DEVELOPMENT OF GENE EDITING OF DEAD END GENE IN STRIPED CATFISH (*PANGASIANODON HYPOPHTHALMUS*)

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การพัฒนาเทคโนโลยี gene editing สำหรับ dead end gene ในปลาสวาย (*Pangasianodon hypophthalmus*)

นางสาวกุลนันทน์ บุญเชิด

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การพัฒนาเทคโนโลยีการปลูกถ่ายเซลล์สืบพันธุ์เพื่อผลิตพ่อแม่ปลาอุ้มบุญ เพื่อประโยชน์ในการประยุกต์ใช้ในอุตสาหกรรมการเพาะเลี้ยงสัตว์น้ำและการอนุรักษ์พันธุ์ปลา โดยในการเพิ่มประสิทธิภาพการปลูกถ่ายเซลล์สืบพันธุ์นั้น จำเป็นต้องผลิตปลาผู้รับที่ไม่มีเซลล์สืบพันธุ์แรกเริ่ม (Primordial germ cell; PGC) ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์ที่จะพัฒนาเทคนิค CRISPR/Cas9 ในการยับยั้งการแสดงออกของยีน dead end (*dnd*) ในปลาสวาย (*Pangasianodon hypophthalamus*) เพื่อลดจำนวน PGC ในลูกปลาสวายวัยอ่อน โดยแบ่งการทดลองออกเป็น 2 การทดลอง ได้แก่ การทดลองที่ 1 การโคลนและการ characterization ยีน *dnd* และการศึกษาแสดงออกของยีน *dnd* ในเนื้อเยื่อต่าง ๆ ของปลาสวาย และ การทดลองที่ 2 การพัฒนาเทคนิค CRISPR/Cas9 เพื่อยับยั้งการแสดงออกของยีน *dnd*

การทดลองที่ 1 ได้ทำการโคลนและการ characterization ยีน *dnd* และการศึกษาแสดงออกของยีน *dnd* ในเนื้อเยื่อต่าง ๆ ของปลาสวาย molecular characterization ของยีน *dnd* และการแสดงออกยีน *dnd* ในเนื้อเยื้อต่างๆ จากการศึกษาได้ทำการโคลนยีนบางส่วนของ cDNA พบว่ายีน *dnd* มี 1,513 bp ประกอบด้วย ORF ที่ 1,137 bp encode ได้กรดอะมิโน 399 residues และในส่วนของ 3’-UTR มี 371 bp ประกอบไปด้วย consensus motifs ซึ่งมีความสอดคล้องกันในปลาหลาย ๆ species ประกอบไปด้วย RNA recognition motif (RRM), N-terminal regions (NR), และ C-terminal regions (CR1-4) สำหรับการวิเคราะห์สายวิวัฒนาการ (Phylogenetic analysis) ที่เกี่ยวกับโปรตีน Dnd นั้นพบว่าโปรตีน Dnd ในปลาสวายจัดอยู่ในกลุ่มของปลากระดูกแข็ง ผลการวิเคราะห์ RT-PCR พบการแสดงออกของ *dnd* mRNA เฉพาะที่รังไข่และอัณฑะเท่านั้น และพบการวิเคราะห์ Quantitative RT-PCR พบว่ายีน *dnd* มีการการแสดงออกสูงในรังไข่และอัณฑะเช่นกัน

ในการทดลองที่ 2 การพัฒนาเทคนิคการยับยั้งยีน *dnd* โดยใช้ CRISPR/Cas9 เพื่อหาวิธีการที่เหมาะสม จึงได้ออกแบบ sgRNA 3 ตำแหน่ง บริเวณ 5’ UTR ร่วมกับการหาความเข้มข้นที่เหมาะสมของ sgRNA (100 และ 200 ng/µl) และ Cas9 (100, 250 และ 500 ng/µl) จากการทดลองได้ทำการฉีด sgRNA+Cas9 ใน fertilized egg ของปลาสวาย จากการทดลองไม่พบความแตกต่างของอัตราการฟัก อัตราการรอด และไม่พบความแตกต่างของการเจริญเติบโต sgRNA/Cas9 สามารถทำ

ให้เกิด indel mutation ดังนี้ deletion (1-16 nt), insertion (1-13 nt) and substitution (1-13 nt) การวิเคราะห์ทางสัณฐานวิทยาในระยะ 60 วันหลังจากฟักพบว่า ในทุกกลุ่มการทดลองของการฉีด sgRNA/Cas9 สามารถเหนี่ยวนำให้จำนวนเซลล์ PCG ลดลง นอกจากนั้นยังพบว่าความเข้มข้นที่ 200 ng/µl ของ sgRNA3 และความเข้มข้นที่ 500 ng/µl ของ Cas9 สามารถลดจำนวนเซลล์ PCG ได้มากที่สุด ซึ่งสอดคล้องกับการวิเคราะห์ด้วย situ hybridization โดยใช้ probe antisense *dnd* และ *vasa* อีกด้วย ในทุกกลุ่มการทดลองของการฉีด sgRNA/Cas9 เกิดการลดการแสดงออกของยีน *dnd, cxcr4b, dazl, nanos1*, *nanos2* และ *vasa* นอกจากนั้นยังพบการลดลงของการแสดงออกของยีนอย่างมีนัยสำคัญทางสถิติในปลาที่ฉีด sgRNA3 200 ng/µl และ Cas9 500 ng/µl (P<0.05). การวิเคราะห์ค่า GSI ในปลาอายุ 1 ปี พบว่า GSI ลดลงอย่างมีนัยสำคัญทางสถิติในปลาที่ฉีดด้วย sgRNA และ Cas9 ที่ความเข้มข้น 500 ng/µl นอกจากนี้ได้มีการศึกษาสัณฐานวิทยาของรังไข่ และอัณฑะ ในปลาอายุ 1 ปี พบว่ารังไข่มีจำนวน previtellogenic oocyte น้อยมาก และในอัณฑะพบ spermatogonia จำนวนน้อยมากเมื่อเทียบกับกลุ่มควบคุมเช่นกัน จึงสรุปได้ว่า CRISPR/Cas 9 ที่ออกแบบยับยั้งบริเวณ 5'-UTR สามารถลดการแสดงออกของยีน *dnd* และสามารถลดจำนวนเซลล์ PGC ได้ จากผลการทดลองสามารถสรุปได้ว่า ตำแหน่ง sgRNA ที่ใกล้ start codon มากที่สุดจะมีประสิทธิภาพมากที่สุดเช่นกัน

สาขาวิชาเทคโนโลยีและนวัตกรรมทางสัตว์ ลายมือชื่อนักศึกษา กุลนันทน์ บุญเชิด

ปีการศึกษา 2565 ลายมือชื่ออาจารย์ที่ปรึกษา

KUNLANAN BOONCHERD : DEVELOPMENT OF GENE EDITING OF DEAD END GENE IN STRIPED CATFISH (*PANGASIANODON HYPOPHTHALMUS*)
THESIS ADVISOR: ASSOC. PROF. DR. SURINTORN BOONANUNTANASARN, Ph.D., 90 PP.

Keyword: Striped catfish/Crispr/Cas9/Dead end gene/ *Pangasianodon hypophthalmus*/Germ cell

To establish surrogate broodstock technology in fish, germ cell transplantation has been developed for its application in aquaculture industries and fish conservation approaches. To increase the efficiency of germ cell transplantation, production of primordial germ cell-less (PGC) fish for use as recipient larvae is required. This study therefore aimed to produce germ cell-less fish larvae using the CRISPR/Cas9 mediated dead end (*dnd*) knockout technique in striped catfish (*Pangasianodon hypophthalamus*). There were two experiments including 1) cloning and characterization of *dnd* and its expression in various tissues and 2) development of CRISPR/Cas9 mediated *dnd* knockout techniques.

In the Experiment 1, molecular characterization of *dnd* and its expression in various tissues was investigated. The partial cDNA of *dnd* contained 1,137 bp of open reading frame encoding 399 amino acid residues and 371 bp of partial 3'-UTR. Characterization of *dnd* found that *dnd* contains all the predicted consensus motifs that are shared among *dnd* in other fish species, including the RNA recognition motif (RRM), N-terminal regions (NR), and four C-terminal regions (CR1-4). Phylogenetic analysis demonstrated that Dnd clustered within the teleost Dnd. Reverse transcription polymerase chain reaction (RT-PCR) indicated that *dnd* mRNA occurred only in the testes and ovaries. Quantitative RT-PCR showed that *dnd* was highly expressed in the ovaries and testes.

In Experiment 2, to optimize CRISPR/Cas9 induced *dnd* knockout, three sgRNAs including sgRNA1, sgRNA2 and sgRNA3 were designed to target exon encoding 5'-UTR. The combination of two concentration of each sgRNA (100 or 200 ng/µl) and three concentrations of Cas9 (100, 250 or 500 ng/µl) were microinjected into fertilized eggs

of striped catfish. Our results showed that there were no significant differences in the hatching rate of fertilized eggs and survival rates of embryos as well as growth of fish among various concentration of each sgRNA and Cas9 protein (P>0.05). These sgRNAs/Cas9 were able to generate indel mutation including deletion (1-16 nt), insertion (1-13 nt) and substitution (1-13 nt). Using histological study, all sgRNA/Cas9 led to a decreased number of PGC in gonad obtained from 60-dph fry, and fry injected with sgRNA3 targeting 5'UTR near to start codon at 200 ng/µL and Cas9 at 500 ng/µL showed the lowest PGC number. The reduction of PGC number was confirmed by in situ hybridization using antisense *dnd* and *vasa* probe. All combinations of sgRNA/Cas9 reduced expression of germ cell marker genes including *dnd, cxcr4b, dazl, nanos1*, *nanos2* and *vasa*, and significant lowest expression levels were observed in gonad obtained from fish injected with sgRNA3 at 200 ng/µL and Cas9 at 500 ng/µL (P<0.05). In addition, at 1 year of age, significant lower GSI was observed in fish injected with all sgRNAs at various concentration and Cas9 at 500 ng/µL (P<0.05). Moreover, compared to control fish without sgRNA/Cas9 injection, different morphological appearances in ovary and testis were observed in sgRNA/Cas9 injected fish, i.e., few previtellogenic oocyte in ovary and spermatogonial cell-less testes. Taken together, CRISPR/Cas 9 targeting *dnd1* knockout at 5'-UTR was achieved which resulted in down-regulation of *dnd1* and lowered PGC, and the sgRNA targeted exon nearest start codon showed the highest knockout activity.

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Kunlanan Booncherd

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

℃	=	Degree Celsius
3’ UTR	=	3’untranslated regions
5’ UTR	=	5’untranslated regions
ANOVA	=	Analysis of Variance
AS-ON	=	Antisense oligonucleotides
bp	=	Base pair
cDNA	=	Complementary DNA
CDS	=	Coding sequence
CRD	=	Completely Randomized Design
CRISPR	=	Clustered regularly Interspaced Short Palindromic Repeats
DPF	=	Day post fertilizes
DPH	=	Day post hatching
FITC	=	Fluorescein isothiocyanate
g	=	Gram
gDNA	=	Genomic DNA
Gfp	=	Green fluorescent protein
HCG	=	Human chorionic gonadotropin
HR	=	Homologous recombination
IU kg-1	=	International Unit/ kilogram-1
Kg	=	kilogram
KO	=	Gene knockout
M	=	Mole
mg/ml	=	Milligram per milliliter
ml	=	Milliliter
mM	=	Millimolar
mm	=	Millimeter
MO-ON	=	Morpholino phosphorodiamidate
mRNA	=	Messenger RNA

LIST OF ABBREVIATIONS (Continued)

ng	=	Nanogram
NHEJ	=	Non-homologous end joining
nt	=	Nucleotide
PAM	=	Proto-spacer Adjacent Motifs
PGC	=	Primordial germ cell
pmol	=	Picomole
rpm	=	Revolutions per minute
sgRNA	=	Small guide RNA
siRNA	=	Small interfering RNA
tacrRNA	=	Trans-activating crRNA
TALENs	=	Transcription activator-like effector nuclease
U/µl	=	Unit per microliter
µg/ml	=	Microgram per milliliter
µl	=	Microliter
µm	=	Micrometer
ZFNs	=	Zinc-finger nucleases

CHAPTER I
INTRODUCTION

1.1 Introduction

Germ cell transplantation is a technique for transferring translatability germ cell from donor fish into recipient gonad. Generally, germ cells that exhibit translatability are undifferentiated germ cells including primordial germ cell (PGCs), oogonia and spermatogonia. In order to develop germ cell transplantation, information of early gonadal development in fish larvae is important and a prerequisite factor. For example, study on primordial germ cell (PGC) migration process is necessary to determine the optimum period for germ cell transplantation. Therefore, characterization of PGC is required to investigate the regulation process of PGC development. Several genes have been demonstrated to be involve in PGC migration such as vasa (Kobayashi et al., 2000), nanos (Kprunner et al., 2001) and dead end. Among these genes, dead end (*dnd*) has been revealed to be essential for formation and migration of PGCs in loach (Fujimoto et al., 2010), goldfish (Goto et al., 2012) and Japanese medaka (Kurokawa et al., 2007). In vertebrates, dead end protein binds to 3'-UTRs (untranslated regions) of germ cells specific RNAs, to protects them against miRNA-mediated degradation (Baloch et al., 2019).

Generally, during early gonadal development, endogenous PGCs migrate toward and colonize in genital ridge of fish larvae. The process of germ cell transplantation mimics early gonadal development in fish larvae. The transplanted germ cell migrates and incorporates in the genital ridge of recipient fish. Therefore, the transplanted germ cells need to compete with the migration of endogenous germ cell. The inhibition of endogenous germ cell migration would eventually increase the efficiency of donor germ cell migration. Since *dnd* was revealed to be essential for formation and migration of PGCs (Wargelius et al., 2016), inhibition of *dnd* expression would lead to decrease endogenous germ cell migration and incorporation in genital ridge of fish larvae. Inhibition of gene expression have two

technologies consists of gene knockdown and gene knockout. Gene knockdown or antisense technology able to inhibit only mRNA target in translation process. Therefore, gene knockdown technology is transient inhibition of gene expression. In addition, gene knockout or gene editing technology has become high effective tools for modification, insertion, deletion or replace the target gene of genome organisms. Indeed, the gene knockout investigates the effects of gene loss by comparing differences in phenotypes between the knockout organism and normal organism. Recently, the CRISPR-Cas9 system has been a tool allow researchers to direct edit or modulate the DNA functions of any organism of choice, enabling them to identify and reveal the functionality of the genome at the system levels. (Gupta and Shukla, 2017). The gene editing technology provide as gene knockout technology to inhibit gene expression. Therefore, gene editing to inhibit *dnd* function would provide as a useful tool to generate sterile fish which would be able to be used as recipient fish for germ cell transplantation.

In Pangasiid, there are a member of fish species which have been important species in term of economical aquaculture. Some have been considered as endangered species. Therefore, biotechnological approaches to improve the reproduction in the Pangasiid are needed to develop to preserve these fish population for not only aquaculture purpose but also conservation. striped catfish (*Pangasianodon hypophthalmus*) has been an economically important species as a white flesh fish product on the world market and the full-cycle cultivation of the striped catfish has been conducted. Therefore, striped catfish will be used as a model for generate sterile fish for development to use as recipient for approach germ cell transplantation in Pangasiid.

1.2 Objectives

This study aims to develop gene editing technique for knock-out of *dnd* in striped catfish (*Pangasianodon hypophthalmus*). There are two specific objectives:

1.2.1 To clone and characterize *dnd* cDNA and its partial genomic DNA in striped catfish and analyze its expression in various tissues.

1.2.2 To develop *dnd* knockout technology using CRISPR/Cas9 and study its effects on *dnd* expression and germ cell number.

1.3 Hypothesis

1.3.1 Characteristic of *dnd* in striped catfish are conserved with teleost *dnd*.

1.3.2 CRISPR/Cas9 technology is able to produce indel mutation in *dnd*.

1.3.3 Indel mutation in *dnd* could interfere with expression of *dnd* which results in decrease in number of germ cell in gonad of striped catfish.

1.4 Scope of the study

The gene editing technique was developed to target *dnd* using CRISPR/Cas9. The genotyping of fish which was disrupted of *dnd* determined. In addition, the expression level of *dnd* was examined. The effects of gene editing of *dnd* on PGCs number were counted.

1.5 Expected outcome

The gene editing to inhibit *dnd* function would enable to generate recipient sterilize fish for used as recipient for germ cell transplantation. In addition, gene editing of *dnd* would able to increase the efficiency of surrogate broodstock to produce the Pangasiid offspring.

1.6 Keywords

Gene editing, Crispr/cas9, dead end gene, *Pangasianodon hypophthalmus*, Germ cell, Sterilize recipient fish.

CHAPTER II

LITERATURE REVIEWS

2.1 Striped catfish

In Pangasiid, there are a number of fish species which have been important species in term of economical aquaculture. Some have been considered as endangered species. Among them, the striped catfish has been an economically important species as a white flesh fish product on the world market. Indeed, recently, Viet Nam has become the top producer pangas catfish. In addition, the Mekong giant catfish (*Pangasianodon gigas*) is the largest freshwater fish in the word. It has been classified as critically endangered species. Therefore, biotechnological approaches to improve the reproduction in the Pangasiid are needed to develop to preserve these fish population for not only aquaculture purpose but also conservation. For example, development of germ cell transplantation in these fish would enable the generation of surrogate broodstock to reproduce fish which are difficult to breed in farm. Since the full-cycle cultivation of the striped catfish has been conducted, the striped catfish could be a fish to be used as recipient fish for germ cell transplantation in Pangasiid. The germ cell transplantation and surrogate broodstock technologies would enable to expand wild population of several endangered Pangasiid, particularly the Mekong giant catfish in the future.

Striped catfish (*Pangasianodon hypophthalmus*) (Figure 2.1 and Table 2.1) is belonging in family of Pangasiidae The striped catfish has large mouth, and its upper jaw is thoroughly hidden by the lower jaw when the mouth is closed. The pelvic fin contains 8 spines. The body length can reach up to 130 cm. and wight can reach up maximum to 44 kg. The striped catfish conclude 45-46 vertebrae and has two pairs of barbels: one pair at on upper jaw and another one on under jaw. These barbels in young striped catfish are longer than that of head length. In old fish, barbels are shorter than head length. The body color of the striped catfish is grey and little dark-blue color on the dorsal surface. The sides of body are upward mixed sliver-blue.

There is little yellow color at the pelvic fins. The dorsal and caudal fins are black-grey, the caudal fins have adjunct a little red color. (Robert and Vidthayanon, 1991).

Table 2.1 Taxonomy of the striped catfish.

Kingdom	**Animalia**
Phylum	Chordata
Class	Actinopterygii
Order	Siluriformes
Family	Pangasiidae
Genus	Pangasianodon
species	Pangasianodon hypophthalmus

Reference: Sauvage, 1878.

The habitats of striped catfish have been reported to found in large rivers throughout Mekong, Chao Phraya, and Mekong basins of Southeast Asia, risen in Cambodia, Laos, Thailand and Viet Nam (Robert and Vidthayanon, 1991). Generally, there living within condition pH 6.5-7.5 and temperature 22-26 ℃. The striped catfish is benthopelagic and omnivorous, feeding on plant, algae, zooplankton, insects, crustaceans and fish. The production of striped catfish has been full-cycle cultivation. The striped catfish can be maintained until maturation, and they can be broodstock to produce seed in commercial farm. Female takes 3 years to reach maturity or body weight reach to 3 kg, and male usually become mature at 2 years (FAO, 2010).

Figure 2.1 Striped catfish (*Pangasianodon hypophthalmus*).

2.2 Germ cell transplantation in striped catfish

Germ cell transplantation is a technique for transferring translatability germ cell from donor fish into recipient gonad. Generally, germ cells that exhibit translatability include primordial germ cell (PGCs), oogonia and spermatogonia. The transplanted germ cell could colonize in recipient gonad, and the recipient fish is grown through maturation to become broodstock. These broodstock produce donor-offspring, and the broodstock is therefore call surrogate broodstock. The surrogate broodstock technology has been intensively developed to increase efficiency of seed production for aquaculture production or conservation endangered fish species (Yoshizaki et al., 2012). Germ cell transplantation consist of 2 methods. First, germ cell transplantation was performed using adult fish as a recipient. This technology was demonstrated in adult tilapia. The transplanted was transfer through the urogenital papilla. The transplanted germ cell that was labeled with PKH26 was observed under fluorescence microscopy to incorporate in the seminiferous tubules of recipient fish (Lacerda et al., 2018). In fact, when the recipient was injected live cell of another species, germ cell transplantation in adult fish might have problem with immune rejection. Second, germ cell transplantation by use larvae as a recipient is another available method. Because in newly hatching embryo, the immune system remains undifferentiated, thus, the germ cell transplanted into larvae recipient would not be immune rejected (Manning and Nakanishi., 1996). The primordial germ cells (PGCs) are a progenitor cell of germ cell, PGCs in development embryo stage will migrating there into genital ridges (future side of gonad) by chemotaxis using pseudopodia (Yoshizaki et al., 2012). and PGCs become incorporated germ cell and differentiate in recipient gonad to become oogonia in female and spermatogonia in male. In the experiment of spermatogonia transplantation of rout into sterile Salmon fish. The Salmon recipients produced only donor-derived egg and sperm of Trout. The spermatogonia transplantation was successfully to preserve fish genetic resources (Tsukamoto et al., 2008). In another experiment, allogenic transplantation of spermatogonia was conducted in Nibe croaker (Nibea mitsukurii). Donor germ cell was microinjected into peritoneal cavity of larvae. The incorporated germ cell was observed in the gonad of recipient for 3 weeks (Takeuchi et al., 2009). Nowadays, transplantation in fish is an effective of production technology in conservation

endangered fish or important aquaculture species. However, in order to improve the efficiency of germ cell transplantation, the recipients for transplantation were created as sterile triploid recipients for increase efficiency colonization of donor-derived germ cell. However, the triploidization leads to reduction survival rate and the rate of sterile will not high in every species (Yoshizaki and Yazawa, 2019). Therefore, other techniques such as gene knockdown or gene knockout of particular genes that are especial for germ cell development in fish larvae would help to produce sterile recipient larvae. These sterile larvae could be use as recipient fish to improve germ cell transplantation efficiency.

2.3 Molecular information of dnd gene

Normally, the mRNA of gene contains three parts including 5'untranslated regions (5'UTR), coding sequence (CDS) and 3'untranslated regions (3' UTR) (Vilela and McCarthy, 2003). Although the function of 5'UTR (the region upstream of coding sequence) is not fully understood, it has been demonstrated that 5'UTR is essential for translation of mRNA. The region of 5' UTR is recognized by small subunit ribosome binding and start translation (Penalva, L. O. F. and Sanchez, 2003). The CDS contains RNA that is translated into amino acids to produce the function protein (Höglund et al., 1990). Generally, the CDS of the same gene among vertebrate are very conserved. The region of 3' UTR is revealed to involve in tissue specific gene expression and the level of gene expression (Höglund et al., 1990). For mRNA of *dnd* in various fish species (16 species), *dnd* mRNA comprised of the 5'UTR which contained 10-435 nucleotides (nt). The *dnd* mRNA comprised of the 3'UTR and poly A tails which contained 53-945 nucleotides (nt). The CDS contains 1,094-1,235 nucleotides (nt) that encodes amino acid 365-401 residuals (Table 2.2). The length of CDS appeared to be similar among fish species which enable to use as the guideline to predict the length of CDS of *dnd* in other fish species.

Characterization of Dnd proteins from various fish showed that Dnd proteins contains five conserved motifs including N-terminal region (NR), RNA recognition motif (RRM), C-terminal region 1-4 (CR1-4) (Figure 2.2). For example, Dnd protein of Japanese medaka (*Oryzias latipes*) (GQ184560) contains NR motif, and the amino acid of NR

includes LTQVNGQRKYGGPPD VWDGPPP. The amino acid of RRM comprised of EVFISQTPRDVYEDLLIPLFSSVGALWEFRLMMNFSGQNRGFAYAKGTAAIANDAIHLLHGYPLGPGARL SV.

The amino acid of C-terminal region 1 consist of SIEKRQLCIQNLPASTRQEELLQVL. In addition, the amino acid of C-terminal region 2 includes AVVAFSSHHAASM AKKALGEEFKKQFCLDISIKW. The amino acid of C-terminal region 3 comprised of GDPHYEMLFSHAGPDGFLYFTYKVHVPGAP TTFPGFVMIL. The amino caid of C-terminal region 4 enclose EEARRAAAQQVLQK (Liu et al., 2009) (Table 2.3). These motif characteristics were conserved among fish species including in Turbot (Scophthalmus maximus) (Lin et al., 2013), Chinese sturgeon (Acipenser sinensis) (Yang et al., 2015) and Tambaqui (Colossoma macropomum) (Vasconcelos et al., 2019).

Table 2.2 Molecular information of *dnd* mRNA form NCBI.

