THE STUDY OF ESTROGEN RECEPTOR IN SANDWORM *PERINEREIS NUNTIA*

BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
IN BIOMEDICAL SCIENCES
FACULTY OF SCIENCE

GRADUATE SCHOOL, RANGSIT UNIVERSITY
ACADEMIC YEAR 2017

การศึกษายีนสร้างโปรตีนคล้ายตัวรับเอสโตรเจนในพ่อแม่พันธุ์เพรียงทราย *PERINEREIS NUNTIA*

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โดย

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วิทยานิพนธ์ฉบับนี้เป็นส่วนหนึ่งของการศึกษาตาม

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31 พฤษภาคม 2561

Thesis entitled

THE STUDY OF ESTROGEN RECEPTOR IN SANDWORM

PERINEREIS NUNTIA

by

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was submitted in partial fulfillment of the requirements

for the degree of Master of Biomedical Sciences

Rangsit University

Academic Year 2017

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May 31, 2018

ACKNOWLEDGEMENTS

I would like to express my gratitude to all those who gave me the possibility to complete this thesis. First, my sincere grstitude and deep appreciation are expressed to my major advisor, Dr.Gun Anantasomboon from Rangsit University. I'm appreciated assistance for a good knowledge of educate and extensive support with care in the subject also document review instruction and good suggestions including the document review and edit all of the defects until the research and the preparation of this thesis is complete. My sincere appreciation is also expressed to Assoc.Prof.Dr.Kanokpan Wongprasert, my chairman, for her advice, guidance, and kidness through the course of this study. I am gratefully indebted to Asst.Prof.Pol.Lt.Dr.Acharawan Thongmee my co-advisor, for her guidance, continuous discussion, and many suggestions to make me carrying out my successful study. I am also gratefully to Asst.Prof.Dr.Patamaporn Sukplang, my committee, for advise and suggestions for doing this research.

Finally, I would like to thank my parents and friends for encouragement, assistance and always support in all aspects and helped me a lot in finalizing this project within the limited time frame

Chotip Phooim

Researcher

กิตติกรรมประกาศ

วิทยานิพนธ์ฉบับนี้ สำเร็จลุล่วงไปได้ด้วยดี ด้วยความกรุณา และ ความอนุเคราะห์อย่างสูง จากบุคลากรผู้ทรงคุณวุฒิหลายท่าน ทั้งนี้จึงใคร่ขอกราบขอบพระคุณ อาจารย์ที่ปรึกษาวิทยานิพนธ์ ดร.กัญ อนันตสมบูรณ์ อาจารย์ประจำมหาวิทยาลัยรังสิต ที่ได้ประสิทธิประสาทวิชาความรู้ และให้การสนับสนุนในทุก ๆ ด้านด้วยความเอาใจใส่ รวมทั้งให้ข้อแนะนำ การตรวจทานเอกสาร การให้ข้อเสนอแนะ และแก้ไขข้อบกพร่องต่าง ๆ ในทุกขั้นตอนของการทำงานวิจัย จนทำให้การศึกษาวิจัย และการจัดทำวิทยานิพนธ์ฉบับนี้เสร็จสมบูรณ์

ขอกราบขอบพระคุณ รศ.ดร.กนกพรรณ วงศ์ประเสริฐ ที่ให้คำแนะนำ และข้อเสนอแนะในการทำงานวิจัยนี้

ขอกราบขอบพระคุณ ผศ.รตท.หญิง ดร.อัจฉราวรรณ ทองมี ให้ความช่วยเหลือ และให้การสนับสนุนในการศึกษาวิจัยครั้งนี้ในทุก ๆ ด้าน ด้วยความเมตตากรุณา รวมทั้งให้ความรู้ ข้อแนะนำ และการแก้ไขปัญหาต่าง ๆ ทางห้องปฏิบัติการในการศึกษาวิจัย และ จัดทำวิทยานิพนธ์ฉบับนี้ จนเสร็จสมบูรณ์

ขอขอบคุณ ผศ.ดร.ปถมาพร สุกปลั่ง ที่ให้คำแนะนำข้อเสนอแนะในการทำงานวิจัยนี้

สุดท้ายนี้ ขอขอบคุณบิดา มารดา และเพื่อนทุกคน ที่ให้กำลังใจ ความช่วยเหลือ และให้การสนับสนุนในทุกด้านตลอดมา จนกระทั่งงานวิจัยนี้สำเร็จลุล่วงด้วยดี

ช่อทิพย์ โพธิ์อิ่ม

ผู้วิจัย

5607500 : MAJOR: BIOMEDICAL SCIENCES;

M.Sc. (BIOMEDICAL SCIENCES)

KEYWORDS : ESTROGEN RECEPTOR, SANDWORM, *PERINEREIS NUNTIA*

CHOTIP PHOOIM: THE STUDY OF ESTROGEN RECEPTOR IN SANDWORM *PERINEREIS NUNTIA*. THESIS ADVISOR: GUN ANANTASOMBOON, Ph.D., THESIS CO-ADVISOR: ASST.PROF.POL.LT.ACHARAWAN THONGMEE, Ph.D., 70 p.

The objective of this research is to study the estrogen receptor (*ER*) gene fragment in marine sandworm, *Perinereis nuntia,* broodstocks. The presumption of this study is that the reproductive sandworm may generate and use estrogen to control inter-cellular group for reproductive functions as was reported in Korean lugworm, *P. aibuhitensis* and other vertebrate species. Actions of estrogen at its target cells are commonly known since it binds with the ER. Six brooders of sandworm at 5-6 months of age and 5 pre-reproductive animals at 2-3 months old were examined. Histological study was used for identifying the reproductive stage of sandworms. Genomic DNAs were also extracted and tested for the estrogen receptor gene by using PCR and DNA sequencing analyses. Histological study revealed that the coelomic cavity of brooders was fulfilled with a numerous of ova or sperms. Result from PCR amplification of estrogen receptor gene fragment in all six sandworm broodstocks exhibited PCR product at molecular weight of 200 bp. However, the retrieved PCR fragment was not identity with *ER* sequence of *P. aibuhitensis*. For ER protein synthesis and deposition, indirect immunohistochemistry was determined and revealed positive immunoreactions specific for ER-beta with clusters of cells closed to the stump of parapodia of the brooders. An intense immunoreaction also deposited at the cytoplasm of the ovum. The results from this study indicate that ER found in sandworm broodstocks was likely related to the process of reproductive cell generation and the completion of reproductive system. The expressed ER could be used as biomarker to verify the completion of reproductive growth in *P. nuntia*. Moreover, the research should be continuously studied for more information on a complete sequence of *ER* gene, as well as time-course of ER expression by this mentioned gene in sandworms.

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คำสำคัญ : ESTROGEN RECEPTOR, SANDWORM, *PERINEREIS NUNTIA*

ช่อทิพย์ โพธิ์อิ่ม : การศึกษายีนสร้างโปรตีนคล้ายตัวรับเอสโตรเจนในพ่อแม่พันธุ์เพรียงทราย *PERINEREIS NUNTIA* (THE STUDY OF ESTROGEN RECEPTOR IN SANDWORM *PERINEREIS NUNTIA*) อาจารย์ที่ปรึกษา: ดร.กัญ อนันตสมบูรณ์, อาจารย์ที่ปรึกษาร่วม: ผศ.รตท.หญิง ดร.อัจฉราวรรณ ทองมี, 70 หน้า.

การศึกษาวิจัยนี้มีวัตถุประสงค์เพื่อศึกษายีนตัวรับฮอร์โมนเอสโตรเจน (Estrogen Receptor Gene) ในเพรียงทรายสายพันธุ์ *Perinereis nuntia* วัยเจริญพันธุ์ โดยมีสมมติฐานว่าเพรียงทรายสายพันธุ์นี้มีการสร้างและใช้ฮอร์โมนเอสโตรเจนเพื่อควบคุมการทำงานระหว่างกลุ่มเซลล์ของระบบสืบพันธุ์เหมือนกับรายงานที่ตรวจพบกับเพรียงทรายสายพันธุ์ *P. aibuhitensis* และสัตว์มีกระดูกสันหลังทั่วไป ซึ่งฮอร์โมนเอสโตรเจนจะออกฤทธิ์ที่เซลล์เป้าหมายโดยการจับกับตัวรับฮอร์โมนเพศชนิดนี้ ตัวอย่างพ่อแม่พันธุ์เพรียงทรายอายุ 5-6 เดือนเพศละ 6 ตัว และเพรียงทรายก่อนวัยเจริญพันธุ์อายุ 2-3 เดือนจำนวน 5 ตัว ถูกนำมาตรวจสอบชิ้นเนื้อเพื่อยืนยันระยะเจริญพันธุ์ของเพรียงทรายที่นำมาศึกษา แยกสกัดตัวอย่าง DNA เพื่อตรวจสอบยีนตัวรับเอสโตรเจนโดยวิธี PCR และ DNA sequencing ผลการศึกษาเนื้อเยื่อพบว่าพ่อแม่พันธุ์เพรียงทรายแต่ละเพศมีการผลิตเซลล์ไข่และอสุจิจำนวนมากสะสมภายในช่องว่างกลางลำตัว ผลการตรวจสอบชิ้นส่วนของยีนตัวรับเอสโตรเจนจากตัวอย่างเพรียงทรายวัยเจริญพันธุ์ทั้ง 6 ตัว ได้แถบผลผลิต PCR มีขนาดโมเลกุลประมาณ 200 bp โดยพบว่ามีลำดับเบสไม่ตรงกับตัวรับฮอร์โมนเอสโตรเจนของเพรียงทรายสายพันธุ์ *P. aibuhitensis* เมื่อตรวจสอบตำแหน่งที่มีการสร้างและสะสมของโปรตีนตัวรับเอสโตรเจนโดยวิธี Indirect Immunohistochemistry พบผลบวกจากปฏิกิริยาอิมมูโนฮีสโตเคมีของแอนติบอดีจำเพาะต่อตัวรับฮอร์โมนเอสโตรเจนชนิดเบต้ากับกลุ่มเซลล์ที่อยู่บริเวณโคนรยางค์ของแต่ละปล้อง (Parapodia) ทั้งสองข้างของพ่อแม่พันธุ์เพรียงทรายทั้งเพศผู้และเพศเมียและยังพบผลบวกที่ชั้นเปลือกนอกของไข่ จากผลการศึกษาชี้ให้เห็นว่าตัวรับฮอร์โมนเอสโตรเจนที่ตรวจพบในพ่อแม่พันธุ์เกี่ยวข้องกับการเจริญพันธุ์ในเพรียงทรายโดยทำหน้าที่กระตุ้นให้เกิดความสมบูรณ์ของระบบสืบพันธุ์และกระบวนการสร้างเซลล์สืบพันธุ์ ทั้งนี้ตัวรับฮอร์โมนเอสโตรเจนอาจใช้เป็น Biomarker สำหรับตรวจสอบความสมบูรณ์ด้านการเจริญพันธุ์ของเพรียงทราย อย่างไรก็ตามควรทำการศึกษาลำดับเบสทั้งหมดของยีนรวมถึงการแสดงออกของยีนตัวรับชนิดนี้เพิ่มเติม

ลายมือชื่อนักศึกษา ..... ลายมือชื่ออาจารย์ที่ปรึกษา .....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

CONTENTS

		Page
ACKNOWLEDGEMENTS		**i**
ABSTRACT		**iii**
CONTENTS		**v**
LIST OF TABLES		**vii**
LIST OF FIGURES		**viii**
ABBREVATIONS		**xii**
CHAPTER 1	**INTRODUCTION**	**1**
	1.1 Background of the problem	1
	1.2 Objectives of the research	2
	1.3 Research hypothesis	3
	1.4 Benefits of the research	3
CHAPTER 2	**LITERATURE REVIEWS**	**4**
	2.1 *Perinereis nuntia*	4
	2.2 Estrogen receptor	8
	2.3 Structure of ER receptors	9
	2.4 Signaling of ERs	11
CHAPTER 3	**MATERIALS AND MATHODS**	**16**
	3.1 Animal	16
	3.2 Fixation	16
	3.3 Histological analysis	16
	3.4 PCR analysis	18
	3.5 In situ hybridization	21
	3.6 Immunohistochemistry	21

CONTENTS (CONT.)

		Page
CHAPTER 4	**RESULTS**	**23**
	4.1 Histopathology of *Perinereis nuntia* brood stocks	23
	4.2 Validation of genes that regulate the production of estrogen receptor by PCR analysis	30
	4.3 In situ hybridization	45
	4.4 Immunohistochemistry	46
CHAPTER 5	**DISCUSSION AND CONCLUSION**	**52**
REFERENCES		**56**
APPENDICES		**65**
	Appendix A Reagent Preparation	66
BIOGRAPHY		**70**

LIST OF TABLES

Table		Page
3.1	The concentration and sequence of estrogen receptor gene fragment of marine sandworm *P. nuntia* broodstocks primers	19
3.2	The preparation of master mix (BIOLINE; MyTaqTM HS DNA Polymerase)in one PCR tube was composed of Master mix to 12.5 µl. and DNA template 1 µl.	19

LIST OF FIGURES

Figure		Page
2.1	Diagram showing the major morphology features of a generalized polychaete	5
2.2	*Perinereis nuntia*	7
2.3	Chemical structures and biosynthesis pathways for endogenous estrogens and the endogenous selective estrogen receptor modulator (SERM) 27-hydroxycholesterol (27HC)	9
2.4	The structure of estrogen receptor	10
2.5	Domain organization of human ERα and ERβ	10
2.6	Model representing the mechanistically distinct molecular pathways used in the regulatory actions of ERs	12
4.1	Photomicrographs of premature stage of *Perinereis nuntia* (3-4 months of age) stained with H&E reveal normal histology without deposition of sex cells in the coelomic cavity (C). Parapodia (P), Muscular wall (M), Intestine (I), Stomach (S), Cranial ganglion (G), Intersegmental septum.	24
4.2	Photomicrographs of female *P. nuntia* broodstock (5-6 months of age) stained with H&E exhibit the deposition of developing eggs (*) in the coelomic cavity (C). Muscular wall (M), Intestinal epithelium (I), Ventral nerve cord (N), Ventral blood vessel (V), Dorsal blood vessel (DV).	25
4.3	Photomicrographs of female *P. nuntia* broodstock show abundant of the developing eggs (*) deposited in the coelomic cavity (C). Muscular wall (M), Intestinal epithelium (I)	26

LIST OF FIGURES (CONT.)

Figure		Page
4.4	Photomicrographs of female *P. nuntia* broodstock reveal abundant of the mature ovums (*) deposited in the coelomic cavity (C). The mature ovum has a thicken layer of jelly coat. Longitudinal muscle (M), Intersegmental septum (S), Parapodia (P).	27
4.5	Photomicrographs of male *P. nuntia* broodstock (5-6 months of age) stained with H&E exhibit abundant of sperm (*, arrowheads) deposited in the coelomic cavity (C). Longitudinal muscle of body wall (M), Intestinal lumen (I), Parapodia (P).	28
4.6	Photomicrographs at higher magnification of parapodium of the male broodstock reveal the deposition of abundant sperms (arrow) in the coelomic cavity (C). Hemocyte (H).	29
4.7	PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f0, ER_r5 primer pairs and annealing at 53 °C	31
4.8	PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f0, ER_r5 primer pairs and annealing at 50 °C	32
4.9	PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f0, ER_r5 primer pairs and annealing at 49 °C	33
4.10	PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f0, ER_r5 primer pairs and annealing at 46 °C	34
4.11	PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f ER_r1 primer pairs and annealing at 53 °C	35

LIST OF FIGURES (CONT.)

