated to the dorsocaudal part of the peritoneal cavity, while more migrating PGCs (up to 15 cells) had settled in the larvae at 5-9 dph (Fig. 4.6A, B, C, D; Fig. 4.7). In larvae at 10 dpf, migrated PGCs had settled at the position of the presumptive genital ridge. Although not the case for all cells, surrounding somatic cells of PGC were observed in some fish larvae (Fig. 4.6E, F). PGCs at 15 dpf were enclosed within gonadal somatic cells and the genital ridge had formed (Fig. 4.6G, H). Multilayers of gonadal somatic cells enclosed the PGCs in larvae at 20 dpf (Fig. 4.6I, J). The proliferation of PGCs inside the genital ridge was first observed in larvae at 25 dpf (Fig. 4.6), while clustering of PGCs were observed in larvae at 30 dpf (Fig. 4.6 K, L).

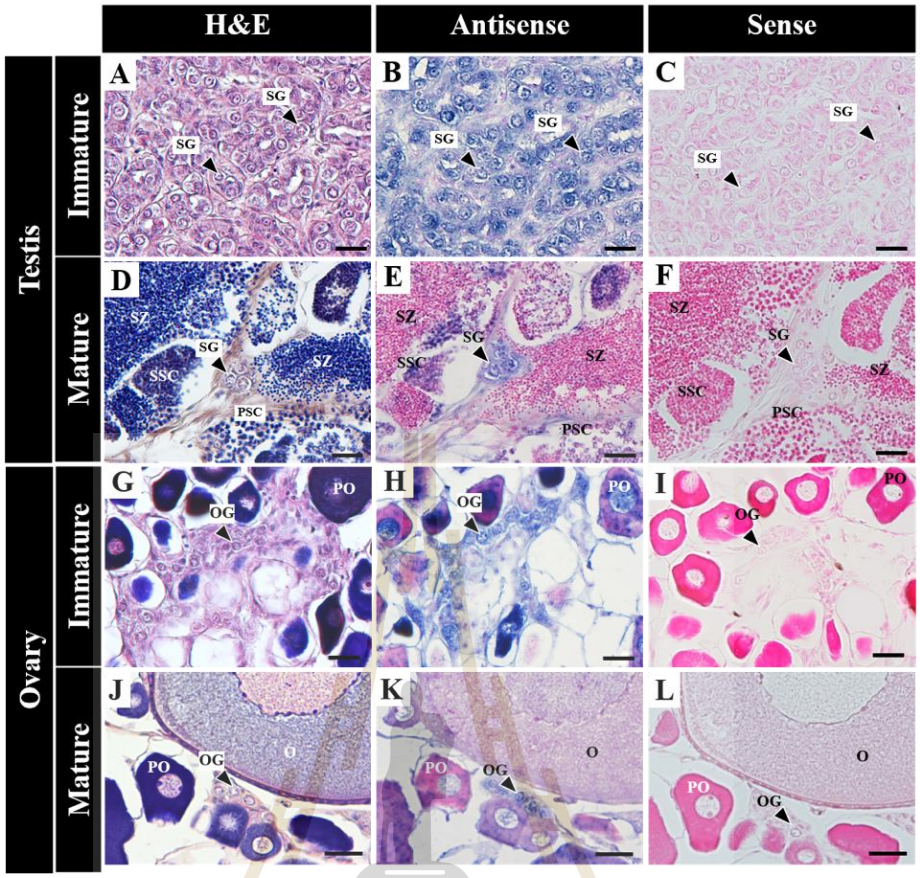

Figure 4.5 Histological characterization and *in situ* hybridization using *Phy-vasa* antisense and sense probes. Immature testis (A), mature testis (D), immature ovary (G) and mature ovary (J) stained with H&E. *In situ* hybridization with the antisense probe was performed for immature testis (B), mature testis (E), immature ovary (H) and mature ovary (K). The immature testis (D), mature testis (F), immature ovary (I) and mature ovary (L) were each hybridized with a sense probe. Scale bars represent 20 µm (A-F) and 50 µm (G-L). OG, oogonia; PO, previtellogenic oocyte; O, maturing oocytes; SG, spermatogonia; PSC, primary spermatocyte; SSC, secondary spermatocyte; SZ, spermatozoa.

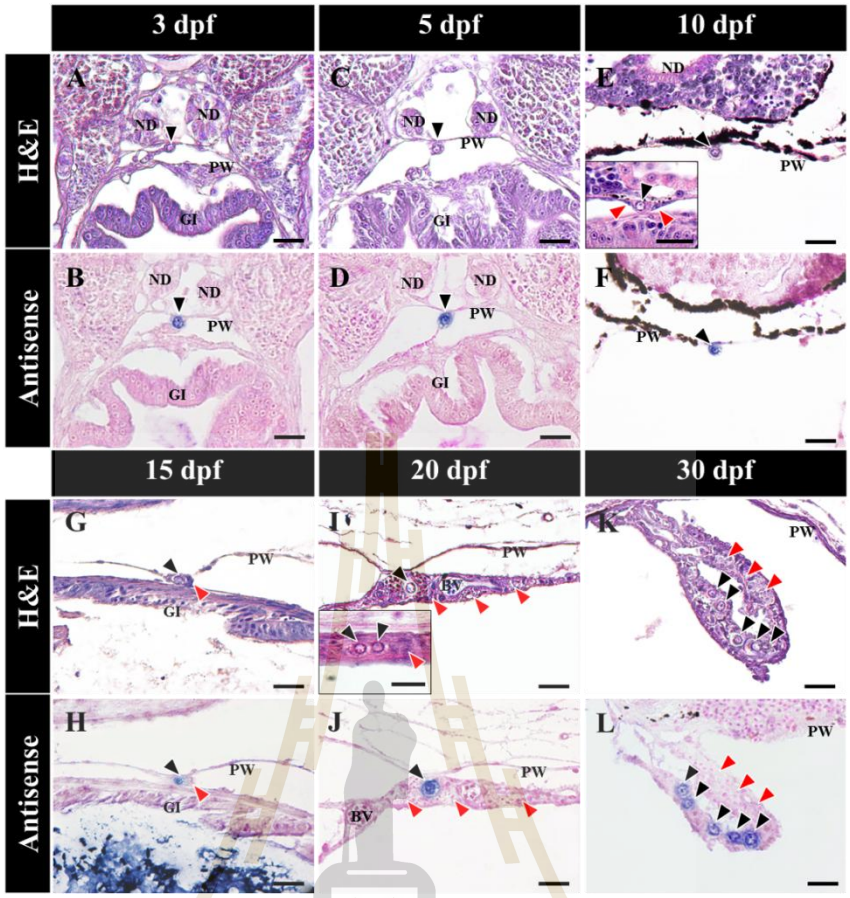

Figure 4.6 Histological characterization and *in situ* hybridization using a *Phy-vasa* antisense probe. Serial transverse sections of striped catfish larvae at 3, 5, 10, 15, 20, 25 and 30 dpf stained with H&E (A, C, E, G, I, K, respectively) and hybridized with antisense probe (B, D, F, H, J, L, respectively). The somatic cells that surrounded PGC were observed in few larvae at 10 dpf (E; rectangular). The proliferation of PGCs were first observed in larvae at 25 dpf (I; rectangular). PGCs and gonadal somatic cells are indicated by black and red arrowheads, respectively. *Phy-vasa*-expressing PGCs were specifically observed under the peritoneal wall (PW). Scale bars represent 20 µm. ND, nephric duct; GI, gastrointestinal tract.

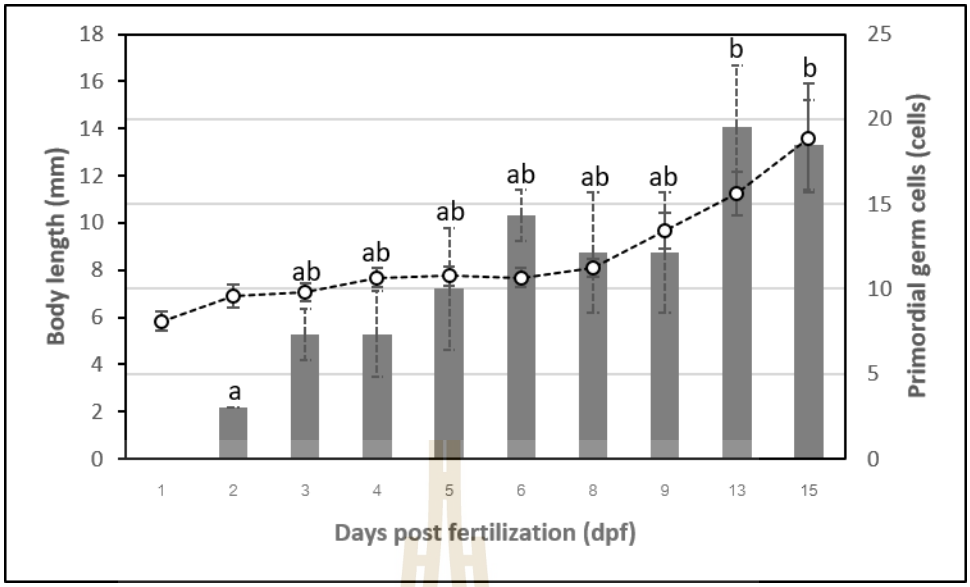

Figure 4.7 Numbers of primordial germ cells (PGCs; grey bars) per individual and total length (black dots) of the striped catfish during early gonad development. Serial sagittal sections were analyzed to evaluate PGC numbers (mean ± standard deviation [SD]) at each stage of larval development (n=5). Body length was determined as total length (mean ± SD) (n=5). A significant nonlinear relationship was observed between larval age 9 dph) (x) and the number of PGCs (y) ($Y = -0.732x^2 + 2.775x - 2.012$, $R^2 = 0.9175$, $P<0.05$) or total length (mm) (y) ($Y = 0.1027x^2 - 0.4381x + 7.0047$, $R^2 = 0.9297$, $P<0.05$)

4.5 Discussion

Molecular germ cell markers have provided a useful tool for studying reproductive development in a variety of animals including fish, particularly during early development of the gonads. In mammals, PGC proliferation occurs during migration while proliferation of PGCs in fish was demonstrated to take place after the completion of the PGC migration process (reviewed in Yoshizaki et al., 2011; De Felici, 2013). Although PGCs generally have unique characteristics among teleost, gene markers to confirm their morphological characterization during the migration process is still needed. The *vasa* gene has been characterized and used as a powerful molecular germ cell marker for reproductive research. In this study, *Phy-vasa* gene expression was characterized in striped catfish, and its specific expression was observed in germ cells in both mature and immature gonads. In addition, *Phy-vasa* was used to identify PGCs which enabled the description of early gonadal development, which provides valuable information for exploring the further development of germ cell transplantation in Pangasiid fish.

In this study, cDNA encoding *vasa*-like sequences was cloned and characterized from the striped catfish *P. hypophthalmus* (*Phy-vasa*). The deduced amino acid of Phy-Vasa contained eight consensus sequence motifs of DEAD-box proteins, which has crucial roles in binding ATP and RNA, as well as in ATP hydrolysis for unwinding RNA (Schmid and Linder 1992; Pause et al., 1993; Rocak and Linder 2004; Cordin et al., 2006). In addition, a Q-motif was observed in the aa sequence of Phy-Vasa, which was implicated as a conserved motif in DEAD-box protein family members (Tanner et al., 2003; for review, see Cordin et al., 2006; 2003). The Phy-Vasa aa sequences comprised consensus sequences typical of DEAD-

box helicases in other Vasa proteins. For instance, Phy-Vasa contained RGG- and RG-repeat-rich N-terminal regions which have been suggested to be essential for RNA binding (Kiledjian and Dreyfuss, 1992; Liang et al., 1994; Castrillon et al., 2000). In addition, consensus D, E and W residues were observed next to the initiation and stop codons. Phylogenetic-tree construction demonstrated that Phy-Vasa clustered with the Vasa family of proteins and was clearly distinct from the PL 10 family of proteins. Further classification revealed that Phy-Vasa clustered with proteins in the order of Siluriformes, which is closely related to the channel catfish (*Ictalurus punctatus*) (Shang Su et al., 2015), African sharptooth catfish (*Clarias gariepinus*) (Raghuveer et al., 2010) and Southern catfish (*Silurus meridionalis*) (Chong-Jiang et al., 2013). Together, the predicted aa sequence of Phy-Vasa shared most consensus motifs with other Vasa family and DEAD-box proteins. Thus, the characterized structure of *Phy-vasa* suggested that it does, in fact, fulfill the essential features of a typical *vasa* homolog.

vasa expression has been demonstrated to be specific to germ cells. Our RT-PCR analysis showed that *Phy-vasa* transcripts were only detectable in the testis and ovary, which was in agreement with the results obtained from other fish species, including rainbow trout (Yoshizaki et al., 2000), medaka (Shinomiya et al., 2000), tilapia (Kobayashi et al., 2002), gibel carp (Xu et al., 2005), ride field eel (Ye et al., 2007), the Pacific Bluefin tuna (Nagasawa et al., 2009), other carp species (Li et al., 2010), African sharptooth catfish (Raghuveer et al., 2010), European sea bass (Blazquez et al., 2011), rare minnow (Cao et al., 2012), *Solea senegalensis* (Pacchiarini et al., 2013), toadfish (Ubeda-Manzanaro et al., 2014), Japanese flounder (Wu et al., 2014), half-smooth tongue sole (Huang et al., 2014) and brown-marbled

grouper (Boonanuntanasarn et al., 2016). In addition, using a specific *Phy-vasa* probe, in situ hybridization demonstrated that *Phy-vasa* expression was obviously found in the spermatogonial cells of both immature and mature testis, as well as the oogonial cells in immature and mature ovaries. Moreover, *Phy-vasa* expression in the PGC was observed during their gonadal development. Our observations suggested that *Phy-vasa* is expressed specifically in germline cells, which is in agreement with the findings demonstrated in *Drosophila*, humans and fish (Hay et al., 1988; Fujiwara et al., 1994; reviewed in Raz 2000, Boonanuntanasarn et al., 2016). Therefore, together with the predicted structure of *Phy-vasa*, which contains characteristics of a typical *vasa* homolog, *Phy-vasa* expression also correlates with the predicted function of *vasa* and its specific expression in immature germline cells.