Accession	Species	Common name	CDS (nt)	Amino acid (residuals)
GQ184560	*Oryzias latipes*	Japanese medaka	1,118	373
XM_035650077	*Scophthalmus maximus*	Turbot	1,202	401
KY426013	*Colossoma macropomum*	Tambaqui	1,193	398
JN712911	*Salmo salar*	Atlantic salmon	1,100	367
KF128758	*Thunnus orientalis*	Pacific bluefin tuna	1,142	381
KM655832	*Acipenser sinensis*	Chinese sturgeon	1,190	397
XM_026918857	*Pangasianodon hypophthalmus*	Striped catfish	1,139	380
KM044011	*Gobiocypris rarus*	Chinese rare minnow	1,166	389
XM_017722544	*Pygocentrus nattereri*	Red-bellied piranha	1,172	391
MK887177	*Oncorhynchus mykiss*	Rainbow trout	1,094	365
AB531494	*Misgurnus anguillicaudatus*	Oriental weatherfish	1,118	373
AY225448	*Danio rerio*	Zebrafish	1,235	412
KP171240	*Thunnus maccoyii*	Southern bluefin tuna	1,145	382
MH283870	*Oryzias celebensis*	Celebes medaka	1,121	374
LC317114	*Nibea mitsukurii*	Nibe croaker	1,142	381
KP641680	*Carassius gibelio*	Silver crucian carp	1,127	376

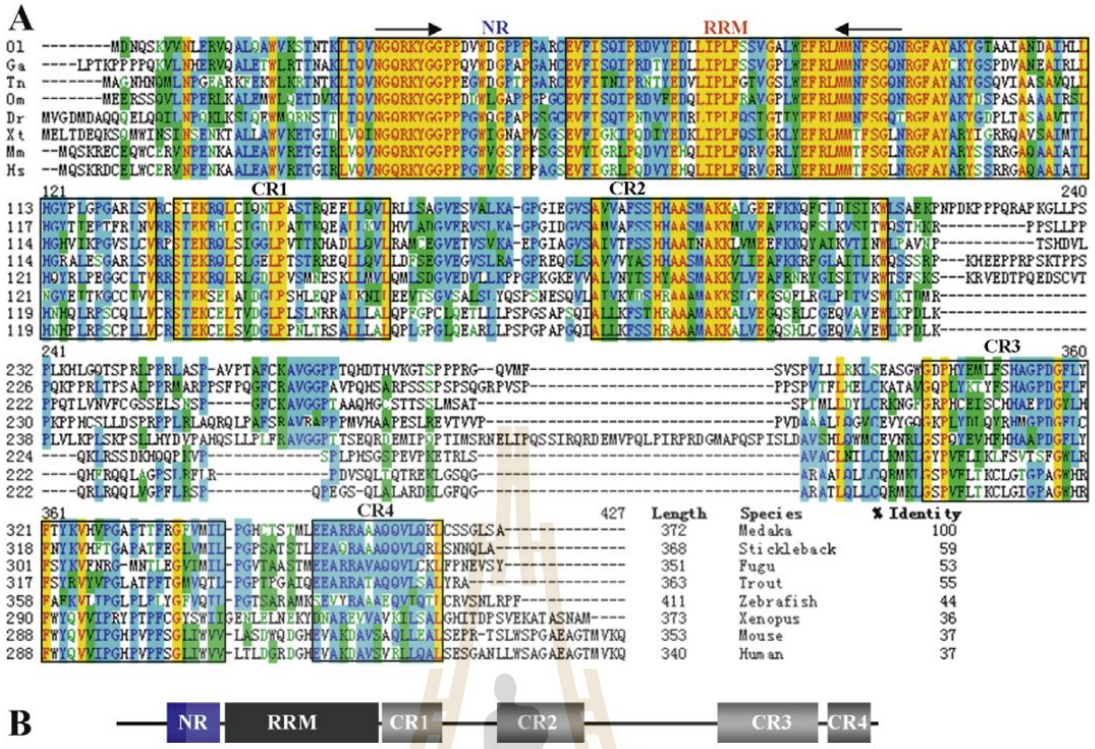

Figure 2.2 Six conserved motifs of dnd proteins contains N-terminal region (NR) RNA recognition motif (RRM) and C-terminal region 1-4 (CR1-4). (A) show the multiple amino acid alignment of Japanese medaka *dnd* and other fishes. (B) show the schematic structure of conservation of Dnd protein (Liu et al., 2009).

Table 2.3 Characterization of dnd protein of Japanese medaka (*Oryzias latipes*).

Conserved motifs	Amino acid
N-terminal region (NR)	LTQVNGQRKYGGPPD VWDGPPP
RNA recognition motif (RRM)	EVFISQTPRDVYEDLLIPLFSSVGALWEFRLMMNF
	SGQNRGFAYAKGTAAIANDAIHLLHGYPLGPGARLSV
C-terminal region 1	SIEKRQLCIQNLPASTRQEELLQVL
C-terminal region 2	AVVAFSSHHAASMAKKALGEEFKKQFCLDISIKW
C-terminal region 3	GDPHYEMLFSHAGPDGFLYFTYKVHVPGAPTTFPGF
	VMIL
C-terminal region 4	EEARRAAAQQVLQK

2.4 Tissue distribution of *dnd* expression

Tissue distribution of *dnd* expression was analyzed using reverse transcription PCR (RT-PCR). Most transcription analysis was conducted by sampling various tissue including head kidney, stomach, gut, liver, intestine, eye, gall bladder, pyloric, caeca, skin, muscle, gill, heart, spleen, kidney, ovary, testis and blood. In Turbot, the *dnd* gene expression was detected only in testis and ovary and weak in brain but the expression of *dnd* mRNA was not detected in heart, kidney, gill, spleen, liver, gut and muscle (Lin et al., 2103). Likewise, the *dnd* expression was detected only in testis and ovary, but the expression of *dnd* mRNA was not detected in brain, eye, gill, kidney, liver and spleen in Japanese medaka (Liu et al., 2009). The expression of *dnd* was detected only in testis and ovary but the expression of *dnd* was not detected in brain, muscle, heart, spleen, liver, kidney, stomach and gut in Pacific bluefin tuna (*Thunnus orientalis*) (Yazawa et al., 2013). Similarly, finding in Tumbaqui (Vasconcelos et al., 2019) and in Atlantic salmon (*Salmo salar*) (Nagasawa et al., 2013) In addition, qRT-PCR was conducted and demonstrated that the *dnd* expression was high in ovary and testis in Chinese sturgeon (Yang et al., 2015). Taken together, in various fish, *dnd* was expressed only in testis and ovary suggesting that *dnd* would be benefit for application for germ cell specific gene marker.

2.4.1 *dnd* expression in testis and ovary in mature fish

The expression of *dnd* was provided as gene marker to identify developmental stage of germ cells. For instant, in Chinese sturgeon, using in situ hybridization, *dnd* antisense probe was able to identify oogonia and oocyte, and the expression of *dnd* was weaker in oogonia comparing to that in oocyte. In testis, the expression of *dnd* was detected in spermatogonia, spermatocyte and spermatid. Strong signal of *dnd* expression was observed spermatogonia while weak signal of *dnd* expression was found in spermatid (Yang et al., 2015). In Turbot, *dnd* antisense probe was able to detect oogonia, primary oocyte, secondary oocyte (previtellogenic oocyte), but the signal became weak in the third oocyte (early vitellogenic oocyte). The signal of expression was weaker in oocyte that are more developed. In testis, the expression of *dnd* was observed in spermatogonia, spermatocyte but not at spermatid (Lin et al., 2013). In Japanese medaka, *dnd* antisense could be used as probe to identify developmental stage of ovary. The expression of *dnd* was detected

throughout oogenesis from oogonia to maturing oocytes. In addition, *dnd* antisense probe was able to characterize all stage of cells in spermatogenesis, and the weak signal was detected in sperm (Liu et al., 2009). In tambaqui, *dnd* antisense probe was able to identify in testis, *dnd* probe was used to detect spermatogonia, spermatocyte and spermatid (Vasconcelos et al., 2019). The expression of *dnd* in various developmental stages of differentiated germ cell in ovary and testis are summarized in suggesting that *dnd* might have important function in germ cell development in both male and female.

2.4.2 *dnd* expression during embryogenic stage

The expression of the *dnd* RNA in germ cell were reported in various vertebrates such as xenopus, chicken and mouse. In addition, *dnd* was revealed to express in germline during embryogenesis (Weidinger et al., 2003). Moreover, the specifically expression of *dnd* enables the use of *dnd* as germ cell gene marker for identifying PGC in various fish such as in Japanese medaka (Liu et al., 2009), Chinese sturgeon (Yang et al., 2015). The *dnd* gene was also used to identify type A spermatogonia in pacific bluefin tuna (Yazawa et al., 2013). The expression *dnd* was detected at high level during early development, and its expression was reported to decrease in late embryonic development. For example, in Japanese medaka, *dnd* mRNA was detected in high level in embryo during 2-cell, 8-cell, morula and blastula stages. The expression of *dnd* was decreased at gastrula stage, and *dnd* mRNA was not observed in fry (Liu et al., 2009). Consistently, in Atlantic salmon, *dnd* expressed at high level in embryo at 2-cell stage. On the other hand, the expression of *dnd* was gradually decreased during blastula stage (Nagasawa et al., 2013). Moreover, in Turbot embryo, *dnd* mRNA was detected in unfertilized egg. The expression of *dnd* was found in fertilized egg and in embryo at hatching stage (lin et al., 2103). In addition, the qRT-PCR was performed in Chinese sturgeon, and it was also reported that *dnd* expression was high during unfertilized egg, morula, blastula and gastrula stage. However, the expression of *dnd* was decreased at neurula. Particularly, the *dnd* expression was detected as high level in unfertilized egg, showing that *dnd* mRNA was the maternal RNA (Yang et al., 2015). Combined together, *dnd* might have important role in embryonic germ cell development.

The Dnd protein is an RNA binding protein which is a germplasm component. The *dnd* is essential for migration and formation of primordial germ cells (PGCs). In vertebrates, the *dnd* binds to 3'-UTRs (untranslated regions) of germ cells specific RNAs, thus, it protects these specific RNAs against miRNA-mediated degradation. The *dnd* can contribute to maintain the function of PGCs (Baloch et al., 2019). In Salmon, zygotic *dnd* RNA was essential for PGC migration to presumptive gonad (Wargelius et al., 2016). Therefore, the role of *dnd* protein was involved in formation and migration of germ cell during early embryonic development. Additionally, loss of *dnd* function in mouse caused germ cell free sterile gonad (Youngren et al., 2005). According to *dnd* knockout in embryo sterlet (*Acipenser ruthenus*) by CRISPR/Cas9 technology led to lacked PGCs, that can bring to use as sterile recipient fish for conservation in sturgeon (Baloch et al., 2019) or *dnd* knockdown in sterlet by antisense morpholino oligonucleotide (AMO) technology (Linhartová et al., 2015). Therefore, interference with *dnd* gene expression would be used as a useful tool for inhibition of PGC migration. Interference with gene expression could be achieved using gene knockdown and gene knockout via gene editing.

Gene interferences have been investigated for studying gene function, inhibition of target expression for specific purpose. Antisense technology includes two mechanisms: 1) gene targeting for mRNA resulting in degradation of target mRNA and 2) gene knockdown by blocking ribosome to bind to target mRNA. Therefore, the blocking of ribosome binding led to inhibition of translation process. Gene knockdown or antisense technology is a technique to inhibit target gene expression which could be achieve at transcription and/or translation levels. Development of interferences technology in fish have been carried out using gene knockdown using a number of antisense molecules. The antisense molecules including antisense oligonucleotides, antisense RNA (AS-ON), double-stranded RNA, small interfering RNA (siRNA) and ribozyme. Firstly, the AS-ONs using single-strand DNA consist of 15-25 nucleotides, which are designed to contain nucleotides complementary to target mRNA. AS-ON consists of native oligonucleotide and various modified nucleotide. The most use of AS-ONs have been the modification of oligonucleotide because of high stability, solubility and binding capacity. The modified AS-ONs used in fish

include Phosphorothioate DNA (PS-ON), Morpholino phosphorodiamidate (MO-ON), Peptide nucleic acid (PNA) and Trans-4-hydroxyl-proline (PNA). Second, double-stranded RNA and small interfering RNA (siRNA) have been used to interfere with target expression by inducing cleavage of target mRNA. The siRNA is the short molecule of double-stranded RNA which is cleaved by dicer.

These siRNA incorporated into a multicomponent nuclease complex which induce the degradation of specific target mRNA. Third, ribozyme (R_z) or catalytic RNA is a specific RNA for cleavage activity. R_z contains secondary structure which specifically induces cleavage of target RNA at conserved NUH (Boonanuntanasarn, 2008). In the experiment of Yoshizaki et al. (2016), knockdown of *dnd* gene was performed using antisense MO-ON in Salmonids. Knockdown of *dnd* led to produce germ cell-deficient fish which were used as recipient fish for germ cell transplantation. The knockdown recipient fish could be used to increase germ cell transplantation efficiency (Yoshizaki et al., 2016). In another the experiment, knockdown of *dnd* gene was conducted using of antisense MO-ON in sterlet. Gene interfering with *dnd* led to produce sterile fish which was also used for recipient fish for germ cell transplantation of Sturgeon (Linhartová et al., 2015). The concentration of *dnd*-AMO, that were injcted into 1-4 cell stage sterlet embryos were tested 100-1000 μM. the concentration were observed in *dnd*-AMO concentration 250 μM result in 63.3% after hatching survival rate is optimum concentration which led to significant development restrain of PGCs (Linhartová et al., 2015) (Table 2.4). Although gene knockdown of *dnd* gene resulted in good result of inhibitory effects of *dnd* gene expression, the gene knockdown technology was temporary for inhibition of target expression, and the effects were disappeared when the antisense molecules were absent. Therefore, permanent gene inhibitory effects such as gene knockout are needed for long-term development of gene interfering effects.

2.5 Gene knockout

Gene knockout (KO) is a genetic engineering to modify organism's gene to be nonfunction. Gene knockout has been demonstrated to be an useful tool to study gene function. Indeed, the gene knockout investigates the effects of gene loss by comparing differences in phenotypes between the knockout organism and normal

organism. In order to develop gene knockout technology, the genome editing technology has become high effective tools for modification, insertion, deletion or replace the target gene of genome organisms. Recently, genome editing technologies which includes Zinc-finger nuclease (ZFNs), Transcription activator-like effector nuclease (TALENs) and Clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) have been developed in fish. These technologies induced DNA double-strand breaks (DSBs) by function of endonuclease which determines their site specificity. Zinc-finger nuclease (ZFNs) consists of specific DNA binding protein, that made form tandem zinc finger binding motif combined with non-specific chevage domain of the restriction endonuclease (FokI). Zinc finger is recognized region at double strand DNA that, including 6-18 bp. combine with FokI nuclease at the target region or spacer conclude 5-6 bp. ZFNs are able to cleave double strand at specific sites. Subsequently, DNA usually repaired by non-homologous end joining (NHEJ) and homologous recombination (HR) (Mashimo, 2014). In Japanese medaka, transgenic carrying green fluorescent protein *(gfp)* gene was interrupted by ZFNs. The ZFNs induced mutation in *gfp* in germ cell and was able to inherited to next generation. Experiment of (Ansai and Kinoshita., 2012). In addition, similar to ZFNs, transcription activator-like effector nuclease (TALENs) is artificial nuclease technology. However, TALE proteins are highly conserved repeat domain. Naturally, TALE proteins occurring in bacteria photogenic plant (*Xanthomonas spp.*). TAL effector connected with FokI nuclease. TALENs are able to binding with DNA and induced cleavage at target region in pair. In tilapia (*Oreochromis niloticus*), somatic mutation using 6 pairs of TALENs for 6 targets gene including *dmrt1, foxl2, cyp19a1a, gsdf, igf3,* and *nrob1b* were investigated. The maximum rate of indel mutation efficiency up to 81% at the target loci (Li et al., 2013).

2.6 CRISPR/Cas 9

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) or CRISPR/Cas has been an efficient tool to modulate or edit genetic information. The CRISPR/Cas has been allowed for studying gene function and uncovering the crucial biological mechanisms. The CRISPR/Cas9 technology have derived from adaptive defense mechanism of *Streptococcus pyogenes* for against

bacteriophage genome infection (Heler et al., 2014). The mechanism of CRISPR/Cas9 immunity is begun when bacteria recorded genetic information of the invading virus. The short fragment of the virus genome is integrated into CRISPR locus within the host genome. Afterward, CRISPR locus was being transcript through endonucleolytic cleavage become CRISPR RNA (crRNA). The crRNA contains spacer, short sequence that is homologous with a sequence form invaders element at 5' end and CRISPR repeat sequence at 3' end. During the process, the small noncoding RNA is called Trans-activating crRNA (tacrRNA) which bind to the repeat sequence of crRNA. The hybrid RNA structure is a guide which is called small guide RNA (sgRNA). The hybrid RNA directs Cas9 to cleave complementary DNA (around 20 nucleotides) target sequence which is adjacent to Proto-spacer Adjacent Motifs (PAM). Cas9 ribonucleoprotein (RNP) binds to sgRNA and then cleaves target DNA, which is complementary to crRNA. The searching DNA sequence of invader is recognized by Cas9 protein and PAM (Gasiunas et al., 2012). The Cas9 proteins are different in diverse species of bacteria or archea that recognize different PAM For example, *S. pyogenes* Cas9 (SpCas9) recognizes 5'-NGG-3' PAM that is widely used for editing the genome of various organism. The SpCas9 and PAM have the interaction between guanines of PAM and arginine in SpCas9. The phosphate of DNA backbone 5' of PAM interacts with phosphate-lock loop of Cas9. The Cas9 nuclease which cleaves the DNA strand contains two different domains: HNH and RuvC domains. The HNH domain cleaves the DNA strand which is complementary with crRNA. The RuvC domain cleaves the DNA strand which is not complementary with crRNA (Gasiunas et al., 2012). After the CRISPR/Cas9 mechanism is finish, the cell will be repaired its damage DNA by one of two pathways. First, non-homologous and joining (NHEJ) is the error-pone pathway, that randomly performs insertions and deletions (indels) or probably substitutions at DSB site. Second, homology directed repair (HDR) is the error-free pathway. This process contains the donor template of interest inserted by homology arms. The HDR pathway provides the precise gene modification, such as gene knockout, gene knock in, gene insertion/correction/replacement. Generally, two pathways happen after DNA replication but before cell division (Cromie et al., 2001). In bacteria or archaea, CRISPR-Cas systems have been classified into 3 types (I, II, III). Each type utilizes a different mechanism. The types II CRISPR system has been the

most normally applied to genome editing because only requiring just one Cas protein and two RNA components. In order to develop gene editing in fish, the optimum concentration of sgRNA and Cas9 was important factor for fish survival and gene editing efficiency. Gene editing of *nanos2* and *nanos3* was developed in tilapia by microinjection of sgRNA and Cas9 into fertilized egg at 1 cell stage. The optimum concentration of sg*nanos2*RNA at 50 ng/µl and Cas9 at 500 ng/µl which resulted in highest fish survival (38 fish in total 300 fertilized eggs) at 14 days after haching (dph). This concentration of sgRNA and Cas led to highest indel. In addition, similar concentration of sgRNA and CAS of *nanos3* (sg*nanos3*RNA50 ng/µl and Cas9 500 ng/µl) was demonstrated which led to 22 survival fish of 300 microinjected eggs at 14 dph (Li et al., 2014) (Table 2.4). In Japanese medaka, mutagenesis using CRISPR/cas9-midiated RNA-guide endonuclease (RGEN), the concentration of CRISPR/Cas9 (sgRNA in the range of 1-25 ng/µl and Cas9 in the range of 0-100 ng/µl) had no effects on survival rate. However, decreasing of dosage could reduce the off-target (Ansai and Kinoshita, 2014) (Table 2.4).

2.7 The experiment of CRISPR/Cas9 in animals

Recently, CRISPR/Cas9 technology has been used to study gene fuction several animals. The CRISPR/Cas9 technology could be use as useful tool to perform mutation in organism. Genome editing technology is able to applied for various research fields. For example, in mammals, gene editing has been applied for therapeutic research. For example, rat was used as an animal model for genome editing by microinjrction or eletroporation methods for transfer Cas9/sgRNA into fertilized embryo at 1-cell stage. For instance, the rats was used for conducting gene editing for the Parkinson's desease by knocking out Tyrosine Hydroxylase gene in brain via injection of Adeno-Associated virus (AAV) vectors expressing SpCas9 and sgRNA (Back et al., 2019). In addition, CRISPR/Cas9 technology has been applied in extensively purpose in various fishes. In sterlet, knockout of *dnd* was established to generate germ cell-free fish. The germ cell-free fish was aimed to use as recipeint fish for development surrogate broodstock technology. The result was showed that the number of PGCs were decreased in the CRISPR/Cas9 injected embryo. The injected fish became sterile. Deformity in some of sterile fish were detected. However, when

CRISPR/Cas9 method was compared with using UV and AMO for gene knockout, it was found that the servival rate and hatching rate of the CRISPR/Cas9 injected embryo was highest (Baloch et al., 2019). In tilapia, CRISPR/Cas9 system was used to disturpt several heritable genes including, *nanos2, nanos3, dmrt1* and *foxl2.* The result was showed that average mutation rates were 31% for *nanos2,* 24% for *nanos3,* 44% for *dmrt1* and 50% for *foxl2.* The maximum mutation effective could reach to 95% in *nanos2* and *foxl2.* Moreover, tilapia was observed for germ cell-deficinet in gonad also (Li et al., 2014). In Atlantic salmon, CRISPR/Cas9 system was used to knockout the target of two genes that are involved in pigmentation inculding *tyrosinase (tyr)* and *solute carrier family 45, member 2 (slc45a2).* The embryo had the mutation rate 40% for *slc45a2* and 22% for *tyr.* Moreover, the complete lack of pigmantation phenotype was observed in CRISPR*slc45a2*/Cas9 injected embryo (Edverdsen et al., 2014). Successful targeted mutagenesis using CRISPR/cas9-midiated RNA-guide endonuclease (RGEN) in Japanese medaka. The off-target alteration in the RGEN-injected fish were found which could be reduce by decreasing dosage of sgRNA (Ansai and Kinoshita, 2014).

Table 2.4 Different concentration of sgRNA/Cas9 and *dnd*-MO (co-injected with FITC) on the survival.

treatment	sgRNA/cas9 (ng/µl)	Total no. of embryos/larvae/eggs	Total no. of survival				Species	Ref.
			14 days after injection	After hatching	1 dpf	3 dpf		
dnd-MO 1000 µM		30		20.05%[d]			*Acipenser ruthenus* (sterlet)	Linhartová et al., 2015
dnd-MO 750 µM		30		20.0%[d]				
dnd-MO 500 µM		30		40.0%[c]				
dnd-MO 250 µM		30		63.3%[b]				
dnd-MO 100 µM		30		73.3%[a]				
Control FITC		30		63.3%[b]				
noninjected		30		63.3%[b]				
Control	0/100	18			18	16	*Oryzias latipes* (*Japanese* medaka)	Ansai and Kinoshita, 2014
sgRNA 1	25/100	22			21	19		
sgRNA 2a	5/100	16			16	16		
	10/100	21			21	21		
	25/100	20			20	20		
sgRNA 2b	25/100	20			20	20		
sgRNA 3a	1/100	22			21	21		
	10/100	21			21	20		
	25/100	20			20	19		
	25/0	22			21	20		

Table 2.4 Different concentration of sgRNA/Cas9 and *dnd*-MO (co-injected with FITC) on the survival (continued).

treatment	sgRNA/cas9 (ng/µl)	Total no. of embryos/larvae/eggs	Total no. of survival				Species	Ref.
			14 days after injection	After hatching	1 dpf	3 dpf		
sgRNA 3a	25/10	21			21	21	*Oryzias latipes*	Ansai and
	25/30	22			22	22	*(Japanese*	Kinoshita, 2014
sgRNA 3b	25/100	12			12	11	medaka)	
nanos2	50/100	300	100					
nanos2	50/300	300	66					
nanos2	50/500	300	38				*Oreochromis*	
nanos2	150/800	300	21				*niloticus*	Li et al., 2014
nanos3	50/100	300	81				(tilapia)	
nanos3	50/300	300	65					
nanos3	50/500	300	22					
nanos3	150/800	300	15					

Superscript especially experiment of Z. Linhartová et al. (2015) in values in each column are not significantly different (t test, $P > 0.05$), Abbreviations: FITC, fluorescein isothiocyanate

2.8 Visualization of gonad after injected with dnd-AMO and CRISPR/ Cas9 gene

The study of gene editing using CRISPR/cas 9 to inhibit function of *nanos2* and *nanos3*. Indeed, these genes are essential for development of germ cell in testis and oocyte in adult (Kprunner et al., 2001). Indeed, *nanos2* and *nanos3* were expressed in germ cell of tilapia (*Oreochromis niloticus*). It was revealed that the 3' UTR of *vasa* controls the specific expression in germ cell. The green fluorescent protein gene fused to 3' UTR of vasa produce green fluorescent protein in germ cell; therefore, the gfp -labeled germ cells were located in embryo. After injection for 72 hours post fertilization, no germ cells were observed. In addition, the result was confirmed by immunohistochemistry (IHC) with *vasa*. The result of immunohistochemistry (IHC) showed that, the expression of *nanos2* and *nanos3* were not detected in embryo at 72 hr postfertilization (Li et al., 2014) (Figure 2.3). In sterlet, gene knockdown using AMO of *dnd* was established to generate germ cell-free fish. Histological study was investigated in gonad at 60, 150, and 210 days post fertilization (dpf). The results showed that at 60 dpf fish, the morphants gonad were empty spaces. In 150 dpf fish, gonad of control fish was occupied by germ cells and consists of surrounded somatic cells while that of morphants fish are increase size and were occupied by somatic cell and large number of blood vessels. In 210 dpf fish, gonad of morphants comprised only of blood vessel and somatic cells without germ cells when compare with control gonad that comprise of large number germ cell (Linhartová et al., 2015). In Rainbow trout (*Oncorhynchus mykiss*), gene knockdown using AMO was used to establish germ cell-deficient fish by knockdown of *dnd*. The effects of knockdown of *dnd* in larvae that were received *dnd*-AMO were determined by histology and IHC of germ cells were performed with anti-*gfp*. In the larvae that received *dnd*-AMO showed no *gfp*-positive germ cell in gonad in both females and males at 6-month-old fish, suggesting that knockdown of *dnd* interfered with germ cell development (Yoshizaki et al., 2016).