Figure		Page
4.12	PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f ER_r1 primer pairs and annealing at 50 °C	36
4.13	PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f ER_r1 primer pairs and annealing at 49 °C	37
4.14	PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f ER_r1 primer pairs and annealing at 46 °C	38
4.15	PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f 3 ER_rb primer pairs and annealing at 53 °C	39
4.16	PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f 3 ER_rb primer pairs and annealing at 50 °C	40
4.17	PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f 3 ER_rb primer pairs and annealing at 49 °C	41
4.18	PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f 3 ER_rb primer pairs and annealing at 49 °C	42
4.19	Photomicrographs of control immunohistochemistry with mouse mammary tissue exhibit positive reaction of anti-estrogen receptor-beta (ER-beta) at the cytoplasm of epithelial cells of lactiferous gland as red-brownish color (L).	47

LIST OF FIGURES (CONT.)

Figure		Page
4.20	Photomicrographs of immunoperoxidase labeling for ER-beta with female broodstock (5-6 months of age) reveal positive reaction with the developing egg cells (*) accumulated in the coelomic cavity (C). Cuticular wall (Cu), Muscle tissue (M).	48
4.21	Photomicrograph at higher magnification of female sandworm broodstock with indirect immunohistochemistry shows ER-beta deposition at the cytoplasm of developing eggs.	49
4.22	Photomicrographs of immunoperoxidase labeling for ER-beta deposition in female broodstock reveal positive reaction at the mucosal cell layer of intestine (I).	50
4.23	Photomicrographs of indirect immunohistochemical labeling of female (a) and male (b) broodstocks exhibit positive reaction of anti-estrogen receptor-beta at the cluster of cells as red-brownish color (arrow, *), at the tip of parapodia. This feature corresponds to the reproductive stage of egg and sperm productions and deposition, found in the coelomic cavity.	51

ABBREVIATIONS

Abbreviation	Meaning
%	Percent
^{o}C	Degree celcius
bp	Base pair
ddNTPs	Dideoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
dH_2O	Distilled water
ER-β	Estrogen receptor-beta
ER-α	Estrogen receptor-alpha
EtOH	Ethyl alcohol
g	Gram
LM	Light microscope
$MgCl_2$	Magnesium chloride
ml	milliliter
mRNA	Messenger ribonucleic acid
min	Minute
ng	Nanogram
nM	Nanomolar
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
Primer F	Forward primer
Primer R	Reverse primer
RNA	Ribonucleic acid
rpm	Revolution per minute
s	Seconds
Taq	*Thermes Aquaticus*
µl	microliter

CHAPTER I

INTRODUCTION

1.1 Background of the problem

The Polychaete used for marine aquaculture in Thailand are sandworms (*Perinereis sp.*) and mudworms (*Marphysa.sp*) (Meunpol, Meejing, and Piyatiratitivorakul, 2005). The *Perinereis nuntia* has been used as food in aquaculture businesses and as fishing bait. For the live feeds, polychaetes, as same as single feed diet or as a part of a combination feed, are normally used for broodstock due to their ability to enhance ovarian maturation (Lytle, J., Lytle, T., & Ogle, 1990; Middleditch et al., 1980) due to their high levels of protein, lipid, PUFA, and other hormonally active compounds (Dall, Smith, & Moore, 1991; Lytle et al., 1990; Marsden, McGuren, Hansford, & Burke, 1997; Middleditch et al., 1980; Naessens et al., 1997). preparation of PUFAs like AA, EPA, DHA, and the high n3:n6 ratio that related in fertilization, hatch rates, and spawning frequency in penaeid shrimp brood stock (Kangpanich, Pratoomyot, Siranonthana, & Senanan, 2016). There may be certain ingredients in the polychaete that play significant role in inducing ovarian maturation, in addition to the high content of PUFA. One of the hormones that may play significant role in the reproductive process of crustaceans is prostaglandins (PGs), a group of oxygenated polyunsaturated C20 fatty acids and a derivative of arachidonic acid (Spaziani, Mattson, Wang, & McDougall, 1999) suggested that the prostaglandin found in the polychaete tube-worm *Americonuphis reesei* Fauchald (Onuphidae) accelerate gonad maturation in penaeid shrimp. PGs exert their effects primarily through cell surface G protein-coupled receptors (Sales, Boddy, and Jabbour, 2008) and also though nuclear receptors (Bhattacharya et al., 1999; Helliwell, Berry, O'Carroll, & Mitchell, 2004). Prostaglandin F2α (PGF2α) plays important role in the ovarian development and spawning in fish, crayfish, and peneaid shrimp (Bell,

Farndale, Bruce, Navas, & Carillo, 1997; Kornthong et al., 2014; Murdoch, Hansen, & McPherson, 1993; Priddy & Killick, 1993; Spaziani, Hinsch, & Edwards, 1995; Stanley-Samuelson, 1994; Yano, 1995; Yui, Imataka, Nakamura, Ohara, & Naito, 2015), It may induce signaling cascades after binding to its specific receptor, resulting in the stimulation of mitogen-activated protein kinase pathway via phospholipase C-mediated protein kinase C activation (Regan, 2003; Sales et al., 2008) With this background, this study was therefore aimed at determining the levels of PGs in *P. cf. nuntia* and PG receptors in ovarian tissue of *P. monodon.*

However, it did not study significant role in reproduction, development, growth and sexual differentiation. Invertebrates, estrogen plays significant roles in those reproduction, development, growth and sexual differentiation, exerting its actions through the ligand-induced transcriptional activation of the estrogen receptor (ER) (Hassan, Salama, Arafa, Hamada, & Al-Hendy, 2007; Nelson & Habibi, 2013; Wallen, Tomas, Visakorpi, Holli, & Maenpaa, 2005). Previous reports demonstrate that the ER orthologues have been cloned and characterized in mollusks, amphioxus, annilids (Eick & Thornton, 2011; Kajiwara et al., 2008; Lv et al., 2017; Nelson & Habibi, 2013). Compared with vertebrates, there are limit information about the functions of invertebrates ERs and its tissue specific for ER gene expression. Therefore, The objective of this study was to determine the present of estrogen receptor sequence in marine polychaete *Perinereis nuntia* which is known to be a primary mediator signaling for estrogen as well as the tissue-specific expression of estrogen receptor (ER).

1.2 Objectives of the research

To study the estrogen receptor (ER) sequence in marine sandworm *Perinereis nuntia* brood stocks.

1.3 Research hypothesis

1.3.1 Estrogen receptor gene fragment of marine sandworm *P. nuntia* brood stocks should be found and characterized for its nucleotide sequences.

1.3.2 The present of ER gene in *P. nuntia* may indicate the ER translation in specific tissues.

1.3.3 ER plays a key role in primary mediator of estrogen signaling in polychaetes and involves in its reproductive function.

1.4 Benefits of the research

1.4.1 The discover of estrogen receptor (ER) sequence in marine sandworm *Perinereis nuntia* broodstock which may exert as primary mediator of estrogen signaling in polychaete.

1.4.2 This is description of the mechanism of action of sex hormone-like substances that may be present in marine sandworm by binding to estrogen receptor.

CHAPTER 2

LITERATURE REVIEWS

2.1 *Perinereis nuntia*

2.1.1 Classification

Perinereis nuntia is an invertebrate polychaete worm. (Hamdy, Dorgham, El-Rashidy, & Atta, 2014). There lives in the intertidal zone to deep sea. (Kawsar et al., 2009)

Domain: Eukaryota

Kingdom: Animalia

Phylum: Annelida

Class: Polychaeta

Subclass: Errantia

Order: Phyllodocida

Suborder: Nereidiformia

Family: Nereididae

Subfamily: Nereidiformia

Genus: Perinereis

Species: Perinereis nuntia

2.1.2 General morphology (Kristian, 1977)

The body of polychaetes can be divided into three parts: Head, Trunk, and Tail.

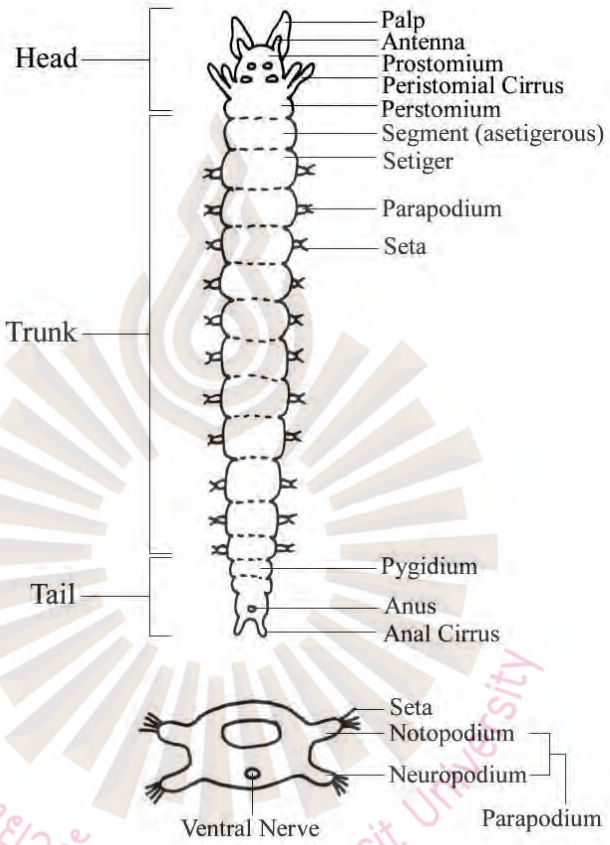

Figure 2.1 Diagram showing the major morphology features of a generalized polychaete (adapted from Fauchald 1977). Top: Entire worm.
Bottom: Cross section of stage
Source: Winslow, 2007

2.1.2.1 Head

a. Prostomium

It is a pre-segmental part of the body anterior to the mouth that includes antennae and palps. Antennae are sensory. Palps may be sensory or used as feeding appendages. Some species have one or two pairs of eyes on prostomium.

b. Peristomium

It is the first distinct post-prostomal region around the mouth that includes tentacular cirrus and proboscis.

c. Pharynx

It is the anterior part of digestive tract for feeding and sometimes for burrowing. Most polychaetes have eversible pharynx.

2.1.2.2 Trunk

Each segment generally has its own local nerve center called ganglion and a pair of nephridia for excretion.

a. Parapodia

The parapodia are the flatlike projections on both sides of each segment for locomotion and gas exchange. It can be biramus with both notopodia (upper division) and neuropodia (lower division) or uniramus with only neuropodia.

b. Setae

It is the chitinous bristle bearing on the parapodia and used for locomotion, feeding and building tubes. There are many kinds of setae: simple, compound, capillary, limbate, bifurcate, trifurcate, pinnate, harpoon, pectinate and spatulate, etc.

2.1.2.3 Tail

The posterior section of the body is the truncated or tapered pygidium which contains a dorsal or terminal anus and it has a Cirri

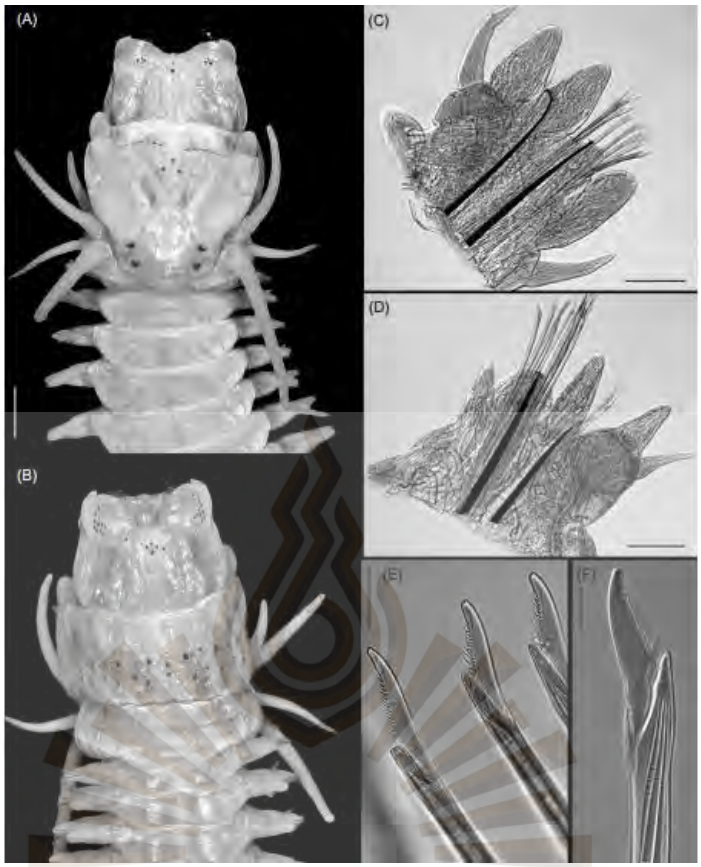

Figure 2.2 *Perinereis nuntia*. (A) Anterior end with pharynx everted, dorsal view; (B) anterior end with pharynx everted,ventral view; (C) parapodium, anterior view; (D) posterior parapodium, posterior view; (E) subacicular neurochaetae from parapodium7; (F) subacicular neuropodial heterogomph falciger from posterior parapodium. A-F: NTM W19042. Scalebars: A, B = 0.5 mm; C, D =0.2 mm; E, F = 0.02 mm.
Source: Glasby & Hsieh, 2006

Feedind Habit and Behavior. *Perinereis nuntia* is omnivorous and feeds on organic matter accumulated by rivers or the tide. They perfect organic matter of animal organic. They feed actively when the bottom is slightly covered with water, on the surfaces of wet sand or rocks. They do not feed under dry conditions. They extrude about half of their bodies out of their nest holes when feeding and carry food into their nest holes as soon as they catch it. The worms usually keep their head upward in the nest hole. When they excrete, they put the end of their tails out of the nest hole and, with head down, contracts the body to retract the tails into the original position after

excreting. They live over a relatively wide range of temperature, as feeding is observed between 5-35 °C.On the other hand, they have little tolerance to lower salinity. A worm which was living in seswater swells up in freshwater, turns red and quickly becomes inactive (Poltana, 2005)

2.1.3 Life cycle and mating behavior

Polychaeta are sexual reproduction. Females produce a pheromone attracting and signalling the males to shed sperm which in turn stimulates females to shed eggs, this behavior is known as swarming. Gametes are spawned through the metanephridia or body wall rupturing (termed as "epitoky", wherein a pelagic, reproductive individual, "epitoke", is formed from a benthic, nonreproductive individual, "atoke"). After fertilization, most eggs become planktonic; although some are retained in the worm tubes or burrowed in jelly masses attached to the tubes (egg brooders). Life Cycle: Eggs develop into trocophore larva, which later metamorph into juvenile stage (body lengthened), and later develop into adults. (Ruppert, Fox, & Barnes, 2004)

2.2. Estrogen receptor

Steroid hormones are function of reproductive biology in vertebrates and are largely regulated nuclear receptor family of intracellular receptors, and membrane estrogen receptors. Sex steroid hormones, such as progestogens, androgens, and estrogens, play important roles in reproductive, including development of sexual organs. (Heldring et al., 2007; Klinge, 2001; Kong, Pike, & Hubbard, 2003; Lee et al., 2012). There are chemically related compounds derived from androgen precursors but containing a defining aromatic and hydroxyl group at the 17 position (Figure 2.3). 17β-estradiol is the main physiologic hormone, but it is speculated that estriol, estetrol and estrone may play important roles during pregnancy and post-menopause (Lee et al., 2012)