Gonad development in fish larvae generally begins with the migration of PGCs from the extragonadal area toward the site of the future genital ridge. Subsequently, surrounding somatic cells are formed, and PGCs perform proliferation and sex differentiation to produce functional gametes (for review, see Braat et al., 1999; Yoshizaki et al., 2002). The *vasa* gene was demonstrated to be specifically expressed at high level in immature germ cells (Yoshizaki et al., 2000). Our results showed that *Phy-vasa* mRNA-positive signals were observed in PGCs that contained a large, rounded nucleus. Therefore, to study the development of the gonad during larval growth in striped catfish, we characterized the PGC to confirm previous histological studies using in situ hybridization with a *Phy-vasa* probe. Few PGCs were observed at the presumptive genital ridge of larvae between 2-9 dpf, suggesting that PGCs begin the migration towards the presumptive genital ridge around 2 dpf (2.7±0.6 mm total length (TL) to 9 dpf (9.7±0.8 mm TL). At 10 dpf, formation of surrounding

somatic cells of PGCs was observed in a few larvae, though not all, suggesting that migration of PGCs could be observed most prominently in larvae at 2-10 dpf. Gonadal somatic cells entirely covered the PGCs at 15 dpf.

Subsequently, the multilayer formation of surrounding somatic cells in PGCs was observed at 20 dpf, demonstrating that the formation of the genital ridge, which contains both PGCs and somatic cells likely occurred in larvae at 10-20 dpf (14.2±5.2 mm TL). At 25 dpf, proliferation of PGCs was observed, and at 30 dpf, the observation of a cluster of PGCs suggested that this process took place between 25-30 dpf (30.7±1.5 mm TL). In Atlantic halibut (*Hippoglossus hippoglossus*), PGCs were observed in 10.0 mm fork length (FL) larvae and their migration was seen in 21.0 mm FL larvae (Hendry et al., 2002). In *Spratelloides gracilis*, PGCs were found in yolk-sac larvae (4.0 mm notochord length) and proliferation of PGCs in this species occurred in larvae at a size of 13.7 mm (Hatakeyama et al., 2005). In the Nibe croaker, PGC migration occurred in larvae at 12 dpf (4 mm TL) and PGC proliferation began 23 dpf (8 mm TL) (Takeuchi et al., 2009). In tilapia (*Oreochromis niloticus*), migration of PGCs toward the genital ridge was observed in larvae at 6-8 dpf. Subsequently, a single layer of surrounding somatic cells was observed at 11 dpf (Farlora et al., 2014). In the brown marbled grouper (*Epinephelus fuscoguttatus*), the PGC migration process occurred between 9 and 15 dph. Later, at 15-27 dph, the genital ridge formed and the proliferation stage of PGC occurred after 32 dph. Taken together among various fish species, the early gonadal development processes in larvae are conserved, although the timing and duration can vary.

By using in situ hybridization with a *Phy*-vasa probe, we confirmed PGCs presence in the demonstration of gonadal development in striped catfish larvae. Our

findings provide useful information for studying the development of germ cell transplantation by using the striped catfish larvae as the recipient fish. We showed that the optimum period for germ cell transplantation into recipient larvae was when endogenous PGCs are migrating. Furthermore, it should be before the process of surrounding of somatic cells with PGCs is complete. This period of gonadal development in fish larvae was demonstrated to be successful with high efficiency for conducting germ cell transplantation in the Nibe croaker, chub mackerel and yellowtail (Takeuchi et al., 2009; Morita et al., 2012). Therefore, this study suggested that germ cell transplantation using the striped catfish as recipient larvae would have to occur between 2 and 9 dpf.

In conclusion, *Phy-vasa* was cloned and characterized from the striped catfish. The *Phy-vasa* was specifically expressed in germline cells. Using *Phy-vasa* as molecular marker, PGC migration during gonadal development in the striped larvae was demonstrated.

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CHAPTER V

PRODUCTION OF TRIPLOID STRIPED CATFISH (*PANGASIANODON HYPOPHTHALMUS*)

5.1 Abstract

This study aimed to produce triploid striped catfish. Different temperatures and duration of treatment for heat-shock and cold-shock were employed to investigate the optimum method to produce triploid fish. Our results showed that cold-shock at 7.5°C for 30 min was the optimum process to obtain 90% triploid fish with 35.34% of hatching rate and 20% of survival rate of larvae at 7 days post fertilization. Characterization of red blood cells (RBC) was conducted to determine triploidization. Triploid RBC has significantly larger nuclear sizes. The result of flow cytometry of RBC was showed two discrete peak of DNA content in which the DNA content of RBC of triploid fish was approximately 1.5 times of that of diploid fish. Hematological study was demonstrated that there were no significantly different between triploid and diploid fish. In addition, there were no significant differences in growth performances between diploid and triploid fish. The gonadosomatic index (GSI) and histological study were determined in male and female at 7, 10, 13 and 16 months of ages. The larger GSI in diploid fish were observed in ovary since 7 months of ages, and the larger GSI in testis of diploid fish was found at 13 and 16 months of ages. Moreover, histological study demonstrated that testis of diploid and triploid fish at ages of 7 and 10 months appeared to be similar development. At ages of 13 and 16

months, testis of diploid fish contained differentiated spermatocytes and few spermatozoa. However, triploid testis contained most spermatogonia which were similar to that observed in diploid testis at age of 7 and 10 months. The ovary of diploid and triploid fish at ages of 7 month contained most oogonia and primary oocytes. At ages of 10, 13 and 16 months, ovary of diploid fish contained most previtellogenic oocytes (PVO). However, triploid ovary contained most oogonia and primary oocytes. Few PVOs were observed in triploid ovary at age of 13 and 16 months. In conclusion, for this experimental period, growth performance, hematological indices and early gonadal development of diploid and triploid fish were similar. However, gametogenesis of triploid fish appeared to be disorder.

5.2 Introduction

Germ cell transplantation (GCT) have been established in fish including Nibe croaker, nile tilapia, rainbow trout, Siberian sturgeon, chub mackerel, and yellowtail (Farlora et al., 2014; Morita et al., 2012; Pšenička et al., 2015; Takeuchi et al., 2009; Takeuchi et al., 2003; Yazawa et al., 2010). GCT technique is performed by isolating of immature germ cells including primordial germ cells (PGCs), type A spermatogonia (ASG) and type A oogonia (AOG) from donor fish and subsequently transplanting them into desired recipient larvae. The technique for GCT in fish simply mimics the principle of gonadal development in fish. During the early stage of development, PGCs are developed outside of the gonad. PGCs migrate toward the genital ridge with pseudopodia by chemotaxis (Raz, 2004). After PGCs have settled in genital ridge, PGCs are surrounded by somatic cells and start proliferation (Yoshizaki et al., 2002). The transplanted germ cells were subsequently developed for

sex differentiation and gametogenesis (Kagawa, 2013). For example, Morita et al. (2015) investigated xenogenic transplantation by using yellowtail (*Seriola quinqueradiata*) and jack mackerel (*Trachurus japonicus*) as donor and recipient fish, respectively. Only 0.033% offspring were donor-derived offspring. Xenogenic transplantation was achieved by using rainbow trout (*Oncorhynchus mykiss*) and masu salmon (*Oncorhynchus masou*) as donor and recipient fish, respectively. The percentages of donor derived-offspring were 0.2-91.3% of total offspring (Okutsu et al., 2006). Combined together, GCT was achieved to obtain donor-derived offspring. However, the migration of the transplanted germ cell has to compete with that of endogenous germ cell. Therefore, the efficiency of GCT or the percentage of donor-derived offspring was variable. In order to improve the efficiency of production of donor-derived offspring, sterile fish or fish that cannot produce their own gamete but could support the development of the transplanted germ cells is needed.

Triploid fish with three complete sets of chromosomes instead of two sets in diploid individuals can be generated by preventing the extrusion of the second polar body during the second meiotic division in embryonic development (Benfey, 1999). Triploidization techniques were developed in several aquaculture-related fish species. It was demonstrated that gonad development in triploid undergoes normally for mitotic division. However, gametogenesis was arrested because of inducing a meiotic disorder. It was reported in Nibe croaker (*Nibea mitsukurii*) that ovarian growth is greatly retarded, but testis grows to near normal size (Takeuchi et al., 2018). This difference in gonadal growth is due to differences between females and males in both the number and size of gametes produced. Diploid females produce relatively small numbers of large oocytes whereas diploid males produce relatively large numbers of

small spermatozoa. Many of the studies have reported that viable spermatozoa could be produced by triploids, but at greatly reduced numbers, resulting in very dilute milt. Abnormalities in spermatozoan morphology have also been described (Kawamura et al., 1995; Kitamura et al., 1991; Nakamura et al., 1993). When milt from triploid males is used to fertilize normal haploid eggs, the resultant progeny typically begin development but die at embryonic and larval stages. Spermatozoa from triploids are generally aneuploid (Allen et al., 1986; Benfey et al., 1986), resulting in aneuploid embryos (Ueda et al., 1987). However, Van Eenennaam et al. (1990) showed that some euploid offspring (i.e., having chromosome numbers equal to a complete multiple of haploid) could be obtained in grass carp from crosses between triploid males and diploid females. A different situation has been described in triploid red sea bream (*Pagrus major*), which apparently produce only euploid spermatozoa (Kawamura et al., 1995). Most of these spermatozoa are haploid, but some diploid, triploid, hexaploid, and heptaploid spermatozoa were also observed. In addition, no progeny of Nibe croacker from crosses between triploids and diploids survived later than 24 h subsequent to hatching (Takeuchi et al., 2018). Taken together, triploid fish could be a recipient fish for GCT since it could contribute normal gonad development but disorder in functional gamete production. Therefore, the triploid fish could be effectively used as surrogate recipients in GCT.

Currently, the family Pangasiidae comprises of 21 recognized species, some of Pangasiid catfish are economically important and/or endangered species. Among Pangasiid catfish, the Mekong giant catfish, *Pangasianodon gigas*, has been attracted globally because it is the world's largest freshwater fish. The maturation of the Mekong giant catfish species was reported at more than 15 years old and at 37 kg of

body weight (Phayao, 2000). The Mekong giant catfish has been considered to be Critically Endangered (IUCN) and is already listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). The striped catfish, *Pangasianodon hypophthalmus*, occasionally becomes maturity at 900 g of body weight. The striped catfish at 2 years old can generally be broodstock and able to produce functional gamete. In addition, its full cycle culture is well-developed. Moreover, the striped catfish is belonged to the same genus is considered to be evolutionarily related with the Mekong giant catfish. Therefore, the striped catfish could be a good candidate to be a recipient for GCT of the Mekong catfish.