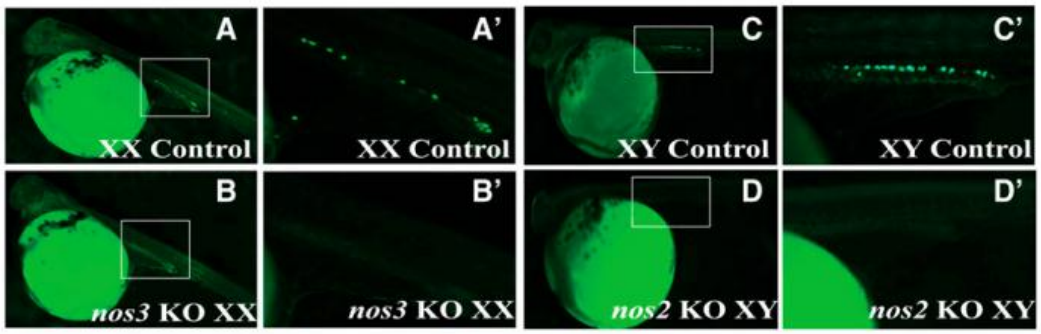

Figure 2.3 Mutation of *nanos2* and *nanos3* by CRISPR/Cas9 resulted in germ-cell-deficient gonads. In vitrosynthesized eGFP-*vasa* 3'UTR mRNA was injected into fertilized eggs to label germ cells. GFP-labeled germ cells were located in the gonadal primordium (box9) in the normal XX and XY embryos at 72 hr postfertilization (A and C) while no GFP-labeled germ cells were observed in embryos co-injected with nanos3 (B) or nanos2 (D) gRNA, Cas9, and eGFP-*vasa* 3'UTR mRNA at the same stage. (A', B', C', and D') Magnification of the boxed areas in A, B, C, and D, respectively (Li et al., 2014).

CHAPTER III

MATERIALS AND METHODS

This study has 2 experiments consist of

Experiment I Molecular characterization and tissue expression of *dnd* in striped catfish.

Experiment II Gene dnd editing by CRISPR/Cas 9

3.1 Broodstock management

Mature male and female mature fishes of the striped catfish were reared within a cage (7×15 m) in the earthen ponds at SUT farm, Suranaree University of Technology, Nakhon Ratchasima, Thailand. Fish were fed daily, twice a day, at a rate of 2% of the total body weight per day with 25% crude protein commercial pellets.

3.2 Research station

SUT Farm (fisheries station) and equipment building F14 in Suranaree University of Technology.

3.3 Venue of the study

Suranaree University of Technology Farm (Aquaculture), Instrument Building 14, Science and Technology Equipment Center Suranaree University of Technology.

3.3.1 Equipment and chemicals

- Cage size 7×15 square mater (m^2)
- Fiber tank size 100, 500 liters (L)
- Glove and bucket
- 100 ppm of clove oil
- 300 IU kg^{-1} human chorionic gonadotropin (HCG)
- pituitary gland
- Operating scissor
- Forceps

- Micropipette size 1000, 200, 10 μl
- Microtip
- Microcentrifuge tube 1.5 ml
- PCR tube size 200 μl
- Tube size 15, 50 ml
- Rack
- Real time PCR pate 384 well
- Vortex mixer
- Refrigerated centrifuge
- Dry bath
- Nanodrop spectrophotometer
- -80°C ultra-low temperature freezer
- -20°C temperature freezer
- Thermal cycler for Polymerase chain reaction (PCR)
- Light Cycler® 480 Real time PCR system
- Gel chamber
- Gel documentation system
- UV transilluminator
- Erlenmeyer flask size 250, 500, 1000 ml
- Para film paper
- Incubator
- Incubator checker
- Alcohol lamp
- Sterilized toothpick
- Spreader
- Loop
- Petridis
- Microwave
- PCR EX Taq kit (Takara)
- PCR LA Taq kit (Takara)
- PCR GoTaq® DNA polymerase (Promega)
- QIAEX II Gel Extraction Kit (Qiagen)

- pGEM® T-Easy vector system I (Promega)
- DNase
- RNase
- T4 DNA ligase
- Proteinase K
- TNES buffer
- Phenol-Chloroform-isoamyl (25:24:1 at pH 8)
- Cell Suspension Solution (25mM Tris-Cl pH 8, 50 mM glucose, 10 mM EDTA)
- Lysis buffer (0.2 M NaOH, 1% SDS)
- Neutralization Buffer (3 M potassium, 5 M acetate, about pH 5)
- *E. coli* (DH5 α)
- Trizol reagent
- Isopropanol
- Ethanol
- Phenol
- Chloroform
- Sodium acetate
- Ethylene diaminetetraacetic acid (EDTA)
- Dimethyl sulfoxide (DMSO)
- ImProm-II™ Reverse Transcription system Kit (Promega, Madison, WI, USA)
- Light Cycler® 480 SYBR Green I Master (Roche Applied Science, Indianapolis, IN)
- DNase free water
- RNase free weter
- Agarose powder
- ViSafe Red Gel Stain bands
- 0.5X TBE buffer
- 50 mg/ml Ampicillin
- 20 mg/ml Isopropyl-ß-D-thiogalactopyranoside (IPTG)
- 0.04 mg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal)

- Stereomicroscope Olympus SZX10.
- Thin-walled Glass capillary size 1×90 mm

3.4 Experiment I Molecular characterization and tissue expression of *dnd* in striped catfish

To cloning and characterize *dnd* from striped catfish and demonstrated its specific expression in the gonads along with its expression levels in the testes and ovaries of adult fish.

3.4.1 Cloning of the partial *dnd* cDNA

1) Design primer

Specific primers for nested PCR were designed according to DNA sequence of *Pangasianodon hypophthalmus* DND microRNA-mediated repression inhibitor 1 (*dnd1*), transcript variant X2, mRNA (XM_026918857.2). Amplification of 1 fragment for *dnd* cDNA which have the expected size as 1,107 bp.

2) Cloning of a fragment of *dnd* cDNA

In order to clone the dead end form complementary DNA, the fragment (expected size as 1,107 bp) was used primer GSP-dnd-F1 with GSP-dnd-R1 for primary PCR and GSP-dnd-F2 with GSP-dnd-R2 for nested PCR (Table 3.1), PCR was carried out in a total volume of 50 µl, contains 50 ng of cDNA for first round and first round PCR product dilute in ratio 1:50 was used to template for nested PCR, 2.5 mM of dNTP, 25 mM of $MgCl_2$, 10 pmol of primers, 10X LA taq™ buffer and 5 U/µl LA taq, PCR was amplified use Takara LA taq. The PCR was performed on DNA thermal cycle denaturation at 95°C for 5 min, followed by 35 reaction cycles of 45 s at 95°C, 45 s at 60°C and 3 min at 72°C, with a final elongation step at 72°C for 7 min.

The PCR product was analyzed by agarose gel electrophoresis, add 2 g in 100 ml 0.5x TBE buffer in 500 ml Erlenmeyer flask then dissolve agarose by heating on microwave. Then, to the solubilization 100 ml was added 6 µl of ViSafe Red Gel Stain and maintained at 50 to 60°C until pour agarose solution into the chamber. After agarose was set, remove comb and infuse the gel into 0.5x TBE buffer. Mixing 10 µl of PCR solution containing 10 ng to 2 ng of DNA with 2 µl of gel loading solution. Combine 10 µl of 1 ug DNA marker with 2 µl of gel loading solution.

Loading the sample and standard to individual wells by using micropipette and turn on volage electrophoresis, usually 50-100 V for DNA migrate. When electrophoresis complete, photograph the gel using a gel Documentation for visualize ViSafe Red Gel Stain bands. The amplicon bands with the expected size were gel purification according to commercial kit protocol (The QIAEX II Gel Extraction Kit).

3) Ligation of PCR fragment into vector

The DNA fragments were ligated into pGEM-T easy vector (Figure 3.1) to generate recombinant plasmid. The reaction in total volume of 10 µl contains 3 µl of purified PCR product, 50 ng of vector p-GEM-T, 5 µl of 2X rapid ligation buffer and 1 µl of T4 DNA ligase. Then, the ligation reaction was incubated for 2 hours at 25°C and then at 4°C for overnight.

Table 3.1 The oligonucleotide primers used in amplification *dnd* fragment.

Primer name	5'- sequence -3'	Amplicon size	purpose
Dnd-FN1	GCCACGCCCATTTCCCTCCAGCCG	772	Cloning gDNA
Dnd-RN1	CTTAACAAGCGTGCAGAGAAGAGGA		
Dnd-FN2	TGTTAATGTTGTGGAAACATTACAG	449	Cloning gDNA
Dnd-RN2	TTCTTTTCGGGCACAGGATGCCATG		
Dnd-FN3	GGATCCACAGCGACCCTGACAATTG	607	Cloning gDNA
Dnd-RN3	CCAACACCTTCTTGGCCATGGAGGC		
Dnd-FN4	ACCTACTTTTACCCTTTCCACATGT	556	Cloning gDNA
Dnd-RN3	CCAACACCTTCTTGGCCATGGAGGC		
GSP-dnd-F1	TTGCAGATGTTGAATCCGCAGA	1,509	Cloning cDNA
GSP-dnd-R1	GACAGACCACCTCTGGCAGTTTCTA		
T7-dnd-FN1	T7-GCCACGCCCATTTCCCTCCAGCCG	692	PCR for genotypic analysis
SP6-dnd-RN2	SP6- TTCTTTTCGGGCACAGGATGCCATG		
GSP-dnd-F2	GAAGTCTCTGGAGAAGTGGCTTGAG	1,107	Cloning cDNA, RT-PCR
GSP-dnd-R2	GTACACCGTCCGCTCTTTTGGT		
β-actinF	ACTACC TCA AGATCCTG	517	RT-PCR
β-actinR	TTGCTGATCCACATCTGCTG		

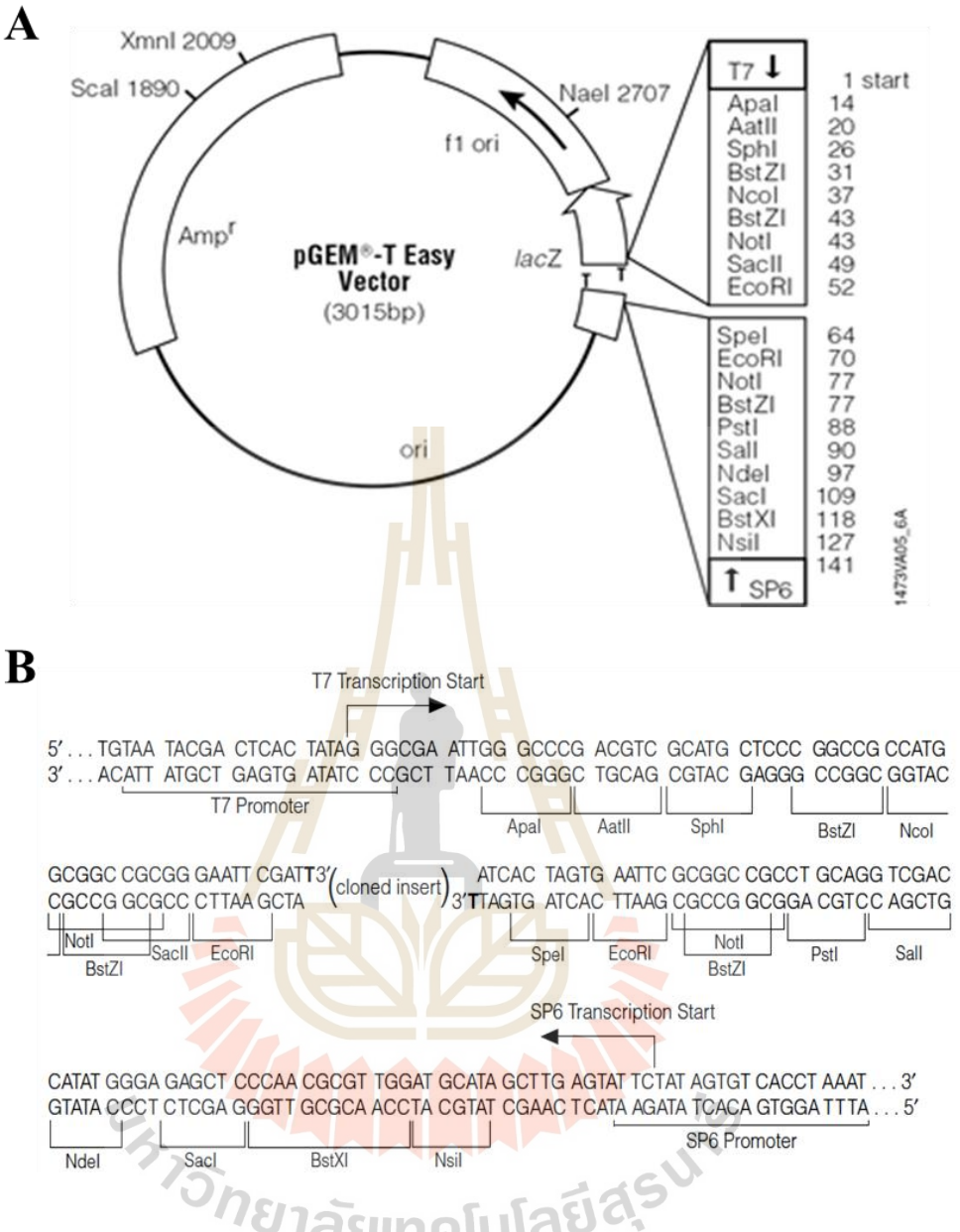

Figure 3.1 Plasmid pGEM®-T Easy vector (A) and the promoter and multiple cloning sequence of the pGEM®-T Easy Vector (B).

4) Transformation recombinant plasmid into competent cell

The ligation reaction was transformed into *E. coli* (DH5 α). Transformation was carried out by adding 6 µl of ligation reaction into 100 µl of competent cell and incubate on ice for 30 min. Heat chock was carried out by incubating at 42°C for 60 sec, then immediately incubating on ice for 3 min. The transformant cells was enriched in 900 µl of SOC by shacking at 37°C. The

transformants was spread on 2XYT plate with 50 mg/ml of ampicillin, 20 mg/ml of IPTG (Isopropyl-ß-D-thiogalactopyranoside) and 0.04 mg/ml of X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) and incubated at 37°C for 16-18 hours. The white colony was selected for plasmid extraction.

5) Plasmid extraction

The growing bacterial colonies on 2XYT plate were transferred with sterile toothpick to 3.5 ml of LB medium and incubate at 37°C with shaking for overnight. After that, bacteria were collected by microcentrifugation at 10,000 rpm for 1 min at 25°C then, pour off supernatant. Plasmid extraction was performed using alkaline-SDS lysis. Add 100 µl of Cell Suspension Solution (25mM Tris-Cl pH 8, 50 mM glucose, 10 mM EDTA) and mix by vortexing. Then, cells were resuspended on ice and add 200 µl of lysis buffer (0.2 M NaOH, 1% SDS) and mix by gentle inversion, then incubate on ice for 5 min, after that add 150 µl of Neutralization Buffer (3 M potassium, 5 M acetate, about pH 5) and mix by gentle inversion, then incubate on ice for 5 min. the pellet was centrifuged for 5 min at 25°C. the supernatant was transferred to new 1.5 ml tube and add 5 µl of 10 mg/ml of RNase for treat the plasmid then incubated at 37°C for 20 min. The plasmid DNA was purified using 400 µl Phenol/chloroform and the upper aqueous phase was separated by centrifugation at 10,000 rpm for 2 min. the aqueous phase was transferred into new 1.5 ml tube and precipitated in 2.5 Volume of 95% ethanol then mixing by inversion several time and keep at -20°C for at least 1 hr. The pellet was centrifuged at 10,000 rpm for 15 min and wash the pellet with 70% ethanol and then recollect the pellet by centrifugation. After that, the plasmid pellet was resuspended in 50 µl sterile distilled water and store plasmid DNA at -20°C. After plasmid extraction method, plasmid solution was performed agarose gel electrophoresis. The concentration of the plasmid DNA was determined using spectrophotometer and performed DNA sequencing (Macrogen).

The partial *dnd* cDNA was used for further analysis. Multiple sequence alignment was conducted using CLUSTAL W (https://www.genome.jp/tp/ tools-bin/clustalw, accessed on 25 February 2023) (Thompson et al., 1994). A phylogenetic tree of Dnd was constructed with 1000 bootstrap replications using the Mega 11 program and the UPGMA method (Sneath et al., 1973; Tamura et al., 2013).

3.4.2 Analysis of tissue expression of *dnd*

First-strand cDNA was synthesized using 1 µg of the total RNA extracted from the brain, gill, muscle, liver, spleen, intestines, and ovaries using the SuperScript III RNAseH-Reverse transcriptase kit (Invitrogen) with random primers (Promega, Madison, WI, USA). A pair of primers (GSP-dnd-F2 and GSP-dnd-R2) was designed based on *Phy-dnd* and used to analyze the expression of *dnd*. The β-actin gene (*actb*) served as an internal control, (β-actinF and β-actinR) (Duangkaew et al., 2019) was used (Table 3.1). RT-PCR analysis was performed in a total volume of 10 µL consisting of 1 µL of cDNA template, 1 µL of dNTPss.l mix (2.5 mM each), 10 pmol of each primer, 2.5 mM $MgCl_2$, 5X GoTaq Flexi buffer, and 0.25 U GoTaq DNA polymerase (Promega). RT-PCR analysis was performed with an initial denaturation step at 95°C for 3 min, followed by 35 reaction cycles of 45 s at 95°C, 30 s at 60°C, and 90 s at 72°C. The final elongation step was carried out at 72°C for 5 min. A plasmid containing *dnd* was used as the positive control. The PCR products of *dnd* and *actb* were verified using agarose gel electrophoresis and RedSafe Nucleic Acid Staining (JH Science, iNtRON Biotechnology, WA, USA).

3.4.3 Quantitative analysis of *dnd* mRNA expression of various tissues

A pair of primers (dnd-RT-F and dnd-RT-R) was designed based on *dnd* to determine its expression level (Table 3.2). Real-time quantitative PCR (qPCR) amplification (in triplicates) was performed using a Light Cycler® 480 SYBR Green I Master Mix (Roche Applied Science, Indianapolis, IN, USA). For normalization, *actb* was used as an internal reference. The primers and annealing temperatures used for *dnd* and *actb* are listed in Table 3.2, which generated amplicons of 208 bp and 95 bp, respectively. Each PCR was performed using a final reaction mix of 10 µL consisting of 1 µL of cDNA template (synthesized from 1 µg of total RNA) or distilled water (negative control), 5 µL of Light Cycler® 480 SYBR Green I Master Mix, 1 µL of 5 µM of each primer, and 2 µL of distilled water. The PCR products were preincubated for 10 min at 95°C, followed by 40 amplification cycles at 95°C for 15 s, 60°C for 10 s, and 72°C for 15 s. The comparative cycle threshold (Ct) method was used to analyze the data. Upon completion of the amplification, PCR was performed to analyze the melting curve. An external standard curve for *dnd* was generated using a plasmid containing *dnd* with known copy numbers. For *actb*, an external standard curve was

generated using pooled cDNA from all samples with serial dilution. Subsequently, the mRNA expression of *dnd* was normalized to that of *actb* using the following equation: log (copy number of dnd1)/dilution (%) of *actb*. Statistical analysis was performed using SPSS for Windows version 25 (SPSS Inc., Chicago, IL, USA). The normalized *dnd* expression was subjected to one-way analysis of variance (ANOVA), followed by Tukey's procedure to rank the groups when significant differences ($p < 0.05$ considered as significant) were observed among the groups.

Table 3.2 Primer for quantitative real time PCR.

Gene	Primer name	5'-sequence-3'	Amplicon size	Accession number
dnd1	dnd-RT-F	TCCCTCGGGACGTGTACGAA	208	XM_026918857.2
	dnd-RT-R	CTCCTCCTGACGGTGAGCCT		
cxcr4b	cxcr4b-RT-F	GGCGCTGAAGACCACCATCA	171	XM_026912811.2
	cxcr4b-RT-R	AAGTAGGCGAGAGCCTCGGT		
dazl	dazl-RT-F	GCTCAGTGGAGGTGCACAGT	198	XM_026928099.2
	dazl-RT-R	AGTGTCAGCAACGTCTGGACT		
nanos1	nanos1-RT-F	GCCAAGCAGGAGCCCAAGAT	169	XM_026917336.2
	nanos1-RT-R	TGATGGTGTGTGCGTTGTCG		
nanos2	nanos2-RT-F	GGCGCACACACGTCACTACT	140	XM_026922235.2
	nanos2-RT-R	CCATGTCAGAGCTGGGCTGAA		
vasa	vasa-RT-F	CGGCAAACCCTTATGTTCAG	210	MK134711.1
	vasa-RT-R	CATTGTTCTCTGCGTACCTG		
beta-actin	actin-RT-F	TGGCAATGAGAGGTTCCG	95	XM_026928832.2
	actin-RT-R	TGCTGTTGTAGGTGGTTTCG		

3.5 Experiment II Gene dnd editing by CRISPR/Cas 9

To investigate cloning a partial gene of genomic DNA and to investigate the effects of CRISPR/Cas9 of *dnd* on indel mutation of *dnd* on gDNA and germ cell development of striped catfish

3.5.1 Cloning of the partial *dnd* gDNA

Specific primers for nested PCR were designed according to DNA sequence of Pangasianodon hypophthalmus whole genome shotgun sequence

(VFJC01000010.1). Amplification of 2 fragments for dnd gDNA which have the expected size as 449 base pair (bp) and 566 bp, respectively (Figure 3.2).

In order to clone the dead end form genomic DNA, the fragment 1 (expected size as 449 bp) used primer Dnd-FN1 with Dnd-RN1 for primary PCR and Dnd-FN2 with Dnd-RN2 for nested PCR and the fragment 2 (expected size as 566 bp.) used primer Dnd-FN3 with Dnd-RN3 for primary PCR and Dnd-FN4 with Dnd-RN3 for nested PCR (Table 3.1), PCR was carried out in a total volume of 50 µl, contains 50 µg/ml of genomic DNA for first round and first round PCR product dilute in ratio 1:50 was used to template for nested PCR , 25 mM of dNTP, 10 pmol of primer, 10X Ex taq ^{TM} buffer and 5 U/µl Ex taq, PCR was amplified by use Takara Ex taq. The PCR was performed on DNA thermal cycle denaturation at 95°C for 5 min, followed by 35 reaction cycles of 45 s at 95°C, 30 s at 65°C and 30s at 72°C, with a final elongation step at 72°C for 7 min. PCR products of the expected size were isolated and purified using the The QIAEX II Gel Extraction Kit. The PCR-amplified DNA fragment was cloned into a pGEM-T Easy plasmid (Promega). The recombinant plasmid was transformation into competent cell then the plasmid was extracted according to 3.4.1 Cloning of the partial *dnd* cDNA. The concentration of the plasmid DNA was determined using spectrophotometer and performed DNA sequencing (Macrogen).

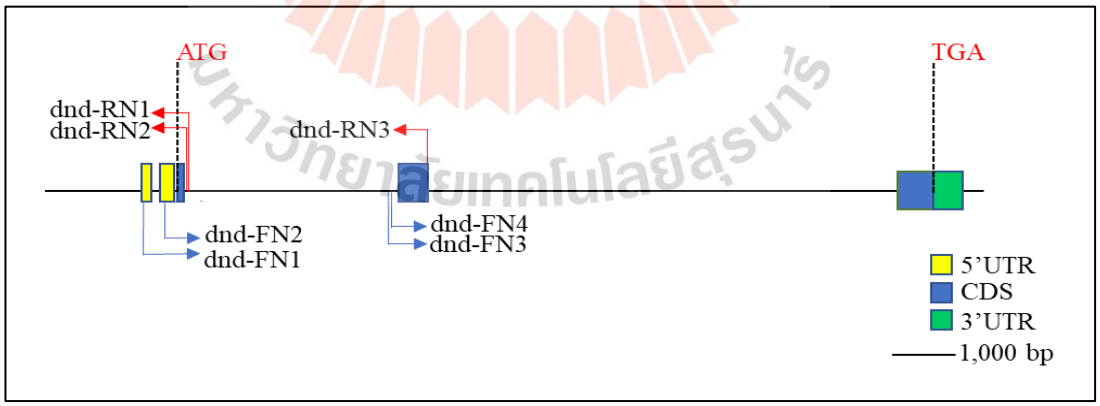

Figure 3.2 Structural map primer of *dnd* gDNA cloning form striped catfish.

3.5.2 CRISPR/Cas9 designation

In order to determine the suitable target sgRNA, three sgRNAs (sgRNA1: TACAGAAAACTTGTACTCCAAGG; sgRNA2: ATTGAATTCACTCTCGAGCACGG and sgRNA3: AACATGTGCACTTGACTACAAGG) (underline determined PAM) were designed to search for PAM target by free program www.crispor.tefor.net. Oligos for sgRNAs were ordered from Macrogen Company (Macrogen Inc., Seoul, Korea), resuspended in TE buffer (pH 7.4), aliquoted and kept at -80°C. Cas9 protein (Alt-R™ Cas9 Nuclease, Integrated DNA Technologies, Inc., Iowa, USA) was diluted with PBS buffer to designed concentration, aliquoted and stored at -80°C. To optimize concentration of sgRNA and Cas9 for microinjection, eighteen mixtures of each sgRNA (100 ng/µL and 200 ng/µL) and Cas9 (100 ng/µL, 250 ng/µL and 500 ng/µL) were mixed with 1.5% isothiocyanate (FITC)-biotin-dextran (M.W.= 500,000).