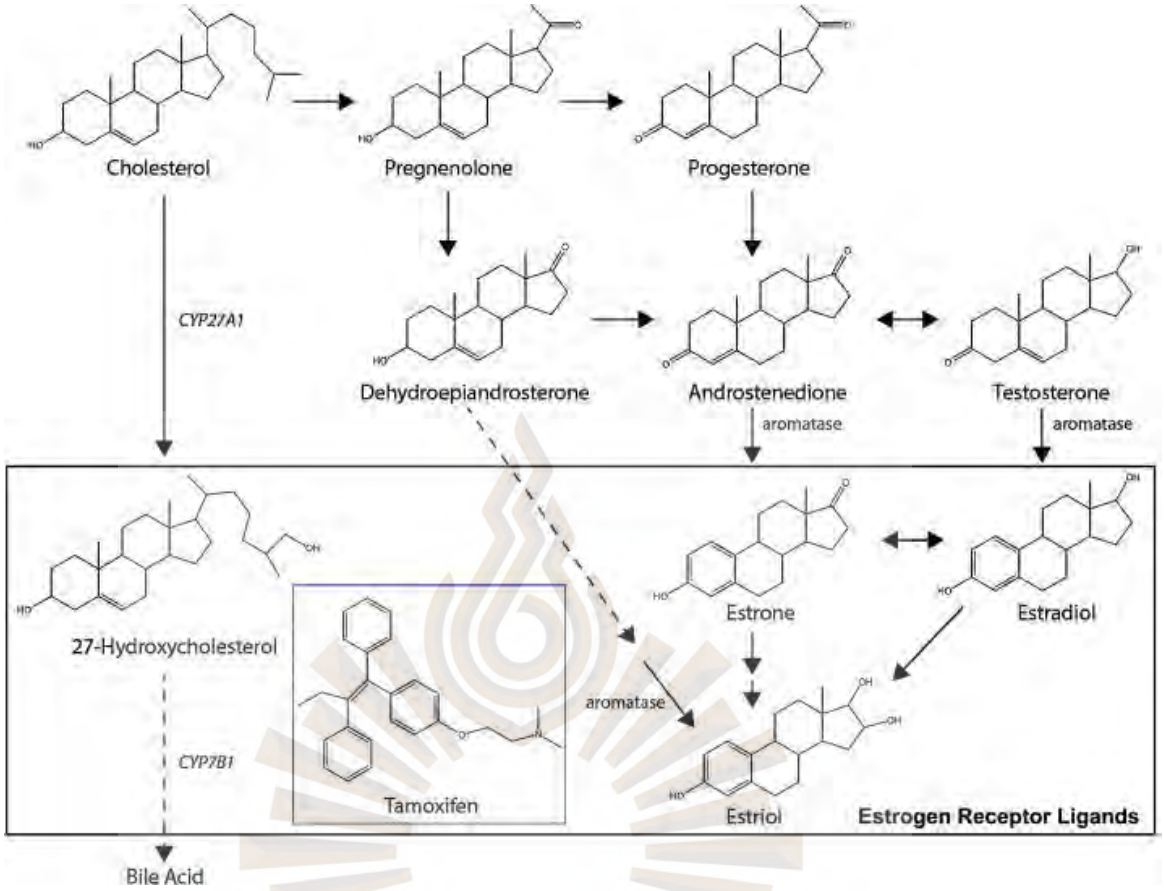

Figure 2.3 Chemical structures and biosynthesis pathways for endogenous estrogens and the endogenous selective estrogen receptor modulator (SERM) 27-hydroxycholesterol (27HC). Tamoxifen, a synthetic SERM is shown for comparison. 27HC is a primary metabolite of cholesterol, hydroxylated by the actions of CYP27A1, and is further metabolized by CYP7B1. Estrogens are formed when they are aromatized by the enzyme aromatase (CYP19) from precursor androgens.

Source: He & Nelson, 2017

2.3 Structure of ER receptors

Estrogen receptors are ligand active enhancer proteins. There are two classified forms of the estrogen receptor as ERα and ER β. Similar to other nuclear steroid receptors, these two receptors are structurally composed of six domains,

including the DNA-binding domain (DBD), that allows ER binding to DNA at estrogen response element (ERE), and ligand-binding domain (LBD), which forms a ligand-binding pocket for reception of estrogenic and various other compounds (Klinge, 2001). ERα is primarily considered to have higher significance in biological roles than ERβ, and have been associated with multitudes of physiological functions (Couse & Korach, 1999).

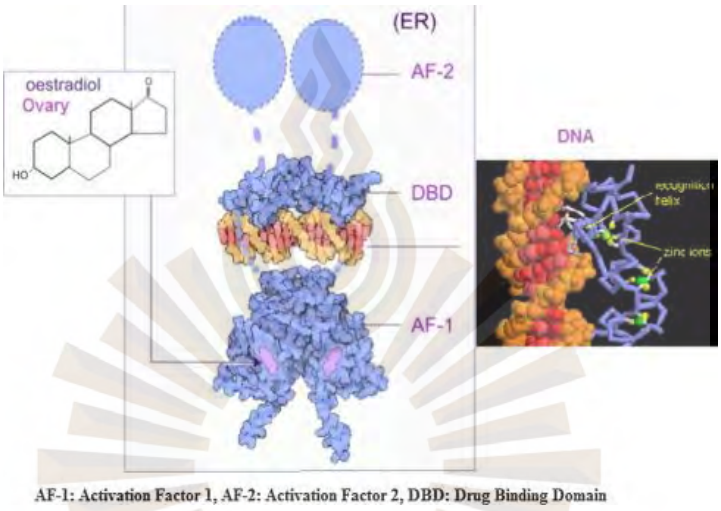

Figure 2.4 The structure of estrogen receptor

Source: Begam, Jubie, & Nanjan, 2017

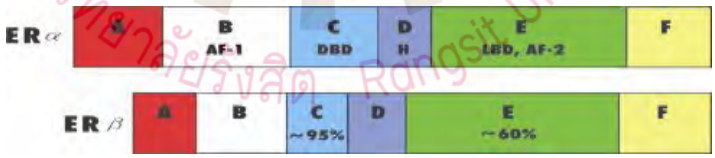

Figure 2.5 Domain organization of human ERα and ERβ. ERs consist of the *N*-terminal region involved in transactivation (A/B domains, AF-1), the DNA binding domain (DBD, C domain), the hinge region involved in dimerization (D domain), the *C*-terminal region containing ligand binding domain (LBD, E/F domain, AF-2) and transactivation function-2 (AF-2). The percentage indicates the homology between ERα and ERβ

Source: Begam et al., 2017

Estrogen receptors have six structural domains referred to as A, B, C, D, E, and F (Figure 2.5). The N-terminal of the A/B domains of ERs consist of activation function-1 (AF-1) which contributes to the transcriptional activity of ERs and is an essential domain of interaction with co-regulators. AF-1 is the least conserved region with only 30% identity between ER α and ER β. Functional studies have shown that ER β has low levels of AF-1 activity. The A/B domains also contain amino acids that are targets of post-transcriptional modifications, including splicing to stimulate AF-1 activity (Lee, Kim, & Choi, 2012; Shao & Brown, 2004) The C domain encodes a centrally located DNA binding domain (DBD), essential for sequence specific binging of ERs to DNA and regulating the expression of target genes (Geserick, Meyer, & Haendler, 2005). The D domain, a hinge region, includes amino acid sequences that stimulate nuclear localization signaling and facilitate post-translational modification of ERs, resulting in the activation of ER signaling in cells. Finally, the E/F domain, located in the C-terminal region, contains a ligand binding domain (LBD). The LBD also contains a dimerization surface and a ligand-dependent activation function-2 (AF-2). AF-2 undergoes a marked conformational change in the presence of different ligands and determines the subsequent binding of coactivators or corepressors. AF-1 and AF-2 control the transcriptional regulatory activity of ERs because activation of ERs is stimulated during cellular responses to the environment (Edwards, 2000). The E/F domains of ER α and ER β share 53% sequence identity and affect cellular responses through ligand-dependent ER activation. The F domain also affects the activity of ERα and ERβ. The differences between the F domains of the ERs may contribute to the ability of ERs to selectively control transcriptional activities of specific target genes (Lee et al., 2012). Out of the six domains, the most conserved domain is the central DNA-binding domain (DBD, region C), followed by the ligand binding domain (LBD, region E) (Shao & Brown, 2004).

2.4 Signaling of ERs

Estrogens and androgens are involved as necessary factors for initiating the important program of morphogenesis and in particular the development of organs. These receptors are needed for communication between epithelium and mesenchyme.

Without this communication, morphogenesis cannot occur. The major players in the cross talk between epithelium and mesenchyme are the epidermal growth factor pathway, which includes epidermal growth factor receptor (EGFR) (stromal & epithelial), the proteases (which activate epithelial and stromal), fibroblast growth factor receptor (FGFR) (stromal and epithelial), the transforming growth factor beta family of peptides (BMPs) (stromal) and their receptors and the indentation signaling pathway which includes the epithelial receptors (Forster et al., 2002).

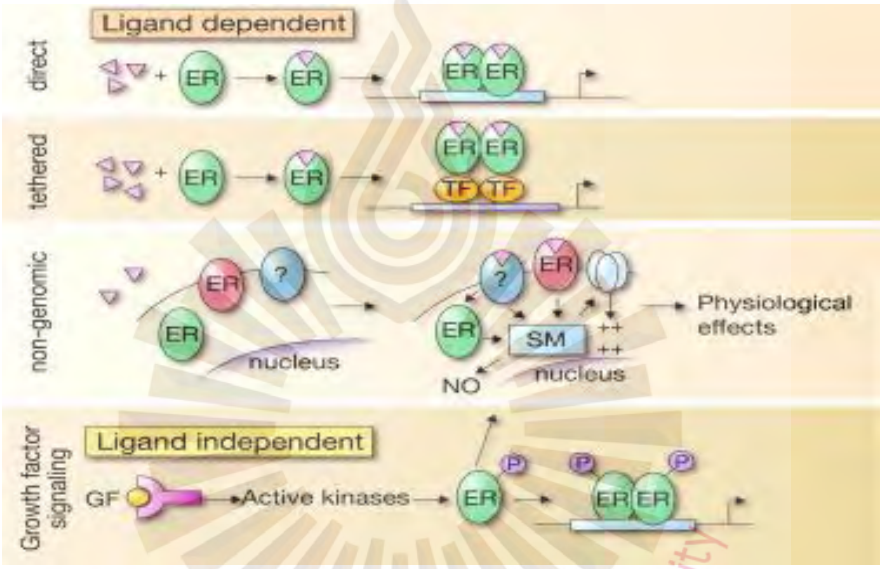

Figure 2.6 Model representing the mechanistically distinct molecular pathways used in the regulatory actions of ERs.
Source: Begam et al., 2017

Two distinct types of signaling are mediated, often referred to as genomic and non-genomic or non-genotropic pathways. In the genomic pathway, estrogens bind to ERs in the nucleus, inducing a conformational change in the receptors that cause dissociation from the chaperones, dimerization and activation of the receptor transcriptional domain (Figure 2.6) (Hall, Couse, & Korach, 2001; Hall & McDonnell, 2005; McGuire, 1978). The classical (direct) pathway includes ligand activation and a direct DNA binding to estrogen response elements (ERE) before modulation of gene regulation. The tethered pathway includes protein-protein interaction with other transcription factors after ligand activation, thus affecting gene regulation by indirect DNA binding. The non-genomic signaling is not as well understood as the genomic

mechanism but has been observed in many tissues. In this signaling, the ligand activates a receptor, possibly associated with the membrane; either it is a classical ERα and ERβ isoform or a distinct receptor or, alternatively, a signal activates a classical ER located in the cytoplasm. After this rather unclear event, signaling cascades are initiated via second messengers (SM) that affect ion channels or increase nitric oxide levels in the cytoplasm ultimately leading to a rapid physiological response without involving gene regulation. The ligand-independent pathway includes activation through other signaling pathways such as growth factor signaling. In this case, activated kinases phosphorylate ERs and activate them to dimerize, bind DNA and regulate genes (Marino, Galluzzo, & Ascenzi, 2006)

Perinereis nuntia is an invertebrate polychaete worm. There are a large class in Phylum Annelid. Worldwide there are known to be over 10,000 species of Polychaeta living in locations ranging from tidal zone to deep sea (Metcalfe & Glasby, 2008). In the primary producer, Polychaeta is one of the most species rich groups (Metcalfe & Glasby, 2008; Zheng et al., 2011). Polychaetes play an important role in the ecological niche. They serve as detritivores, feeding on the sea floor and as prey for larger fish, snails and other predators (Zheng et al., 2010). There are several studies the association about polychaeta as follows;

Techaprempreecha et al. (2011) reported that the total saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) Contents were higher in wild than farmed sandworms, the polyunsaturated fatty acids (PUFA) content was lower in wild than farmed sandworms. But the content of eicosapentaenoic acid (EPA) in farmed sandworms was lower than that of wild sandworms.

Lv et al. (2017) study the association between the full-length cDNA of estrogen receptor (paER) and marine polychaete *Perinereis aibuhitensis***.** *They found that* describes the first molecular characterization of full-length paER and the specimen tissue was expression in *P. aibuhitensis*. The positions of the cysteine residues and the residues around them, which constitute two zincfinger motifs and a P-box, are conserved in both vertebrates and invertebrates.

Leelatanawit et al. (2014) studied the effects of sand polychaetes on growth, survival, reproductive performance and sperm physiology and morphology of domesticated male broodstock P. monodon. After feeding with either polychaetes or commercial pellets for four weeks, growth and survival rates of polychaete-fed group were significantly higher than those of pellet-fed group.

Zheng et al. (2010) studied reproductive toxic effects of sublethal cadmium on the marine polychaete *Perinereis nuntia.* They reported that the sublethal cadmium reproduce toxic on *P. nuntia* by inhibiting sexual maturation, fertilization and zygote hatching, and the increased expression of vitellogenin suggesting cadmium has strong feminization effects on polychaetes.

Meunpol, Iam-Pai, Suthikrai, and Piyatiratitivorakul (2007) found that the steroid hormones in polychaetes have been suspected as being a potential component for prawn reproductive system development. Progesterone (P4) and 17-alpha hydroxyprogesterone (17α-OHP4) were extracted from *Perinereis* sp. and female Penaeus monodon broodstock at various maturation stages.

Gaete, Alvarez, Lobos, Soto, and Jara-Gutierrez (2017) studied the effect of estuarine sediments on the polychaete *Perinereis gualpensis* using oxidative stress biomarkers and to determine the metal concentrations in sediments and their accumulation in *P. gualpensis*. The metal concentrations of Fe Cu, Pb, Zn and Cd were determined in tissues of the organisms and in sediments. They reported the concentrations of metals in sediments were higher in the estuary of the Aconcagua River. In tissues, Pb and Fe were higher in the estuary of the Maipo River, while Cd was detected only in the Catapilco River mouth.

Liu et al. (2015) study the relationships between *Perinereis aibuhitensis* and populations on the coasts of China. They estimated the genetic relationships within *P. aibuhitensis* using Target Region Amplified Polymorphisms (TRAP) and Amplified Fragment Length Polymorphisms (AFLP).The result show. The genetic diversity was low for *P. aibuhitensis* at the population level compared with the species level.

Zhao, Zhou, Li, Y., Li, S. and Yang (2014) studied Molecular cloning and expression of the gene for G protein alpha subunit.They found a G protein alpha subunit gene named Pa G which was isolated from the marine polychaete *Perinereis aibuhitensis*. The full-length cDNA of Pa G was 1832 bp and contained a 205 bp 5 untranslated region (5 UTR), a 565 bp 3 UTR and a 1062 bp open reading frame encoding 353 amino acid residues.

CHAPTER 3

MATERIALS AND METHODS

3.1 Animal

3 female and 3 male broodstock (age 5-6 months) and 3 premature stage (age 3-4 months) were selected and artificially mated in a 15 cm × 30 cm × 10 cm polystyrene box. The number of the males and females were at ratio of 1:1 (3 females and 3 males) and represented almost all of the natural distribution areas of *Perinereis nuntia* in Thailand. The animals were transferred from the field to an ice cooler. They were acclimated under laboratory conditions (17 ± 0.5 °C, salinity 25 ± 1, pH 8.0 ± 0.1). After a week, putting them on ice and then frozen at −70 °C until analysis.

3.2 Fixation

Perinereis nuntia specimens were freeze-dried and various tissues, including the header, middle, and trail were isolated, immediately preserved in LM fixative (10% Neutral formaldehyde) for 2-4 h. to optimize tissue suitability for light microscopy (LM) and for possible research applications including In-situ hybridization (ISH) studies.