Development of triploidization in striped catfish would be not only benefits for GCT. It was reported that sexual maturation is a major problem in aquaculture since fish consumes their metabolic energy for gonadal development before reaching marketable size (Taranger et al., 2010). Therefore, triploid fish would be able to utilize diets solely for growth. Triploid cell nuclei contain, by definition, 50% more DNA than diploid cell nuclei. Nuclear volume is increased in triploids to accommodate this extra genetic material. A corresponding increase in cellular volume typically results due to the approximate maintenance of the diploid ratio of nuclear to cytoplasmic volume. Despite increased cell size, triploid individuals are not, as a rule, larger than diploids. This appears to be due to a reduction in cell numbers in those tissues and organs containing larger cells. Therefore, it was interested to investigate whether development of triploid striped catfish would also improve growth performance of striped catfish or not.

In this study, the optimum condition of production of triploid striped catfish was investigated. Several techniques to determine triploid cell were conducted

including flow cytometry and erythrocyte characteristic. Comparative studies were carried out for hematological, histological of gonad and growth performance.

5.3 Materials and methods

5.3.1 Fish culture and breeding

Stripped catfish used in this study were cultured at the university farm of Suranaree University of Technology, Nakhon Ratchasima, Thailand. Albino broodstock (females 2.5-3.5 kg, males 2-3.5 kg) were maintained in earthen pond ($10\times40\times1.5$ m^3). The broodstock were fed with commercial diet (25% crude protein, 3% crude fat) at 5% body weight at 10:00 and 16.00 every day. Broodstock (1:1, male/female) were transferred for hormonal injection in canvas pond (2 m^3). Female fish were injected hormone for 2 times, first injection by 1 dose of fish's pituitary extract (PE) and 300 IU kg^{-1} of human chorionic gonadotropin (HCG). After 8-12 hours. The second injection was performed with injection of 4 doses of fish's PE and 900 IU kg^{-1} HCG. After the second injection for 10-12 hours, artificial fertilization was conducted by hand-stripping eggs and milt.

5.3.2 Temperature-shock application

Eggs were inseminated with milt, subsequently subjected to experimental conditions. Approximately 9,000 eggs and 500 µl of milt were divided into treatment which different condition. This study consisted of one experiment repeated 6 times trial. For each trial, fertilized eggs were divided into 13 groups: 1 control and 12 treated which contained 2 kinds of temperature-shock including cold-shock and heat-shock. Cold-shock commenced at 1 minute post-fertilization (mpf), and was applied for durations of either 10, 20, or 30 minutes at either 5 or 7.5 °C.

Likewise, heat-shock also commenced at 1 minute mpf, and were applied for durations of either 3, 5, or 10 minutes at either 36 or 39 °C. An untreated control group was rinsed by fresh water at 28ºC and immediately transferred to the hatching tank at 28ºC. For all shocked groups, after the shock duration had finished, each one of the 12 treated groups were rinsed by fresh water at 28ºC and immediately transferred to the hatching tank at 28ºC. The larvae were hatched for 36 hours at 28ºC.

5.3.3 Hatching and survival rates of temperature-shock treated embryos

Subsequent to temperature-shock treatments, fertilized eggs were reared at 28°C in 20-l plastic tanks. In order to evaluate hatching rates in each treatment, total numbers of hatched larvae were counted at 36 hours after fertilization. Consequently, the hatched larvae were then transferred into a new tank (0.5 m^3) at a density of 100 fish per liter with aeration .The larvae were fed with newly hatched brine shrimp (individuals ml^{-1}) from 3-14 days post-hatching (dph) Subsequently, the fry were fed with commercial powdered diets (36% crude protein, 4% crude fat). Survival rates of temperature-shock treated eggs and control eggs were monitored at 7 dph.

5.3.4 Ploidy determination

In order to determine ploidy of treated fish, 10 individuals from each 6 replications of one-month-old (3.5-4.5 cm of total length) were used to measure DNA contents by flow-cytometry as described in Hare and Johnston (2011), with slight modifications. Briefly, 15 µl of blood sample was fixed with 4% of paraformaldehyde (PFA) (Sigma-Aldrich Inc., USA) and kept in 4ºC until it was used. Subsequently, centrifuged the sample at 4,000 rpm for 2 minutes (4ºC) then supernatant was

discarded. Consequently, cell pellet was washed 3 times by 1X of PBS then was centrifuged at 4,000 rpm for 2 minutes (4ºC). Finally, working solution contained 0.1% of TritonX-100 (Sigma-Aldrich Inc., USA) in PBS, RNase A (Thermo Scientific Inc., USA) 20 μg/ml, and propidium iodide (PI) (Sigma-Aldrich Inc., USA) 20 μg/ml were added to the sample. Allow samples to stain in the dark at 4°C for up to 24 hours. The duration of staining must be determined empirically for each organism, but 30 minutes can be used as a starting point then were analyzed by flow-cytometer (BD FACS Calibur™; BD Biosciences, Singapore).

5.3.5 Erythrocyte characteristic

In order to test if the ploidy of striped catfish specimens could be determined using the major axis length of erythrocytes as it in other species of teleosts (Ballarin et al., 2004; Hamasaki et al., 2013), whether or not erythrocyte of triploids was larger than that of diploids was studied. Blood smears were prepared from one-month-old diploids (n=10) and triploids (n=10), whose ploidies had been determined by their respective DNA content results. The stained blood with PI from flow-cytometric analysis was placed onto a clean microscope slide. The major axis length, cell area, major axis nucleus length, and nucleus area of 30 erythrocytes per individuals were measured and photographed under a ×1000 fluorescent microscope. Averages of the values of triploid and diploid erythrocytes were compared. After confirming that major axis length, cell area, major axis nucleus length, and nucleus area of erythrocyte were a valid indicator of striped catfish specimen ploidy, they were used to determine ploidy for the remainder of the present study.

5.3.6 Growth performance

In order to determine growth performance in diploids and triploid striped catfish, forty individuals from each 4 replications were randomized sampling to culture in hapa cages for 12 months. Daily, fish were fed with commercial diet (25% crude protein, 3% crude fat) at 5% body weight at 10:00 and 16.00. Growth performance parameters were observed every month. In addition, hepatosomatic index (HSI), gonadosomatic index (GSI) and hematological parameters were determined every 4 months.

5.3.7 Gonadal histology

Fish used in histological analyses were 7-, 10-, 13-, and 16-month-old diploid and triploid striped catfish. Five individuals from each age group were collected and dissected. Gonadosomatic index (GSI; [gonad weight in grams/body weight in grams] × 100) was used to measure gonadal development of specimens. The gonads of striped catfish were fixed using Bouin's fixative, cut into 5-μm-thick sections using the standard paraffin-embedding method, and stained with hematoxylin-eosin (HE). Histological sections were examined and photographed using a light microscope.

5.3.8 Statistical analysis

Data are presented as means ± SD unless otherwise stated. All data were analyzed one-way analysis of variance (ANOVA) using SPSS for Windows (Release 14) (SPSS Inc. Chicago, IL, USA). When significant differences were observed among the groups, Tukey’s multiple comparison test was performed to rank the groups. Values were considered statistically significant when the calculated *P* values were less than 0.05 ($P<0.05$).

5.4 Results

Table 5.1 showed the hatching and the survival rates of temperature-shock treated embryos. Hatching rates were dramatically decreased in temperature-shock treated groups. At 1 week, the survival rates of embryos which were subjected to 39ºC for 5 and 10 minutes were significantly decreased when compared with non-treated group (control group). The highest hatching rates of treated groups were found in groups subjected to 36ºC for 3 and 5 minutes (83.43±8.63 and 77.72±8.27, respectively). It seemed that at extremely both high and low treated temperatures lowered not only hatching rates but also survival rates.

Table 5.1 Hatching and survival rate of treated and control striped catfish (mean ± standard deviation, n = 6).

Treatment		Hatching rate	Survival rate
Temp. (ºC)	**Shock duration (min)**	**(%)**	**(%)**
5	10	13.01±1.09^{e}	20.83±3.39^{ab}
5	20	11.43±0.94^{e}	23.33±3.50^{ab}
5	30	9.82±0.48^{e}	23.33±3.54^{ab}
7.5	10	38.06±2.44^{d}	19.67±2.49^{ab}
7.5	20	34.35±0.85^{d}	19.67±3.20^{ab}
7.5	30	35.34±0.92^{d}	20.00±2.77^{ab}
36	3	83.43±8.63^{b}	27.00±5.72^{a}
36	5	77.72±8.27^{b}	24.67±3.50^{ab}
36	10	57.85±11.17^{c}	22.67±4.57^{ab}
39	3	13.18±1.77^{e}	19.33±2.43^{b}
39	5	11.18±1.78^{e}	9.00±1.91^{c}
39	10	4.70±1.64^{e}	7.67±2.21^{c}
Control		94.72±2.29^{a}	23.50±2.93^{ab}

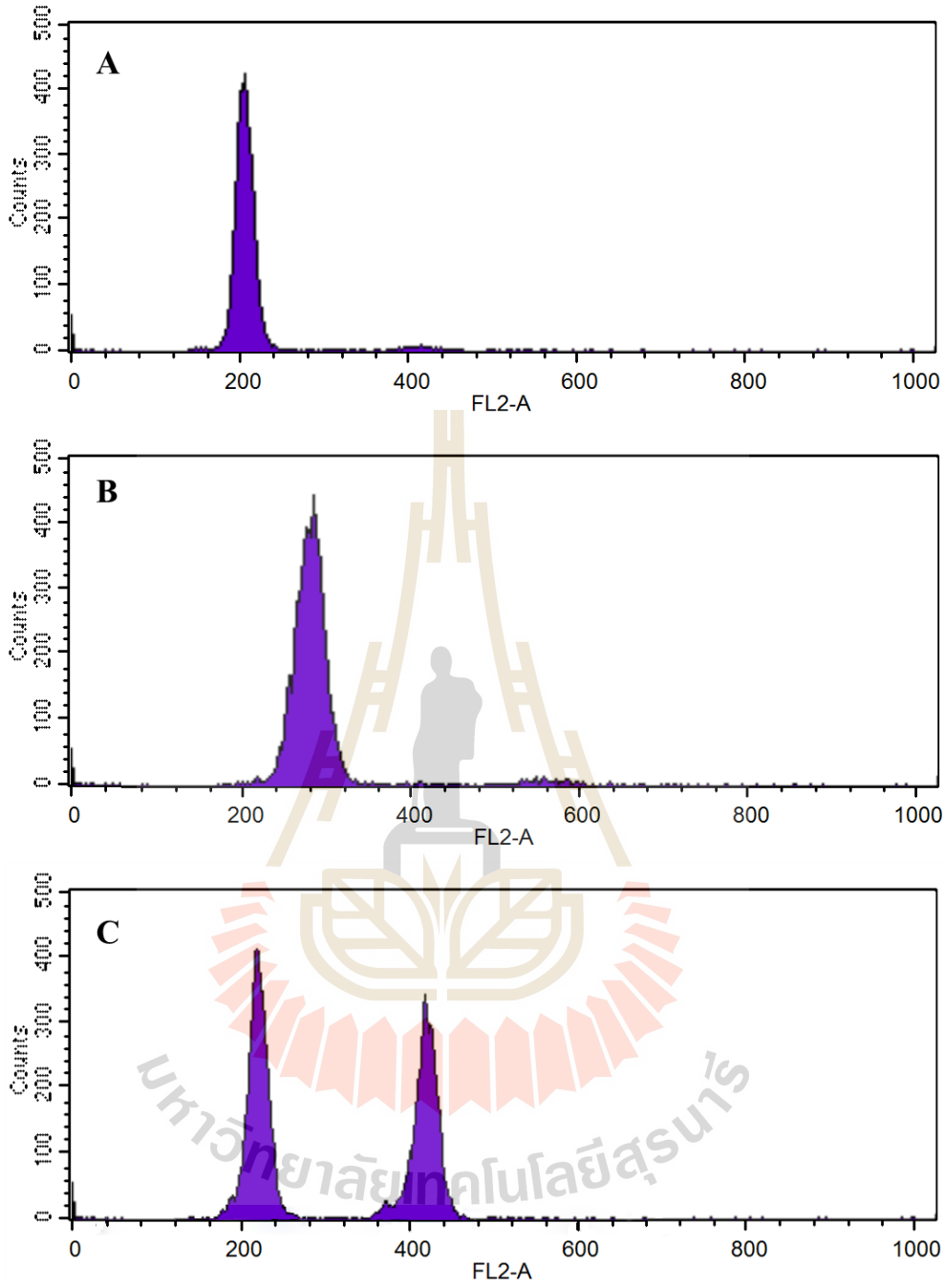

Figure 5.1 Flow cytometric histograms for the relative DNA content of diploid (A) and triploid (B) striped catfish. The cytometric histograms of fish that were treated at 39 C for 10 min. Note that only one was observed.