3.5.3 Fertilization of striped catfish, microinjection and fish culture

The broodstock of striped catfish, females 1.5-2.5 kg: males 1.0-2.5 kg was cultured in an earthen pond and feed with commercial feed 25% crude protein at 2% body weight. In spawning, around March to October, the broodstock was bring to polyethylene pond for artificial fertilization. Firstly, fish was anesthetized with 100 ppm of clove oil and then injected hormone intramuscularly. Female was injected with pituitary gland of equable weight and in male was injected with 300 IU kg^{-1} of human chorionic gonadotropin (HCG). After 8-12 hr, striped catfish was given injection again with four glands of pituitary equable weight in female and 900 IU kg^{-1} of HGCs in male. After 10-12 hr, artificial fertilization was performed by mixing eggs together with milt using dry method. The sperm was activated using 0.85% of NaCl for fertilization

3.5.4 Microinjection, experimental design and fish culture

In order to determine the suitable sgRNA, in experimental design consist of three types of sgRNA and control groups are non-injected and were employed in a Completely Randomized Design (CRD) with 3 replications. Microinjection of sgRNA and Cas9 was conducted in embryo at 1 cell stage. One hundred of fertilized eggs were conducted in each treatment. The experiment were varying concentration between sgRNA consist of 100 and 200 ng/ul and cas9 protein consist of 100, 250 and 500 ng/ul. Microinjection of sgRNAs and Cas9 protein mixture were done under

stereomicroscope Olympus SZX10 (Figure 3.3 and 3.4). After injection, hatching rate at 2 dph was determined, and survival rate at 60 dph was evaluated. The fly stage fishes were maintained in 100 L tank until 60 dph after that the fish were transferred to 500 L tank until 1 year old

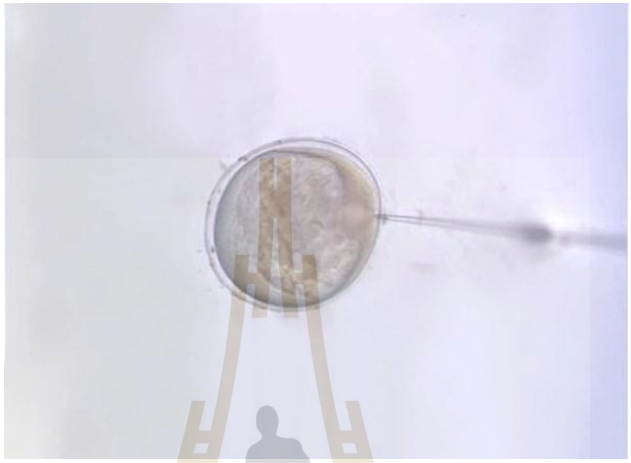

Figure 3.3 Microinjection of sgRNA/Cas 9 complex with FITC into one cell stage embryo.

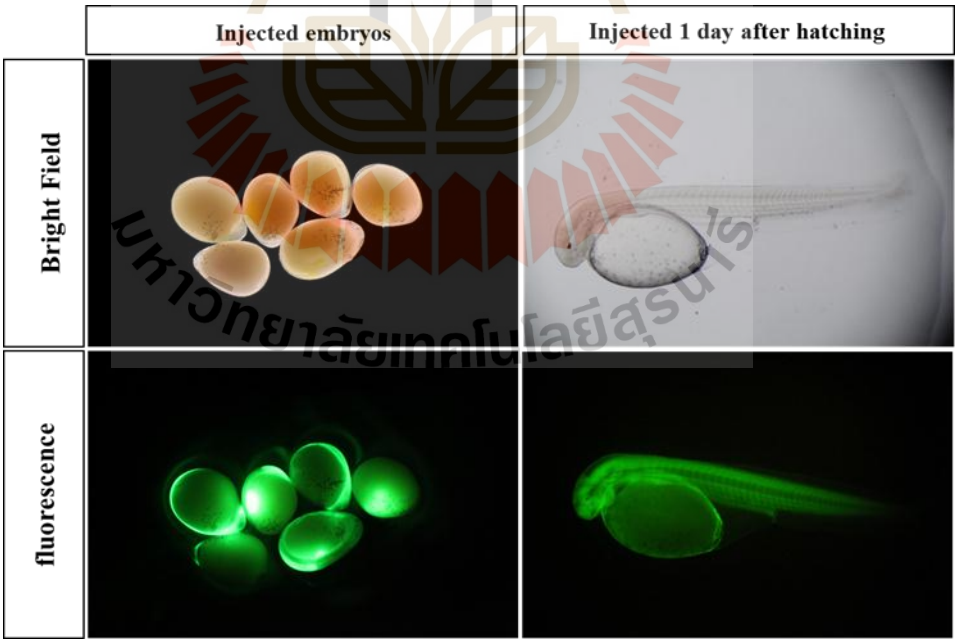

Figure 3.4 Injected embryos showing green fluorescence in germinal disc in fertilized embryos and hatched larvae.

3.5.5 Genomic extraction and genotypic analysis

The injected embryos at 60 DPH were collected fin for genomic DNA extraction. The fins were incubated in 150 ul of TNES and 3 µl of Proteinase K (20 mg/ml) for lysis tissue 2 hr at 55°C. After that, genomic DNA was purified by Phenol-Chloroform-isoamyl (25:24:1 at pH 8) (equal volume) and the gDNA was centrifuged at 12,500 rpm for 5 min at 4°C. The upper aqueous phase was separated, add 150 µl of isopropanol and then incubated at 25°C for 1 hr. The gDNA was centrifuged at 12,500 rpm for 30 min at 4°C and air day. The gDNA was dissolved by 150 µl of 1X TE buffer then 1 µl of RNase (10 mg/ml) was added into gDNA and then incubate at 37°C for 10 min. The gDNA was purified by using 75 µl Phenol/chloroform and the upper aqueous phase was separated by centrifugation at 10,000 rpm for 5 min at 4°C. The upper aqueous phase was separated and precipitated by 1 µl of glycogen and 15 µl of 3M NaOAC and 450 µl of 100% EtOH then incubated at -80°C for overnight. The pellet was separated by centrifuged at 12,500 rpm for 30 min at 4°C. The DNA pellet washed with 1ml 80% EtOH and resuspending the pellet in 25 µl DNase free water. RNase treatment was carried out. The DNA was purified, resuspended in DNase free water and kept at -20°C until use. The concentration of the gDNA was determined using spectrophotometer

In order to amplified the *dnd* form genomic DNA, the fragment (expected size as 692 bp) was used primer T7-dnd-FN1 with SP6-dnd-RN2 (Table3.1), PCR was carried out in a total volume of 10 µl, contains 50 µg/ml of genomic DNA was used to template for PCR, 25 mM of dNTP, 10 pmol of primer, 10X Ex taq TM buffer and 5 U/µl Ex taq, PCR was amplified by use Takara Ex taq. The PCR was performed on DNA thermal cycle denaturation at 95°C for 5 min, followed by 35 reaction cycles of 45 s at 95°C, 30 s at 65°C and 30s at 72°C, with a final elongation step at 72°C for 7 min. the PCR fragment was performed agarose gel electrophoresis. The concentration of the DNA was determined using spectrophotometer and performed DNA sequencing (Macrogen).

3.5.6 Histological analysis

1) Hematoxylin and Eosin staining

The gonads of striped catfish at 60 dph and immature ovary and testis at 1 year old 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 embed process (Table 3.3). Subsequently, the tissues were serial sectioned to a thickness of 5 µm and stained with hematoxylin and eosin (H&E) (Figure 3.5). The number of germ cells were counted in all tissue sections under microscope.

2) In situ hybridization

The gonad of fish at 60 dph was fixed with Bouin's solution at 4°C for 18 hr. and the solution was replaced with 80% EtOH and the gonad was stored at 4°C until use. The fixed gonad of fish at 60 dph was embedded in paraffin wax and cut into serial section at 5 µm intervals, dewax, dehydrate in situ hybridization with *dnd* antisense and vasa antisense probes to analysis the number of PGCs. The probes were performed by digoxigenin (DIG) label, uridine triphosphate (UTP) with T7 RNA polymerase then synthesized in vitro transcription. In situ hybridization as described by (Jangprai et al., 2011). After permeabilization and acetylation, slides were incubated at 65°C for overnight in hybridization solution consist of antisense probe then wash and incubate with anti-DIG alkaline phosphatase conjugated antibody. The colorimetric reaction was developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate and the slide were counterstain with nuclear fast red described by (Duangkaew et al., 2019).

Table 3.3 Embedding process.

Solution	Soaking time
90% ethanol	1 hour or overnight
First absolute ethanol	20 minutes
Second absolute ethanol	20 minutes
Third absolute ethanol	20 minutes
Ethanol and butanol ratio by 4:1	30 minutes
Ethanol and butanol ratio by 3:2	30 minutes
Ethanol and butanol ratio by 2:3	30 minutes
Ethanol and butanol ratio by 1:4	30 minutes
First 100% butanol	30 minutes
Second 100% butanol	30 minutes
Butanol and xylene ratio by 1:1	20 minutes
First 100% xylene	20 minutes
Second 100% xylene	20 minutes
Xylene and paraffin ratio by 1:1	20 minutes
First paraffin	30 minutes
Second paraffin	30 minutes

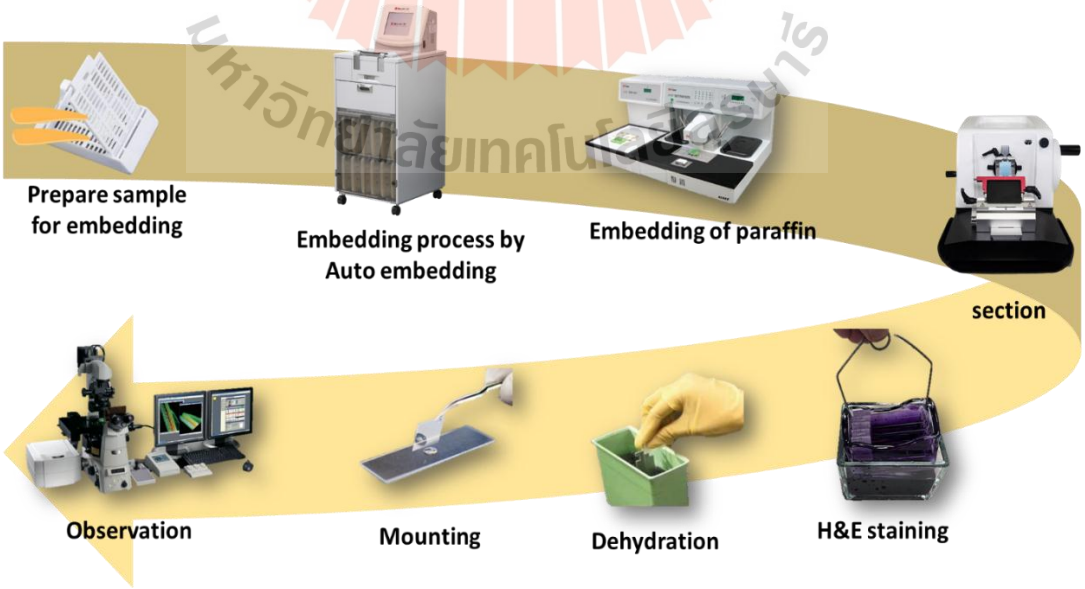

Figure 3.5 Histological process.

3.5.7 Gonadosomatic index (GSI)

The injected fish and control fish were raised until 1 year old. They were measured body weight and measured gonad weight for calculate the gonad mass as a ratio of the body mass. It is represented by the formular;

$$\text{GSI} = \frac{\textbf{Gonad weight}}{\textbf{Body weight}} \times 100$$

The GSI is tool for measuring sexual maturity of animals in correlation to gonadal development in fish.

3.5.8 Total RNA extraction

The injected embryos at 6-month-old were collected gonad tissue for total RNA extraction. The total RNA was isolated from protein used 250 μl of Trizol reagent then gonad was homogenized with pestle and incubate at 25°C for 5 min. the upper aqueous phase was separated by centrifugation at 12,500 rpm for 15 min at 4°C. Precipitation total RNA with 150 μl isopropanol and incubated at 25°C for 30 min and then total RNA was centrifuge at 12,500 rpm at 4°C for 25 min. The pellet was washed with 80% ethanol and resuspending the pellet in 8 μl RNase free water. The concentration of total RNA was determined quantified using spectrophotometer and kept at -80°C until use.

3.5.9 Complementary DNA synthesis

The total RNA was prepared concentration 2 μg for 1 reaction of synthesis complementary DNA (cDNA) and then the total RNA was combined with Oligo(dT) primer (0.5μg/reaction) and heat block at 70°C for 5 min. Immediately chill in ice-water for at least 5 min. Preparing the reverse transcription master mix, master mix was carried out in a total volume of 15 μl in each sample, contains 4 μl of 5X Im Prom II ^{TM} buffer, 4.8 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP, 0.5 μl of 40 U/ul RNase Inhibitor, 1 μl of Reverse Transcriptase and Nuclease-Free Water to a final volume of 15μl. After that, the master mix was added into total RNA tube. The cDNA was performed on dry bath, annealing at 25°C for 10 min, extension at 42°C for 1.30 hr, inactivation at 70°C for 15 min and kept at -20°C until use. RNA was quantified by spectrophotometer and quality was verified on 1% of agarose gel.

3.5.10 Quantitative analysis of *dnd* mRNA expression of gonad tissue

Relative gene expression was determined by quantitative real time PCR on RNA extract from gonad tissue of striped catfish at 6-month-old. The sequence specific primers used in quantitative real time PCR were designed according to DNA sequence of NCBI data base (https://www.ncbi.nlm.nih.gov/) (Table 3.2). For the real time PCR assays of transcripts of genes that involve with germ cell, the Roche Light cycler 480 system was used. The assays were performed using a reaction mix of 6 μl consist of 2 μl of diluted cDNA template, 0.24 μl of each primer, 3 μl of Light Cycler 480 SYBR Green I Master mix and 0.52 μl of DNase/RNase free water. The PCR protocol was initiated at 95°C for 10 min for denaturation of a two-step amplification program (95°C for 15 s; 62°C for 15 s; 72°C for 15 s). Melting curve were systematically monitored (temperature gradient at 1.1°C/10 s from 65°C to 95°C). For the analysis of mRNA levels, relative quantification of target gene expression was performed using the comparative cycle threshold (Ct) method. Upon completion of the amplification, PCR was preformed to analyze the melting curve. An external standard curve for *dnd* was generated using a plasmid containing *dnd* with known copy numbers. For *dazl, vasa, nanos1, nanos2, cxcr4b* and *actin,* an external standard curve was generated using pooled cDNA from all samples with serial dilution. Subsequently, the mRNA expression of *dnd, dazl, vasa, nanos1, nanos2 and cxcr4b* were normalized to that of *actb.* Statistical analysis was preformed using SPSS for Windows version 25 (SPSS Inc., Chicago, IL, USA). The normalized *dnd, dazl, vasa, nanos1, nanos2* and *cxcr4b* expression were subjected to one-way analysis of variance (ANOVA), followed by Tukey's procedure to rank the groups when significant difference ($p < 0.05$ considered as significant) were observed among the groups.

3.6 Statistical analysis

Statistical analysis was performed using SPSS for Windows, version 10. Statistical significance was performed by using one-way analysis of variance (ANOVA) to analyze the hatching rate, the survival rate, The body weight and the total length and the number of germ cell. When significant differences were found among the

groups, Tukey's method was used to rank the group. Throughout the experiment, the effects and differences were announced to be significant when $p < 0.05$.

CHAPTER IV

RESULT

4.1 Experiment I Molecular characterization and tissue expression of *dnd* in striped catfish

4.1.1 Molecular cloning and characterization of Dnd in striped catfish

The partial cDNA of *dnd* cloning contained 1,513 bp (Figure 4.1), which included an ORF encoding 399 amino acid residues and 371 bp of partial 3'-UTR (Figure 4.2). The deduced amino acid sequence of the Dnd protein contained a typical RNA recognition motif (RRM) and five conserved regions, including an N-terminal region (NR) and four C-terminal regions (CR1-4). Multiple alignments of Dnd with other known Dnd protein homologues were highly conserved for typical RRM, NR, and CR1-4 (Figure 4.3). Indeed, Dnd shared the greatest identity (75.47% - 98.68%) with Dnd protein homologues of other fish species (Figure 4.3).

Phylogenetic tree analysis of the Dnd protein with other known vertebrate Dnd protein homologues revealed that the tree was divided into two branches, including the fish branch and that of other vertebrate species, with Dnd being located in the fish branch (Figure 4.4).

4.1.2 Tissue distribution of *dnd*

We analyzed the expression of *dnd* in various tissues, including the brain, gill, muscle, liver, spleen, intestine, testis, and ovary using RT-PCR, and the expression of *actb* was used as an internal standard. The expression of *dnd* was detected only in the testis and ovary, whereas that of *actb* was detected in all the examined tissues (Fig 4.5).

GSP-dnd-F1 **GSP-dnd-F2**
TTGCAGATGTTGAATCCGCAGA **GAAGTCTCTGGAGAAGTGGCTTGAG**

TTGCAGATGTTGAATCCGCAGAGACTGAAGTCTCTGGAGAAGTGGCTTGAGGAAACCAAC 60

ACCACCTTGACGCAGGTTAACGGCCAGCGCAGATATGGTGCTCCTCCTCCGGGATGGAGA 120

GGTCCGACACCAGGTCCAGGCTGCGAGGTCTTTATAAGTCAGATCCCTCGGGACGTGTAC 180

GAAGACCAGCTGATCCCCCTGTTCCAGAGCGTGGCCCCGCTCTACGAGTTCCGCCTCATG 240

ATGAACTTCAGCGGGCAGAACCGAGGCTTCGCCTACGCCAAATACGGAGACACGGCTAGC 300

GCAGCCGCCGCCATCCAGGCTCTAAACCTGTATCCAATCCAGAGTGGCGTCAGGCTCACC 360

GTCAGGAGGAGCACGGAGAAGAGGCAGCTGTGTCTCAGCGATCTGCCTCCCACCATGGAG 420

CGCAACGAGCTGCTGACAGTGCTGCAGCAAATCGCTGACGGAGTGGAGGGCGTCACTATG 480

AGGACCACCGGGCCTAAGGAGAAGGACGTCACTGCGCTCGTGCACTACTCCTCCCACTAT 540

GCTGCCTCCATGGCCAAGAAGGTGTTGGTGCAAGCCTTCAGGAAGCTGTACGGCGTGTCC 600

ATCTCCGTCCGATGGATGTCTGGAAACGCCAAATCCAGACACGAGGAGCATGACGAAGAG 660

AGTGCTCTCGCCCCTCCTGGGCTGAAATCCATGGCCGCGAATTCTTTAACGCCTCCACGC 720

TTCCAGCTCAGCCGAGACCCCGAGCATCCTCCACCTCTTCCTACTCCACCCTCTCCCCTT 780

CACCCGCAGTTCTTCTCGCGGGCAGTAGGGGGTCCAACCCCGCAGGTGACGAGTGTGATG 840

TTACCCCTGAAGCCCAGGAGCGCGGAGGAGCCACCGCACGACTCGGTGCACCAGCTCCGT 900

TGGCTGTGTGAGCTGCACGGGCTCGGCATGCCGCTCTACAACGTGCGCTACGACCACACA 960

GGCCCTGACGGCTTCCTGTATTTTGCCTACAGGGTGGTGGTGCCAGGGCTGGCCATGCCG 1020

TTCTGCGGCGTAGTCCCCGTCCTGCCCAGCACCTGCGCCAACAACATGGAGGCTGAGGTT 1080

GSP-dnd-R2 **GTACACCGTCCGCTCTTTTGGT**

CATCGAGCCGCTGCCAAGCAGCTCCTCAACGCCATGTGGCAGGCGAGAAAACCA**TGA**GCT 1140

TTGACCTCAGATGCATCATCTGTTTTGGGTGAGAATGTGATTTTCTGTAGTAGAACGTGG 1200

TCCTGGATGAGCTTTCCTATGTTTAAACTGTTGAAAATACAAATCTCAGAGATGTCACAG 1260

TCACAGATGGAGCTCGTAGATGTATTAGAGCAGTTAGCATCTCCTCCAAGCTTGTTTCTG 1320

TAGCTGTTCTCTAGCTATTTAGAGGAGAACTAGATAACAGGAACATGGATTGGCAATGTC 1380

TAAACTAAGAAAAAAAAATGAAGTAAGCTCCAAATATAAATAAGTAACTAATAATGAAAT 1440

GSP-dnd-R1 **GACAGACCACCTCTG**

AAATAAAATCTCCTCACATTGCAAAGCTCTGCTAACTTTACTGTCTAGAAACTGCCAGAG 1500

GCAGTTTCTA

GTGGTCTGTC 1510

Figure 4.1 Sequence of primary PCR of *dnd* cloning from complementary DNA.

```
ATGTTGCAGATGTTGAATCCGCAGAGACTGAAGTCTCTGGAGAAGTGGCTTGAGGAAACC    60
  M  L  Q  M  L  N  P  Q  R  L  K  S  L  E  K  W  L  E  E  T    20

AACACCACCTTGACGCAGGTTAACGGCCAGCGCAGATATGGTGCTCCTCCTCCGGGATGG    120
  N  T  T  L  T  Q  V  N  G  Q  R  R  Y  G  A  P  P  P  G  W    40

AGAGGTCCGACACCAGGTCCAGGCTGCGAGGTCTTTATAAGTCAGATCCCTCGGGACGTG    180
  R  G  P  T  P  G  P  G  C  E  V  F  I  S  Q  I  P  R  D  V    80

TACGAAGACCAGCTGATCCCCCTGTTCCAGAGCGTGGCCCCGCTCTACGAGTTCCGCCTC    240
  Y  E  D  Q  L  I  P  L  F  Q  S  V  A  P  L  Y  E  F  R  L    100

ATGATGAACTTCAGCGGGCAGAACCGAGGCTTCGCCTACGCCAAATACGGAGACACGGCT    300
  M  M  N  F  S  G  Q  N  R  G  F  A  Y  A  K  Y  G  D  T  A    120

AGCGCAGCCGCCGCCATCCAGGCTCTAAACCTGTATCCAATCCAGAGTGGCGTCAGGCTC    360
  S  A  A  A  A  A  I  Q  A  L  N  L  Y  P  I  Q  S  G  V  R  L    140

ACCGTCAGGAGGAGCACGGAGAAGAGGCAGCTGTGTCTCAGCGATCTGCCTCCCACCATG    420
  T  V  R  R  S  T  E  K  R  Q  L  C  L  S  D  L  P  P  T  M    160

GAGCGCAACGAGCTGCTGACAGTGCTGCAGCAAATCGCTGACGGAGTGGAGGGCGTCACT    480
  E  R  N  E  L  L  T  V  L  Q  Q  I  A  D  G  V  E  G  V  T    180

ATGAGGACCACCGGGCCTAAGGAGAAGGACGTCACTGCGCTCGTGCACTACTCCTCCCAC    540
  M  R  T  T  G  P  K  E  K  D  V  T  A  L  V  H  Y  S  S  H    200

TATGCTGCCTCCATGGCCAAGAAGGTGTTGGTGCAAGCCTTCAGGAAGCTGTACGGCGTG    600
  Y  A  A  S  M  A  K  K  V  L  V  Q  A  F  R  K  L  Y  G  V    220

TCCATCTCCGTCCGATGGATGTCTGGAAACGCCAAATCCAGACACGAGGAGCATGACGAA    660
  S  I  S  V  R  W  M  S  G  N  A  K  S  R  H  E  E  H  D  E    240

GAGAGTGCTCTCGCCCCTCCTGGGCTGAAATCCATGGCCGCGAATTCTTTAACGCCTCCA    720
  E  S  A  L  A  P  P  G  L  K  S  M  A  A  N  S  L  T  P  P    260

CGCTTCCAGCTCAGCCGAGACCCCGAGCATCCTCCACCTCTTCCTACTCCACCCTCTCCC    780
  R  F  Q  L  S  R  D  P  E  H  P  P  P  L  P  T  P  P  S  P    280

CTTCACCCGCAGTTCTTCTCGCGGGCAGTAGGGGGTCCAACCCCGCAGGTGACGAGTGTG    840
  L  H  P  Q  F  F  S  R  A  V  G  G  P  T  P  Q  V  T  S  V    300

ATGTTACCCCTGAAGCCCAGGAGCGCGGAGGAGCCACCGCACGACTCGGTGCACCAGCTC    900
  M  L  P  L  K  P  R  S  A  E  E  P  P  H  D  S  V  H  Q  L    320

CGTTGGCTGTGTGAGCTGCACGGGCTCGGCATGCCGCTCTACAACGTGCGCTACGACCAC    960
  R  W  L  C  E  L  H  G  L  G  M  P  L  Y  N  V  R  Y  D  H    340

ACAGGCCCTGACGGCTTCCTGTATTTTGCCTACAGGGTGGTGGTGCCAGGGCTGGCCATG    1020
  T  G  P  D  G  F  L  Y  F  A  Y  R  V  V  V  P  G  L  A  M    360

CCGTTCTGCGGCGTAGTCCCCGTCCTGCCCAGCACCTGCGCCAACAACATGGAGGCTGAG    1080
  P  F  C  G  V  V  P  V  L  P  S  T  C  A  N  N  M  E  A  E    380

GTTCATCGAGCCGCTGCCAAGCAGCTCCTCAACGCCATGTGGCAGGCGAGAAAACCATGA    1140
  V  H  R  A  A  A  K  Q  L  L  N  A  M  W  Q  A  R  K  P  *    399

gctttgacctcagatgcatcatctgttttgggtgagaatgtgattttctgtagtagaacg    1200
tggtcctggatgagctttcctatgtttaaactgttgaaaatacaaatctcagagatgtca    1260
cagtcacagatggagctcgtagatgtattagagcagttagcatctcctccaagcttgttt    1320
```

Figure 4.2 The translation of nucleotides sequences of cDNA of *dnd* to amino acids.