3.3 Histological analysis

The specimen tissue was fixed in 10% Neutral formaldehyde for 2-4 h. and then dehydrated in a 95% alcohol, 30 min x 3. and then dehydrated in an Absolute alcohol, 40 min x 3. Clearing tissue with Xylene, 60 min x 2 and then infiltration tissue with paraffin 60-64 °C, 60 min x 3. Specimens in paraffin were put into embedding cassettes to be processed in an embedding machine using vacuum to make

tissues permeable for wax. Then, specimens were transferred into embedding molds, where they were oriented in the desired position and submerged in melted wax (60 °C). A microtome holding cassette was put on top. The mold was transferred onto a cold plate for 30 min. until the wax had hardened. A scalpel was used to remove the mold. The wax block was trimmed to be ready for sectioning with a microtome. Tissue blocks were trimmed with a razor blade. The wax block with the specimen to be cut was then fixed to the microtome via the holding cassette. Specimens for in situ hybridization on sections were cut at 4-6 μm, specimens for histological analysis were cut at 4-6 μm. Ribbons of sections were transferred into a 43 °C water bath, so that sections could spread on the surface of the water. Sections were then transferred onto a slide and dried vertically at room temperature. Ribbons of sections for in situ hybridization on sections were transferred onto prepared slides with a drop of sterile water. These slides were put on a slide warmer (50 °C) for 30 min to spread the sections. Finally, sections were dried vertically at RT. Counterstaining with hematoxylin and eosin of histological sections of *Perinereis nuntia* specimens were used to analyze the morphology and histology. Sections of *Perinereis nuntia* specimens were deparaffinized in xylene (1 x 3min, 1x 3 min), rehydrated in an 3 x absolute alcohol (3 min), 95% alcohol (2 min) and rinsed under running tap water for 3 min. Sections were then stained in hematoxylin for 4 min and washed again under running tap water to get rid of an excess of hematoxylin. Sections were bluing in 1% Ammonia solution in water (5 sec). Slides were washed again in running tap water for 5 min and then stained in eosin (3 min). Finally, sections were rehydrated through an 95% alcohol (45 sec), absolute alcohol (1 min), 3 x acetone solution (1 min) and 2 steps in xylene (7 and 8 min). Slides were mounted using Permount. Morphology of the specimens was examined under a light microscope (Axioskop 2 Plus, Zeiss) and pictures were taken using a digital camera (Leica DC300).

3.4 PCR analysis

3.4.1 Protocol of DNA Extraction

The 25 mg of *Perinereis nuntia* tissue have cut into small slice. After that, took these samples to microcentrifuge tube (size1.5-ml) that were homogenized. The lysis solution consists of Buffer T1 with 180 uL. and Proteinase K with 25 uL were added to homogenize samples into microcentrifuge tube and mixing by vortex for 15 second(MACHEREY-NAGEL, Germany). The samples with lysis solution were incubated at 56 °C for 1–3 hours. The samples were completely disintegrated and then 200 uL of Buffer B3 was added. The samples were vortex and incubated at 70 °C for 10 minutes. Next to briefly vortex, the 210 ul with 96-100% of ethanol was added to sample solutions that were mixed by vortex for 15 seconds. The sample solutions were briefly reloaded to the spin column and centrifuge at 11,000Xg for 1 minute and then, to bring the new clean of spin column 2-ml collection tube. The 500 ul. of buffer BW was added to the spin column and centrifuge at 11,000Xg for 1 minute which was discarded flow through and put the spin column into the same collection tube. The spin column was changed to new clean 2-ml collection tube. The 600 ul. of buffer B5 was added to the spin column and centrifuge at 11,000Xg for 1 minute. To discarded of flow through and then, was put the spin column into the same collection tube which microcentrifuge tube was centrifuged to remove of drop inside of lid at 11,000Xg for 1 minute. To changing, the spin column to new sterile1.5-ml size of microcentrifuge tube which 100 ul. of buffer BE was added to spin column and incubated at room temperature for 5 minutes. The spin column was centrifuged at 11,000Xg for 1 minutes. Samples were kept at -20 °C until required.

3.4.2 Protocol of PCR for estrogen from *Perinereis nuntia*.

Total genomic DNA was extracted from the whole body of *Perinereis nuntia*. The quality and quantity of the DNA were determined using 1.5% agarose gel electrophoresis. To stained with 0.5 µg/ml ethidium bromide and then visualized using the gel documentation. The PCR program was as follows: 94 °C for 2min, 40 cycles of

94 °C for 30 s, 49 °C for 30 s, 72 °C for 1 min; finally, extension at 72 °C for 5 min. submitted to sequencing in both directions.

The sequence of forward and reverse primers for detection of Estrogen receptor gene fragment of marine sandworm *P. nuntia* broodstocks are described in Table 3.1

Table 3.1 The concentration and sequence of Estrogen receptor gene fragment of marine sandworm *P. nuntia* broodstocks primers

Primers	Sequencing	Product (bp)
ER_f0 ER_r5	5'-TGGTGGGTTTCTCCTCCCTCAC 5'-TGGCAGCTCTTGGCGCCGATGT	980
ER_f ER_r1	5'-ATCGGCGCAAGAGCTGCCAAGCTTG 5'-TCGATATCTGCCATATTTCTCCCAC	777
ER_f3 ER_rb	5'-GTGGGAGAAATATGGCAGATATCGA 5'-TGGAGTGGGAATTAAAAACAAGTAA	930

The preparation of master mix for amplified DNA in Estrogen receptor gene fragment of marine sandworm *P. nuntia* brood stocks analysis with 5X Q5 Reaction buffer, *My Taq* ^{Hs} DNA polymerase, 10 µm. F primer, 10 µm. R primer and DNA Polymerase. The details preparation of master mix was shown that Table 3.2

Table 3.2 The preparation of master mix (BIOLINE; MyTaqTM HS DNA Polymerase) in one PCR tube was composed of Master mix to 12.5 µl. and DNA template 1 µl..

Reagents	1XReaction
1) dH2O	7.1
2) 5X Q5 Reaction buffer	2.5
3) My Taq Hs DNA polymerase	0.5
4) 10 um F primer	0.7
5) 10 um R primer	0.7
6) DNA template	1each
Total	12.5 ul.

One PCR tube composed of Master mix 12.5 uL and DNA template 1 µl.

3.4.3 Protocol of Gel electrophoresis

The agarose gel was prepared by 1.5 grams of agarose powder with 100 ml of 1XTBE buffer into erlenmeyer flask and then, melted in microwave. The melt agarose was poured to casting tray for electrophoresis and until agarose gel was set solidification. The completely preparative agarose gel was placed to electrophoresis chamber in 1X TBE with cover agarose gel.

After that, the preparation of loading dye were taken 3 µl. on parafilm with 12.5 µl. of distilled- water to negative control, 12.5 µl. of control to positive control and 12.5 µl. of sample that pipette was up and down. To loaded into an empty well of agarose gel. The gel electrophoresis was ran 30 minutes and then, this agarose gel was stained with ethidiumbromide for 3 minutes and destained with tap to water for 5 minutes. The visualization of DNA was under UV light and the result of DNA agarose gel photograph was recorded.

3.4.4 DNA sequencing

The selective gel from application of PCR product band was cut into centrifuge tube and extracted by NucleoSpin® Gel and PCR Clean-up kit. The weight of gel in centrifuge tube was recorded analysis without weight of the tube. The sample was added with 400 µl of NTI Buffer at 50 °C for 10 minutes on block heater. After that, was taken 700 µl by micropipette to spin column and collected to collection tube that spin down at 11,000Xg for 1 minute. The 700 ml of Buffer NT3 was used dispose and spin down at 11,000Xg for 30 seconds which Buffer NT3 was used again to dispose. To Spin down again and then, the collection solution was poured and centrifuge at 11,000Xg for 1 minute. The collection tube was removed into a new centrifuge tube over the spin columns and at incubated at 70 °C for 5 minutes. Added 40 µl of distilled water to centrifuge tube and centrifuged at 11,000Xg for 2 minutes. The completely DNA extraction of the centrifuge tube and the primer was sent to

GIBTHAI company. After that, the per-formation of DNA sequence was compared to GenBank database.

3.5 In situ hybridization

The *Perinereis nuntia* was embedded to paraffin block that was cut by microtome to 5-6 microns per sections. From *Perinereis nuntia* paraffin sections were used positively charged microscoape slide (EMS, England) and air dried for 3 hours. The sections were deparaffinized in 100% methanol for 10 minutes at room temperature, dehydrated with MeOH in 1X PBS solutions; 75%, 50%, and 25%, respectively for 10 minutes at room temperature. Then, the sections were washed to with PBS for 3 minutes and repeat for 3 times. Each of the tissue sections was treated with proteinase K (1 μg/mL) for 5 minutes at room temperature. The second fixative of tissue sections was done by 4% paraformaldehyde in PBS for 5 minutes and washed with PBS as mentioned before. RNA probes were prepared using digoxigenin (DIG)-RNA labeling and detection kit from Boehringer Mannheim Biochemicals, followed by the manufacture's protocol. The Hybridization and visualization of RNA probes were carried out as described by Schulte-Merker, Ho, Herrmann, and Nusslein-Volhard (1992).

3.6 Immunohistochemistry

Tree micron sections of *Perinereis nuntia* were cut from a paraffin-embedded block and placed on a positively charged microscoape slides (SuperFrost Plus (Menzel®; Braunschweig, Germany)) and air dried for 3 h. The sections were then deparaffinized in xylene and hydrated in graded alcohols and distilled water. Endogenous horseradish peroxidase (HRP) activity was blocked by incubation in methanol containing 0.3% hydrogen peroxide for 20 min at 25 °C. The sections were washed with distilled water, and antigen retrieval was performed using an autoclave in 10 mM citrate buffer (pH, 6.0) and heat (121 °C) for 5 min. The sections were incubated with 1% BSA for 1 h at 25 °C and immunostained using 6 mg/ml of a rabbit anti-ER primary antibody (Roche) for 1 h at 25 °C. The sections were washed with

PBS and incubated with a biotinylated goat anti-rabbit IgG secondary antibody. The sections were incubated with streptavidin-conjugated HRP (Vector Labs.) for 30 min at 25 °C or Avi-TMR particles for 2 h at 25 °C. Sections that were incubated with streptavidin-conjugated HRP were treated with a DAB chromogen reagent (Roche). DAB chromogen treatment was concurrently performed under the same conditions (temperature, time, and substrate concentration) in all tissues. The reaction of all tissues, including low-level ER-expressing samples, was arrested according to the manufacturer's instructions when a strong DAB intensity was recognized in tissues with high-level ER expression. In addition to the staining of biomarkers, the investigation of nuclear morphology using counterstaining with hematoxylin is very important in cancer diagnosis using IHC. All samples were stained with hematoxylin after DAB and Avi-TMR particle staining and mounted in a mounting medium for observation.

CHAPTER 4

RESULTS

For the detection of angiogenesis with gene expression similar to the genes that regulate the estrogen receptor, the study was conducted with 3 female and 3 male broodstock (age 5-6 months) and 3 premature stage (age 3-4 months). In first experiment, To confirm reproductive stages of the male and female brooders and young animal using histological examination. After that to study paER-beta gene fragments and its tissue-specific expression in *P. nuntia*.

4.1 Histological analysis

Histopathology with paraffin sections and hematoxylin and eosin stain of various tissue from male and female *Perinereis nuntia* broodstocks and premature animals, including the header, middle, and trail were isolated, immediately preserved in LM fixative (10% Neutral formaldehyde) optimize tissue suitability for light microscopy (LM). Confirm the reproductive stage of the specimen. Show the cumulative position of the sperm cells and the oocytes was increased.

Histological examinations revealed that in the premature stage of *Perinereis nuntia* (3-4 months of age) we conducted a histological observation of the cells using hematoxylin and eosin stains reveal normal histology without deposition of sex cells (Figure 4.1) but surviving *Perinereis nuntia* broodstocks (5-6 months of age) show developing eggs in coelomic cavity in female. (Figure 4.2) At the same time, we also observed the developing eggs deposited in the coelomic cavity were found abundant of the developing eggs (Figure 4.3) In juvenile genital tissues, female *P. nuntia* broodstock reveal abundant of the mature ovums deposited in the coelomic cavity. The mature ovum has a thicken layer of jelly coat. (Figure 4.4), male *P. nuntia* broodstock reveal abundant of sperm cells are found in the intestinal cavity (coelomic cavity) and

dorsal parapodia on both sides of nearly all body segments. (Figure 4.5) Interestingly,sperm was strongly expressed in a population of cells in each parapodium (Figure 4.6) The germ cell was detected in *Perinereis nuntia* broodstocks of various forms based on their location from the distal end of the parapodium to the coelomic cavity. Most of these cells were found in the coelomic cavity.

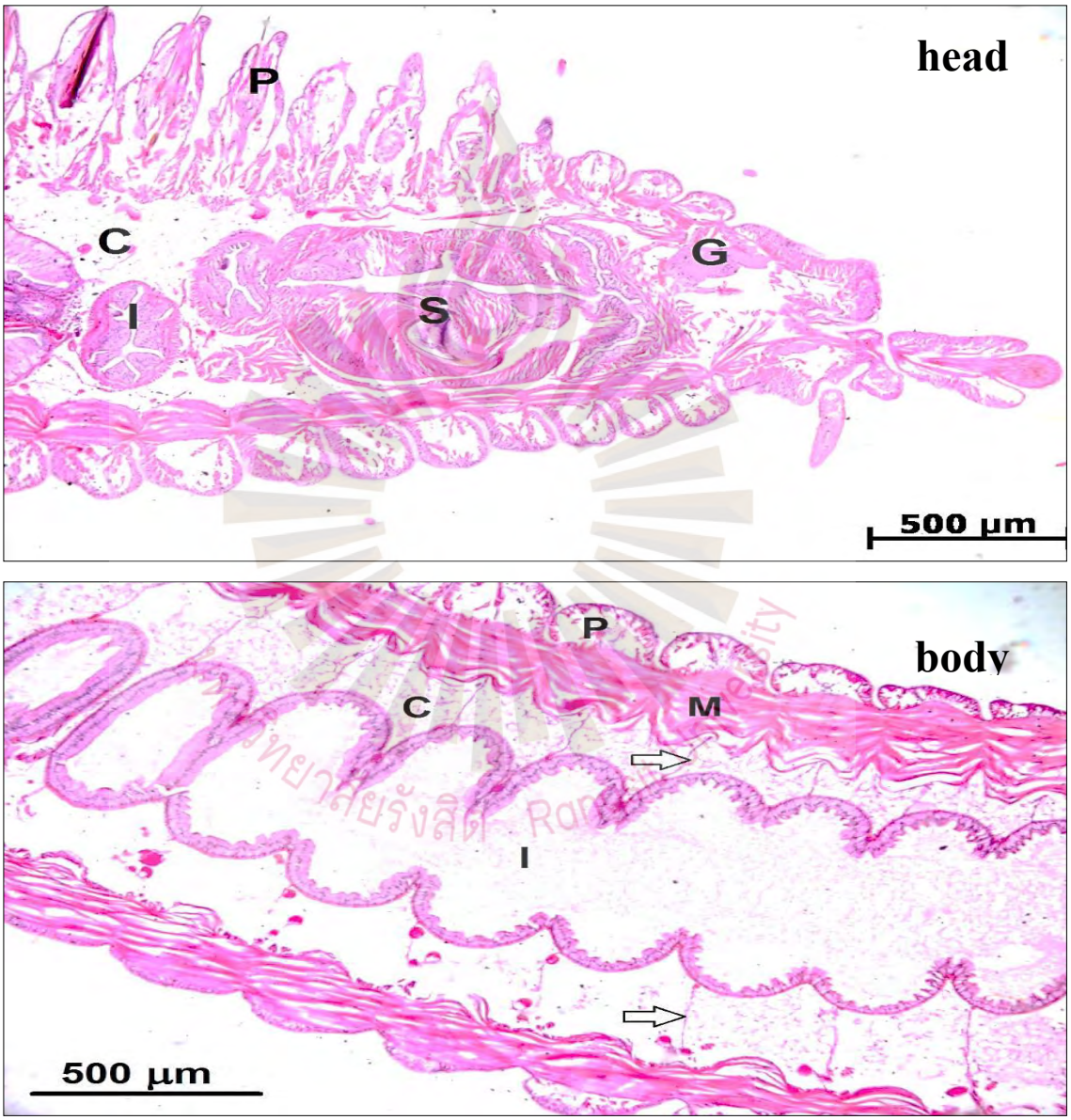

Figure 4.1 Photomicrographs of premature stage of *Perinereis nuntia* (3-4 months of age) stained with H&E reveal normal histology without deposition of sex cells in the coelomic cavity (C). Parapodia (P), Muscular wall (M), Intestine (I), Stomach (S), Cranial ganglion (G), Intersegmental septum.