Table 5.2 Diploid and triploid rate of treated and control striped catfish (mean ± standard deviation, n = 6).

Treatment		Diploid rate (%)	Triploid rate (%)
Temp. (ºC)	**Shock duration (min)**		
5	10	100±0.00	0±0.00
5	20	100±0.00	0±0.00
5	30	100±0.00	0±0.00
7.5	10	100±0.00	0±0.00
7.5	20	100±0.00	0±0.00
7.5	30	10±8.94	90±8.94
36	3	100±0.00	0±0.00
36	5	100±0.00	0±0.00
36	10	100±0.00	0±0.00
39	3	100±0.00	0±0.00
39	5	100±0.00	0±0.00
39	10	100±0.00	0±0.00
	Control	100±0.00	0±0.00

In order to determine the rate of triploidy, flow-cytometry was used to analyze DNA contents in erythrocyte of treated and non-treated striped catfish. Histogram measurements were calculated using peak fluorescence on a FL2-A photomultiplier tube (Fig. 5.1). Diploid and triploid individuals were easily identified by flow-cytometry measurements of the cell suspension. Non-treated of striped catfish were used as diploidy standard that showed FL2-A value approximately 200 units while

triploid striped catfish showed DNA contents more than diploids for 1.5 times that approximately 300 units (Fig. 5.1). The results of diploid and triploid rates were demonstrated in Table 5.2. Only temperature-shock groups subjected to 7.5ºC for 30 minutes was found for 90±8.94% of triploid rate. To confirm the triploid cell, erythrocyte characteristic analysis was conducted by using PI-stained erythrocytes. Major axis length, cell area, major axis nucleus length, nucleus area were observed and measured from the stained erythrocytes by fluorescent microscope (Fig. 5.2, Table 5.3). The major axis length, cell area, major axis nucleus length, nucleus area of sample treated by 7.5ºC for 30 minutes showed significant differences among treatments and higher than control group. The average length of the major cell axis of triploid erythrocytes was 13.01±0.38 μm, which was 1.24 times larger than those of diploids (10.45±0.32 μm) (P<0.05). The average cell area of triploid erythrocytes was 94.41±1.94 μm^2, which was 1.25 times larger than those of diploids (75.30±4.97 μm^2) (P<0.05). The average length of the major nucleus axis of triploid erythrocytes was 6.09±0.17 μm, which was 1.41 times larger than those of diploids (4.32±0.14 μm) (P<0.05). The average nucleus area of triploid erythrocytes was 20.18±1.54 μm^2, which was 1.41 times larger than those of diploids (14.36±0.36 μm^2) (P<0.05). Apparently, the results of major axis length, cell area, major axis nucleus length, nucleus area were higher than control group closed to 1.5 times that related with flow-cytometric result.

To investigate growth performance of triploid striped catfish compared with diploid striped catfish, forty individuals (4 replications) in non-treated group and the group that was treated at 7.5ºC for 30 min were cultured for 16 months. There were no significant differences body weight, weight gain, specific growth rate, feed intake,

feed conversion ratio, condition factor, and hepatosomatic index between control diploid and treated triploid fish at age of 7 and 16 months (Table 5.4). Hematological analysis of diploid and triploid fish was showed in Table 5. There were no significant differences in hemoglobin and hematocrit between diploid and triploid fish at each and every examined period. The red blood cell number appeared to be lower in triploid fish; however, only fish at age of 13 months were significant lower (Table 5.5).

Table 5.3 Erythrocyte characteristics of treated and control striped catfish (mean ± standard deviation, n = 6).

Treatment		Major axis cell length (μm)	Cell area (μm^2)	Major axis nucleus length (μm)	Nucleus area (μm^2)
Temp. (ºC)	Shock duration (min)				
5	10	10.60 ± 0.19^{b}	78.89 ± 2.48^{b}	4.33 ± 0.06^{b}	13.78 ± 0.70^{b}
5	20	10.88 ± 0.31^{b}	78.71 ± 3.04^{b}	4.51 ± 0.14^{bc}	14.09 ± 0.36^{b}
5	30	10.80 ± 0.35^{b}	76.49 ± 3.56^{b}	4.27 ± 0.09^{bc}	13.94 ± 0.58^{b}
7.5	10	10.96 ± 0.22^{b}	77.26 ± 3.72^{b}	4.32 ± 0.11^{bc}	13.77 ± 0.31^{b}
7.5	20	10.87 ± 0.30^{b}	73.19 ± 5.23^{b}	4.37 ± 0.18^{bc}	13.70 ± 0.26^{b}
7.5	30	13.01 ± 0.38^{a}	94.41 ± 1.94^{a}	6.09 ± 0.17^{a}	20.18 ± 1.54^{a}
36	3	10.95 ± 0.16^{b}	77.48 ± 4.25^{b}	4.27 ± 0.15^{bc}	13.92 ± 0.43^{b}
36	5	10.76 ± 0.09^{b}	74.51 ± 3.10^{b}	4.38 ± 0.12^{bc}	13.84 ± 0.30^{b}
36	10	10.79 ± 0.23^{b}	78.11 ± 3.63^{b}	4.30 ± 0.10^{bc}	13.54 ± 0.61^{b}
39	3	10.69 ± 0.13^{b}	79.56 ± 7.98^{b}	4.33 ± 0.31^{bc}	13.41 ± 0.19^{b}
39	5	10.53 ± 0.25^{b}	74.10 ± 3.46^{b}	4.24 ± 0.11^{c}	13.89 ± 0.55^{b}
39	10	10.65 ± 0.19^{b}	77.66 ± 2.87^{b}	4.36 ± 0.11^{bc}	14.09 ± 0.50^{b}

Means with different small superscripts in each column differ significantly from each other ($P<0.05$).

GSI of gonad of diploid and triploid were showed in Fig. 5.3. In males, during the periods at 7- to 10-month-old, the GSI of triploids was similar to that of diploids. Compared with the diploid control, GSI of triploid males was lower at ages 13 and 16 months (Fig. 5.3). In addition, GSI of female triploids at 7-, 10-, 13- and 16- months of age were lower when compared to that of diploid fish.

Table 5.4 Growth performance of diploid and triploid striped catfish (mean ± standard error, n = 4).

Parameter	Initial		Final	
	2N	3N	2N	3N
Body weight (g)	29.58±2.80	25.10±3.48	460.94±105.11	389.33±79.23
Condition factor (%)	1.71±0.07	1.58±0.22	1.74±0.23	1.39±0.22
Hepatosomatic index (%)	1.38±0.20	1.32±0.10	0.91±0.11	1.16±0.08
Weight gain (%)	-	-	9.25±8.16	11.56±5.14
Specific growth rate (%/day)	-	-	0.29±0.24	0.84±0.76
Feed intake (g/day)	-	-	0.61±0.14	0.52±0.11
FCR (kg of feed/kg of animal)	-	-	2.89±1.62	1.58±0.51

No significant difference were observed among ploidy.

Table 5.5 Hematological parameters of diploid and triploid striped catfish (mean ± standard error, n = 4).

Age (months)	Hemoglobin (g/dl)		Hematocrit (%)		RBC ($\times 10^6$ cell/µl)	
	2N	3N	2N	3N	2N	3N
7	8.70±1.00	7.57±0.51	27.68±2.77	23.61±1.23	1.73±0.18	1.25±0.07
10	8.77±0.43	7.70±0.70	34.55±2.76	36.13±1.28	1.86±0.14	1.55±0.05
13	10.12±0.83	8.71±0.52	42.37±0.90	39.62±0.86	1.82±0.01^a	1.35±0.04^b
16	19.00±0.77	17.99±0.76	42.78±1.17	40.52±1.25	1.88±0.06	1.68±0.27

Means with different small superscripts in each parameter differ significantly from each other among age ($P < 0.05$).

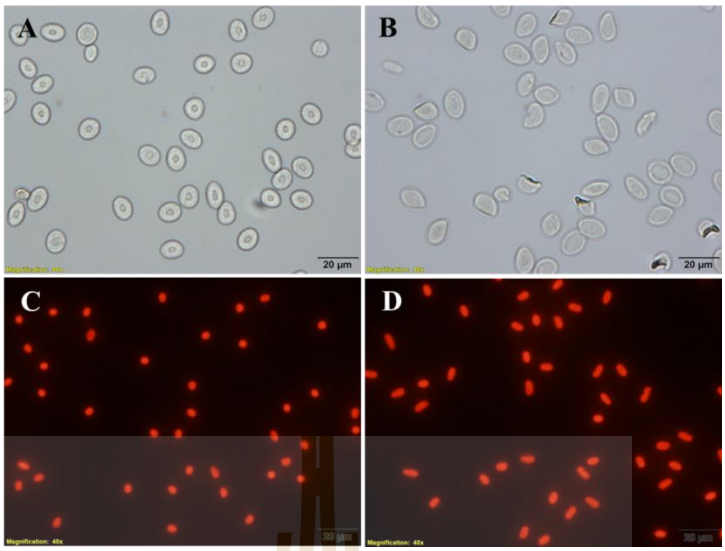

Figure 5.2 Erythrocytes of diploid (A and C) and triploid (B and D) of striped catfish were observed by bright field (A and B) and fluorescent view (D and D). Scale bars = 20 µm.

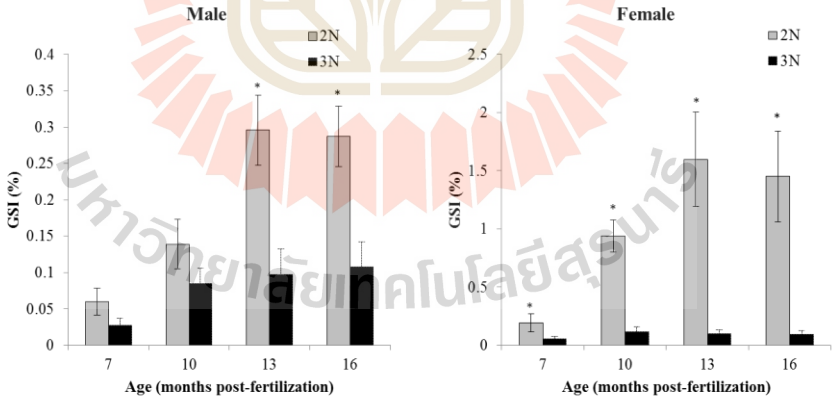

Figure 5.3 Gonadosomatic index (GSI) of male and female of diploids and triploids at 7, 10, 13 and 16 months of ages.

Histological study of testis and ovary of diploid and triploid at various ages including 7, 10, 13 and 16 months of ages were showed in Fig. 5.4. Testis of diploid fish at ages of 7 and 10 months contained most spermatogonia which were similar to that observed in triploid fish at the same ages (Fig. 5.4A, B, E, F). At ages of 13 and 16 months, testis of diploid fish contained differentiated spermatocytes. Few spermatozoa were observed. However, triploid testis contained most spermatogonia which were similar to that observed in triploid testis at age of 7 and 10 months. Histological study of ovary of diploid and triploid at various ages including 7, 10, 13 and 16 months of ages were showed in Fig. 5.4I, J, K, L, M, N, O, P). The ovary of diploid and triploid fish at ages of 7 month contained most oogonia and primary oocytes. At ages of 10, 13 and 16 months, ovary of diploid fish contained most previtellogenic oocytes (PVO); however, triploid ovary contained most oogonia and primary oocytes. Few PVOs were observed in triploid ovary at age of 13 and 16 months.