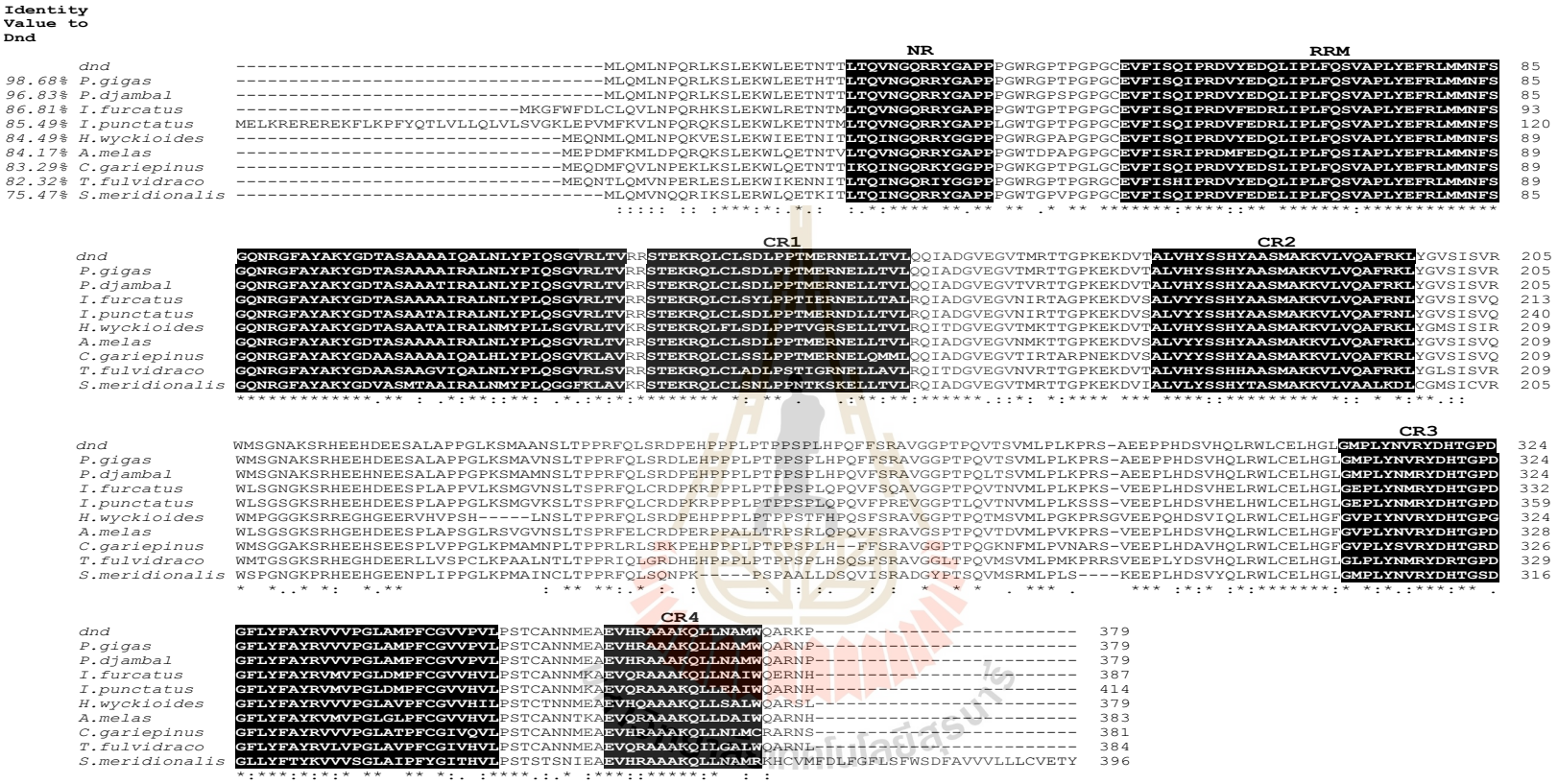

Figure 4.3 Multiple alignments of the Dnd amino acid sequence with known vertebrate protein homologues. The amino acid sequences were aligned using CLUSTAL W, and the percentage identities of Dnd with the other Dnd sequences that had the greatest sequence identity are shown in front of the sequence. Gaps that were introduced to maximize sequence homology are indicated by dashes. The conserved motifs, including NR, RRM, CR1, CR2, CR3, and CR4, are indicated by black boxes. Fully, highly, and less conserved amino acid

residues are indicated by (*), (:), and (.), respectively. The GenBank accession numbers of the Dnd protein homologues are as follows: *Pangasianodon gigas*, MCI4381563.1; *Pangasius djambal*, MCJ8735689.1; *Ictalurus furcatus*, XP_053498456.1; *Ictalurus punctatus*, XP_017340221.1; *Hemibagrus wyckioides*, KAG7324468.1; *Ameiurus melas*, KAF4081039.1; *Clarias gariepinus*, XP_053342036.1; *Tachysurus fulvidraco*, XP_027020922.2; *Silurus meridionalis*, XP_046715472.1

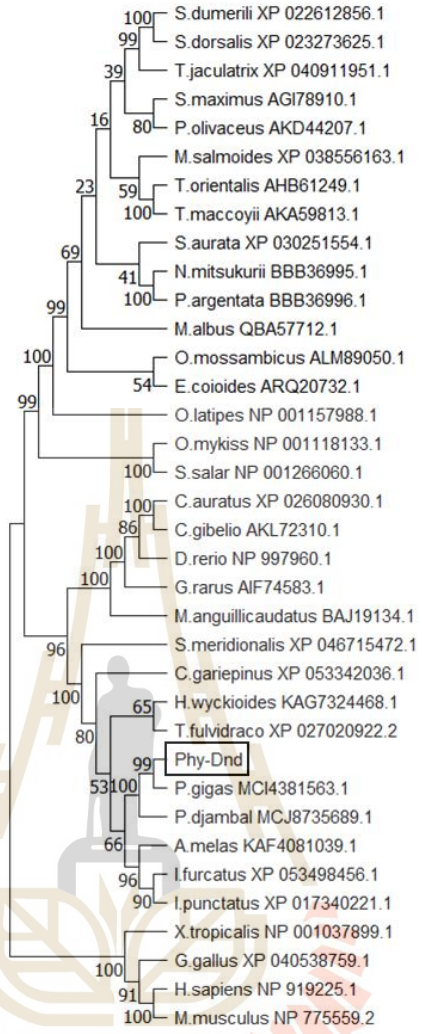

Figure 4.4 Phylogenetic tree of Dnd from different fish species and higher vertebrates. The tree was constructed using 1000 bootstrap replications with MEGA 11 using the UMPGA method (Sneath et al., 1973; Tamura et al., 2013). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. The GenBank accession numbers of the Dnd proteins are provided in brackets.

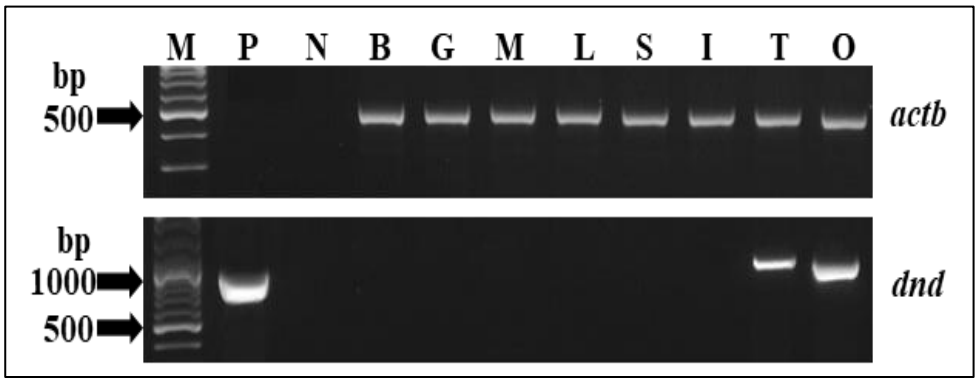

Figure 4.5 Reverse-transcription polymerase chain reaction (RT-PCR) of actb, and *dnd* mRNAs in various tissues of striped catfish. cDNA was synthesized using total RNA isolated from the brain (B), gill (G), muscle (M), liver (L), spleen (S), intestine (I), testis (T) and ovary (O). Distilled water was used as a negative control (N) and *dnd* plasmid (P) was used as a positive control. M represents the DNA marker.

4.1.3 Expression levels of *dnd* in various tissues

Real time quantitative PCR was performed to evaluate the relative expression levels of *dnd* in the adult stage. Figure 4.6 shows that *dnd* was highly expressed in the ovaries and testes while very low expression levels were detected in the brain, gills, muscle, liver, spleen, and intestine. Note that brain, gill muscle, liver, spleen, and intestine were obtained form 1 female and 2 males (Fig 4.6)

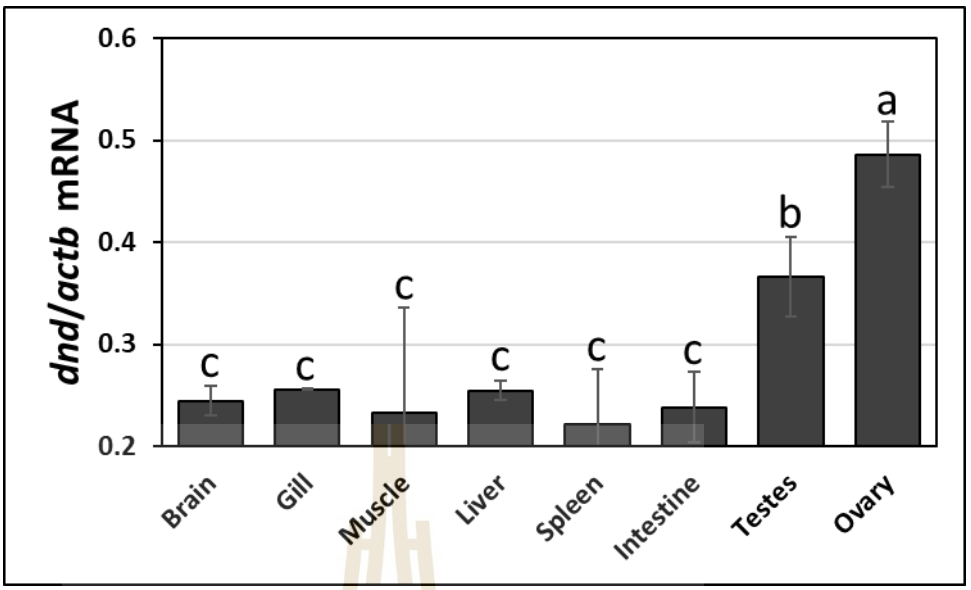

Figure 4.6 Expression levels of *dnd* in various tissues of striped catfish at mature stages. The expression level of *dnd* was normalized with the expression of *actb*. The values are expressed as mean + SD from 3 samples after triplicate qPCR analysis. One-way ANOVA followed by Tukey's range test was performed to rank the expression level in different tissues. Different letters in the bar graph indicate significant differences ($p < 0.05$).

4.2 Experiment II Gene dnd editing by CRISPR/Cas 9

4.2.1 *dnd* genome and designation of sgRNA and PAM

According to genome database accession number VFJC01000010.1 and cDNA sequences (XM_026918857.2), *dnd* (14,660 bp) consisted of 4 exons and 3 introns (Figure 3.1). Analysis of DNA sequences in 5'UTR and coding sequence (CDS) in exon 3 showed in Figure 4.7 and in exon 4 in Figure 4.8, respectively. Three guide RNA (sgRNA) were designed to on 5'UTR (Figure 4.9).

CRISPR/Cas9 technology requires a custom single guide RNA (sgRNA) that contain crRNA (target region) is 20 nucleotides and tracrRNA that essential and interact for cleavage by Cas9. The target specific crRNA position was designed from sequence of *dnd* genomic DNA cloning. The target position was determined on exon 3 which is located upstream of start codon (Figure 4.9A). In order to determine the suitable target crRNA, three target specific crRNA position (target site1: TACAGAA

AACTTGT ACTCCAAGG; target site2: ATTGAATTCACTCTCGAGCACGG and target site3: AACAT GTGCACTTGACTACAAGG) (underline determined PAM) were designed to search for PAM target by free program www.crispor.tefor.net. Three targets specific sgRNA position were selected. Importantly, the target region of sgRNA is 20 nucleotides must be adjacent to PAM site, for the *S. pyogenes* Cas9 (SpCas9) which recognized 5'-NGG-3'. The PAM position should be close to the required mutation location. In addition, the target site should be lower are off-target effects in the genome and the required mutation position should has specificity that measures the uniqueness of a guide in the genome.

Structure of sgRNA that contain crRNA, crRNA is a complementary to target region sequence complex with tracrRNA, tracrRNA is stem loop that required for cleavage by Cas9 protein. Ordinary, in prokaryote tracrRNA which play a role in maturation crRNA. The tracrRNA complementary which repeat position of pre-crRNA and bring to cut by RNase III. After selected the target region around 20 nucleotides that adjacent to PAM by free program finished. The complete structure of three sgRNAs were combined. Initially, 20 nucleotides of target region were complementary such as Target site1: 5'-TACAGAA AACTTGTACTCCA-3' were complementary to 3'-ATGTCTTT TGAACATGAGGT-5' and were transcribed to crRNA 5'-UACAGAA AACUUGUACUCCA-3'. Subsequently, the crRNAs were combined with stem loop of tracrRNA become to complete sgRNA (Figure 4.9B) and complex with Cas9 protein and FITC used to injection in to the one cell stage embryos.

4.2.2 Hatching rate and survival rate at 60 dph

Hatching was occurred around 36 hours post fertilization (hpf), and the hatching rate were showed in Table 4.1. The results showed that no significant differences in hatching rate were observed in all sgRNAs/Cas-injected eggs and normal saline-injected egg ($P>0.05$). However, the hatching rates of all sgRNAs/Cas-injected eggs and normal saline-injected egg appeared to be lower compared with control eggs un-injection ($P<0.05$). To continue culture fish at suitable stocking density, the hatched larvae were transferred to nursing tank (5 larvae/L). Survival rate was determined at 60 dph. There were no significant differences in survival rate among experimental groups (Table 4.1).

```
GCCACGCCCATTTTCCCTCCAGCCGTTTGTTGGGTGTCGCAGTGAGAAACGTGCAAAAAA    60

AACAAAACAAAACAAAAAAAAAAACAATTATTTTCCTTAACGTTAATCTTGCCATAACTC    120

TGTTGGAAAACCGGAAAGAAGGAACAAGACatgttgcaggtaaatgctgatacaagtttg    180

Tttacaccaaactgcatcactggatgcatcataagtttggagtgtgtttgtttgtttatt    240
                                  TGTTAATGTTGTGGAAACATTACAG dnd-FN2
tatttgtttgtttatttctgaaATGTAATGTTAATGTTGTGGAAACATTACAGAAAACTT    300

GTACTCCAAGGCTTTTAATTGAATTCACTCTCGAGCACGGATTCACAATCTGTCGAATAT    360

TATTTCAATTTTTTTTTCTACAAACCATTAAGCGCGCGTAAAACATGTGCACTTGACTAC    420

AAGGGTGTATTGGCTAATGTGTGGGGAACTTGAACTTTAATGTGAATGTGAAAGGGTTGG    480

GGTTGTATTTCTGCTTGCAGATGTTGAATCCGCAGAGACTGAAGTCTCTGGAGAAGTGGC    540

TTGAGGAAACCAACACCACCTTGACGCAGGTTAACGGCCAGCGCAGATATGGTGCTCCTC    600

CTCCGGgtatgtgcgcgcattgggggtttctccaatcaaaaccttctttcagttccttttt    660
dnd-RN2 TTCTTTTCGGGCACAGGATGCCATG    CTTAACAAGCGTGCAGAGAAGAGGA dnd-RN1
acatggcatcctgtgcccgaaaagaattttcctcttctctgcacgcttgttaag           714
```

Figure 4.7 Sequence of primary PCR of fragment 1 *dnd* cloning from genomic DNA. Abbreviations: UPPERCASE– Exon region; lowercase – Intron region.

```
GGATCCACAGCGACCCTGACAATTG dnd-FN3          ACCTACTTTTACCCTTTCC
ggatccacagcgaccctgacaattgcttctaaagtacatttacctacttttacccttttcc    60
ACATGT dnd-FN4
acatgttacttttctcatgacttgcatctacttctctctgttctaactcatttttttct    120

tttcttactccccccttcttcctcatctaccaggATGGAGAGGTCCGACACCAGGTCCAG    180

GCTGCGAGGTCTTTATAAGTCAGATCCCTCGGGACGTGTACGAAGACCAGCTGATCCCTC    240

TGTTCCAGAGCGTGGCCCCGCTCTACGAGTTCCGCCTCATGATGAACTTCAGCGGGCAGA    300

ACCGAGGCTTCGCCTACGCCAAATACGGAGACACGGCTAGCGCAGCCGCCGCCATCCGGG    360

CTCTAAACCTGTATCCAATCCAGAGTGGCGTCAGGCTCACCGTCAGGAGGAGCACGGAGA    420

AGAGGCAGCTGTGTCTCAGCGATCTGCCTCCCACCATGGAGCGCAACGAGCTGCTGACAG    480

TGCTGCAGCAAATCGCTGACGGAGTGGAGGGCGTCACTATGAGGACCACCGGGCCTAAGG    540
                                                  CCAACACCTTCTTGGC
AGAAGGACGTCACTGCGCTCGTGCACTACTCCTCCCACTATGCTGCCTCCATGGCCAAGA    600
CATGGAGGC Dnd-RN3
AGGTGTTGG                                                        609
```

Figure 4.8 Sequence of primary PCR of fragment 2 *dnd* cloning from genomic DNA. Abbreviations: UPPERCASE– Exon region; lowercase – Intron region.

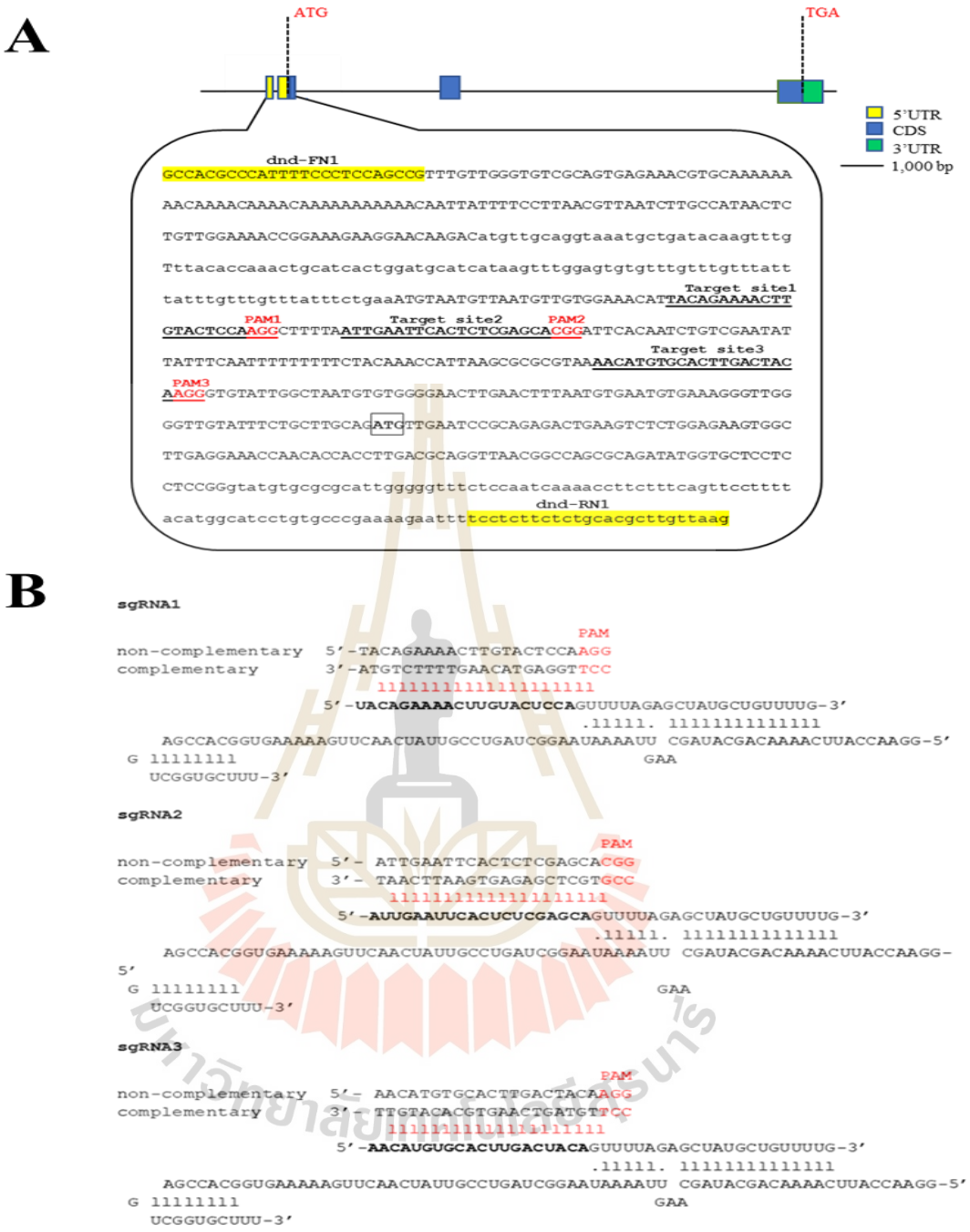

Figure 4.9 Structural map primer of *dnd* cloning form striped catfish genomic DNA for nested PCR (A). Structure of three sgRNA, consist of target specific crRNA sequence in bold letters and stem loop of tracrRNA (B).

Table 4.1 Hatching rate and survival rate at 60 dph of fishes injected with varying amounts of Cas9 protein and sgRNA (Mean±SD; N=3).

No. of sgRNA	sgRNA(ng/ul)	cas9(ng/ul)	hatching rate	survival rate at 60 dph
control un-inject			47.60±0.36[a]	51.67±2.23
normal saline - injected			32.58±1.70[b]	51.00±0.90
sgRNA1	100	100	35.12±1.04[b]	52.85±1.16
		250	33.12±0.59[b]	53.18±1.73
		500	35.37±1.06[b]	54.24±0.80
	200	100	32.31±3.29[b]	51.19±4.36
		250	34.22±4.83[b]	51.51±4.46
		500	37.02±5.09[b]	49.89±5.01
sgRNA2	100	100	36.19±2.05[b]	52.09±2.29
		250	32.97±6.62[b]	50.72±3.15
		500	36.98±6.34[b]	50.57±1.00
	200	100	31.38±5.19[b]	53.80±1.70
		250	34.10±2.82[b]	52.06±2.08
		500	33.86±2.90[b]	52.30±3.68
sgRNA3	100	100	36.56±4.23[b]	53.90±2.81
		250	34.40±4.29[b]	50.22±2.06
		500	33.58±3.27[b]	50.85±3.09
	200	100	33.12±2.09[b]	51.18±2.94
		250	33.04±4.59[b]	*52.18±2.28*
		500	33.24±4.05[b]	51.43±2.97
p-values			0.009	0.891

4.2.3 Growth of experimental fish at 60 dph

The body weight and total length were determined at 60 dph. The body weight of fish appeared to be similar among sgRNAs/Cas9- and normal saline-injected groups and control un-injected groups ($P>0.05$). Also, the total length of fish appeared to be similar among experimental groups ($P>0.05$) (Table 4.2)

Table 4.2 The body weight and the total length at 60 dph of fishes injected with varying amounts of Cas9 protein and sgRNA (Mean±SD; N=3).

No. of sgRNA	sgRNA(ng/ul)	cas9(ng/ul)	body weight (g)	body Length (cm)
control un-inject			13.50±2.34	12.93±0.40
normal saline injected			12.58±0.78	12.57±0.51
sgRNA1	100	100	12.43±1.34	12.60±0.61
		250	13.40±1.87	12.77±0.68
		500	12.90±2.42	12.60±0.92
	200	100	14.43±2.28	13.03±0.96
		250	13.27±2.34	12.90±0.66
		500	14.67±1.19	12.90±0.66
sgRNA2	100	100	13.80±1.54	13.40±0.46
		250	14.53±0.25	13.17±0.21
		500	13.07±1.39	12.57±0.40
	200	100	12.73±2.08	12.47±0.50
		250	12.67±2.15	12.90±0.46
		500	13.50±1.28	12.60±0.61
sgRNA3	100	100	12.77±1.50	12.40±0.69
		250	13.70±1.76	12.90±0.36
		500	13.03±0.85	12.33±0.49
	200	100	12.8±1.01	12.50±0.78
		250	13.47±1.36	12.87±0.50
		500	12.30±2.72	12.33±0.85
p-values			0.941	0.813

4.2.4 Visualization of gonad at 60 dph after sgRNA/Cas9 injection

The injected and control fish were raised in 100 L tank until 60 days post hatching then the fishes were corrected gonads. Gonads were embedded and serial section to a thickness of 5 μm then the gonad were determined germ cell visualization using antisense-*dnd*-cRNA probe and antisense-*vasa*-cRNA probe for in situ hybridization. For the result showed that, the strongest signals were detected

with antisense *dnd* and *vasa* probe. It was observed in tissue gonad of control group. By contrast, both of antisense probe were detected a few signals in embryos injected with mixture of sgRNA+Cas9 protein (Figure 4.10 B, B' and C, C'). Moreover, the gonads were embedded and serial section to a thickness of 5 µm then stained with hematoxylin and eosin for observation and counting number of germ cell. The gonad was observed in localization of germ cell, in the control group were filled with primordial germ cells. On the other hand, the injected sgRNA/cas9 group were found a few of primordial germ cell when compare with control group (Figure 4.10 A and A').