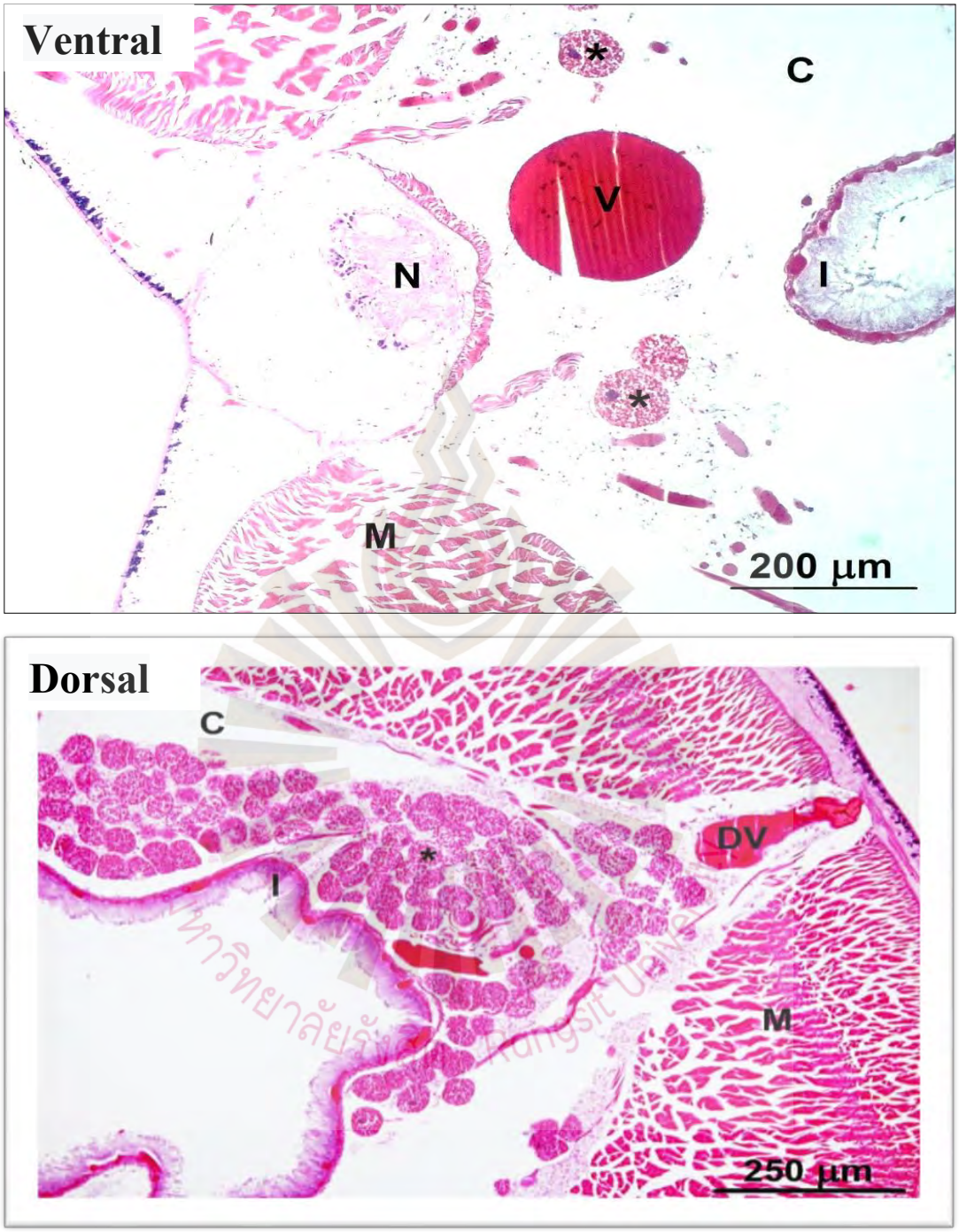

Figure 4.2 Photomicrographs of female *P. nuntia* broodstock (5-6 months of age) stained with H&E exhibit the deposition of developing eggs (*) in the coelomic cavity (C). Muscular wall (M), Intestinal epithelium (I), Ventral nerve cord (N), Ventral blood vessel (V), Dorsal blood vessel (DV).

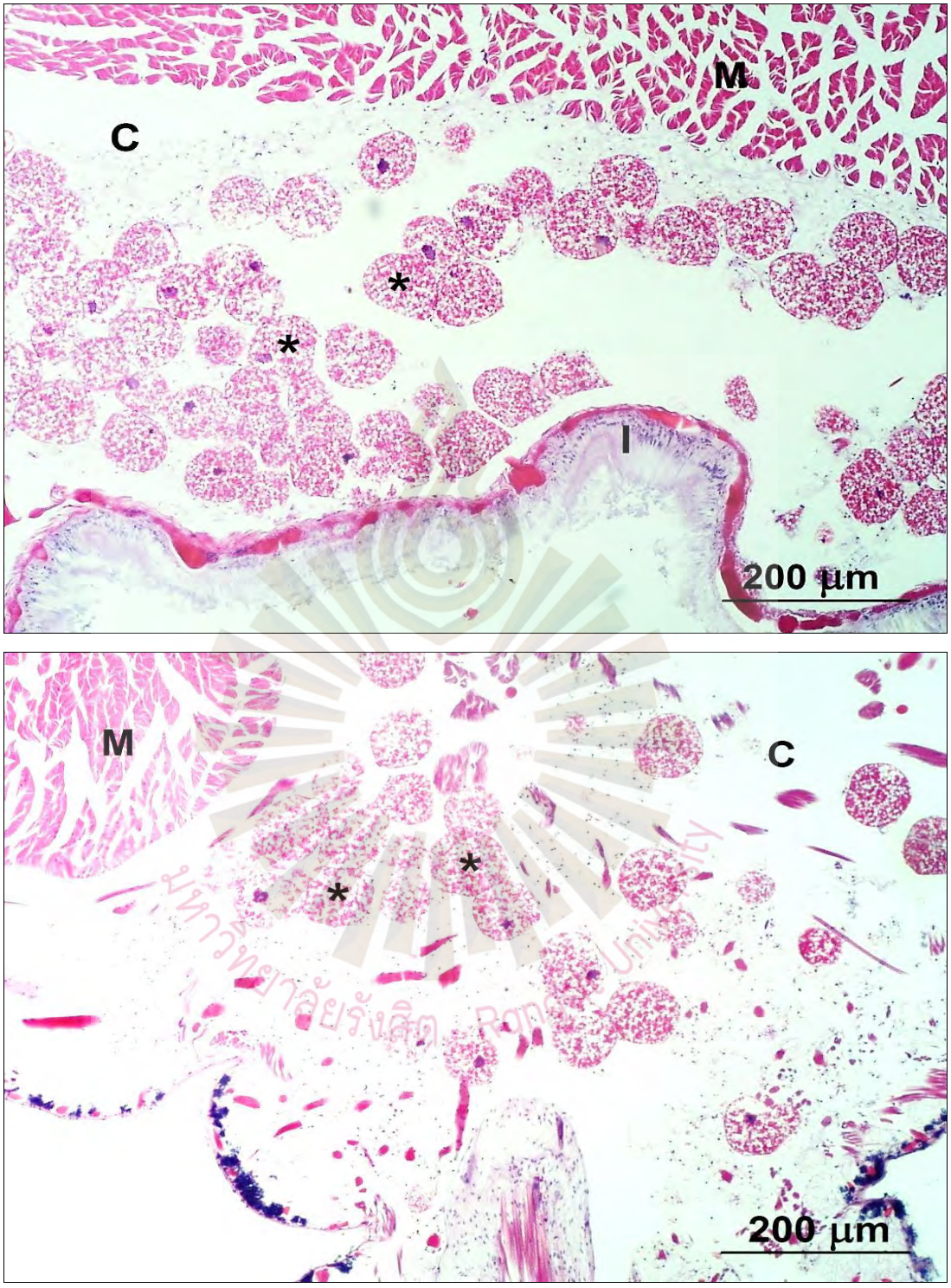

Figure 4.3 Photomicrographs of female *P. nuntia* broodstock show abundant of the developing eggs (*) deposited in the coelomic cavity (C). Muscular wall (M), Intestinal epithelium (I)

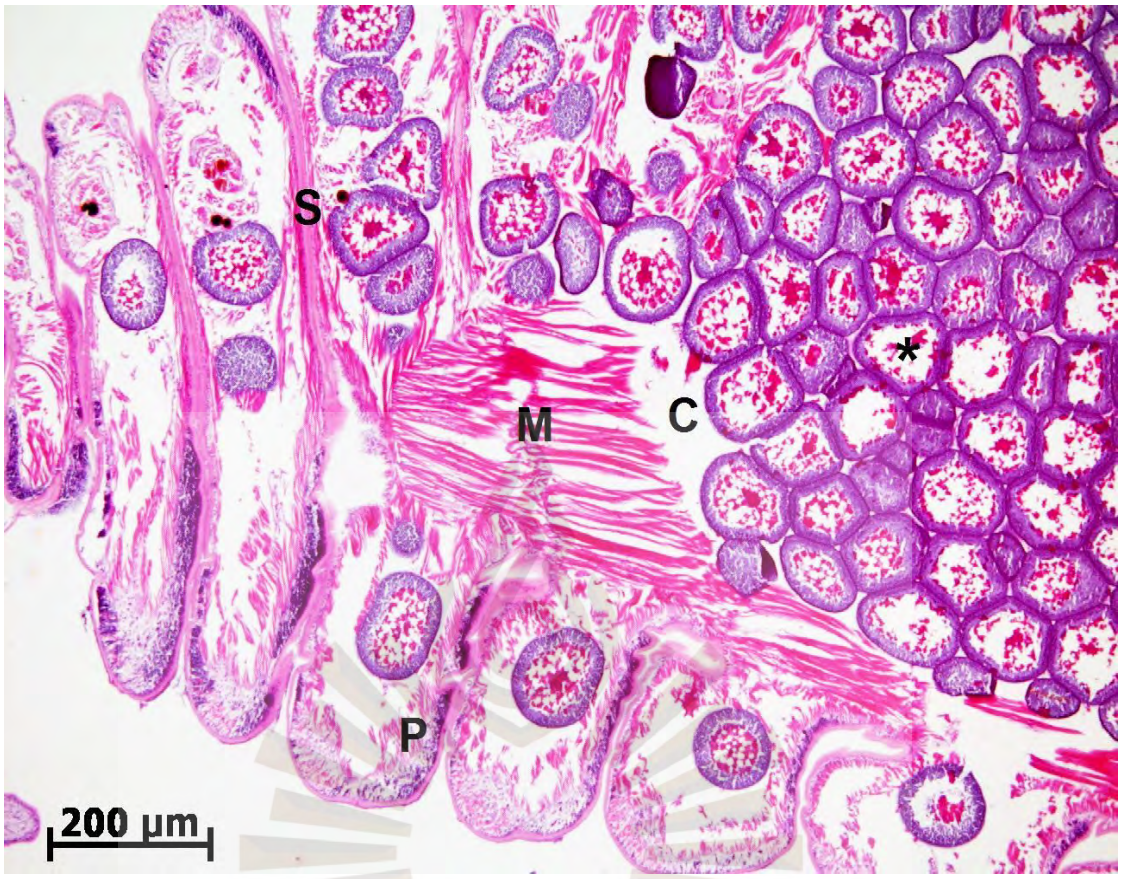

Figure 4.4 Photomicrographs of female *P. nuntia* broodstock reveal abundant of the mature ovums (*) deposited in the coelomic cavity (C). The mature ovum has a thicken layer of jelly coat. Longitudinal muscle (M), Intersegmental septum (S), Parapodia (P).

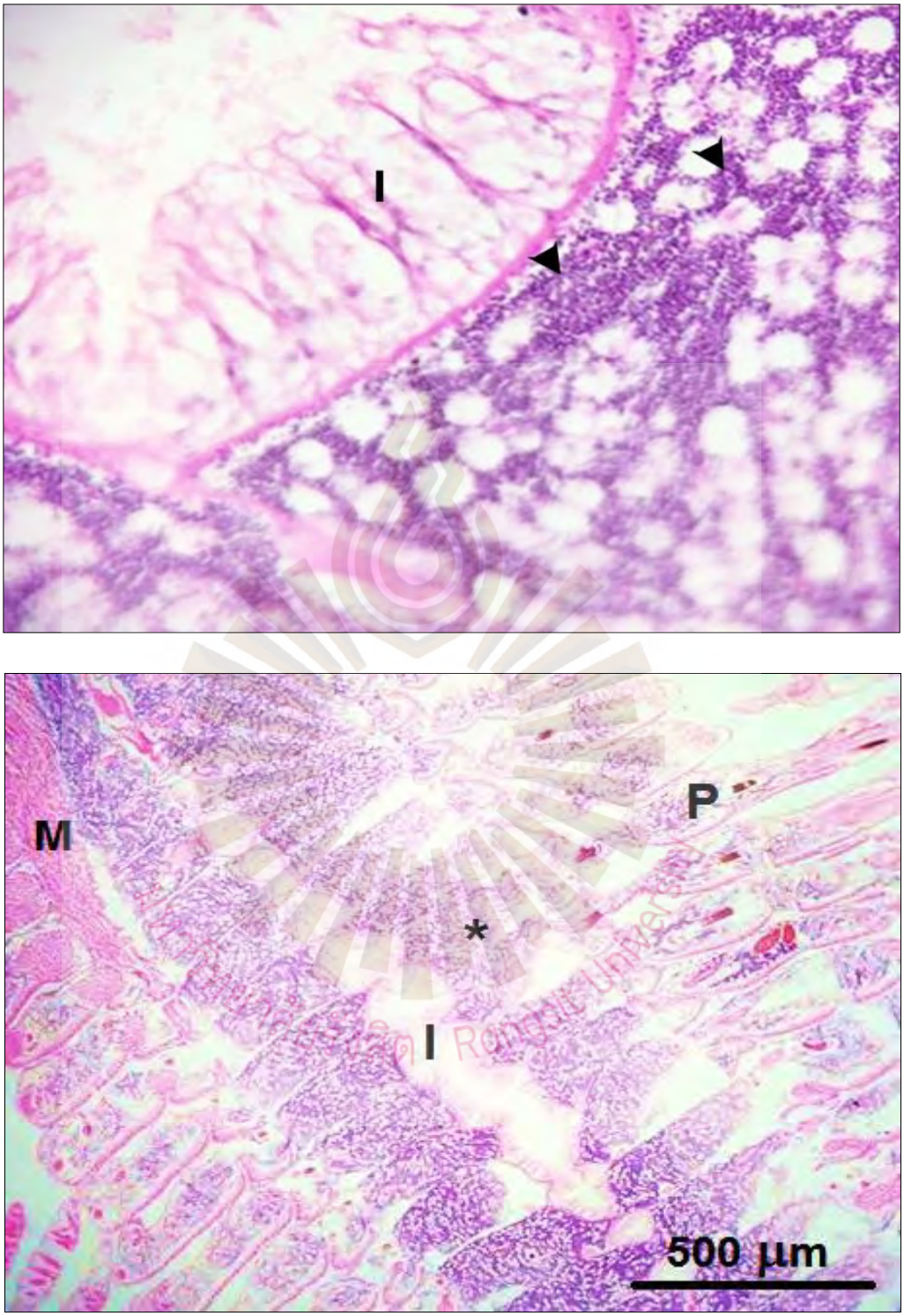

Figure 4.5 Photomicrographs of male *P. nuntia* broodstock (5-6 months of age) stained with H&E exhibit abundant of sperm (*, arrowheads) deposited in the coelomic cavity (C). Longitudinal muscle of body wall (M), Intestinal lumen (I), Parapodia (P).