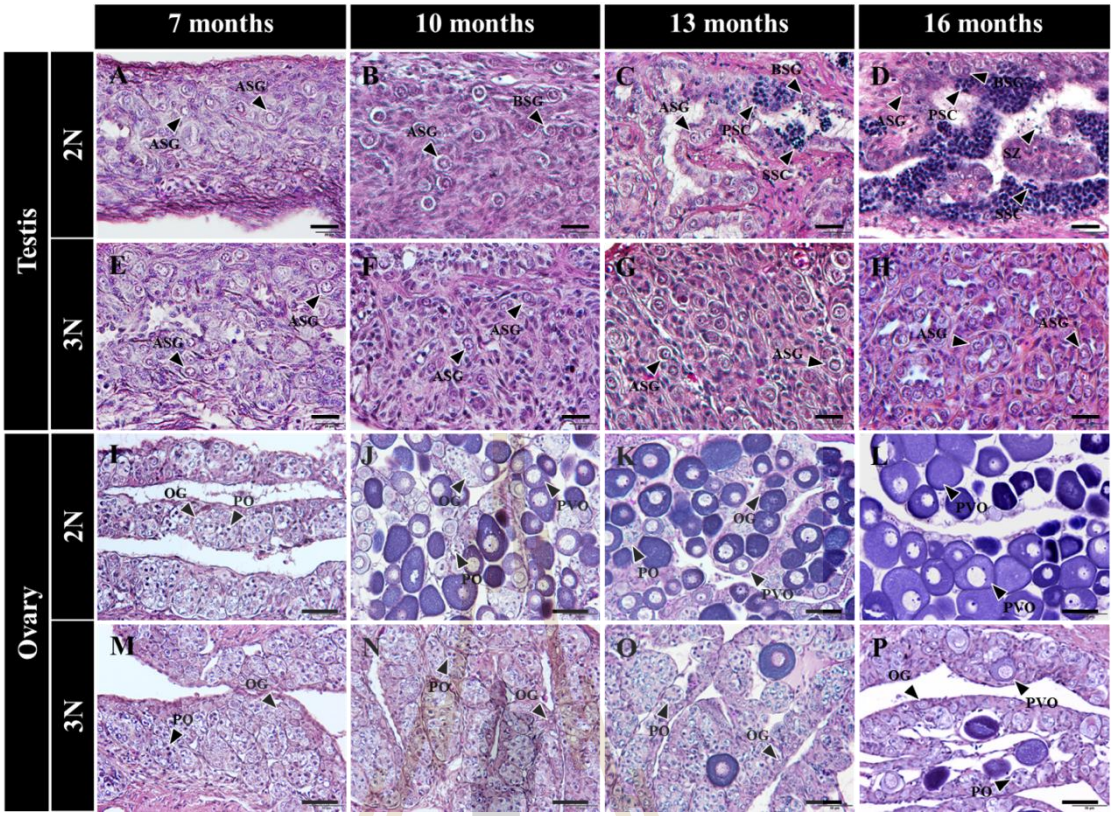

Figure 5.4 Testicular and ovarian histology of diploid and triploid striped catfish sampled at 7 (A, E, I and M), 10 (B, F, J and N), 13 (C, G, K and O), and 16 (D, H, L and P) months of ages. ASG, type A spermatogonia, BSG, type B spermatogonia, PSC, primary spermatocyte, SSC, secondary spermatocyte, SZ, spermatozoa, OG, oogonia, PO, primary oocyte, PVO, previtellogenic oocyte. Scale bars = 20 μm (A-H), 50 μm (I-P)

5.5 Discussion

Result of this study showed the optimal condition for triploidization in striped catfish was incubating fertilized eggs at 7.5ºC for 30 minutes after fertilization which yielded 90% of triploids. In saugeye (*Sander canadensis*), after fertilizing for 5 min, the fertilized embryos were incubated at 31°C for 15 minutes which produced 86.7± 9.4% triploid fish (Garcia-Abiado et al., 2002). In contrary, the optimal triploidization procedure for Eurasian perch (*Perca fluviatilus*) is a heat shock of 30°C ongoing for 25 minutes, induced 5 minutes after fertilization which resulted 100% triploids (Rougeot et al., 2003). In yellow perch (*Perca flavescens*), a heat shock of 28°C for 25 minutes by initiating at 5 minutes after fertilization which yielded 100% triploids (Malison et al., 1993). In brown trout (*Salmo trutta*), a heat shock of 28°C ongoing for 10 minutes by initiating at 5-15 minutes after fertilization at 11°C. This heat shock resulted in 88.2-100% triploids. However, Malison et al. (2001) reported that heat shock did not produce a complete triploid population in walleye (*Sander vitreus*) and recommended hydrostatic pressure shock at 8000 psi applied at initiation time of 4 minutes for duration of 30 minutes. Taken together, heat shock or cold shock could be applied to conduct triploidization in which optimum temperature, period of incubating and duration time after fertilization depended on fish species.

Our findings showed that lower hatching rate and survival rates were observed in fish which were treated with heat or cold shocks. These findings were similar to the results observed in brown trout (*Salmo trutta*) (Lahnsteiner and Kletzl, 2018), lake char (*Salvelinus umbla*) (Lahnsteiner and Kletzl, 2018) Nibe croaker (*Nibea mitsukurii*) (Takeuchi et al., 2018) and silver catfish (*Rhamdia quelen*) (Morón-

Alcain et al., 2017). The early embryogenesis is the critical periods for biological processes and pathways and organogenesis which therefore affects the hatching rate and the survival rates at later stages (Drolet et al., 1991; Kjørsvik et al., 2011).

Since triploid fish contained additional one chromosome set, triploids individual showed DNA content larger than diploids approximately 1.5 times. Our findings showed that triploid fish contained DNA contents and had larger nucleus size of RBC that that of the diploid fish. These results were consistent with the observations in grass carp (*Ctenopharyngodon idella*) (McCarter, 1988), brown trout (*Salmo trutta*) (Preston et al., 2013) and African catfish (*Clarias gariepinus*) (Turan and Guragac, 2014) that were detected by flow-cytometry. In addition, the red blood cells of triploid walking catfish (*Clarias batrachus*) (Venkatachalam et al., 2012) and Nibe croaker (*Nibea mitsukurii*) (Takeuchi et al., 2018) had larger nuclear size than that of diploids.

Although hemoglobin, hematocrit and number of RBC of triploid fish appeared to lower than that of diploid fish, there were no significant different ($P>0.05$). However, it was reported that an increase in the size of erythrocytes in triploid teleost led to decrease in its number (Řehulka et al., 2004). Described by Benfey (1999). The authors explained from the findings that the reduced number of erythrocytes in triploid organisms might be a result of a homeostatic mechanism of triploids to compensate the increase of the cell volume, caused by accommodation of extra genetic material.

In the present study, there were no significant difference in growth performance between diploid and triploid through the experimental period. Similarly, no significant difference of growth performance between triploid and diploid Atlantic

cod were reported (*Gadus morhua*) (Vargas et al., 2016). The rate of muscle fiber growth does not differ between triploids and diploids in both juvenile and adult (Yamashita, 1993). The comparative growth responses of triploid and diploid fish were variable. For example, in European sea bass, the growth performance of fish at 30 months of age was no significant different when compared to that of the control diploid fish. However, after 30 months of age, triploid fish were significantly higher. The higher growth of triploid fish might be because of increase in cell size (Felip et al., 2001). In addition, growth performance of triploids was higher than that of diploids such as Atlantic salmon (*Salmo salar*) (Oppedal et al., 2003). Furthermore, growth performance of triploids was lower than that of diploid rainbow trout (*Oncorhynchus mykiss*) (Karayucel et al., 2018).

Reduced gonadal growth in triploids may allow increased energy allocation to somatic growth. However, growth advantage might not be achieved because of the reduction of levels of gonadal steroids, which have an anabolic effect (Reviewed in Benfey, 1999). Triploid fish in this study showed significantly lower GSI in both male and female compared to diploid fish. Our results were similar to the observation in several fish. Many fishes were reported similar with current study such as brown trout (*Salmo trutta*) (Kızak et al., 2013), sea bass (*Dicentrarchus labrax*) (Felip et al., 2001), rainbow trout (*Oncorhynchus mykiss*) (Krisfalusi et al., 2000), turbot (*Scophthalmus maximus*) (Cal et al., 2006), Nile tilapia (*Oreochromis niloticus*) (Razak et al., 1999), silver barb (*Puntius gonionotus*) (Koedprang and Na-Nakorn, 2000), and yellowtail tetra (*Astyanax altiparanae*) (Do Nascimento et al., 2017).

Our histological study demonstrated that development of testis and ovary in triploids were retarded when compared to that of diploid fish. In diploids, oocytes

generally arrest at first meiotic metaphase undergo tremendous growth during vitellogenesis (Rocha et al., 2008). It appeared that early gonad development occurred but did not progress through first meiotic prophase in triploid female. Therefore, small ovaries with a small number of primary and previtellogenic oocytes were observed. Also, meiosis could not take place in gametogenesis in triploid male. Consequently, most germ cells in triploid testis were spermatogonia. These findings were consistent with several studies such as brown trout, sea bass, rainbow trout, turbot, Nile tilapia, silver barb and yellowtail tetra (Kızak et al., 2013; Felip et al., 2001; Krisfalusi et al., 2000; Cal et al., 2006; Razak et al., 1999; Koedprang and Na-Nakorn, 2000; do Nascimento et al., 2017). This data suggested that gametogenesis was impaired in triploids (Takeuchi et al., 2018).

5.6 References

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CHAPTER VI

GERM CELL TRANSPLANTATION IN PANGASIID FISH: THE PRELIMINARY DEVELOPMENT TECHNOLOGY USING THE MEKONG GIANT CATFISH (*PANGASIANODON GIGAS*) AS DONOR AND THE STRIPED CATFISH (*PANGASIANODON HYPOPHTHALMUS*) AS RECIPIENT

6.1 Abstract

This study aimed to develop germ cell transplantation (GCT) in Pangasiid catfish. The Mekong giant catfish (*Pangasianodon gigas*) were the large freshwater fish which takes long time to become mature and required large facility to raise as broodstock. The immature Mekong giant catfish were used for isolating of spermatogonia (SG) and oogonia (OG) which were used as donor cell for transplantation. The striped catfish (*Pangasianodon hypophthalmus*) in which full-cycle culture has been well established was used as recipients. In addition, triploidization was conducted to produce sterile recipients which were also used as recipient of GCT. GCT was performed by microinjected of SG or OG into the peritoneal cavity of either diploid or triploid larvae. The colonization rate was observed after 28 days post transplantation (dpt). The colonization rates of SG and OG in the gonad of diploid fish were 80.00±16.33% and 90.00±20.00%, respectively.

Similarly, the colonization rates of SG and OG in the gonad of triploid were 79.16±8.33% and 83.33±19.24%, respectively. Reverse Transcription-Polymerase chain reaction-restriction fragment length polymorphism was performed to confirm the incorporation of germ cell of the Mekong catfish in the gonad of the striped catfish. These findings suggest that the transplanted immature germ cell of the Mekong giant catfish migrated toward and incorporated into the genital ridge of the recipient striped catfish larvae. Therefore, our study showed the preliminary success of GCT in which the recipient larvae have been cultivated until the maturation for further investigation of germ line transmission.

6.2 Introduction

Germ cell transplantation (GCT) technology has been established in several fish including Nibe croaker, nile tilapia, rainbow trout, Siberian sturgeon, chub mackerel, and yellowtail. (Farlora et al., 2014; Morita et al., 2012; Pšenička et al., 2015; Takeuchi et al., 2009; Takeuchi et al., 2003; Yazawa et al., 2010). Transgenic rainbow trout which carried green fluorescent protein (*gfp*) driven by the *vasa* promoter (Yoshizaki et al., 2000) was used as donor fish to develop GCT in salmonid. Primordial germ cells (PGCs) which were isolated from donor rainbow trout were introduced into the peritoneal cavity of masu salmon (*Oncorhynchus masou*) recipient larvae (Takeuchi et al., 2004). The transplanted PGCs could migrate toward and incorporated in the genital ridge of recipient masu salmon. In addition, the donor PGCs proliferated and differentiated. The recipient salmon produced trout sperms, and they could produce donor-derived rainbow trout using the masu salmon as the surrogate parents. Although the efficiency of donor-derived offspring was low

(0.4%), this was the first report demonstrating interspecific GCT in fish (Takeuchi et al., 2003, 2004). Since the PGCs were isolated from fish larvae, and there were small number. Therefore, GCT using donor spermatogonia and oogonia were developed and demonstrated to be the appropriate donor cells for establishment of GCT in fish (Okutsu et al., 2006). Indeed, type A spermatogonia was demonstrated to be donor cell which efficiently migrated toward and colonized in the gonad of recipient larvae (Yano et al., 2009).

The technique for GCT in fish simply mimics the principle of gonadal development in fish. During the early stage of development, PGCs are developed outside of the gonad, but genital ridge is formed with somatic cells. PGCs migrate toward the genital ridge with pseudopodia by chemotaxis (Raz, 2004). After PGCs have settled in genital ridge, PGCs are surrounded by somatic cells and start proliferation (Yoshizaki et al., 2002). Later, sex differentiated-mechanism occurs to produce functional gametes. Therefore, GCT in fish has been conducted by introducing the PGCs or spermatogonia into peritoneal cavity of recipient larvae. The donor PGC or spermatogonia will migrate and incorporate toward the genital ridge of recipient fish. Then, recipient somatic cells enclose the migrated PGCs or spermatogonia and support the donor cell proliferation and differentiation. In general, more closely related species between donor and recipient fish will enable more successful of transplantation efficiency. As a result, recipient fish produces donor-derived offspring, enabling surrogate broodstock technology. This technique is considered to be a powerful tool for applying to a number of fish species in which artificial seed production is a major constraint such as fish with large body size and endangered species.