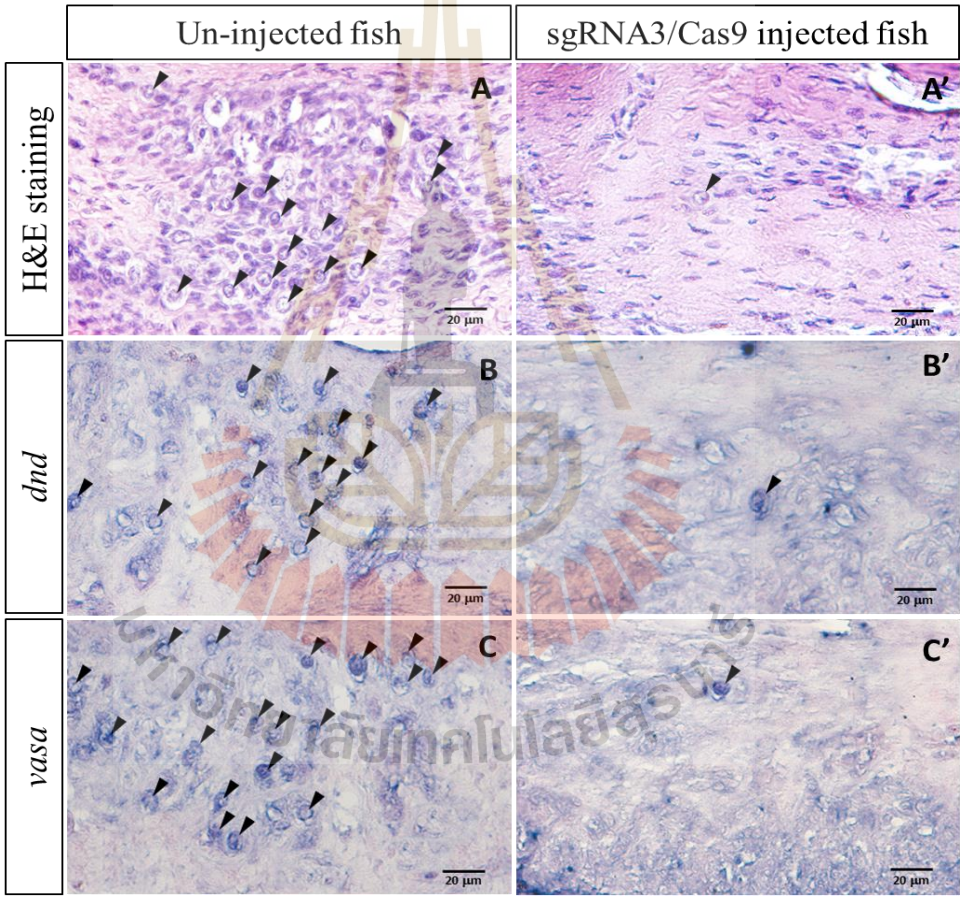

Figure 4.10 Histological and in situ hybridization using *dnd* and *vasa* antisense probe of 60 dph *dnd* knockout and control fish. The left panel shows control fish (A, B, C). The right panel shows dnd knock out fish (A', B', C'). A and A': histological H&E staining. B and B': in situ hybridization antisense *dnd* probe. C and C': in situ hybridization antisense vasa probe. Back arrowheads indicate germ cells.

4.2.5 The number of germ cell of fishes injected with varying amounts of Cas9 protein and sgRNA

The gonad at 60 dph was embedded and serial sectioned to 5 μm for staining with hematoxylin and eosin. Observation under microscope for counted the number of germ cells. From the result, statistical analyses shown a significant different between control fishes and all of varying concentration of sgRNA together with Cas9 protein. The result indicated that the number of germ cell in control fishes were higher than sgRNA/Cas9 injected. Accordingly, knock out *dnd* has effect on reduction number of germ cell in gonad. In addition, from the result shown concentration 200 ng/μl of sgRNA together with 500 ng/μl of Cas9 able to induced greatly reduce germ cell in three sgRNA. Moreover, the results indicated that target sgRNA3 induced reduce germ cell above target sgRNA 1 and 2. Following concentration 200 ng/μl of sgRNA3 and 500 ng/μl of Cas9 have 12.82% to 21.84% that lower number of germ cell (Table 4.3)

4.2.6 Indel mutation

Microinjection of sgRNA1, sgRNA2 and sgRNA3 together with Cas9 protein into one cell stage embryos led to indels mutation, which were determined by Sanger sequencing performed at 60 dph. The injected fish were showed non homologous end joining (NHEJ) repairing of double strand breaks DNA, the sequencing was showed indel mutation including insertion, deletion and substitution. The indel was showed in all treatment; sgRNA1, sgRNA2 and sgRNA3 and varying concentration sgRNA/Cas9 protein. Our result was observed deletion ranging from 1-16 nucleotides, insertion ranging from 1-13 nucleotides and substitution ranging from 1-13 nucleotides. In addition, the injected fish was showed the most of NHEJ repairing is substitution and the deletion, insertion respectively (Table 4.4).

Table 4.3 The number of germ cell of fishes injected with varying amounts of Cas9 protein and sgRNA (Mean ± SD; N=3).

No. of sgRNA	sgRNA(ng/ul)	cas9(ng/ul)	no. of germ cell
control			867.67 ± 19.50^{a}
sgRNA1	100	100	92.00 ± 21.63^{bc}
		250	73.00 ± 11.79^{bcde}
		500	41.67 ± 11.93^{bcde}
	200	100	89.33 ± 2.89^{bc}
		250	78.00 ± 7.55^{bcd}
		500	54.33±6.03^{bcde}
sgRNA2	100	100	81.33 ± 51.48^{bcd}
		250	40.33 ± 12.90^{bcde}
		500	33.00 ± 5.20^{bcde}
	200	100	56.67 ± 24.54^{bde}
		250	31.67 ± 3.79^{bcde}
		500	26.67 ± 7.23^{de}
sgRNA3	100	100	73.00 ± 44.44^{bcde}
		250	33.33 ± 6.81^{bcde}
		500	31.33 ± 9.07^{cde}
	200	100	59.00 ± 20.95^{bcde}
		250	25.33 ± 3.21^{de}
		500	17.33 ± 4.51^{e}
p-values			>0.001

Table 4.4 Conclusion of number of indel mutation fish.

sgRNA	Concentration sgRNA/Cas9 (ng/µl)	No of mutants			Indel mutation
		R1	R2	R3	
sgRNA1	100/100	3/3	2/3	4/4	Deletion/Insertion/Substitution
	100/250	3/3	3/3	2/4	Substitution
	100/500	3/3	3/3	4/4	Deletion/Insertion/Substitution
	200/100	3/3	3/3	4/4	Insertion/Substitution
	200/250	3/3	3/3	4/4	Substitution
	200/500	2/3	3/3	4/4	Deletion /Substitution
sgRNA2	100/100	3/3	3/3	4/4	Insertion/Substitution
	100/250	3/3	3/3	4/4	Deletion/ Substitution
	100/500	3/3	3/3	4/4	Deletion/ Substitution
	200/100	3/3	3/3	4/4	Deletion/Insertion/Substitution
	200/250	2/3	3/3	3/4	Deletion/ Substitution
	200/500	3/3	3/3	4/4	Substitution
sgRNA3	100/100	3/3	3/3	4/4	Deletion /Substitution
	100/250	3/3	3/3	4/4	Insertion/Substitution
	100/500	3/3	3/3	4/4	Deletion /Substitution
	200/100	3/3	3/3	4/4	Deletion /Substitution
	200/250	3/3	3/3	4/4	Insertion/Substitution
	200/500	3/3	3/3	4/4	Insertion/Substitution

4.2.7 Gene expression analysis

The injected and the control fish at 6-month-old were sampling gonad then the gonad was extracted total RNA and performed quantitative RT-PCR. In our study, we used the germ cell marker gene for determined the level gene expression including *cxcr4b*, *dazl*, *nanos1*, *nanos2*, *vasa* and *dnd*. The result showed that the level of germ cell gene marker expression was observed same trended, the target sgRNA3 was showed the lower mRNA expression level when compare with sgRNA1 and sgRNA2. The result showed significant difference in varying concentration of

sgRNA and Cas9 protein. Moreover, in concentration 500 ng/µl of Cas9 protein led to lowest mRNA expression level (Figure 4.11 and 4.12). Suggesting that the optimum condition for decreasing mRNA level of gene that play important role for PGCs survival and migration is concentration 200 ng/µl of sgRNA3 and 500 ng/µl of Cas9 protein.

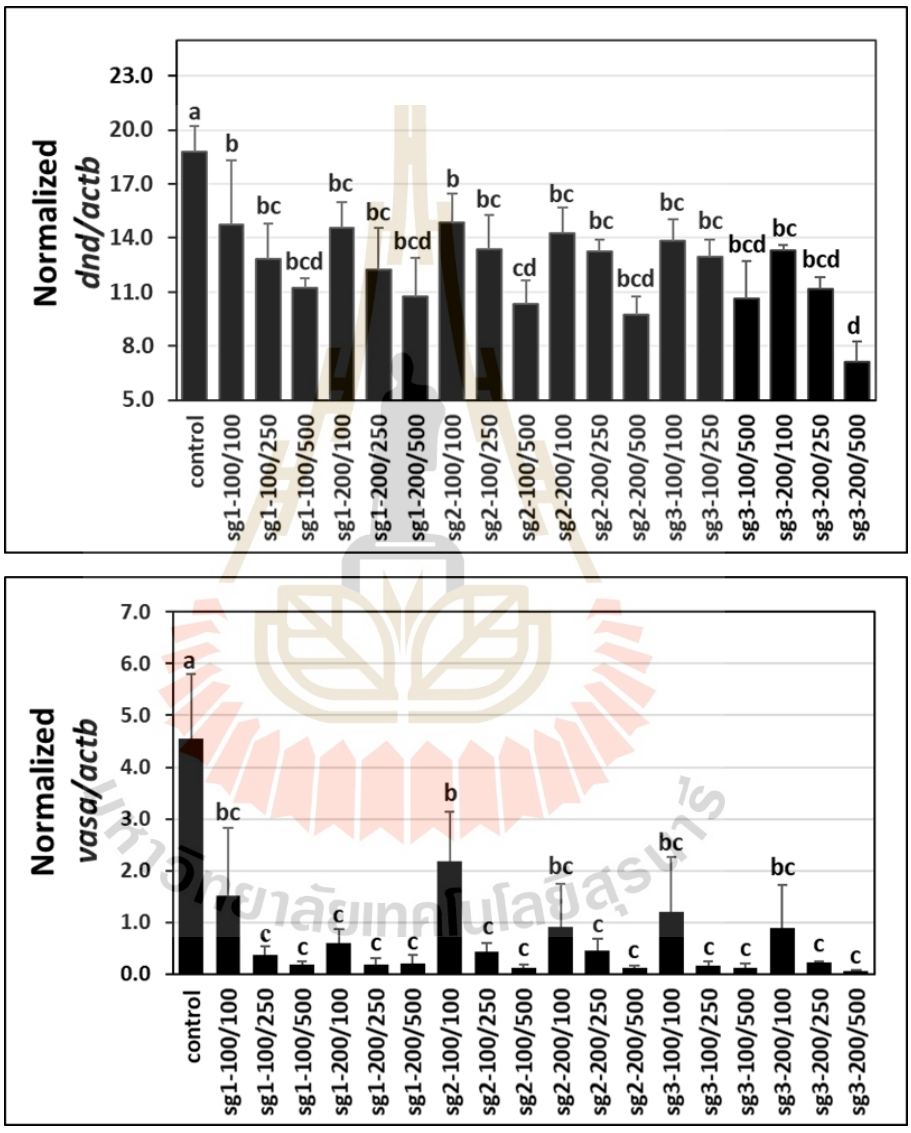

Figure 4.11 Expression levels of *dnd* and *vasa* of gonad tissue of striped catfish at 6-month-old.

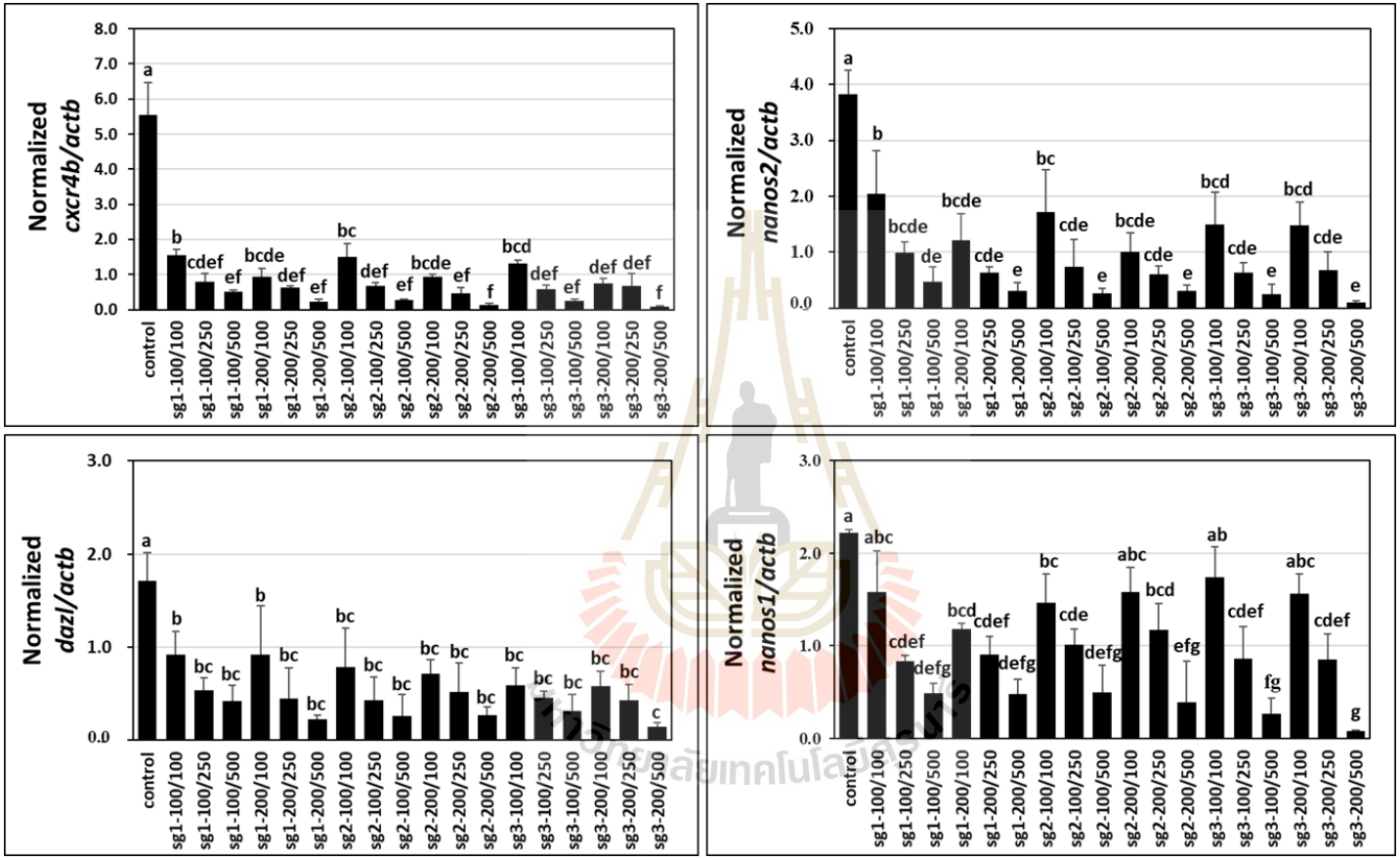

Figure 4.12 Expression levels of *cxcr4b, dazl, nanos1* and *nanos2* of gonad tissue of striped catfish at 6-month-old.

4.2.8 Gonadosomatic index (GSI)

The control and injected fish at 1 year old were weighed total body and gonad for calculate the gonadosomatic index (GSI). From the result was showed the injection of 200 of ng/µl of sgRNA1, sgRNA2 and sgRNA3 together with 500 ng/µl of Cas9 protein into one cell stage embryos led to decrease GSI significantly when compared to control group. However, the concentration 100 and 250 ng/µl of Cas9 protein had no effect on GSI (Table 4.5). Suggesting that injection of 200 ng/µl of sgRNA conjunction with 500 ng/µl of Cas9 into one cell stage embryos has effect on gonadal development letter stage.

Table 4.5 Gonadosomatic index (GSI) at 1 year old (Mean ± SD; N=3).

No. of sgRNA	sgRNA(ng/ul)	cas9(ng/ul)	GSI
control			0.213±0.018[a]
sgRNA1	100	100	0.213±0.014[a]
		250	0.207±0.010[a]
		500	0.157±0.007[b]
	200	100	0.217±0.013[a]
		250	0.196±0.006[a]
		500	0.155±0.004[b]
sgRNA2	100	100	0.218±0.007[a]
		250	0.197±0.007[a]
		500	0.155±0.001[b]
	200	100	0.201±0.009[a]
		250	0.206±0.008[a]
		500	0.151±0.003[b]
sgRNA3	100	100	0.215±0.004[a]
		250	0.206±0.012[a]
		500	0.149±0.003[b]
	200	100	0.201±0.003[a]
		250	0.203±0.004[a]
		500	0.144±0.003[b]
p-values			>0.001

4.2.9 Visualization of immature testis and ovary at 1 year old after sgRNA/Cas9 injection

Histological at this stage, in female was showed filled with previtellogenic oocyte and oogonia and connective tissue, blood vessels in immature ovary of control group. Oppositely, a few of previtellogenic oocyte was observed at localization of germ cell in sgRNA/Cas9 injected fish. Similarly, in male was permitted with spermatogonia and connective tissue, blood vessels in immature testis of control group. On the other hand, testis tissue was contained a few spermatogonia and many holes at localization of germ cell were observed in sgRNA/Cas9 injected fish (Figure 14.3).

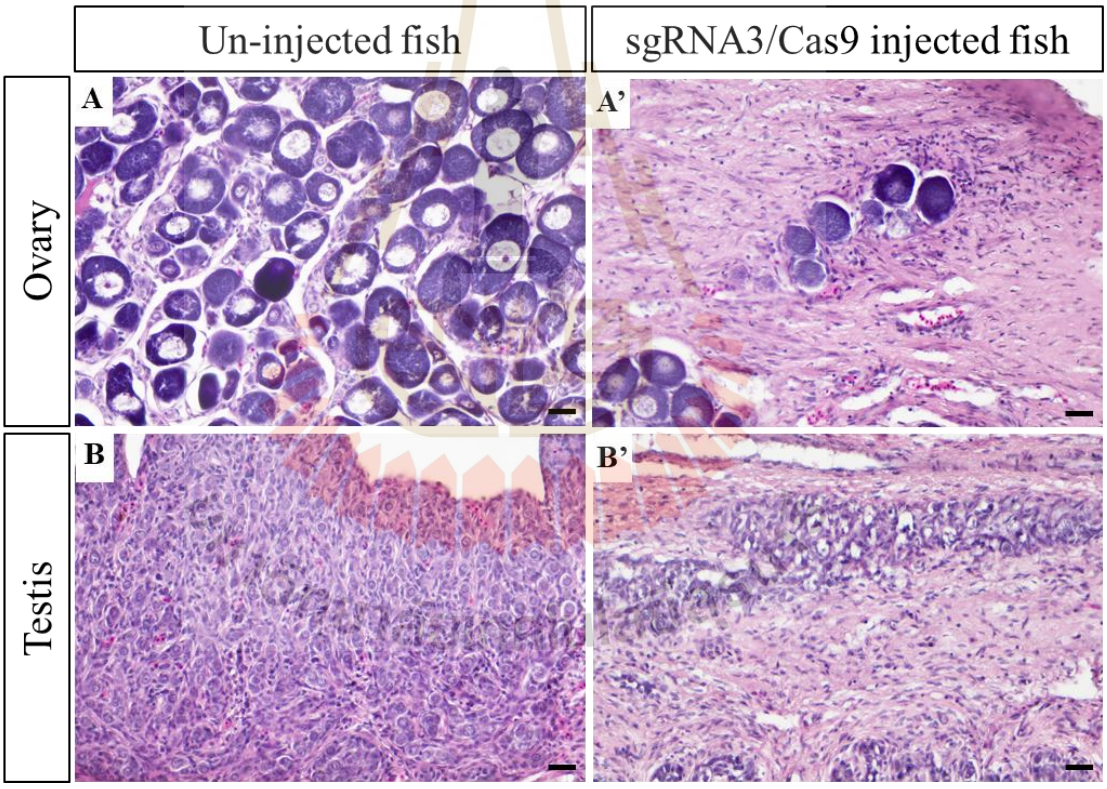

Figure 4.13 Histology of gonad at 1-year-old of striped catfish. The left panel shows control un-injected fish (A and B). The right panel shows knock out fish (A' and B'). Sections of immature ovary (A and A') and immature testis (B and B'). Scale bar represent 20 μm.

CHAPTER V
DISCUSSION

For experiment I, germ cell markers can serve as useful tools for the investigation of gonadal development and molecular reproductive research in fish. Among the germ cell markers, *dnd* which encodes an RNA-binding protein, plays an important role in the migration, survival, and proliferation of PGC (Weidinger et a., 2003; Baloch et al., 2021). In the present study, we cloned and characterized *dnd* and demonstrated its specific differential expression levels in the ovary and testes. Our information on *dnd* can be useful for further studies on its function during gonad development and its application in reproductive biotechnology.

Previous characterization of *dnd* in olive flounder (Wang et al., 2015), gibel carp (Li et al., 2016), medaka (Liu et al. 2009) and turbot (Lin et al., 2013) demonstrated that *dnd* contains six conserved motifs, including the NR, RRM, and CR1-4. In addition, characterization of Dnd proteins has revealed conserved RRM1-2 motifs and DSRM (double-stranded RNA recognition motifs) in starry flounder (Yoon et al., 2021), rare minnow (*Gobiocypris rarus*) (Duan et al., 2015) and tambaqui (*Colossoma macropomum*) (Vasconcelos et al., 2019). In this study, the deduced amino acid sequence of Dnd contained a typical RRM and five conserved regions (NR and CR1-4). The RRM is reported to be the RNA-binding domain that is necessary for the regulation of protein localization in germ cells (Slanchev et al., 2009) and has been reported to play an important role in PGC development and survival (Liu et al., 2009; Slanchev et al., 2009). Overall, Dnd contained the essential motifs of a typical Dnd homologue. Phylogenetic analysis showed that Dnd clustered with the Dnd families of teleost and showed the highest identity with *Pangasianodon gigas* and *Pangasius djambal* in the family Pangasiidae. Taken together, Dnd showed a consensus sequence of the typical domain structures of Dnd. It showed high sequence homology with other teleosts and clustered with teleost Dnd proteins

Using RT-PCR, our results showed that *dnd* specifically expressed in the ovaries and testes of striped catfish. It has been demonstrated that *dnd* specifically expressed in the testes and ovaries of gibel carp (Li et al., 2016), medaka (Liu et al., 2009), turbot (Lin et al., 2013), rere minnow (Duan et al., 2015), and tampaqui (Vasconcelos et al., 2019), Atlantic salmon (*Salmo salar*) (Nagasawa et al., 2013), and pacific bluefin tuna (*Thunnus orientalis*) (Yazawa et al., 2013). In addition, we determined the expression levels of *dnd* in the testes, ovaries, and other tissues. Notably, very low expression of *dnd* was found in the brain, gill, muscle, liver, spleen, and intestine, suggesting that *dnd* might not play an essential role in these tissues. Using qPCR analysis, our findings showed that in adult striped catfish, the expression of *dnd* in the ovary was higher than that in the testes.

This high expression of *dnd* in the ovary suggests its accumulation as a maternal RNA in eggs corroborating with other studies (Baloch et al., 2021). Similarly, higher expression in ovary, comparing to that in testis, was demonstrated in olive flounder (Wang et al., 2015) and starry flounder (Yoon et al., 2021). However, variable dimorphic expression of *dnd* between male and females has been observed in turbot, depending on age (Lin et al., 2013). Therefore, differential expression of *dnd* between the testis and ovary in fish might depend on the fish species and developmental age.

For experiment II, development of germ cell transplantation to establish surrogate broodstock technology in fish has been achieved, and its application has been provided for breeding process in not only aquaculture industries but also conservation approaches

Microinjection has been widely used to deliver biological substances into fertilized embryos at early developmental stages in fish; however, there were reports of microinjection injury at early embryonic development. For example, microinjection led to lower hatching rate of embryos in channel catfish (*Ictalurus punctatus*) and sterlet (*Acipenser ruthenus*) but not for survival rate at later stage (Coogan et al., 2022; Baloch et al., 2019). However, there were no significant differences in survival rate of control uninjected and injected embryos/larvae at 1 and 4 dpf in fathead minnow (*Pimephales promelas*) (Maki et al., 2020). In this study, in order to develop gene editing technique, we microinjected sgRNA/Cas9 complexes into embryo at 1

cell stage. Our result showed that, compared with un-injected eggs, microinjection with either normal saline or sgRNA complexes (various concentration of sgRNA and Cas9 protein) had lower hatching rate. However, there were no significant differences in hatching rates among microinjected embryos, suggesting microinjection technique but not for sgRNA complexes caused lower hatching rate. Since there were enough injected larvae, we continued experiment to investigate the effects of gene editing with sgRNA/Cas9 complexes through 60 dph. Our findings showed that there were no significant differences in survival rates of embryos as well as growth (similar body weight and body length) among various concentration of sgRNA and Cas9 protein. Similarly, there were no significant differences in survival rate of embryos that were injected with different concentration sgRNA and Cas9 complex in starlet and medaka (Baloch et al., 2019; Ansai and Kinoshita, 2014). Gene editing for *nanos* in Nile tilapia showed that high concentration of sgRNA injection decreased survival rate (Li et al., 2014). Taken together, the concentration of sgRNA and Cas 9 complex used in this study were not toxic to fish, we therefore continued to examine the effective of various sgRNAs with their dosages and Cas9 concentration

To simply knockout gene of interest, CRISPR/Cas9-induced insertion and deletion (indel) mutations were widely applied in coding sequence to generate loss of function due to frameshift mutation and/or creating a premature stop codon (Fernandez et al., 2018). For instance, efficient gene knockout in fish using CRISPR/Cas9 system was conducted to generate double stranded break (DSB) and subsequently indel mutation by targeting protein coding sequence near to start codon (Ansai and Kinoshita, 2014; Wargelius et al., 2016; Baloch et al., 2019; Coogan et al., 2022). Recently, gene targeting in non-coding sequences which are important regions for regulating gene expression was achieved and purposed to be an efficient approach for gene knockout (Li et al., 2019). For instance, since 5'UTR and 3'UTR are the important regions controlling gene expression, point mutations in 5'UTR or 3'UTR regions influenced transcription and translation levels which allow for the investigation of 5'UTR or 3'UTR functions of a gene of interest (Zhao et al., 2017; Lim et al., 2021). It was revealed that CRISPR/Cas9-mediated indel mutation in 5'UTR lowered the expression of carotenoid isomerase gene which resulted in changes in conversion level of prolycopene to lycopene in tomato (Jayaraj et al., 2021). Our

study showed that all three different sgRNAs which were designed in 5'UTR region (76-129 upstream of start codon) of *dnd* were able to generate indel mutation including deletion (1-16 nt), insertion (1-13 nt) and substitution (1-13 nt). Application of CRISPR/Cas9 targeting to coding region was demonstrated to cause a deletion of 9 nucleotides of tyrosinase gene in fathead minnow (*Pimephales romelas*) (Maki et al., 2020) and a deletion ranging from 1-10 nucleotides of *egfp* in chinook salmon (*Oncorhynchus tshawytscha*) (Dehler et al., 2016). Unexpectedly, these indel formation had impact on lowering *dnd* mRNA for all sgRNA1-3 but not for eliminating of *dnd* transcription, and sgRNA3 (with highest concentration of both sgRNA and Cas9) had lowest level of *dnd* transcript ($P<0.05$). It might be because of mosaic mutation which was commonly observed when using CRISPR/Cas9 system in embryos (Mehravar et al., 2019). Therefore, efficient CRISPR/Cas9-mediated indel mutation could be achieved by targeting the exon region of 5'UTR which had impact on reduction of gene expression.