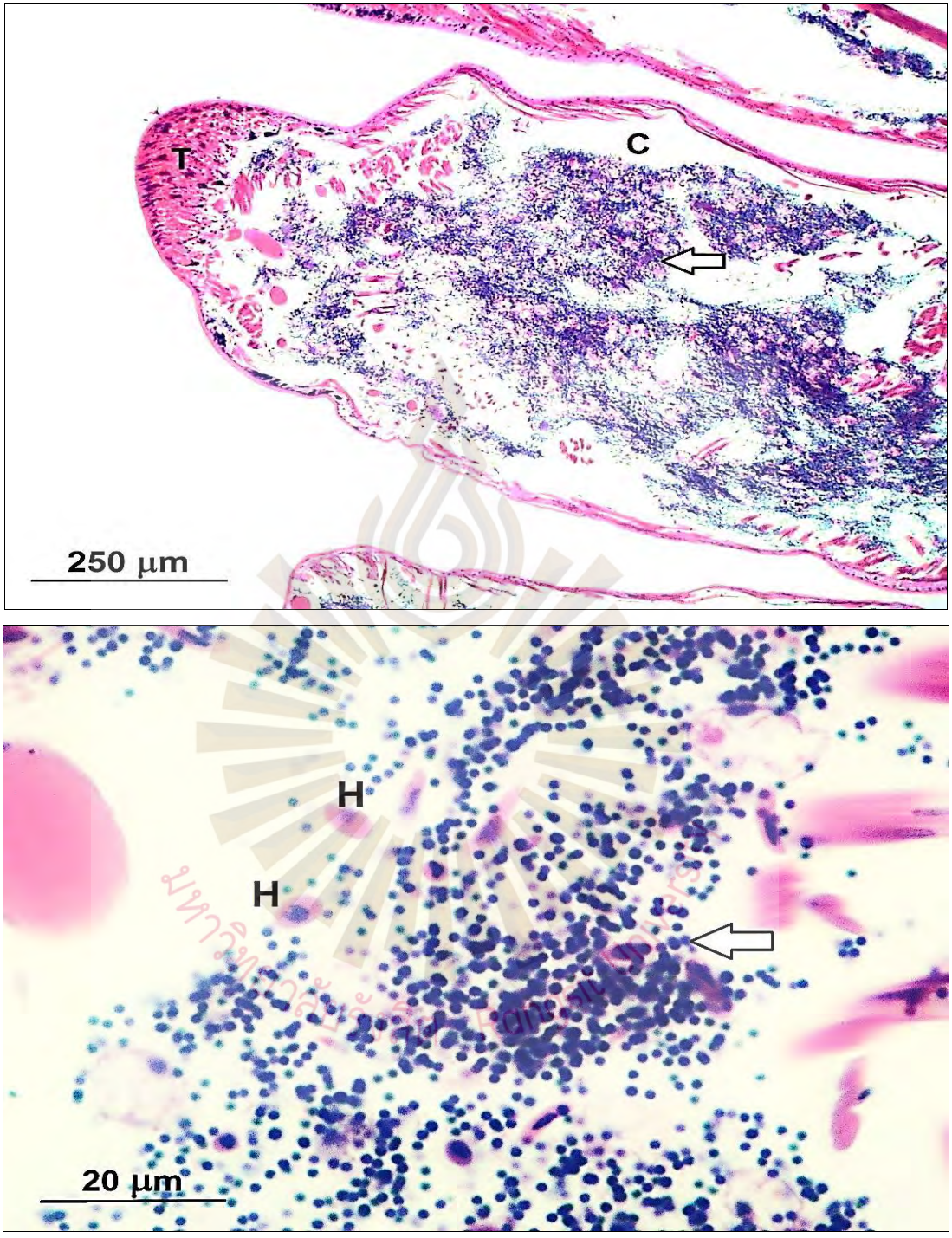

Figure 4.6 Photomicrographs at higher magnification of parapodium of the male broodstock reveal the deposition of abundant sperms (arrow) in the coelomic cavity (C). Hemocyte (H).

4.2 Validation of genes that regulate the production of estrogen receptor by PCR analysis

The specificity of the primer kit was determined by the specificity of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks. The analytical temperature was determined by the annealing temperature during PCR reaction, DNA template control, and to mediator of estrogen signaling in *Perinereis nuntia*. The total DNA was extracted from tree brood stocks.Reaction test with PCR method and investigate in 1.5% agarose gel-electrophoresis.

4.2.1 PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks

The PCR products of estrogen receptor gene (ER) fragment of marine sandworm *Perinereis nuntia* brood stocks when analysis the specificity of the primer based on previous research published. (Lv et al., 2017) Total genomic DNA was extracted from the whole body of *Perinereis nuntia.* The quality and quantity of the DNA were determined using 1.5% agarose gel electrophoresis. The sequence of forward and reverse primers for detection of Estrogen receptor gene fragment of marine sandworm *P. nuntia* broodstocks are described in 3 primer pairs, ER_f0 (5'-TGGTGGGTTTCTCCTCCCTCAC) ER_r5 (5'-TGGCAGCTCTTGGCGCCGATGT), ER_f (5'-ATCGGCGCAAGAGCTGCCAAGCTTG) ER_r1 (5'-TCGATATCTGCCA TATTTC TCCCAC) and ER_f3 (5'-GTGGGAGAAATATGGCAGATATCGA) ER_rb (5'-TGGAG TGGGAATTAAAAACAAGTAA)

4.2.2 PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks are described in ER_f0, ER_r5 primer pairs

The examples of PCR products were not found in 1.5% agarose gel electrophoresis from male and female sexually brood stock of *Perinereis nuntia* was

also measured, ER expression was analyzed with annealing temperature at 53 °C. There were shown in figure 4.7

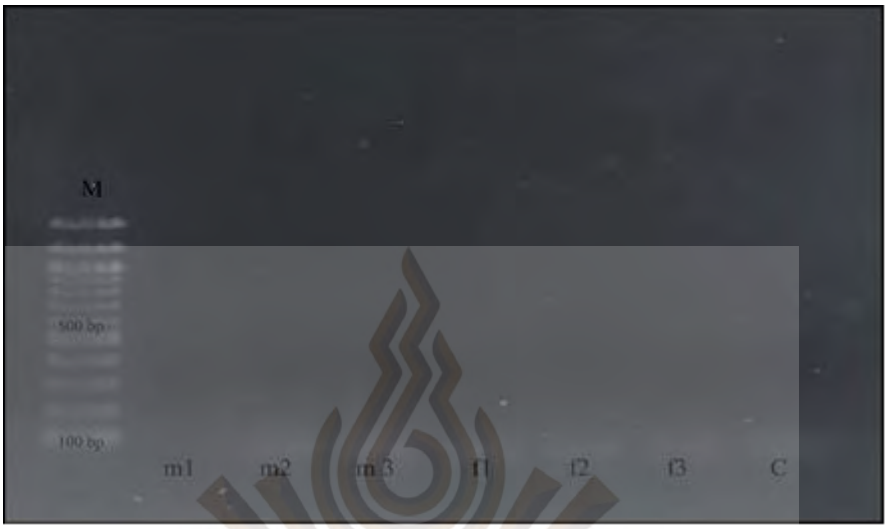

Figure 4.7 PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f0, ER_r5 primer pairs and annealing at 53 °C
M=100 bp marker

Lane 1 100 bp Marker
Lane 2 Male1 Lane 3 Male2
Lane 4 Male3 Lane 5 Female1
Lane 6 Female2 Lane 7 Female3
Lane 8 positive control (overy+uterus)

When study ER expression was analyzed with annealing temperature at 50 °C. The examples of PCR products were not found in 1.5% agarose gel electrophoresis from male and female sexually brood stock of *Perinereis nuntia* was also measured, There were shown in figure 4.8

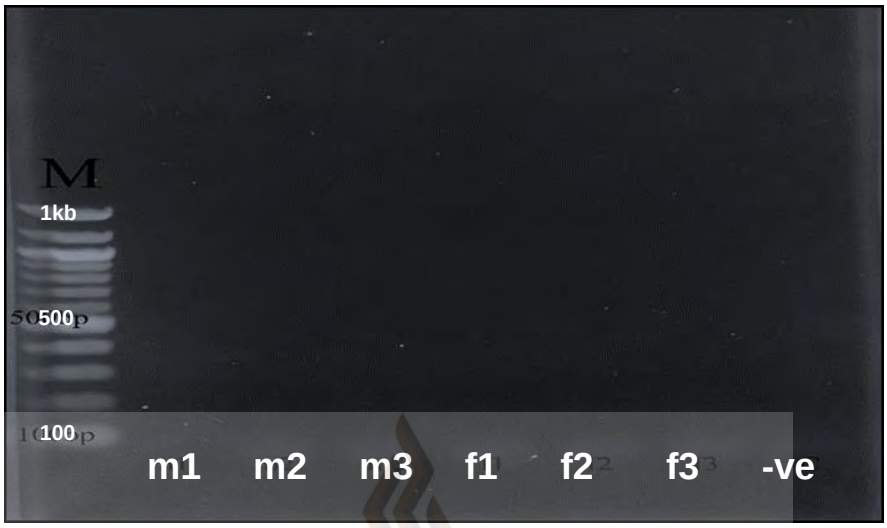

Figure 4.8 PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f0, ER_r5 primer pairs and annealing at 50 °C
M=100 bp marker

Lane 1 100 bp Marker
Lane 2 Male1
Lane 3 Male2
Lane 4 Male3
Lane 5 Female1
Lane 6 Female2
Lane 7 Female3
Lane 8 positive control (overy+uterus)

When study ER expression was analyzed with annealing temperature at 49 °C. The examples of PCR products were not found in 1.5% agarose gel electrophoresis from male and female sexually brood stock of *Perinereis nuntia* was also measured, There were shown in figure 4.9

Figure 4.9 PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f0, ER_r5 primer pairs and annealing at 49 °C
M=100 bp marker

Lane 1 100 bp Marker
Lane 2 Male1
Lane 3 Male2
Lane 4 Male3
Lane 5 Female1
Lane 6 Female2
Lane 7 Female3
Lane 8 positive control (overy+uterus)
Lane 9 Positive control (testis)

When study ER expression was analyzed with annealing temperature at 46 °C. The examples of PCR products were not found in 1.5% agarose gel electrophoresis from male and female sexually brood stock of *Perinereis nuntia* was also measured, There were shown in figure 4.10

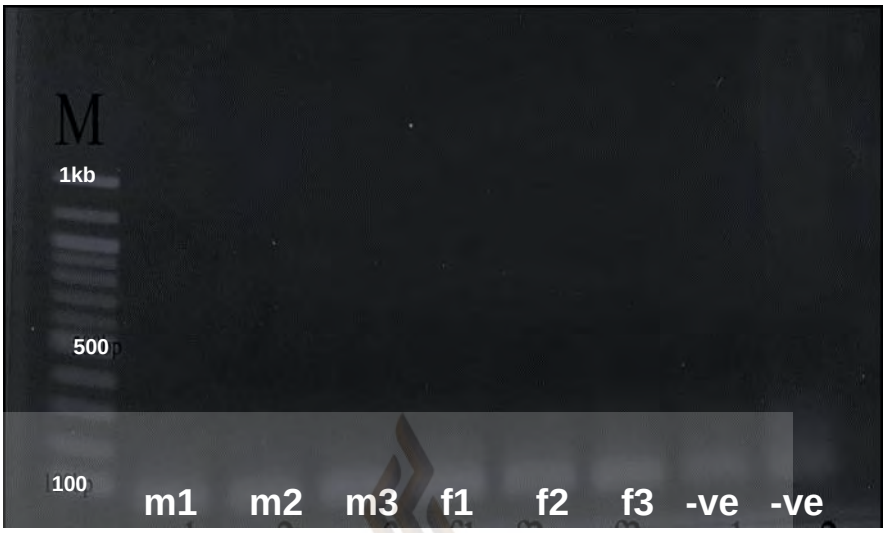

Figure 4.10 PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f0, ER_r5 primer pairs and annealing at 46 °C
M=100 bp marker

Lane 1 100 bp Marker
Lane 2 Male1 Lane 3 Male2
Lane 4 Male3 Lane 5 Female1
Lane 6 Female2 Lane 7 Female3
Lane 8 positive control (overy+uterus) Lane 9 Positive control (testis)

This research cannot find the PCR products from male and female sexually brood stock of *Perinereis nuntia* were digested with ER_f0, ER_r5 primer although design annealing temperature (53, 50, 49, 46 °C)

4.2.3 PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks are described in ER_f ER_r1 primer pairs

The examples of PCR products were not found in 1.5% agarose gel electrophoresis from male and female sexually brood stock of *Perinereis nuntia* was

also measured, ER expression was analyzed with annealing temperature at 53 °C. There were shown in figure 4.11

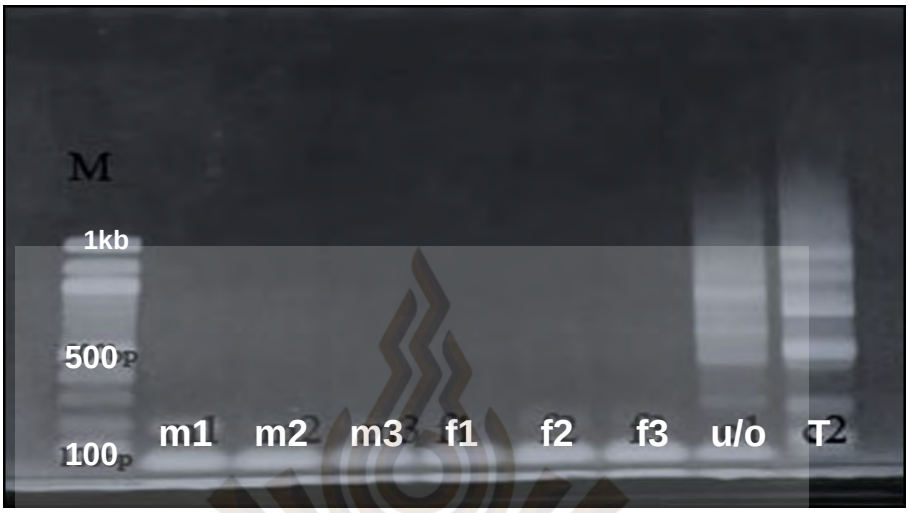

Figure 4.11 PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f ER_r1 primer pairs and annealing at 53 °C
M=100 bp marker

Lane 1 100 bp Marker
Lane 2 Male1
Lane 3 Male2
Lane 4 Male3
Lane 5 Female1
Lane 6 Female2
Lane 7 Female3
Lane 8 positive control (overy+uterus)
Lane 9 Positive control (testis)

When study ER expression was analyzed with annealing temperature at 50 °C. The examples of PCR products were smear band found in 1.5% agarose gel electrophoresis from male and female sexually brood stock of *Perinereis nuntia* was also measured, There were shown in figure 4.12