In general, PGCs are found in young fish or fish larvae which contain only small number of PGCs. It also needs the biological skills to isolate PGCs from fish larvae. As a result, using PGC for transplantation would be a promising technique for basic study but not for aquaculture industries. Spermatogonia could behave stem cell activity resembling PGCs (Yoshida et al., 2007). A high number of spermatogonial cells are found in testis through life cycle of male fish in a number of fish (Kubota et al., 2003). Therefore, isolation of spermatogonial cells is much more practical than that of PGC.Transplantation of spermatogonial cell of rainbow trout with *gfp*-labeled was conducted in masu salmon larvae (Okutsu et al., 2006). The migration and incorporation of donor spermatogonia from rainbow trout could be achieved in masu salmon larvae. Further, the incorporated spermatogonia were be able to proliferate and generate active sperm and produce spermatogonial-derived offspring (18.9 %) (Okutsu et al., 2006). When spermatogonia were transplanted into female larvae, they could produce functional eggs, demonstrating sexual plasticity property of spermatogonia (Okutsu et al., 2006). Oogonial cells from rainbow trout were also transplanted into masu salmon larvae. Subsequently, donor oogonia was reported to incorporate and differentiate in recipient salmon. Oogonia cell was also be able to produce oogonia-derived sperm in male recipient and generate rainbow trout offspring, demonstrating sexual plasticity property of oogonia (Yoshizaki et al., 2010). Combined together, both spermatogonia and oogonia have characteristics of PGC-like cell and sexual plasticity to produce donor-derived gametes. Thus, this research project intends to use both spermatogonia and oogonia as the donor cell since GCT technology using spermatogonia and oogonia provide a wide range of applicability in not only basic biological researches but also aquaculture industries.

GCT was also carried out in marine fish. In order to develop GCT methodology, microinjection of spermatogonia into allogenic larvae was first conducted in Nibe croaker (*Nibea mitsukurii*) (Takeuchi et al., 2009). The maintenance of Nibe croaker broodstock is well-developed which enable the establishment of surrogate broodstock in marine fish. Later, xenogenic GCT was demonstrated by introduction of spermatogonia of chub mackerel (*Scomber japonicus*) and yellow tail (*Seriola quinqueradiata*) into larvae of Nibe croaker (Higuchi et al., 2011; Yazawa et al., 2010). In addition, the study of oogonia transplantation was also reported that ovarian germ cells transplantation from transgenic Pearl danio (*Danio albolineatus*) into sterile *Danio* hybrid recipients was successfully (Wong et al., 2011). This technique provides application for conventional breeding to increase a number of broodstock carrying germ cells with desirable genetic traits (Morita et al., 2012).

Another approach was to confirm the surrogate broodstock that could produce the donor-derived offspring. Albino-strain which is easily classified by color property was used as recipient fish in zebrafish (Lin et al., 1992). As a result, when the recipient fish were maturation, they could produce the donor-offspring which possessed different color from parents. Furthermore, the albino-strain was also used as donor fish in GCT of rainbow trout (Okutsu et al., 2006).

GCT has potential applications for the preservation of endangered species and seed production for commercially important large body species. Currently, the family Pangasiidae comprises of 21 recognized species, some of which are economically important and/or endangered species. Among which Mekong giant catfish, *Pangasianodon gigas,* has been attracting global interest because it is the world's

largest scaleless freshwater catfish and was listed as a critically endangered species by IUCN. Although, Mekong giant catfish can be bred in captivity, the culture of the broodstock (more than 40 kg in size) needs large pond, labour cost, special skill, and long rearing period (at least 10 years) (Mattson et al., 2002). Therefore, it is worth to apply the surrogate technology to produce the Mekong giant catfish seed for both conservation and aquaculture aspects. In this study, Mekong giant catfish was used as donor fish. Striped catfish, *Pangasianodon hypophthalmus*, was used as a recipient because breeding and rearing technology of this species has been well established and its life-cycle is short (2-3 years to maturation). More importantly, it is genetically closest to Mekong giant catfish comparing to other Pangasiids. This study aimed to develop GCT in the Pangasiid catfishes. (family Pangasiidae). The Mekong giant catfish was used as donor fish for isolating of spermatogonia and oogonia. The recipient larvae of the striped catfish of both diploid and triploid were used as recipient larvae, and the colonization rate was determined.

6.3 Materials and methods

6.3.1 Recipient fish

Stripped catfish used in this study were cultured at farm of Suranaree University of Technology, Nakhon Ratchasima, Thailand. Broodstock (females 2.5-3.5 kg, males 2-3.5 kg) were maintained in earthen pond (10×40×1.5 m^3). The broodstock were fed with commercial diet (25% crude protein, 3% crude fat) at 5% body weight at 10:00 and 16.00 every day. Broodstock (1:1, male/female) were transferred for hormonal injection in canvas pond (2 m^3). Female fish were injected hormone for 2 times, first injection by 1 dose of fish's pituitary extract (PE) and 300

IU kg^{-1} of human chorionic gonadotropin (HCG). After 8-12 hours. the second injection was performed with injection of 4 doses of fish's PE and 900 IU kg^{-1} HCG. After the second injection for 10-12 hours, artificial fertilization was conducted by hand-stripping eggs and milt. Eggs were inseminated with milt, subsequently washed and transferred to hatching tank. The larvae hatched within 36 hours. According to the Chapter 5, larvae at 4-5 dpf were used for transplantation.

In this study, triploid larvae were also used as recipient larvae. In order to produce triploid recipients, hormonal injection and artificial fertilization were carried out as describe as the above. After fertilization, the fertilized-eggs were incubated at 7.5°C for 30 minutes before transferring into hatching tank. Also, larvae at 4-5 dpf were used for transplantation.

6.3.2 Donor fish, fish sampling and histological study

The Mekong giant catfish (1.5-2.5 kg) was used as donor fish. The donor-fish were maintained in earthen pond ($10 \times 40 \times 1.5$ m^3) and fed with commercial feed (25% crude protein, 3% crude fat) at 5% body weight at 10:00 and 16.00 every day.

Histological study of gonad of the Mekong giant catfish was carried out. Donor fish were incubated with cold-water (4°C) before sampling. Both male and female fish were collected gonadal tissues for histological study. The gonadal somatic index (GSI = gonad weight × 100 × body $weight^{-1}$) of male and female fish were 0.127±0.004% and 0.004±0.02%, respectively. Testis and ovary were collected and fixed in Bouin's fixative solution at 4°C for 24 h. After washing with 80% ethanol, the fixed specimens were dehydrated using an ethanol series and then embedded in paraffin, sectioned to a thickness of 5 µm, and stained with hematoxylin and eosin (H&E).

6.3.3 Mekong giant catfish gonad collection and gonad dissociation

Donor testis and ovary were taken from the Mekong giant catfish. Isolation of gonadal cell was performed according to protocol described in Morita et al. (2012). Both testis and ovary that was collected from giant catfish was dissociated with collagenase IV and dispase II. Briefly, testis or ovary (0.3 mg/ml dissociation buffer) were minced and incubated with the mixture of 0.2% collagenase IV (Roche Diagnostics, Mannheim, Germany), 0.17% dispase II (Sanko Junyaku Co., Ltd., Tokyo, Japan),10% FBS (Gibco Invitrogen Co.) and 900 U ml^{-1} DNase I (Roche Diagnostics) in L-15 medium (pH 7.8 with Hepes, Gibco Invitrogen Co., Grand Island NY, USA) for 90 minutes at 26°C. The dissociated cell suspension was rinsed with L-15 medium containing 10% FBS to eliminate enzymatic activity. In order to discard non-dissociated cell clumps, the cell suspension was filtrated through a nylon mesh (42-μm) (Tokyo Screen Co., Ltd.). After centrifugation at 200 × g for 10 min at 4°C, the resulting pellets were suspended in the L-15 medium containing 10% FBS (Sigma-Aldrich, Inc.). Before transplantation, the cell suspensions were labeled with PKH26 (Sigma-Aldrich, Inc.) according to the instruction manual and stored at 4°C until use for transplantation.

6.3.4 GCT into recipient and determination of colonization

Immature germ cell isolated from donor Mekong giant catfish labeled PKH26 were used for GCT. Before transplantation, the diploid and triploid recipients were anesthetized with MS-222 at 0.1 g/ml; 0.75 mg/ml BSA (Sigma-Aldrich, Inc.). Approximately 20,000 cells of PKH26 labeled germ cell were microinjected into peritoneal cavity of suitable age of striped catfish larvae. After transplantation for 4 weeks, 5 fish per cage were sampled and observed under a fluorescent microscope to

analyze the colonization of germ cells in their genital ridge (model BX-51N-34FL; Olympus).

6.3.5 PCR-RFLP of *vasa*

In this study, PCR-RFLP was used for determination of the germ cells or gametes of the donor Mekong giant catfish inside the recipient striped catfish. Two years old of transplanted fish were anaesthetized with 500 ppm of 2-phenoxyethanol. The gonadal tissues were randomized to collect for analysis of *vasa* gene. Total RNA was extracted from ovary and testis (approximately 100 mg) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNase I (Promega, Madison, WI, USA), according to the manufacturer's instructions. First-strand cDNA was synthesized using 2 µg of total RNA using the ImProm-II™ Reverse Transcription System Kit (Promega). Two gene-specific primers including Pgvasa-F1 (5'- GACTGGGAAGATGATCAGAGCCCTG -3') and Pgvasa-R4 (5'- CCCTG GTAGGAGCCACGATGATTAC -3') were designed according to previous experiment. PCR was carried out in a total volume 25 µl consisting of 2.5 µl of cDNA, 400 µM of each dNTP, 2.5 pmol of each primer, 2.5 mM $MgCl_2$, 1X LA Taq™ buffer, and 1.25 U LA Taq (Takara Shuzo, Shiga, Japan). The PCR was conditioned at 95°C for 3 min, then 35 reaction cycles of 45 seconds at 95 °C, 45 seconds at 62°C, and 1.5 minutes at 72°C. The final elongation step was carried out at 72°C for 5 min. PCR products were purified and subsequently digested by Sal *I* (Takara Bio Inc.). The digested PCR product were performed 0.7% agarose gel electrophoresis.

6.4 Results

Histological study of testis and ovary of the Mekong giant catfish (GSI of male and female fish were 0.127±0.004 and 0.004±0.02%, respectively) were showed in Fig. 6.1A-B. At this growth stage of the Mekong giant catfish, testis contained high number of spermatogonia. These spermatogonia were identified as type A spermatogonia (ASG) with large, round nuclei which size approximately 8-10 μm (Fig. 6.1A). The ovary of the Mekong giant catfish contained various stage of ovarian cells such as oogonia and previtellogenic oocyte (PVO) (Fig. 6.1B). Therefore, Mekong giant catfish at this growth stage was used for GCT throughout this study.

In order to determine the colonization of GCT, donor cells were stained with the fluorescent membrane dye PKH26, enabling the temporary following of donor cells in the recipient. The spermatogonia cells which were stained with PKH26 had red fluorescence (Fig. 6.1C-D). Approximately 20,000 donor gonadal cells labeled with PKH26 fluorescent dye were transplanted into the peritoneal cavity (Fig. 6.2A). No fluorescent signal was observed in control, non-transplanted fish (Fig. 6.1B-C) whereas abundant PKH26-labeled donor cells were observed in the peritoneal cavity of recipient larvae (Fig. 6.1D-E).

The incorporation of transplanted germ cells in the recipient gonads was observed at 28 days post transplantation (dpt) by fluorescence microscopy. With DAPI staining, the germ cells were observed as large size of cells with weak DAPI due low desity of chromosome in nucleus in both control uninjected and transplanted fish (Fig. 6.3C, D). No fluorescent germ cell was observed in gonads of the control uninjected fish (Fig. 6.3C).