Application of CRISPR/Cas9 targeting genes that play important role for primordial germ cell survival and migration such as *dnd* and *nanos* (Li et al., 2014; Wargelius et al., 2016; Baloch et al., 2019) could produce embryos lacking primordial germ cell (PGC). For example, CRISPR/Cas9 which was applied for disruption of coding sequence of *nanos2* and *nanos3* led to deletion at DSB ranging from 2-14 nt and 5 nt insertion. These genes disruption resulted in PGC-deficient gonads in tilapia (Li et al., 2014). In addition, double genes knockout in Atlantic salmon (*Salmo salar L.*) using CRISPR/Cas9, targeting at CDS of *dnd* and *slc45a2* (*albino/alb*) showed PGC ablation in gonad (Wargelius et al., 2016). Coincidentally, in sterlet embryo, CRISPR/Cas9 mediated knockout *dnd1* by targeting in CDS induced embryo lacking PGCs (Baloch et al., 2019). Moreover, in rainbow trout, *dnd* knockout using CRISPR/Cas9 (targeting in CDS) was generated, and complete absent of germ cells were observed in fish at 1 year of age (Fujihara et al., 2022). Our study presented that, when compared with control uninjected fish, all sgRNA/Cas 9 complexes led to decreased number of PGCs, demonstrating that 5'UTR could be used for CRISPR/Cas9 targeting. Using histological study and in situ hybridization with *dnd* antisense RNA probe, reduction of PGC was observed in gonad obtained from 60-dph fry. Again, embryo injected with sgRNA3 (with highest concentration of sgRNA and Cas9) which

were designed to target 76 nt upstream of start codon had lowest PGC number ($P<0.05$). These findings suggested that sgRNA targeting 5'UTR and closed to start codon showed promising gene knockout effect

To confirm the effects of CRISPR/Cas9 targeting *dnd* on reduction of PGC number, qRT-PCR for genes that were reported to be germ cell marker. For example, germline specific expression of *vasa* was revealed, and its expression was used as germ cell marker to identify PGC in fish (Boonanuntansarn et al., 2016; Kobayashi et al., 2000; Duangkaew et al., 2019). Likewise, *CXCR4* is a crucial chemokine receptor that plays key roles in PGC migration in zebrafish (Li et al., 2016a). In addition, *dazl* mRNA was detected in germ cell at various developmental stages including PGC in orange-spotted grouper (*Epinephelus coioides*) (Qu et al., 2020). As germ cell marker, *nanos* used to identify as a PGCs marker in rainbow trout (*Oncorhynchus mykiss*) (Bellaiche et al., 2014) and orange-spotted grouper (Sun et al., 2017). The reduction in germ cell number in this study were correlated with the expression of these germ cell markers including *cxcr4b*, *dazl*, *nanos1*, *nanos2* and *vasa*. In addition, for in situ hybridization, not only *dnd* but also *vasa* antisense probe, reduction of number of *vasa*-positive germ cells were found and correlated with *dnd*-positive germ cells. These findings supported that all sgRNAs that targeting *dnd* in this study could exert gene knockout effects, and sgRNA3 showed strongest gene inhibition impact.

Previous study of *dnd/alb* knockout in Atlantic salmon produced germ-cell less fish. While the one-year-old control fish showed typical development of testes and ovary, their *dnd/alb* knockout fish showed atypical testicular and ovicular development, i.e., 1) ovary without primary oocytes and pre-meiotic oogonial cells, and 2) spermatogenic tubuli containing only Sertoli cell in testes (Wargelius et al., 2016). In addition, in *dnd* knockout rainbow trout, although *dnd* knockout hatchling larvae had similar PGC number to that of wild-type fish, *dnd* knockout fish at one year of age showed complete absent of germ cell (Fujihara et al., 2022). In this study, both control uninjected and sgRNA/Cas9-injected fish were continued grow through 1 year of age in hatchery condition. While normal development of immature testes and ovary (GSI = 0.213 ± 0.018) were observed in control uninjected juvenile fish, sgRNA/Cas9-injected fish showed germ cell-less testes or ovary. Additionally, significantly lower GSI were founded in sgRNA/Cas9-injected fish with highest Cas9-

injected dosage. Overall, we developed effective CRISPR/Cas 9 mediated reduction of germ-cell in striped catfish fry. Germ cell-deficient larvae have been proposed to be used as recipient fish for transplantation of immature germ cell to improve the efficiency of germ cell transplantation. For instance, knockdown of *dnd* using morpholino oligonucleotide (MO) could produce germ cell-deficient grass puffer (*Takifugu alboplumbeus*), rainbow trout and masu salmon (Yoshizaki et al., 2016; Yoshikawa et al., 2020). The germ cell-deficient recipient larvae of grass puffer and masu salmon could be used as recipient fish for xenogenic transplantation of donor tiger puffer (*T. rubripes*) and rainbow trout, respectively which matured normally and produced functional donor-derived gametes (Yoshizaki et al., 2016 Yoshikawa et al., 2020). As a useful tool for aquaculture and conservative aspect, previous reports demonstrated cryopreservation of whole testes of striped catfish which showed transplantability characteristics (Boonanuntanasarn et al., 2023). The optimized method to produce effective CRISPR/Cas 9 mediated germ cell-less striped catfish would be provided for the further use as recipient fish for germ cell transplantation technology in Pangasiid fish.

In conclusion, we demonstrated that CRISPR/Cas 9 targeting *dnd* at 5'-UTR could produce indel mutation including deletion, insertion and substitution. Effective *dnd*-knockout showing significant lower *dnd* transcript which resulted in reduction of PGC. The sgRNA targeted exon nearest start codon showed the highest knockout activity. The reduction of PGC was correlated with lower expression of genes (*cxcr4b, dazl, nanos1, nanos2* and *vasa*) that were specifically expressed in germ cell

CHAPTER VI
CONCLUSION

The partial cDNA of *dnd* contained 1,137 bp of open reading frame encoding 399 amino acid residues. The deduced amino acid sequence of the Dnd protein contained a typical RNA recognition motif (RRM) and five conserved regions, including an N-terminal region (NR) and four C-terminal regions (CR1-4). Phylogenetic analysis demonstrated that Dnd clustered within the teleost Dnd. Reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR showed that *dnd* mRNA was highly expressed in the ovaries and testes.

Injection of the mixture of sgRNA (100-200 µg/ul) and Cas9 protein (100-500 µg/ul) into one call stage embryos has no effect on hatching rate of fertilized eggs and survival rate of fish larvae as well as body weight and body length of fry. Injection of sgRNAs targeting at 5'UTR of *dnd* and Cas9 induced indel mutation. Gene knockout using sgRNAs targeting at 5'UTR of *dnd* led to reduction in the level mRNA expression in germ cell marker genes including *cxcr4b*, *dazl*, *nanos1*, *nanos2*, *vasa* and *dnd*. Gene knockout using sgRNA/Cas9 reduced number of PGCs, and lowest number of PGC was observed in sgRNA3/Cas9-injected fish with highest concentration. CRISPR/Cas 9 targeting *dnd* at 5'-UTR could produce indel mutation including deletion, insertion and substitution. The gonadosomatic index (GSI) was decreased in fish injected with 200 µg/ul of sgRNAs and 500 µg/ul of Cas9. In fish at 1 year old, sgRNA/Cas9 injected fish showed low number of previtellogenic oocyte oogonia in female and reduction of spermatogonia in male, compared with control un-injected fish. Combined together, CRISPR/Cas 9 targeting *dnd1* knockout at 5'-UTR was achieved which resulted in down-regulation of *dnd1* and lowered PGC, and the sgRNA targeted exon nearest start codon showed the highest knockout activity.

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APPENDIX

Your input sequence is 420 bp long. It contains 37 possible guide sequences.
Shown below are their PAM sites and the expected cleavage position located -3bp 5' of the PAM site.
Click on a match for the PAM NGG below to show its 20 bp-long guide sequence. (Need help? Look at the CRISPOR manual)
Colors **green**, **yellow** and **red** indicate high, medium and low specificity of the PAM's guide sequence in the genome.

Gene Models: Do not show Update
Input sequence not in genome, cannot show genome variants.

Download for: SerialCloner (free) - ApE (free) - GenomeCompiler - Benchling - SnapGene - Geneious - Vector NTI - LaserGene - Genbank - FASTA

Predicted guide sequences for PAMs

Ranked by default from highest to lowest specificity score (Hsu et al., Nat Biot 2013). Click on a column title to rank by a score.
If you use this website, please cite our paper in NAR 2018. Too much information? Look at the CRISPOR manual.

Download as Excel tables: Guides / Guides, all scores / Off-targets / Saturating mutagenesis assistant

Position/ Strand	Guide Sequence + *PAM* + Restriction Enzymes Only G- Only GG- Only A-	MIT Specificity Score	CFD Spec. score	Predicted Efficiency Show all scores Doench '16	Mor.-Mateos	Outcome Out-of-Frame	Lindel	Off-targets for 0-1-2-3-4 mismatches + next to PAM	Genome Browser links to matches sorted by CFD off-target score exons only No match, no chrom filter
145 / rev	CATGTTTTACGCGCGCTTAA *TGG* Not with U6/U3 Enzymes: *Tru1I* Cloning / PCR primers	99	98	38	31	72	86	0 - 0 - 0 - 0 - 13 0 - 0 - 0 - 0 - 0	4:intron:CASC2 4:intergenic:RP11-109J4.1-AC022120.1 4:intergenic:RP11-415C15.2-IGBP1P5 show all...

Your input sequence is 420 bp long. It contains 37 possible guide sequences.
Shown below are their PAM sites and the expected cleavage position located -3bp 5' of the PAM site.
Click on a match for the PAM NGG below to show its 20 bp-long guide sequence. (Need help? Look at the CRISPOR manual)
Colors green, yellow and red indicate high, medium and low specificity of the PAM's guide sequence in the genome.

Gene Models: Do not show Update
Input sequence not in genome, cannot show genome variants.

Download for: SerialCloner (free) - ApE (free) - GenomeCompiler - Benchling - SnapGene - Geneious - Vector NTI - LaserGene - Genbank - FASTA

Predicted guide sequences for PAMs

Ranked by default from highest to lowest specificity score (Hsu et al.,
If you use this website, please cite our paper in NAR 2018. Too much

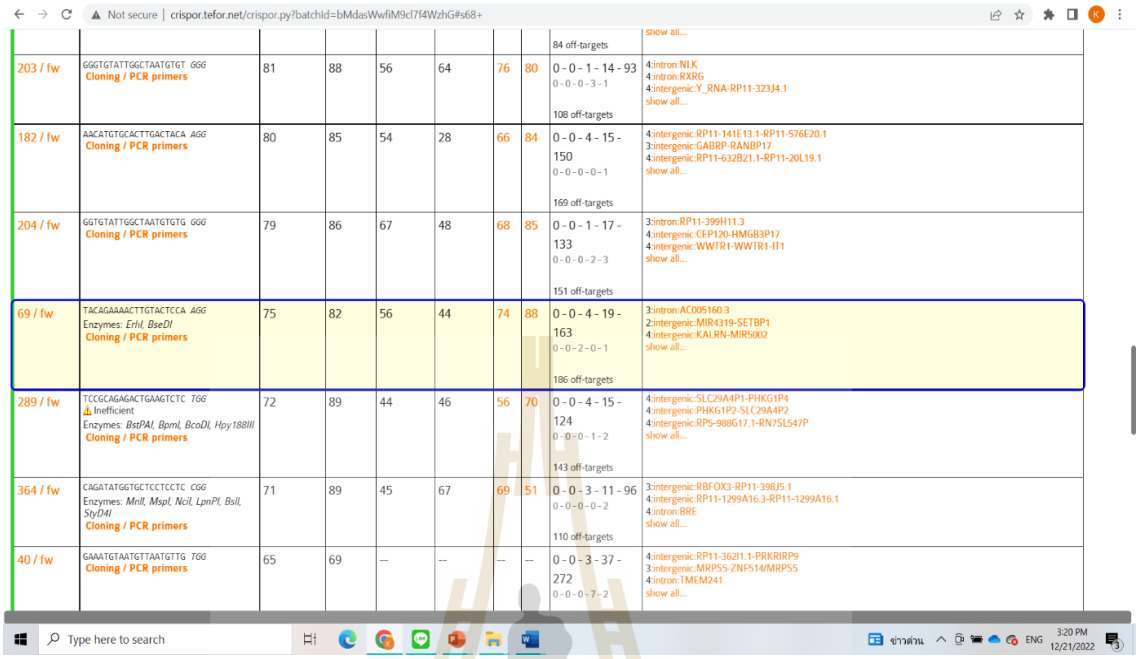
Prediction of the DNA sequence after strand break repair. Click to sort the table by frameshift/out-of-frame scores. Hover over the score names to show information about a particular score. Click a score number to see the predicted indel pattern around the guide.

Download as Excel tables: Guides / Guides, all scores / Off-targets /

Position/ Strand	Guide Sequence + *PAM* + Restriction Enzymes Only G- Only GG- Only A-	MIT Specificity Score	CFD Spec. score	Predicted Efficiency Show all scores Doench '16	Mor.-Mateos	Outcome Out-of-Frame	Lindel	Off-targets for 0-1-2-3-4 mismatches + next to PAM	Genome Browser links to matches sorted by CFD off-target score exons only No match, no chrom filter
145 / rev	CATGTTTTACGCGCGCTTAA *TGG* ⚠ Not with U6/U3 Enzymes: *Tru1I*	99	98	38	31	72	86	0 - 0 - 0 - 0 - 13 0 - 0 - 0 - 0 - 0 13 off-targets	4:intron:CASC2 4:intergenic:RP11-109J4.1-AC022120.1 4:intergenic:RP11-415C15.2-IGBP1P5 show all...

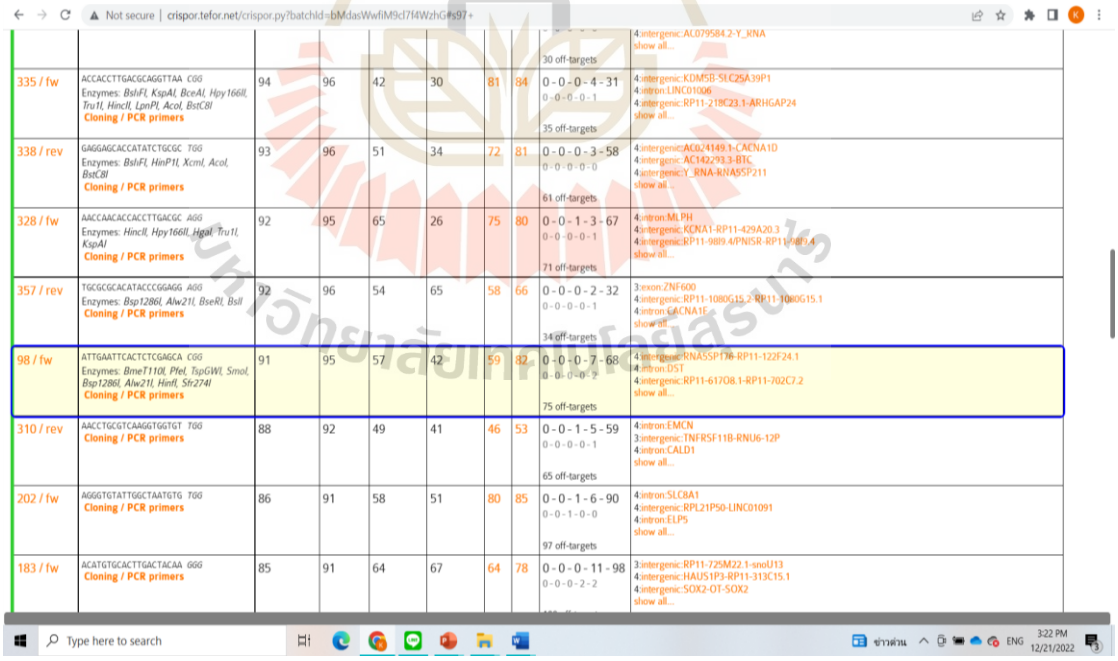
www.crispor.tefor.net/crispor.py?batchId=bMdasWwfiM9cl7f4WzhG&sortBy=oof

PAM1



								84 off-targets	show all...
203 / fw	GGGTGTATTGGCTAATGTGT GGG Cloning / PCR primers	81	88	56	64	76	80	0 - 0 - 1 - 14 - 93 0 - 0 - 0 - 3 - 1 108 off-targets	4:intron:NLK 4:intron:RXRG 4:intergenic:Y_RNA-RP11-323J4.1 show all...
182 / fw	AACATGTGCACTTGACTACA AGG Cloning / PCR primers	80	85	54	28	66	84	0 - 0 - 4 - 15 - 150 0 - 0 - 0 - 0 - 1 169 off-targets	4:intergenic:RP11-141E13.1-RP11-576E20.1 3:intergenic:GABRP-RANBP17 4:intergenic:RP11-632B21.1-RP11-20L19.1 show all...
204 / fw	GGTGTATTGGCTAATGTGTG GGG Cloning / PCR primers	79	86	67	48	68	85	0 - 0 - 1 - 17 - 133 0 - 0 - 0 - 2 - 3 151 off-targets	3:intron:RP11-399H11.3 4:intergenic:CEP120-HMGB3P17 4:intergenic:WWTR1-WWTR1-IT1 show all...
69 / fw	TACAGAAAACTTGTACTCCA AGG Enzymes: ErhI, BseDI Cloning / PCR primers	75	82	56	44	74	88	0 - 0 - 4 - 19 - 163 0 - 0 - 2 - 0 - 1 186 off-targets	3:intron:AC005160.3 2:intergenic:MIR4319-SETBP1 4:intergenic:KALRN-MIR5002 show all...
289 / fw	TCCGCAGAGACTGAAGTCTC TGG ⚠ Inefficient Enzymes: BstPAI, BpmI, BcoDI, Hpy188III Cloning / PCR primers	72	89	44	46	56	70	0 - 0 - 4 - 15 - 124 0 - 0 - 0 - 1 - 2 143 off-targets	4:intergenic:SLC29A4P1-PHKG1P4 4:intergenic:PHKG1P2-SLC29A4P2 4:intergenic:RP5-988G17.1-RN7SL547P show all...
364 / fw	CAGATATGGTGCTCCTCCTC CGG Enzymes: MnlI, MspI, NciI, LpnPI, BsiI, StyD4I Cloning / PCR primers	71	89	45	67	69	51	0 - 0 - 3 - 11 - 96 0 - 0 - 0 - 0 - 2 110 off-targets	3:intergenic:RBFOX3-RP11-398J5.1 4:intergenic:RP11-1299A16.3-RP11-1299A16.1 4:intron:BRE show all...
40 / fw	GAAATGTAATGTTAATGTTG TGG Cloning / PCR primers	65	69	--	--	--	--	0 - 0 - 3 - 37 - 272 0 - 0 - 0 - 7 - 2	4:intergenic:RP11-362I1.1-PRKRIRP9 3:intergenic:MRPS5-ZNF514/MRPS5 4:intron:TMEM241 show all...

PAM2



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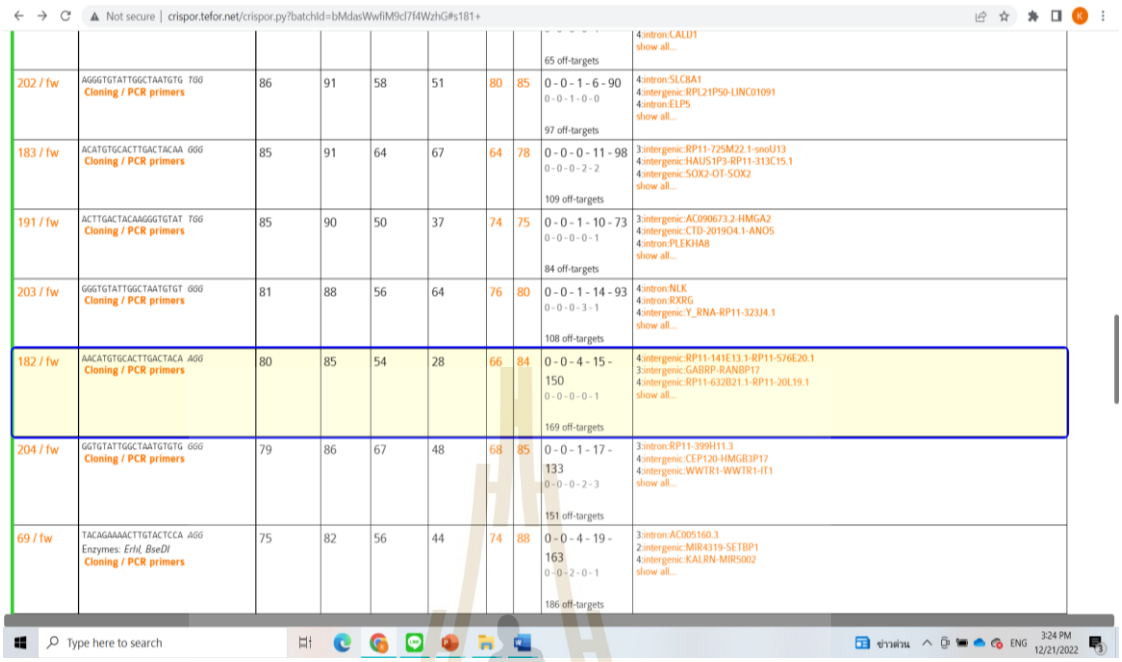
335 / fw	ACCACCTTGACGCAGGTTAA CGG Enzymes: BsiEI, KspAI, BceAI, Hpy166II, Tru1I, HincII, LpnPI, AcoI, BstC8I Cloning / PCR primers	94	96	42	30	81	84	0 - 0 - 0 - 4 - 31 0 - 0 - 0 - 0 - 1 35 off-targets	4:intergenic:KDM5B-SLC25A39P1 4:intron:LINC01006 4:intergenic:RP11-218C23.1-ARHGAP24 show all...
338 / rev	GAGGAGCACCATATCTGCGC TGG Enzymes: BsiEI, HinP1I, XcmI, AcoI, BstC8I Cloning / PCR primers	93	96	51	34	72	81	0 - 0 - 0 - 3 - 58 0 - 0 - 0 - 0 - 0 61 off-targets	4:intergenic:AC024149.1-CACNA1D 4:intergenic:AC142293.3-BTC 4:intergenic:Y_RNA-RNA5SP211 show all...
328 / fw	AACCAACACCACCTTGACGC AGG Enzymes: HincII, Hpy166II, HgaI, Tru1I, KspAI Cloning / PCR primers	92	95	65	26	75	80	0 - 0 - 1 - 3 - 67 0 - 0 - 0 - 0 - 1 71 off-targets	4:intron:MLPH 4:intergenic:KCNA1-RP11-429A20.3 4:intergenic:RP11-98I9.4/PNI5R-RP11-98I9.4 show all...
357 / rev	TGCGCGCACATACCCGGAGG AGG Enzymes: Bsp1286I, Alw21I, BseRI, BslI Cloning / PCR primers	92	96	54	65	58	66	0 - 0 - 0 - 2 - 32 0 - 0 - 0 - 0 - 1 34 off-targets	3:exon:ZNF600 4:intergenic:RP11-1080G15.2-RP11-1080G15.1 4:intron:CACNA1E show all...
98 / fw	ATTGAATTCACTCTCGAGCA CGG Enzymes: BmeT110I, PfeI, TspGWI, SmoI, Bsp1286I, Alw21I, HinfI, Sfr274I Cloning / PCR primers	91	95	57	42	59	82	0 - 0 - 0 - 7 - 68 0 - 0 - 0 - 0 - 2 75 off-targets	4:intergenic:RNA5SP196-RP11-122F24.1 4:intron:DST 4:intergenic:RP11-617O8.1-RP11-702C7.2 show all...
310 / rev	AACCTGCGTCAAGGTGGTGT TGG Cloning / PCR primers	88	92	49	41	46	53	0 - 0 - 1 - 5 - 59 0 - 0 - 0 - 0 - 1 65 off-targets	4:intron:EMCN 3:intergenic:TNFRSF11B-RNU6-12P 4:intron:CALD1 show all...
202 / fw	AGGGTGTATTGGCTAATGTG TGG Cloning / PCR primers	86	91	58	51	80	85	0 - 0 - 1 - 6 - 90 0 - 0 - 1 - 0 - 0 97 off-targets	4:intron:SLC8A1 4:intergenic:RPL21P50-LINC01091 4:intron:ELP5 show all...
183 / fw	ACATGTGCACTTGACTACAA GGG Cloning / PCR primers	85	91	64	67	64	78	0 - 0 - 0 - 11 - 98 0 - 0 - 0 - 2 - 2	3:intergenic:RP11-725M22.1-snoU13 4:intergenic:HAUS1P3-RP11-313C15.1 4:intergenic:SOX2-OT-SOX2 show all...