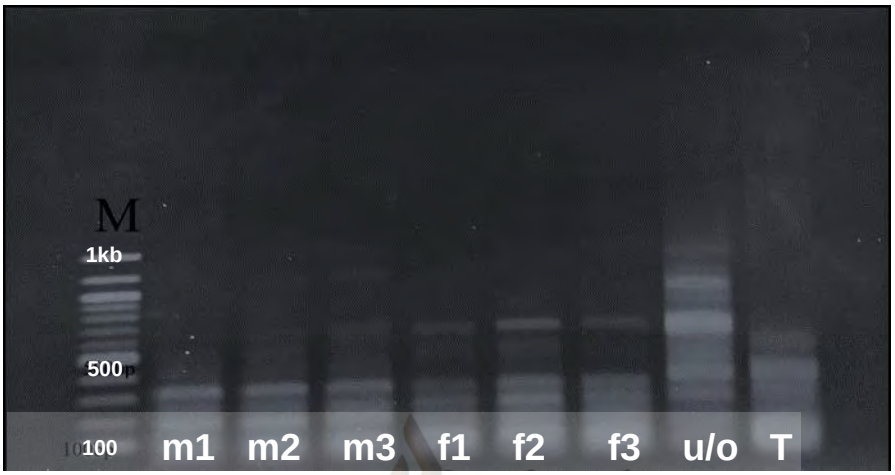

Figure 4.12 PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f ER_r1 primer pairs and annealing at 50 °C
M=100 bp marker

Lane 1 100 bp Marker

Lane 2 Male1 Lane 3 Male2

Lane 4 Male3 Lane 5 Female1

Lane 6 Female2 Lane 7 Female3

Lane 8 positive control (overy+uterus) Lane 9 Positive control (testis)

When study ER expression was analyzed with annealing temperature at 49 °C. The examples of PCR products were multiple band alignment found in 1.5% agarose gel electrophoresis from male and female sexually brood stock of *Perinereis nuntia* was also measured, There were shown in figure 4.13

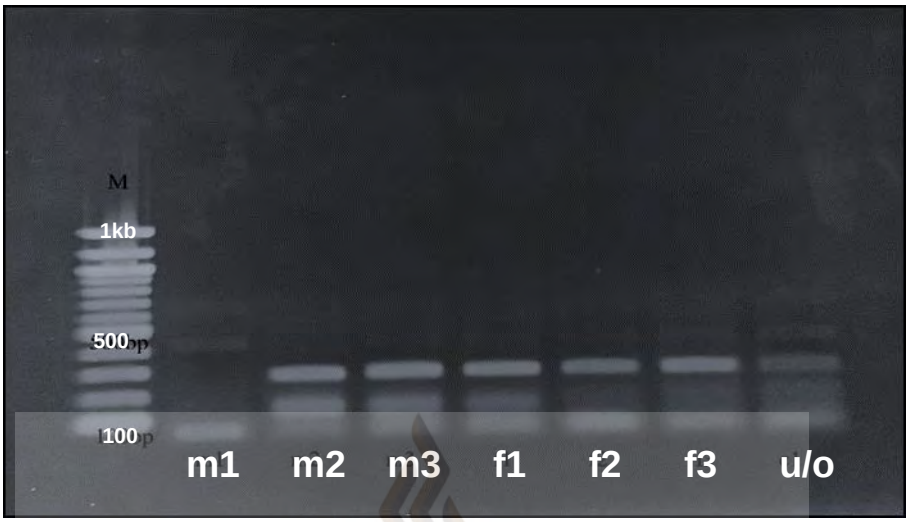

Figure 4.13 PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f ER_r1 primer pairs and annealing at 49 °C
M=100 bp marker

Lane 1 100 bp Marker
Lane 2 Male1
Lane 3 Male2
Lane 4 Male3
Lane 5 Female1
Lane 6 Female2
Lane 7 Female3
Lane 8 positive control (overy+uterus)

When study ER expression was analyzed with annealing temperature at 46 °C. The examples of PCR products were multiple band alignment found in 1.5% agarose gel electrophoresis from male and female sexually brood stock of *Perinereis nuntia* was also measured, there were shown in figure 4.14

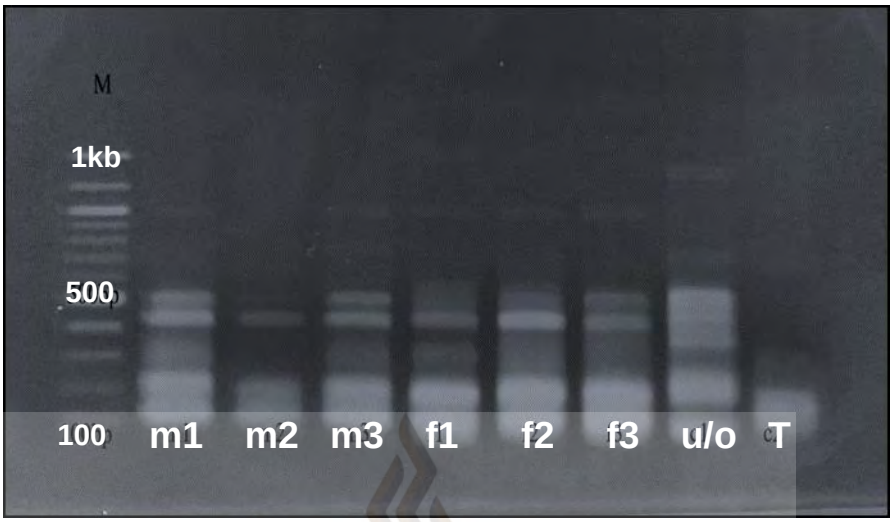

Figure 4.14 PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f ER_r1 primer pairs and annealing at 46 °C
M=100 bp marker

Lane 1 100 bp Marker
Lane 2 Male1 Lane 3 Male2
Lane 4 Male3 Lane 5 Female1
Lane 6 Female2 Lane 7 Female3
Lane 8 positive control (overy+uterus) Lane 9 Positive control (testis)

This research cannot find the PCR products from male and female sexually brood stock of *Perinereis nuntia* were digested with ER_f, ER_r1 primer although design annealing temperature (53, 50, 49, 46 °C). There have multiple band PCR products.

4.2.4 PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks are described in ER_f3, ER_rb primer pairs

The examples of PCR products were smear band in 1.5% agarose gel electrophoresis from male and female sexually brood stock of *Perinereis nuntia* was

also measured, ER expression was analyzed with annealing temperature at 53 °C. There were shown in figure 4.15

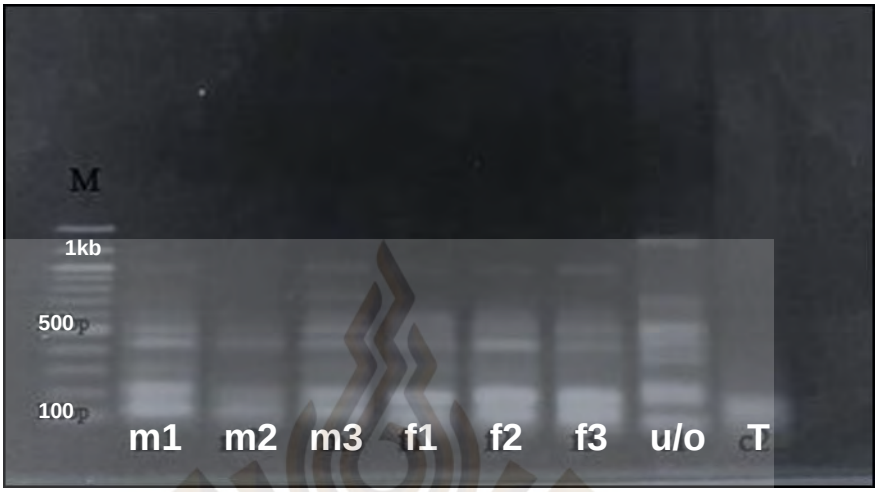

Figure 4.15 PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f 3 ER_rb primer pairs and annealing at 53 °C
M=100 bp marker

Lane 1 100 bp Marker
Lane 2 Male1 Lane 3 Male2
Lane 4 Male3 Lane 5 Female1
Lane 6 Female2 Lane 7 Female3
Lane 8 positive control (overy+uterus) Lane 9 Positive control (testis)

When study ER expression was analyzed with annealing temperature at 50 °C. The examples of PCR products were multiple band alignment found in 1.5% agarose gel electrophoresis from male and female sexually brood stock of *Perinereis nuntia* was also measured, There were shown in figure 4.16

Figure 4.16 PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f 3 ER_rb primer pairs and annealing at 50 °C
M=100 bp marker

Lane 1 100 bp Marker
Lane 2 Male1 Lane 3 Male2
Lane 4 Male3 Lane 5 Female1
Lane 6 Female2 Lane 7 Female3
Lane 8 positive control (overy+uterus) Lane 9 Positive control (testis)

When study ER expression was analyzed with annealing temperature at 49 °C. The examples of PCR products were found to be 200 bp and 400 bp in 1.5% agarose gel electrophoresis from male and female sexually brood stock of *Perinereis nuntia* was also measured, There were shown in figure 4.17 when repeat again the examples of PCR products were found to be 200 bp in 1.5% agarose gel electrophoresis shows in figure 4.18

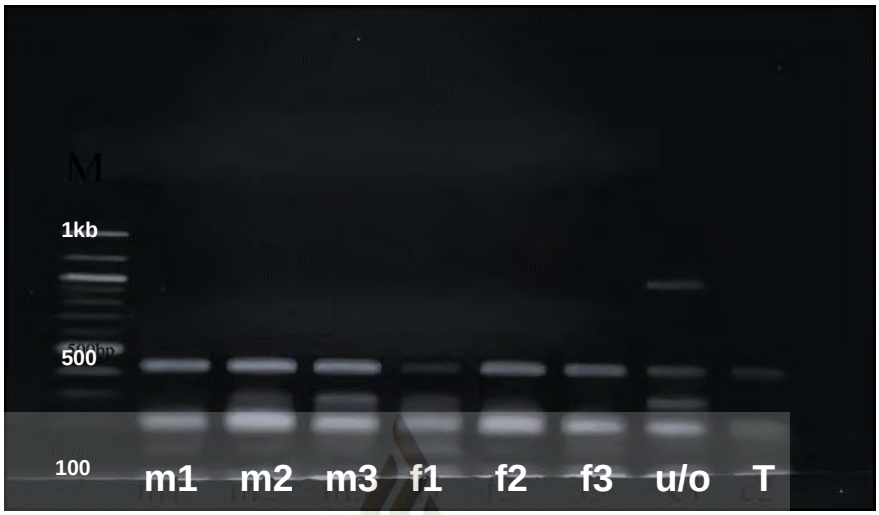

Figure 4.17 PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f 3 ER_rb primer pairs and annealing at 49 °C
M=100 bp marker

Lane 1 100 bp Marker

Lane 2 Male1 Lane 3 Male 2

Lane 4 Male3 Lane 5 Female1

Lane 6 Female2 Lane 7 Female3

Lane 8 positive control (overy+uterus) Lane 9 Positive control (testis)

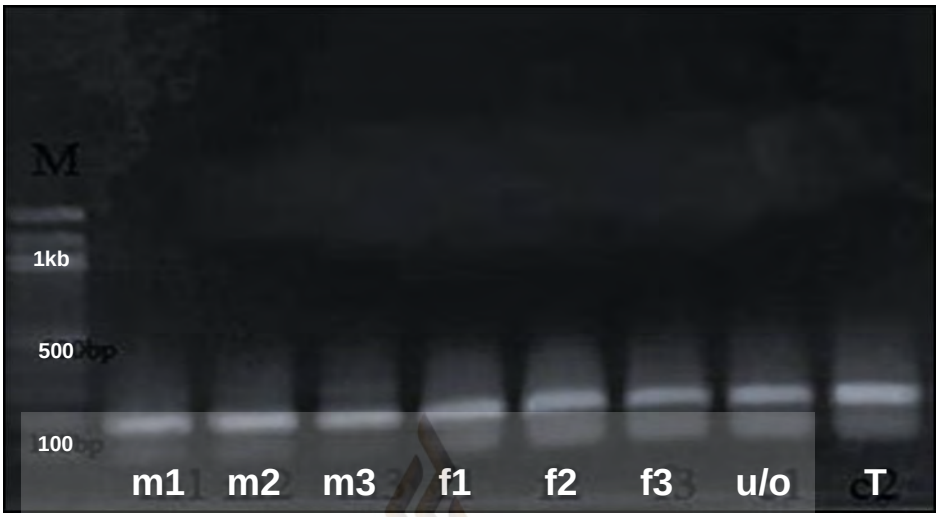

Figure 4.18 PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks amplified with ER_f 3 ER_rb primer pairs and annealing at 49 °C
M=100 bp marker

Lane 1 100 bp Marker
Lane 2 Male1 Lane 3 Male2
Lane 4 Male3 Lane 5 Female1
Lane 6 Female2 Lane 7 Female3
Lane 8 positive control (overy+uterus) Lane 9 Positive control (testis)

The PCR products after restriction and run on 1.5% agarose from male and female sexually brood stock of *Perinereis nuntia* were digested with ER_f3, ER_rb primer, annealing 49 temperature. There are presented 3 different patterns consist, two fragments 200 and 400 bp of the wild type homozygous *Perinereis nuntia* in first agarose gel and a single 200 bp fragment for the wild type homozygous *Perinereis nuntia* in secound agarose gel The examples of restriction fragments or genotypes of *Perinereis nuntia* were shown in figure 4.17 and figure 4.18, respectively. To apply PCR Product to cut the gel into the desired band at 200 and 400 bp. Analyze the sequence of nucleotides. used in this experiment to GIBTHAI company. To perform DNA sequencing. The sequence was compared to GenBank database.

4.2.5 Comparison PCR products of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks are described in ER_f3 ER_rb primer pairs

The sequence of the estrogen receptor gene fragment of marine sandworm *Perinereis nuntia* brood stocks was confirmed with the BLAST al-gorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The PCR Product to cut the gel into the desired band at 200 and 400 bp. when to analyze are show in this sequence.