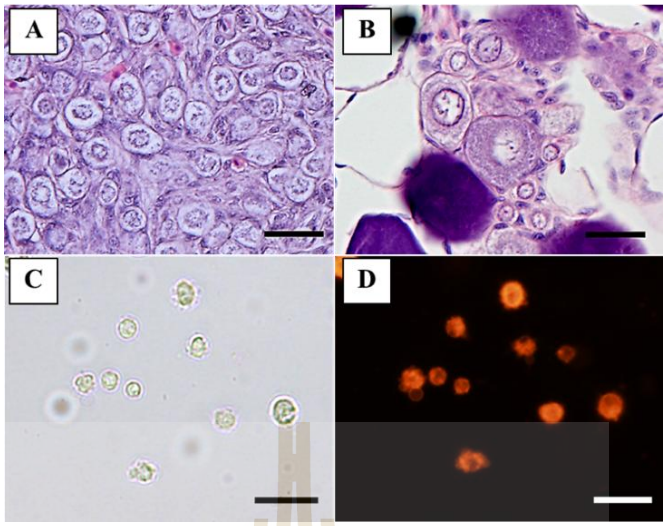

Figure 6.1 Histological study of the Mekong giant catfish testis (A) and ovary (B) stained with H&E. Dissociated gonadal cells (C) labeled with PKH26 (D). (Bar = 20 μm)

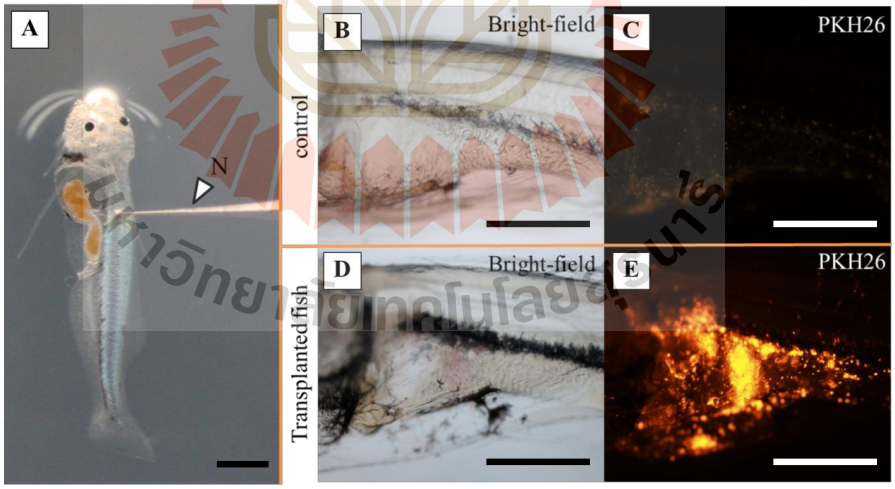

Figure 6.2 GCT in catfish. Intraperitoneal transplantation of the donor cells isolated from the Mekong giant catfish into striped catfish larvae (A). Bright-field (B and D) and fluorescent images (C and E) showing PKH26-labeled cells inside recipient's abdomen. (N = needle, Bar = 1 mm)

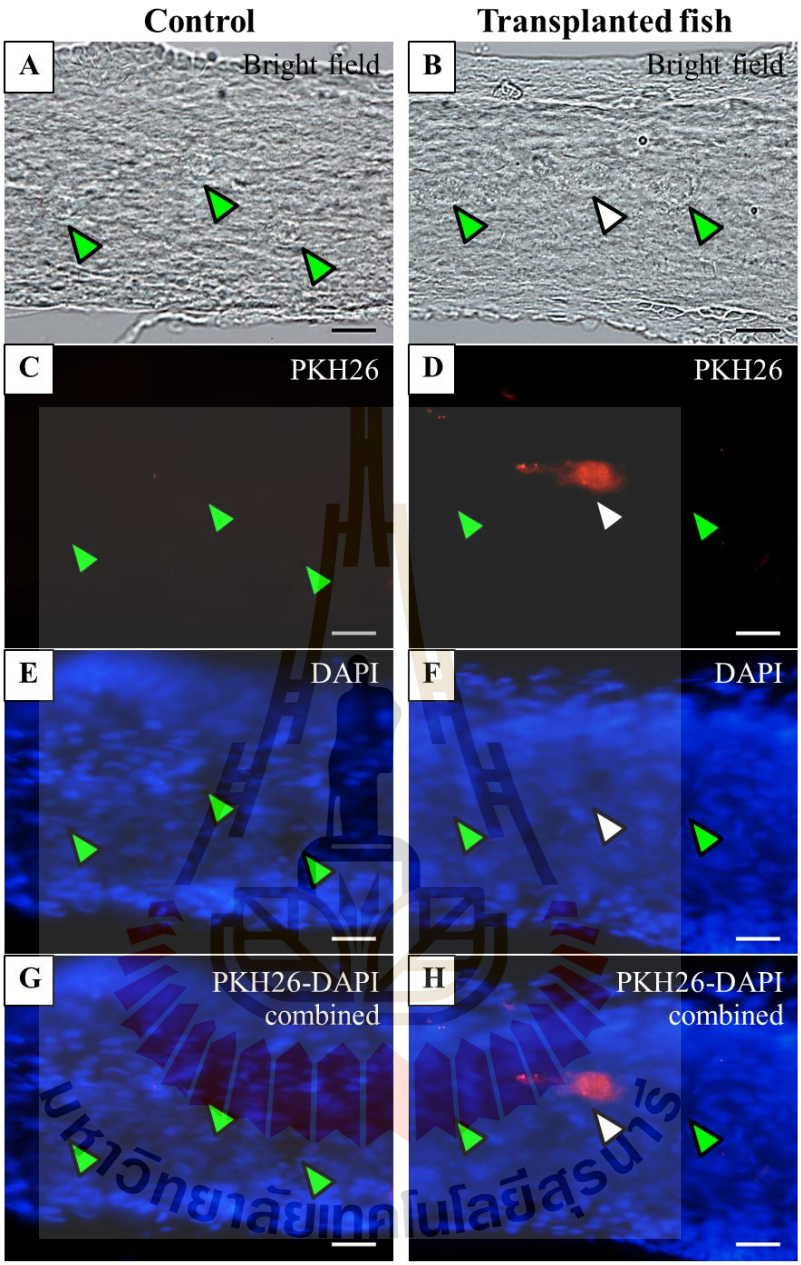

Figure 6.3 Incorporation of transplanted PKH26-labeled cells in recipient genital ridges. Bright-field (A and B) and fluorescent images (C, D, E, F, G and H) showing genital ridges of control (A, C, E and G) and transplanted (B, D, F and H) fish at 28 dpt. White and green arrowheads indicate germ cells of donor and recipient fish, respectively. (Bar = 20 μm).

Table 6.1 Survival rates and colonization rates 28 days following spermatogonial cells transplantation in diploid recipients (mean ± standard deviation, n = 4).

Replication	No. of transplanted	No. of survived	No. of colonized	Survival rate (%)	Colonization rate (%)
Spermatogonia					
Transplanted 1	97	20	4/5	20.62	80
Transplanted 2	105	21	5/5	20.00	100
Transplanted 3	114	38	4/5	33.33	80
Transplanted 4	86	27	3/5	31.40	60
Average				26.34±7.01	80.00±16.33
Non-transplanted					
Control 1	97	25	-	25.77	-
Control 2	105	24	-	22.86	-
Control 3	114	36	-	31.58	-
Control 4	86	25	-	29.07	-
Average				27.32±3.81	-

Table 6.2 Survival rates and colonization rates 28 days following oogonial cells transplantation in diploid recipients (mean ± standard deviation, n = 4).

Replication	No. of transplanted	No. of survived	No. of colonized	Survival rate (%)	Colonization rate (%)
Oogonia					
Transplanted 1	116	30	5/5	25.86	100
Transplanted 2	100	20	5/5	20.00	100
Transplanted 3	96	22	3/5	22.92	60
Transplanted 4	89	34	5/5	38.20	100
Average				26.75±8.00	90.00±20.00
Non-transplanted					
Control 1	116	39	-	33.62	-
Control 2	100	40	-	40.00	-
Control 3	96	37	-	38.54	-
Control 4	89	32	-	35.96	-
Average				37.03±2.82	-

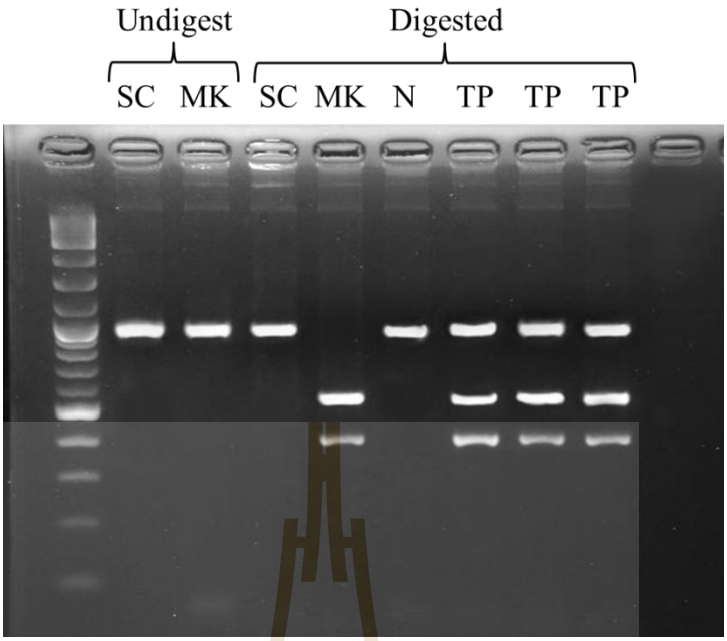

Figure 6.4 RT-PCR-RFLP of Molecular *vasa*-marker analysis of striped catfish (SC), Mekong giant catfish (MK), non-transplanted fish (N) and transplanted fish (TP). By RT-PCR-RFLP with *Sal* I, the amplicon of the striped catfish was not digested and had 931 bp in size. The amplicon of the Mekong catfish which was digested with *Sal* I results in Three DNA fragment at 397 and 534 in size. The amplicon of the striped catfish which were transplanted with donor cells isolated from the Mekong giant catfish. After cleaving with *Sal* I, three DNA fragment at 397, 534, and 931 bp in size were observed.

Table 6.3 Survival rates and colonization rates 28 days following spermatogonial cells transplantation in triploid recipients (mean ± standard deviation, n = 4).

Replication	No. of transplanted	No. of survived	No. of colonized	Survival rate (%)	Colonization rate (%)
Spermatogonia					
Transplanted 1	100	46	5/6	46.00	83.33
Transplanted 2	100	36	4/6	36.00	66.67
Transplanted 3	100	28	5/6	28.00	83.33
Transplanted 4	100	33	5/6	33.00	83.33
Average				35.75±7.59	79.16±8.33
Non-transplanted					
Control 1	100	31	-	31.00	-
Control 2	100	36	-	36.00	-
Control 3	100	47	-	47.00	-
Control 4	100	29	-	29.00	-
Average				35.75±8.06	-

Table 6.4 Survival rates and colonization rates 28 days following oogonial cells transplantation in triploid recipients (mean ± standard deviation, n = 4).

Replication	No. of transplanted	No. of survived	No. of colonized	Survival rate (%)	Colonization rate (%)
Oogonia					
Transplanted 5	100	41	6/6	41.00	100.00
Transplanted 6	100	35	4/6	35.00	66.67
Transplanted 7	100	40	4/6	40.00	66.67
Transplanted 8	100	26	6/6	26.00	100.00
Average				35.5±6.85	83.33±19.24
Non-transplanted					
Control 5	100	38	-	38.00	-
Control 6	100	41	-	41.00	-
Control 7	100	42	-	42.00	-
Control 8	100	39	-	39.00	-
Average				40.00±1.82	-

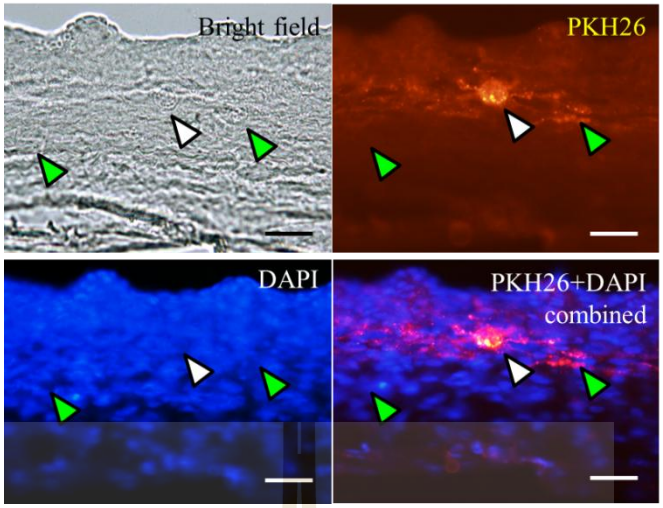

Figure 6.5 Incorporation of transplanted PKH26-labeled cells in triploid recipient genital ridges. Bright-field and fluorescent images show genital ridges of transplanted fish at 28 dpt. White and green arrowheads indicate germ cells of donor and recipient fish, respectively. (Bar = 20 μm)

The PKH26-labeled cells were observed in transplanted-recipient gonad (Fig. 6.3F). When combined PKH26 and DAPI, donor-derived PKH26-labeled cells had large nuclei stained with DAPI and co-localized with endogenous germ cells (Fig. 6.3G, H). Table 6.1-6.2 showed the survival rate of transplanted larvae and colonization rate of donor cells inside the genital ridge of recipient fish. In addition, Fig. 6.3 demonstrated the colonization of the PKH26-labeled cells inside the genital ridge of triploid recipient larvae. The PKH26-labeled cells were observed in the genital ridge isolated from triploid recipient larvae (Fig. 6.5), however, no PKH26-labeled cells were found in the genital ridge of control uninjected triploid recipient larvae (data not shown). These results demonstrated that donor cells transplanted into the peritoneal cavity could migrate to the genital ridges triploid recipients. Table 6.3-

6.4 showed the survival rate of transplanted larvae and colonization rate of donor cells inside the genital ridge of recipient triploid fish. Taken together, these results suggested that donor cells were successfully transplanted into the recipient peritoneal cavity. Subsequently, donor-derived cells were incorporated in the genital ridges of recipients.