PAM3



								65 off-targets	4:intron:CALD1 show all...
202 / fw	AGGGTGTATTGGCTAATGTG *TGG* Cloning / PCR primers	86	91	58	51	80	85	0 - 0 - 1 - 6 - 90 0 - 0 - 1 - 0 - 0 97 off-targets	4:intron:SLC8A1 4:intergenic:RPL21P50-LINC01091 4:intron:ELP5 show all...
183 / fw	ACATGTGCACTTGACTACAA *GGG* Cloning / PCR primers	85	91	64	67	64	78	0 - 0 - 0 - 11 - 98 0 - 0 - 0 - 2 - 2 109 off-targets	3:intergenic:RP11-725M22.1-snoU13 4:intergenic:HAUS1P3-RP11-313C15.1 4:intergenic:SOX2-OT-SOX2 show all...
191 / fw	ACTTGACTACAAGGGTGTAT *TGG* Cloning / PCR primers	85	90	50	37	74	75	0 - 0 - 1 - 10 - 73 0 - 0 - 0 - 0 - 1 84 off-targets	3:intergenic:AC090673.2-HMGA2 4:intergenic:CTD-2019O4.1-ANO5 4:intron:PLEKHA8 show all...
203 / fw	GGGTGTATTGGCTAATGTGT *GGG* Cloning / PCR primers	81	88	56	64	76	80	0 - 0 - 1 - 14 - 93 0 - 0 - 0 - 3 - 1 108 off-targets	4:intron:NLK 4:intron:RXRG 4:intergenic:Y_RNA-RP11-323J4.1 show all...
182 / fw	AACATGTGCACTTGACTACA *AGG* Cloning / PCR primers	80	85	54	28	66	84	0 - 0 - 4 - 15 - 150 0 - 0 - 0 - 0 - 1 169 off-targets	4:intergenic:RP11-141E13.1-RP11-576E20.1 3:intergenic:GABRP-RANBP17 4:intergenic:RP11-632B21.1-RP11-20L19.1 show all...
204 / fw	GGTGTATTGGCTAATGTGTG *GGG* Cloning / PCR primers	79	86	67	48	68	85	0 - 0 - 1 - 17 - 133 0 - 0 - 0 - 2 - 3 151 off-targets	3:intron:RP11-399H11.3 4:intergenic:CEP120-HMGB3P17 4:intergenic:WWTR1-WWTR1-IT1 show all...
69 / fw	TACAGAAAACTTGTACTCCA *AGG* Enzymes: *ErhI, BseDI* Cloning / PCR primers	75	82	56	44	74	88	0 - 0 - 4 - 19 - 163 0 - 0 - 2 - 0 - 1 186 off-targets	3:intron:AC005160.3 2:intergenic:MIR4319-SETBP1 4:intergenic:KALRN-MIR5002 show all...

Table 4.4 Indel mutation

sgRNA	Concentration sgRNA/cas9 (ng/ul)		Mutation	Indel
sgRNA1	100/100		PAM1	
		WT	AACATTACAGAAAACTTGTACTCCAAGGCTTTT	
		T1R1-1	AACATTACAGAAAACTTGTACTCCAGGGCTCCAAGGTTTTT	8insertion
		T1R1-2	AACTTTAAAAAAAACTGGAAACCCAAGGTTTTT	6substitution
		T1R1-3	AACCTAAAAAAAAACTTTTACCCCAT-GCTTTA	6substitution,1deletion
		T1R1-4	AACATTCCAAAAAACTTGAACCCCAGGGCTTTT	4substitution
		T1R1-5	AACATTACAGTAAACTTGTACCCCAAGGTTTCT	2substitution
		T1R1-6	AACTTTACAGAAAACTTGTACTCCAAGGCTTTT	not mutation
		T1R1-7	AACTTTCCAGAAAACTTGTACCCCAAGGCTTTT	2substitution
		T1R1-8	AACATTACAGAAAACTTGTCCCCCAAGGCTTTT	2substitution
		T1R1-9	AACCTTTCAAAAAAACTTGTTCTCCCAGGCTTTT	3substitution
		T1R1-10	AACATTACAAAAAACTTGGACTCCCAGGCTTTT	3substitution
sgRNA1	100/250		PAM1	
		WT	AACATTACAGAAAACTTGTACTCCAAGGCTTTT	
		T2R1-1	AACCTTACAAAAAACTTGGACCCCCAGGGTTTT	3substitution
		T2R1-2	AACATTCCAAAAAATTTGTAAAACCTGGCTTTT	7substitution
		T2R1-3	AACATTACAAAAAACTTGTACCCCAAGGCTTTT	2substitution
		T2R1-4	AACATTCCAAAAAATTTGTAAAAACAGGCTTTT	8substitution
		T2R1-5	AACATTACAGTATACTTGTACCCCAAGGTTTCT	3substitution
		T2R1-6	AACATTTCAGAAAAATTGGTCTTCCAGGTTTTT	6substitution
		T2R1-7	AACATTACAGAAAACTTGTACTCCAAGGCTTTT	not mutation
		T2R1-8	AACATTACAGAAAACTTGTACTCCAAGGCTTTT	not mutation
		T2R1-9	AACATTCCAAAAAACTTGTAAAACCTGGCTTTT	7substitution
		T2R1-10	AACATTACAGTAAACTTGTACTCCAAGGCTTTT	1substitution

Table 4.4 Indel mutation (continued).

sgRNA	Concentration sgRNA/cas9 (ng/ul)		Mutation	Indel
sgRNA1	100/500		PAM1	
		WT	AACATTACAGAAAACTTGTACTCCAAGGCTTTT	3substitution
		T3R1-1	AACATTACAAAAAACTTGTAAACCAAGGCTTTT	3substitution
		T3R1-2	AACATTACAAAAAACTTGTCCCCCAAGGCTTTT	2substitution
		T3R1-3	AACATTACAAAAAACTTGTACCCCAAGGTTTTT	1substitution,1insertion
		T3R1-4	AACATTACAGAAATCTTTGTACCCCAAAGTTTTT	7substitution
		T3R1-5	GAAACTTCAAAAAATTATTGTTCCAAGGCTTTT	2substitution
		T3R1-6	AACTTTACAAAAAAAATTGTATTCCAAGGTTTTC	2substitution
		T3R1-7	AACTTTACAAAAAACTTGTACCCCAAGGTTTTT	4substitution
		T3R1-8	AACATTGCAGTAAACTTGGAGCCCAAGGCTTTT	9deletion,3substitution
		T3R1-9	AACATTGCAGTAAACTTGG-------CTTCT	4substitution
		T3R1-10	AACTTTACAAAAAACTTGACCCCCAAGGCTTTT	
sgRNA1	200/100		PAM1	
		WT	AACATTACAGAAAACTTGTACTCCAAGGCTTTT	8substitution
		T4R1-1	AATATCCCAGGGAACATGTTCCTCAAGGCCTTA	10substitution
		T4R1-2	AACATCTTGAAAAACAGGGCTTCCAAGGCTTTT	5substitution
		T4RT-3	AACATTACAGAAAACTTGTACTCCAAGGCTTTT	1substitution, 1insertion
		T4R1-4	TACATTGCAGAAAACTTGTACCTCCAAGGTTTCT	7substitution
		T4R1-5	AACATCTTGAAAAACTTGTCTTCCAAGGCTTTT	1insertion
		T4R1-6	AACATTTACAGAAAACTTGTACTCCAAGGCTTTT	7substitution
		T4R1-7	AACATTTTGAAAAACTTGGCTTCCAAGGCTTTT	1substitution
		T4R1-8	AACATTACAGTAAACTTGGACTCCAAGGCTTTT	5substitution
		T4R1-9	AACATTAGAAAAAACTTGTAAAACAGGGCTTTT	3substitution
		T4R1-10	AACATTTCAAAAAAATTGTTCTCCAAGGCTTTT	

Table 4.4 Indel mutation (continued).

sgRNA	Concentration sgRNA/cas9 (ng/ul)		Mutation	Indel
sgRNA1	200/250		PAM1	
		WT	AACATTACAGAAAACTTGTACTCCAAGGCTTTT	4substitution
		T5R1-1	TACATTGCAGTAAATTTGGACTCCAAGGTTTCT	11substitution
		T5R1-2	AACTTTAAAAAATACCGGAAACCTGGGGCTCTA	3substitution
		T5R1-3	AACTTTACAAAAAACTTGTCCCCCAAGGCTTTT	3substitution
		T5R1-4	AACTTTACAAAAAACTTGTCCCCCAGGGCTTTT	3substitution
		T5R1-5	AACTTTACAAAAAACTTGTCCCCCAAGGCTTTT	2substitution
		T5R1-6	AACTTTACAAAAAACTTGTACCCCAAGGCTTTT	2substitution
		T5R1-7	AACTTTACAAAAAACTTGTACCCCAAGGCTTTT	2substitution
		T5R1-8	AACTTTACAAAAAACTTGTACCCCAAGGCTTTT	2substitution
		T5R1-9	AACTTTACAAAAAACTTGTCCCCCAAGGCTTTT	2substitution
		T5R1-10	AACTTTACAAAAAACTTGTACCCCAAGGCTTTT	
sgRNA1	200/500		PAM1	
		WT	AACATTACAGAAAACTTGTACTCCAAGGCTTTT	
		T6R1-1	AACATTACAAAAAACTTGTACTCCAAGGCTTTT	1substitution
		T6R1-2	AACTTTAAAAA-AAACCTGAAACCCAGGGCTTT	10substitution, 1deletion
		T6R1-3	AACATTACAAAAAACTTGTACTCCAAGGCTTTT	1substitution
		T6R1-4	AACTTTACAAAAAACTTGTCCTCCAAGGCTTTT	2substitution
		T6R1-5	AACTTTACAAAAAACTTGTACTCCAGGGCTTTT	1substitution
		T6R1-6	AACATTAAAAAAAACTGGAAACCCGGGGCTCTA	8substitution
		T6R1-7	AACTTTACAAAAAACTTGTCCCCCAGGGCTTTT	2substitution
		T6R1-8	AACTTTACAAAAAACTTGTACCCCAGGGCTTTT	2substitution
		T6R1-9	AACTTTACAAAAAACTTGTACCCCAAGGCTTTT	2substitution
		T6R1-10	AACTTTACAAAAAACTTGTACTCCAAGGCTTTT	1substitution

Table 4.4 Indel mutation (continued).

sgRNA	Concentration sgRNA/cas9 (ng/ul)		Mutation	Indel
sgRNA2	100/100		PAM2	
		WT	TTTTAATTGAATTCTCTCTCGAGCACGGATTCA	
		T7R1-1	TTTTAATTGAATTCACTTTCGAGCATGGATTCT	3substitution
		T7R1-2	TTTTAATTGAATTTTTTATTTCCCCCGGATTCA	8substitution
		T7R1-3	TTTTAATTGAATTCAATTTTGAGCACGGTTTCC	4substitution
		T7R1-4	TTTTAATTGAATTCAATTTTGAGCACGGGTATTTGTTTCC	4substitution,7insertion
		T7R1-5	TTTAAATTGAATTCACTTTTGAGCACGGTTTCA	3substitution
		T7R1-6	TTTTAATTGAATTCTCTCTTTTAATCGGATTCA	6substitution
		T7R1-7	TTTTAATTGAATTCCCCCGGGACCCCGGATTCA	6substitution
		T7R1-8	TTTTTATTAAAGAAAAACCTGGACCCCAAATTCC	12substitution
		T7R1-9	TTTTAATTGAATTCCCCCGGGACCCCGGATTCA	6substitution
		T7R1-10	TTCAAATGGAATAAACTCTTGAGCACGGTTTCC	6substitution
sgRNA 2	100/250		PAM2	
		WT	TTTTAATTGAATTCTCTCTCGAGCACGGATTCA	
		T8T1-1	TTTTAATTGAATTAATTTCGAGCACCGGTTTTC	11substitution
		T8R1-2	TTTTATTTGATTTCTCTCTCGAGCACGGATTTA	2substitution
		T8R1-3	TCCAAGGTTTATTTTTTATTTCCCCCGGAACCA	12subsitution
		T8R1-4	TTCTAATTG----------GATTCA	16deletion
		T8R1-5	TTTTAATTGATTTCTCTCTTTTGCCCGGATTCA	5subsitution
		T8R1-6	TTTTAATTGAATTCTCTCTCTTAATCGAATTCC	6substitution
		T8R1-7	TTTTAATTCCCCCCTCTCTTTTGCACGGATTCA	8substitution
		T8R1-8	TTTTTTGTGATTTCTCTCTTCTAATTGAATTCA	11substitution
		T8R1-9	TTTTAATTGAATTCCCCCGCGACCCCGGATTCA	5substitution
		T8R1-10	TTTTAATTGATTTCTCTCTTTTAATCGAATTTC	8substitution

Table 4.4 Indel mutation (continued).

sgRNA	Concentration sgRNA/cas9 (ng/ul)		Mutation	Indel
sgRNA 2	100/500		PAM2	
		WT	TTTTAATTGAATTCTCTCTCGAGCACGGATTCA	
		T9R1-1	TTTTAATTGAATTATCTTTCGAGCTTGGATTCA	4substitution
		T9R1-2	TTTTAATTGAATTCTCTCTTTTGCACGGATTCA	3substitution
		T9R1-3	ATTAATAATT--TCTCTTTAGGCCGCGGATTCA	10substitution, 2deletion
		T9R1-4	TTTTAATTGAATTCACTTTCGAGCACGGATTCA	2substitution
		T9R1-5	TTTTAAGTGTATTCTTGAACACCCCCGGATTCA	10substitution
		T9R1-6	TTTAAATTGAATTCACTTTCGACCACGGTTTTT	3substitution
		T9R1-7	TTTTCATTGAATTATCTCTGGAGCCCGGATTCA	3substitution
		T9R1-8	TTTTAATTGAACTCTCTCTTTTAATCGGATTCA	7substitution
		T9R1-9	TTCTAATTGA----------ATTCA	15deletion
		T9R1-10	TTTTAATTGAATTCTCTCTTTTGCACGGATTCA	3substitution
sgRNA 2	200/100		PAM2	
		WT	TTTTAATTGAATTCTCTCTCGAGCACGGATTCA	1deletion
		T10R1-1	TTTTA-TTGAATTTTCTCTCGAGCACGGATTCA	11substitution
		T10R1-2	TTCCAAGGGTTTTATTTCACTACCCCCGCGTCC	5substotution
		T10R1-3	TTTTAATTGAATTCTCCGGCGACCCCGGCTTCA	1insertion
		T10R1-4	TTTTAATTGAATTCTTCTCTCGAGCACGGATTCA	7substitution
		T10R1-5	TTTTAATTGAATTCTCCGGCGACCCAGTATTCA	7substitution, 4deletion
		T10R1-6	TTTTGATTTC----CCCCGGGACCCCGGGAAGA	10substitution
		T10R1-7	TCCCAAGGGTTTTCTCTCTCTGCCCCCGCTACC	5substitution
		T10R1-8	TTTTAATTGAATTCTCCCGCGACCCAGGATTCA	11substitution
		T10R1-9	TTTTAAAGGGTTTCTCTCGAATTCCCCCTCGCC	18substitution
		T10R1-10	TTACATTACATTAACTTTGGAACCCAAAGTTAC	

Table 4.4 Indel mutation (continued).

sgRNA	Concentration sgRNA/cas9 (ng/ul)		Mutation	Indel
sgRNA 2	200/250		PAM2	
		WT	TTTTAATTGAATTCTCTCTCGAGCACGGATTCA	
		T11R1-1	TTTTAATTGAATTCTCTCTCGAGCCCGGATTCA	1substitution
		T11R1-2	TTTTAATTGAATTCTCTCTCGAGCACGGATTCA	not mutation
		T11R1-3	TTTTAATTGAATTCACTCTCGAGCACGGATTCT	1substitution
		T11R1-4	TTTTAATTGAATTAACTTTCGAGCACGGATTTT	3substitution
		T11R1-5	GTTTA----AATTCTCTCTCGAGCACGGATTCA	4deletion
		T11R1-6	TTTTAATTGAATTCAAATTTCTGTCC--AACCA	9substitution, 2deletion
		T11R1-7	TTTTAATTGAATTCTCTCTCGAGCCCGGATTCA	1substitution
		T11R1-8	TTTTAATTGAATTCTCTCTCGAGCACGGATTCA	not mutation
		T11R1-9	TTTTAATTGAATTCTCTCT------CGAGCA	8deletion
		T11R1-10	TTTTAATTGATTTCCCCGGGACCCGGATTC	7substitution
sgRNA 2	200/500		PAM2	
		WT	TTTTAATTGAATTCTCTCTCGAGCACGGATTCA	
		T12R1-1	TTTTAATTGAATTCTCTCTCGAGCCCGGATTCA	1substitution
		T12R1-2	TTTTAATTGAATTCCCTCTCGAGCCCGGATTCA	2substitution
		T12R1-3	TCCAAGGTTTTTTTTTTATTTCCCCCGGATGCC	13substitution
		T12R1-4	TTTTAATTGAATTCTCTCTCGAGCCCGGATTCA	1substitution
		T12R1-5	TTTTAATTGAATTCTCTCTCGAGCCCGGATTCA	1substitution
		T12R1-6	TTTTAATTGAATTCTCTTTTGAGCATGGTTTCT	3substitution
		T12R1-7	TTTTAATTGAATTCACTCTCGAGCATGGATTCT	2substitution
		T12R1-8	TTTTAGGTGATTTCTTTATTTCCCCCGGATTCA	10substitution
		T12R1-9	TTTTAATTGAATTATCTTTTGACCACGGATTCA	4substitution
		T12R1-10	TTTTAATTGAATTCTCTCTCGAGCCCGGATTCA	1substitution

Table 4.4 Indel mutation (continued).

sgRNA	Concentration sgRNA/cas9 (ng/ul)		Mutation	Indel
sgRNA 3	100/100		PAM3	
		WT	CGTAAAACATGCGCACTTGACTACAAGGGTGTA	
		T13R1-1	CGTAAAACATGCGCACTTGATTAC---GGTGTA	3deletion
		T13R1-2	CGTAAAACATGCGCACTTGATTACAAGGGTGTA	1substitution
		T13R1-3	CGTAAAACATGCGCACTTGATTACAAGGGTGTA	1substitution
		T13R1-4	CGTAAAACATGCGCACTTGATTACAAGGGTGTA	1substitution
		T13R1-5	CGGAAGAGGGGCCCACTGGGGTAAAGGGGAATG	10substitution
		T13R1-6	CGTTAGACATCAGCACTTGATTACAAAAGTGTA	6substitution
		T13R1-7	CGTAAAACATGCGCACTTGATTACAAGGGTGTA	1substitution
		T13R1-8	CGTAAAACATGCGCACTTGATTACAAGGGTGTA	1substitution
		T13R1-9	CGTAAAACATGCGCACTTGATTACAAGGGTGTA	1substitution
		T13R1-10	CGTAAAACATGCGCACTTGATTACAAGGGTGTA	1substitution
sgRNA 3	100/250		PAM3	
		WT	CGTAAAACATGCGCACTTGACTACAAGGGTGTA	
		T14R1-1	CGTAAAACTGGCCCCCTTGCCTCAAGGGGTGTA	8substitution
		T14R1-2	GATAACAAGGGGGGAATTGGCTAAGGGGGGGGA	13substitution
		T14R1-3	CGTAAAAAAGGCCCCTTTGACTCAAAGGGTGTA	6substitution
		T14R1-4	GATAACAAGGGGGGCATTGGCTAATGGGGGGGGA	13substitution
		T14R1-5	GATAACAAGGGGGGCATTGGCTAAGGGGGGGGA	13substitution
		T14R1-6	CGCTAGACATGAGGGCTGGACTAAAAGGGTGTA	6substitution
		T14R1-7	CGTAAAAATGGCCCCTTTGACTACAAGGGTGTA	6substitution
		T14R1-8	CGTAAGACATGTGCGGTGGATTACAAGGGTGTA	5substitution
		T14R1-9	CGTAAAACATGCGCACTTGAGGACTACATGGGTGGACAGGCGTGTA	2substitution,13insertion
		T14R1-10	CGCACTGGATAACAAGGGGGCATCGGCCAAA	16substitution

Table 4.4 Indel mutation (continued).

sgRNA	Concentration sgRNA/cas9 (ng/ul)		Mutation	Indel
sgRNA 3	100/500		PAM3	
		WT	CGTAAAACATGCGCACTTGACTACAAGGGTGTA	8substitution
		T15R1-1	CGTTAGACATCAGCGGTGTATTACAAGGGTGTC	1substitution
		T15R1-2	CGTAAAACATGCGCACTTGATTACAAGGGTGTA	13substitution,1deletion
		T15R1-3	ATTAAAAGGGGGGATT-GGCCAAAGGGGGGGGA	1substitution
		T15R1-4	CGTAAAACATGCGCACTTGATTACAAGGGTGTA	9substitution
		T15R1-5	CGTAAACCTCGCGAACAACATTACAACTGACTA	9substitution
		T15R1-6	GGTAAAAAAGGGGCAATGGGCTAAAGGGGGGGG	7substitution
		T15R1-7	CGTTAGACATGAGGGGTGTATTACAAGGGTGTA	12substitution
		T15R1-8	GTGAAGAGGAGCCCATTGGTTTATTGGGGAAGG	1substitution
		T15R1-9	CGTAAAACATGCGCACTTGACTACAAGGGTGTA	5substitution
		T15R1-10	CGTAAGACATGTGCGGTGGATTACAAGGGTGTA	
sgRNA3	200/100		PAM3	
		WT	CGTAAAACATGCGCACTTGACTACAAGGGTGTA	
		T16R1-1	CGTAAAACATGCGCACTTGATTACAAGGGTGTA	1substitution
		T16R1-2	CGTAAAACATGCGCACTTGATTACAAGGGTGTA	1substitution
		T16R1-3	CGTAAAACATGCGCAC-------AAGGGTGTA	8deletion
		T16R1-4	CGTAAAACATGCGCACTTGATTACAAGGGTGTA	1substitution
		T16R1-5	CGTAAAACAGGCCCCCTTGACTCAAGGGGTGTA	6substitution
		T16R1-6	CGTAAAACATGCGCACTTGATTACAAGGGTGTA	1substitution
		T16R1-7	CGTAAAACATGCGCACTTGATTACAAGGGTGTA	1substitution
		T16R1-8	CGTAAAACATGCCCCCTTGGCTAAAAGGGTGTA	4substitution
		T16R1-9	CGTAAAACTTGCGCACTTGATTACAAGGGTGTA	1substitution
		T16R1-10	GTAAAAACTTGCCCCCTTGCCTCCAAGGGTGTA	5substitution

Table 4.4 Indel mutation (continued).

sgRNA	Concentration sgRNA/cas9 (ng/ul)		Mutation	Indel
sgRNA 3	200/250		PAM3	
		WT	CGTAAAACATGCGCACTTGACTACAAGGGTGTA	
		T17R1-1	TTAAAAACCGGCACACTTGCCCACTGGGGTACA	7substitution
		T17R1-2	CGTAAAACTGGCCCCTTTGACTACAAGGGTGTA	5substitution
		T17R1-3	CGTAAAAAATGCCCCTTTGCATACAGGGGTGTA	6substitution
		T17R1-4	CGTAAAAATTGCCCCTTTGACTACAGGGGTGTA	6substitution
		T17R1-5	CGTAAAACATGCGCACTTGACTAACAAGGGTGTA	1insertion
		T17R1-6	CGTAAAACATGCGCACTTGATTACAAGGGTGTA	1substitution
		T17R1-7	CGTAAAACATGCCCCTTTGCCTCCAGGGGTGTA	6substitution
		T17R1-8	GGTAAAAAGGGGGCATTGGCTAAAGGGGGGGGA	7substitution
		T17R1-9	GATAACAAGGGGGCATTGGCTAAGGGGGGGGGA	13substitution
		T17R1-10	GCTAACAAGGGGGCATTGGCTAAAGGGGGGGGA	13substitution
sgRNA 3	200/500		PAM3	
		WT	CGTAAAACATGCGCACTTGACTACAAGGGTGTA	
		T18R1-1	CGTAAAACATGCGCACTTGATTACAAGGGTGTA	1substitution
		T18R1-2	CGTAAAACATGCCCCCTTGCCTCCAAGGGTGTA	4substitution
		T18R1-3	CGTAAAACATGCGCCCTTGCCTCCAAGGGTGTA	3substitution
		T18R1-4	CAAACATTGGCCCCATTGGTTTATTGGGGAATG	13substitution
		T18R1-5	CGGAAGTGGGGCCCACTGGTGTATTGGGGAATA	12substitution
		T18R1-6	CGTAAAACAGGGCCCTTTGCTTCCAAGGGTGTA	8substitution
		T18R1-7	CGTAAAACATGGTGTATTGGCTAATGTGTGGGG	10substitution
		T18R1-8	GATAACAAGGGGGGATTGGCACAAGGGGGGGGGA	7substitution, 1insertion
		T18R1-9	CATAAAAAGGGGGGATTGGCTAAAGGGGGGGGGA	12substitution,1insertion
		T18R1-10	CCATAACAAGGGGGGAATTTGGCAAATGGGGGGGG	12substitution,1insertion

BIOGRAPHY

Kunlanan Booncherd born on 20 November 1996 in Huaithalaeng, Nakhon Ratchasima, Thailand. In 2015 graduated high school from Huaithalaeng Pittayakom school, Huaithalaeng, Nakhon Ratchasima. In 2019 graduated the Bachelor's degree in Animal Production Technology, institute of Agricultural technology, Suranaree University of Technology, Nakhon Ratchasima. In 2019 began a master's studies in Biotechnology for Aquaculture, Institute of Agriculture Technology. Suranaree University of Technology, Nakhon Ratchasima, Thailand.