Confirmation of incorporation of the donor cells isolated from the Mekong giant catfish in the gonad of recipient striped catfish were conducted using reverse transcription polymerase chain reaction with restriction fragment length polymorphism (RT-PCR-RFLP). Using RT-PCR-RFLP, the amplicons of testis or ovary of the striped catfish were 931 bp in size. The amplicons of testis or ovary of the Mekong giant catfish were digested with *Sal* I and resulted three DNA fragment at 397, 534, and 931 in size (Fig. 6.4). RT-PCR of *vasa* was carried out in the testis and ovary collected from 120 transplanted fish. The result showed that, in the gonads of nontransplanted fish, no Mekong giant catfish *vasa*-positive gene were detected, whereas Mekong giant catfish *vasa*-positive gene in the gonads of transplanted fish were detected, suggesting that transplanted fish striped catfish gonad contained Mekong giant catfish -derived germ cells (Fig. 6.4). With RT-PCR-RFLP, the Mekong giant catfish *vasa*-positive gene was observed in 27 of total transplanted fish detected, demonstrating the incorporation rate of GCT.

6.5 Discussion

In this study, we showed that the intraperitoneally transplanted xenogenic Mekong giant catfish gonadal cell efficiently incorporated into the gonads of striped catfish larvae. This report is the first to show the successful xenogenic transplantation

of immature germ cell between donor (Mekong giant catfish) and recipient (striped catfish) of Pangasiidae family. Our achievement in this study suggests that common mechanisms underlying migration and differentiation of germ cells during early gonadal development are well conserved in a wide range of teleost species. These conserved developmental mechanisms may prove to be an advantage for GCT in diverse teleosts. GCT would provide broadly applicable in freshwater teleosts. Xenogenic transplantation was previously demonstrated in several fish such as masu salmon (*Oncorhynchus masou*) by using donor PGCs rainbow trout (*O. mykiss*) (Takeuchi et al., 2004), zebrafish (*Danio rerio*) by using donor PGCs of pearl danio (*Danio albolineatus*) or goldfish (*Carassius auratus*) or loach (*Misgurnus anguillicaudatus*) (Saito et al., 2008).

Spermatogonial cell type A (ASG) were demonstrated to be able to use for GCT which could migrated and incorporated in the recipient gonad (Yazawa et al., 2010). Therefore, the donor testis which contained high proportion of ASG would provide a number of ASG for GCT. Consequently, investigation of reproductive development of donor gonad is the prerequisite of development of GCT technology. In general, reproductive development of fish varied among teleost taxa. Some fish become mature at few months such as medaka (Seki et al., 2017), and zebrafish (Wong et al., 2011) to few years for instance Nibe croaker (Takeuchi et al., 2018), and yellowtail (Morita et al., 2012). However, a number of fish become mature in many years which have large body size such as Bluefin tuna (*Thunnus thynnus*) and giant grouper (*Epinephelus lanceolatus*). The Makong giant catfish species in current experiment which the first maturation was reported more than 15 years old and at 37 kg of body weight (Phayao, 2000). The Mekong giant catfish has been considered to

be Critically Endangered (IUCN) and is already listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). The striped catfish occasionally becomes maturity at 900 g of body weight. The striped catfish at 2 years old can generally be broodstock and able to produce functional gamete. In addition, its full cycle culture is well-developed. Moreover, the striped catfish is belonged to the same genus is considered to be evolutionarily related with the Mekong giant catfish. Therefore, GCT of donor germ cell from the Mekong is required to develop to aid its seed production. The striped catfish would be a good candidate to be desired as recipient fish for further becoming surrogate broodstock to produce the Mekong giant catfish-derived offspring.

In this study, PKH26 was used for tracking the transplanted ASG in gonad of transplanted recipients. This study was determined the colonization rate after 28 dpt which demonstrated that the PKH26 could be observed and tracked the transplant cells at this period. Similarly, the PKH26 was reported to be the useful dye to follow the germ cell migration and colonization in a number of GCT experiments. For example, transplantation of PKH26-labelled ASG of yellowtail into the Jack mackerel (*Trachurus japonicus*) and Nibe croaker (*Nibea mitsukurii*) allowed determination of the colonization of the PKH26-labelled ASG at 20 dpt and 21 dpt, respectively (Higuchi et al., 2011; Morita et al., 2015). Allogeneic GCT in Nile tilapia allowed determination of the donor PKH26-labelled ASG at 21 dpt (Farlora et al., 2014). In addition, xenogenic transplantation using Siberian sturgeon (*Acipenser baerii*) as donor and sterlet (*Acipenser ruthenus*) as recipient fish. Colonization of the PKH26-labelled oogonia and ASG could be observed at 6, 30, 50 and 90 dpt (Pšenička et al., 2015).

In the present study, we demonstrated that xenogenic donor spermatogonial and oogonial transplantation. The colonization rate of diploid recipient (80.00±16.33% and 90.00±20.00%, respectively) obtained in this study was high and similar to the colonization rates observed in the triploid recipients (79.16±8.33% and 83.33±19.24%, respectively). No significant difference between xenogenic donor spermatogonial and oogonial transplantation into diploid and triploid recipients was observed. These findings suggest that intraperitoneally transplanted immature germ cell migrated toward the genital ridge of the recipient, and their ability to migrate was not suppressed in a xenogenic and or triploid environment. It has been reported that multiple molecular interactions are involved in germ cell migration toward gonadal anlage during early gonadal development (Molyneaux and Wylie, 2004). Likewise, some studies reported the colonization rate such as in rainbow trout was 43% (Okutsu et al., 2006), chub mackerel was 70% (Yazawa et al., 2010), and Nibe croaker was 81.8% (Higuchi et al., 2011).

There have been demonstrated about the molecular mechanism of germ cell migration toward the genital ridge which were conserved in teleosts and even in a xenogenic environment. For example, interaction between chemokine CXCL12 (stromal cell-derived factor 1 [SDF1]) expressed in somatic cells and its receptor CXCR4 expressed by the germ cells is required for directional PGC migration in teleosts (Doitsidou et al., 2002) and mammals (Molyneaux et al., 2003). In an embryo slice culture, human CXCL12 could attract mouse PGCs expressing CXCR4 (Molyneaux et al., 2003).

In order to determine GCT in sterile recipients to produce surrogate broodstock which could be produced only donor derived-gamete, isolated cells

transplanted into triploid recipients was performed. Approximately 79.16-83.33% of colonization rate was observed. From the study which investigated in triploid rainbow trout, the colonization rate was 65-75% and triploid broodstocks could be produced the donor derived-gamete were obtained (Lee et al., 2015). In addition, testicular cells transplantation into triploid recipients was conducted in medaka which 70% of colonization rate and functional gamete was obtained from transplanted broodstocks (Seki et al., 2017). It confirmed that the sterile condition of recipients will not suppress the survival, proliferation, and differentiation of transplanted cells. Furthermore, the transplanted triploid striped catfish in this study will be cultured until they reach to mature for determination whether functional gamete could be obtained from transplanted triploid striped catfish. In conclusion, this developing technique may suitable to apply with other teleost for going to sustainable aquaculture.

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CHAPTER VII

CONCLUSION

This study developed germ cell transplantation (GCT) technology in Pangasiid fish using the Mekong giant catfish as the donor fish and the striped catfish as recipient fish. In order to establish GCT, several information, techniques and methods were demonstrated as the following:

7.1 Aging effects of spermatogonia on GCT

Spermatogonial stem cells (SSCs) function of spermatogonia was investigated in medaka which is a good experimental fish. This study demonstrated aging-related biological characteristics of type A spermatogonia (ASG) which behave as SSCs in fish. For example, there were aging-associated effects on number of ASG. In addition, low water temperature appeared to retard testis development, and therefore, had effects on ASG number. There were no significant differences in colonization rate of ASG isolated from different ages. In addition, similar germ-line transmission rates were observed. Moreover, water temperature had no significant effects on stem cell function of ASG.

7.2 Cloning and characterization of *vasa* mRNA and its used as molecular marker to determine early development of gonad

Studying reproductive system development during larval development requires a known molecular marker that is specifically expressed in germ cell lineage

in order to confirm histological characterization. This study cloned and characterized *vasa* in the striped catfish, *Pangasianodon hypophthalmus* (*Phy-vasa*). *Phy-vasa* contained all of the predicted consensus motifs that are shared within the *vasa* family in other fish and clustered within the Vasa family. By reverse transcription PCR, *Phy-vasa* mRNA was observed only in the testis and ovary. Using *in situ* hybridization, *Phy-vasa* mRNA was expressed specifically only in germ cells, with strong expression detectable in spermatogonia and oogonia. The migration of primordial germ cells (PGCs) were found most abundantly in larvae, 2-10 days post-fertilization (dpf), and the PGCs were started to be surrounded by gonadal somatic cells around 10-20 dpf. The rapid proliferation of PGCs began in larvae by 30 dpf.

7.3 Production of triploid recipient fish

This study determined the optimal method to produce triploid striped catfish. Cold-shock at 7.5°C for 30 min was the optimum process to obtain 90% triploid fish with 35.34% of hatching rate and 20% of survival rate of larvae at 7 days post fertilization. Triploid RBC has significantly larger nuclear sizes and DNA content when compared to that of diploid fish. There were no significantly difference in hematological parameters between triploid and diploid fish. There were no significant differences in growth performances between diploid and triploid fish. The larger GSI in diploid fish were observed in ovary since 7 months of ages. The ovary of diploid and triploid fish at ages of 7 month contained most oogonia and primary oocytes. At ages of 10, 13 and 16 months, ovary of diploid fish contained most previtellogenic oocytes (PVO). However, triploid ovary contained most oogonia and primary oocytes. Few PVOs were observed in triploid ovary at age of 13 and 16 months. The

larger GSI in testis of diploid fish was found at 13 and 16 months of ages. At ages of 13 and 16 months, testis of diploid fish contained differentiated spermatocytes and few spermatozoa. However, triploid testis contained most spermatogonia.

7.4 Development of GCT in Pangasiid catfish

This study developed germ cell transplantation (GCT) in Pangasiid catfish by microinjecting of SG or OG into the peritoneal cavity of either diploid or triploid larvae. The colonization rates of SG and OG in the gonad of diploid fish were 80.00±16.33% and 90.00±20.00%, respectively. Similarly, the colonization rates of SG and OG in the gonad of triploid were 79.16±8.33% and 83.33±19.24%, respectively.

Therefore, this study provided biological information, techniques and methods for GCT. The preliminary success of GCT was achieved. Culture of the transplanted fish have been carried out to confirm whether our technology could produce surrogate broodstock of the Mekong giant catfish.

BIOGRAPHY

Mr. Rungsun Duangkaew was born on October 22nd, 1989 in Suphanburi Province, Thailand. He graduated bachelor's degree from faculty of Animal Sciences and Agricultural Technology at Silpakorn University majoring in Aquatic Animal Production Technology. After graduation, he obtained the scholarship from Royal Golden Jubilee Ph.D. Program to presence a Doctoral degree at school of Animal Production Technology, Suranaree University of Technology, under superintendence of Associate Professor Dr. Surintorn Boonanuntanasarn. In 2017, he had the chance to perform an experiment in the title of 'Aging-related activity of spermatogonial stem cell in medaka: a vertebrate model for investigation of spermatogonial stem cell property' at Tokyo University of Marine Science and Technology (TUMSAT), Tokyo, Japan under supervision of Professor Dr. Goro Yoshizaki. He conducted the research in the topic of 'Development of germ cell transplantation in Pangasiid catfish' from October 2015 to June 2018.