>3.1_F_296
TCAGGCCAGTACACTGGTTACTAGCTTGCTTACTTGCTGATTTGCTTGCTTA
CTTGCTTAATTGCTGACTTGCTTGCTTACTTACTAAACAATAGTACTTAAGC
CAGGTTTCCACTGATGATTTTCAGCCTATATCAAGGGCAATACATGTGATC
AATACATATTGGGGCGCTAGCGGCGCCACGACAACACCTCACACCACAAA
CCGCGCCTTGTTCCATAAACAAAAACATGACTTCAAATTTTCGACCTTTTT
ACCCACCAGAGGAATCCATCTTCATTCGCCGATAGGACAC

>3.2_F_154
AGCTCATGTGATTACTTTTTTGTGCTGTTTTTGCAACTACGATCACTTCAAC
CCTTATGGGTTCTAGTTGTTATCTCATTTCAGTTTGTTTGACTTGGGCTCCG
AGTACTACTGCTACACTTCCGACATTACTTGTTTTTAATTCCCACTCCAA

>3.3_F_152
CGCGAGTGATTACTTTTTTGTGCTGTTTTTGCAACTACAATCACTTCAACCC
TTATGGGTTCTAGTTGTTATCTCATTTCAGTTTGTTTGACTTGGGCTCCAAG
TACTACTGCTACACTTCCGACATTACTTGTTTTTAATTCCCACTCCAA

>3.4_F_156
GCCGTCTTGTGATTACTTTATTTGTGCTGTTTTTGCAACTACGATCACTTCA
ACCCTTATGGGTTCTAGTTGTTATCTCATTTCAGTTTGTTTGACTTGGGCTC
CAAGTACTACTGCTACACTTCCGACATTACTTGTTTTTAATTCCCACTCCAA

4.2.5.1 Result of multiple alignment test

Comparism the sample of PCR product 3.2, 3.3, 3.4 are very similar but there have six base are different

```
3.2_F_154      -AGCTCATGTGATTACTTTTTTG-
TGCTGTTTTTGCAACTACGATCACTTCAACCCTTAT
3.4_F_156
GCCGTCTTGTGATTACTTTATTTGTGCTGTTTTTGCAACTACGATCACTTCA
ACCCTTAT
3.3_F_152      ---CGCGAGTGATTACTTTTTTG-
TGCTGTTTTTGCAACTACAATCACTTCAACCCTTAT
               * .********** ** ********** *********
```

```
3.2_F_154
GGGTTCTAGTTGTTATCTCATTTCAGTTTGTTTGACTTGGGCTCCGAGTACT
ACTGCTAC
3.4_F_156
GGGTTCTAGTTGTTATCTCATTTCAGTTTGTTTGACTTGGGCTCCAAGTACT
ACTGCTAC
3.3_F_152
GGGTTCTAGTTGTTATCTCATTTCAGTTTGTTTGACTTGGGCTCCAAGTACT
ACTGCTAC
               ********************************************** **********
```

```
3.2_F_154      ACTTCCGACATTACTTGTTTTTAATTCCCACTCCAA
3.4_F_156      ACTTCCGACATTACTTGTTTTTAATTCCCACTCCAA
3.3_F_152      ACTTCCGACATTACTTGTTTTTAATTCCCACTCCAA
               **********************************
```

Comparism the sample of PCR product 3.1 with 3.2, 3.3 and 3.4

```
3.1_F_296
TCAGGCCAGTACACTGGTTACTAGCTTGCTTACTTGCTGATTTGCTTGCTTA
CTTGCTTA
3.4_F_156      --------------------------GCCGTCTTG----------TGATTACTTTATTT
                                ** .**** **.****** .**.

3.1_F_296
ATTGCTGACTTGCTTGCTTACTTACTAAACAATAGTACTTAAGCCAGGTTTC
CACTGATG
3.4_F_156      G-TGCTGTTTT----------
TGCAACTACGATCACTTCAACCCTTATG
          . *****. **          *.:***.... ** * :.*.** ***

3.1_F_296
ATTTTCAGCCTATATCAAGGGCAATACATGTGATCAATACATATTGGGGCG
CTAGCGGCG
3.4_F_156      GGTTCTAGTTGTTATC----------
TCATTTCAGTTTGTTTGACTTGGG---
          . ** ** .**** ***.*.** .*** .**.* *

3.1_F_296
CCACGACAACACCTCACACCACAAACCGCGCCTTGTTCCATAAACAAAAA
CATGACTTCA
3.4_F_156      ----------CTCCAAGTACTACTGCTACACTTCC
                          ****.:**.*.:*:. *****.

3.1_F_296
AATTTTCGACCTTTTTACCCACCAGAGGAATCCATCTTCATTCGCCGATAG
GACAC
3.4_F_156
GACATTACTTGTTTTTAATTCCCA----------CTCCAA--------
          .* :**.: ******. .*** * **.*
```

4.3 In situ hybridization

Because the PCR products after restriction and run on 1.5% agarose from male and female sexually brood stock of *Perinereis nuntia* are presented different sequence then can not analyze in In situ hybridization but to study in Immunohistochemistry

4.4 Immunohistochemistry

To determine the immunohistochemistry revealed positive reactions of anti-estrogen receptor-beta at the clusters of cells close to the stump of parapodia with all of female and male brooders. An intense immunoreaction also deposited at the cytoplasm of ovum, where as non-immunoreactive signal was observed in pre-reproductive specimens. The corresponding sense control experiment is shown in Figure 4.19 control immunohistochemistry with mouse mammary tissue exhibit positive reaction of anti-estrogen receptor-beta (ER-beta) at the cytoplasm of epithelial cells of lactiferous gland as red-brownish color. In the female broodstock (5-6 months of age), we conducted of immunoperoxidase labeling for ER-beta using immunohistochemistry reveal positive reaction with the developing egg cells accumulated in the coelomic cavity (Figure 4.20) In the coelomic cavity, the developing egg cells is composed of cells with abundant cytoplasm and red-brownish colored (Figure 4.21). In the mucosal cell layer of intestine in the female broodstock, also contained of immunoperoxidase labeling for ER-beta using immunohistochemistry reveal positive reaction (Figure 4.22). Indirect immunohistochemical labeling was detected in female and male broodstocks exhibit positive reaction of anti-estrogen receptor-beta in the cell clusters of various forms based on their location from the distal end of the parapodium to the coelomic cavity (Figure 4.23). This feature corresponds to the reproductive stage of egg and sperm productions and deposition, found in the coelomic cavity.

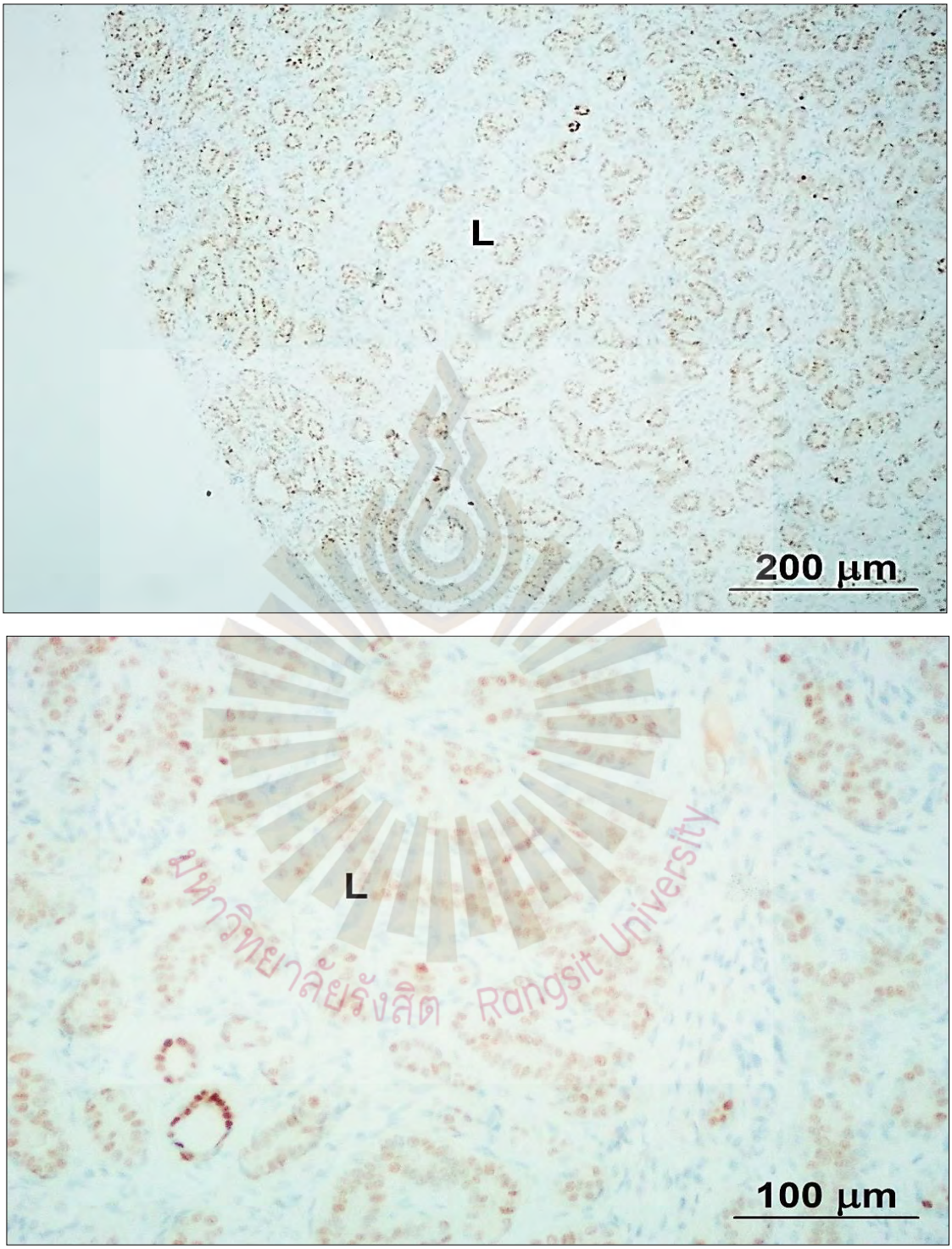

Figure 4.19 Photomicrographs of control immunohistochemistry with mouse mammary tissue exhibit positive reaction of anti-estrogen receptor-beta (ER-beta) at the cytoplasm of epithelial cells of lactiferous gland as red-brownish color (L).

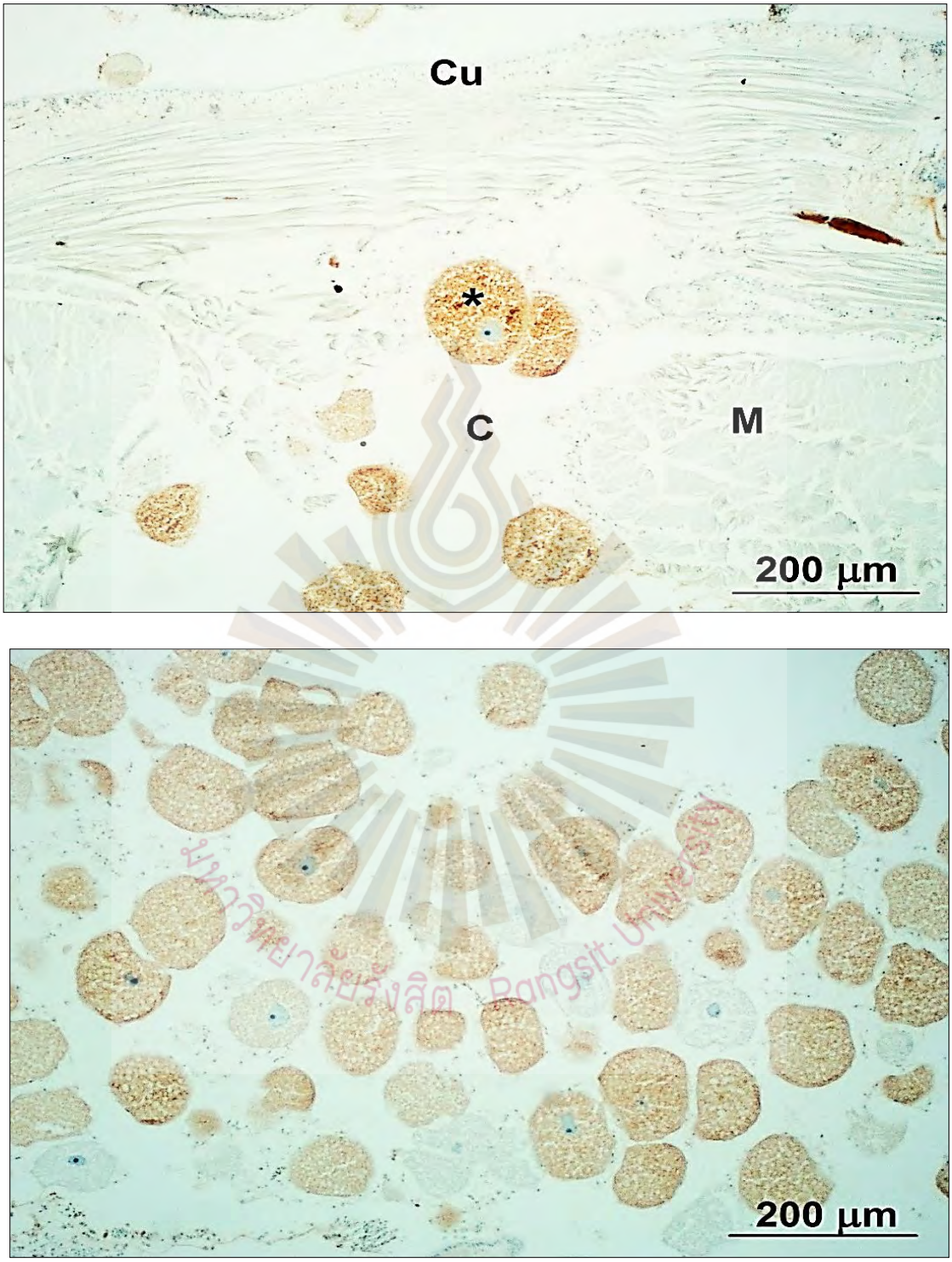

Figure 4.20 Photomicrographs of immunoperoxidase labeling for ER-beta with female broodstock (5-6 months of age) reveal positive reaction with the developing egg cells (*) accumulated in the coelomic cavity (C).
Cuticular wall (Cu), Muscle tissue (M).

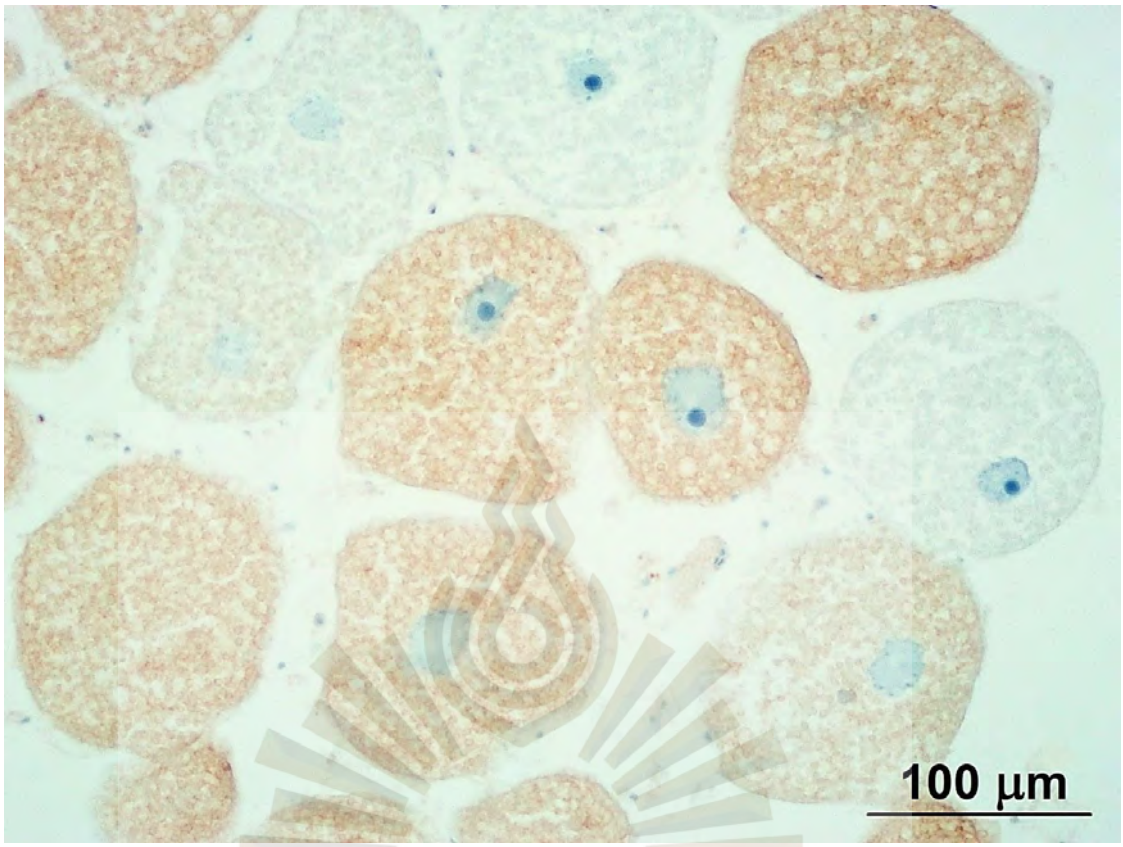

Figure 4.21 Photomicrograph at higher magnification of female sandworm broodstock with indirect immunohistochemistry shows ER-beta deposition at the cytoplasm of developing eggs.

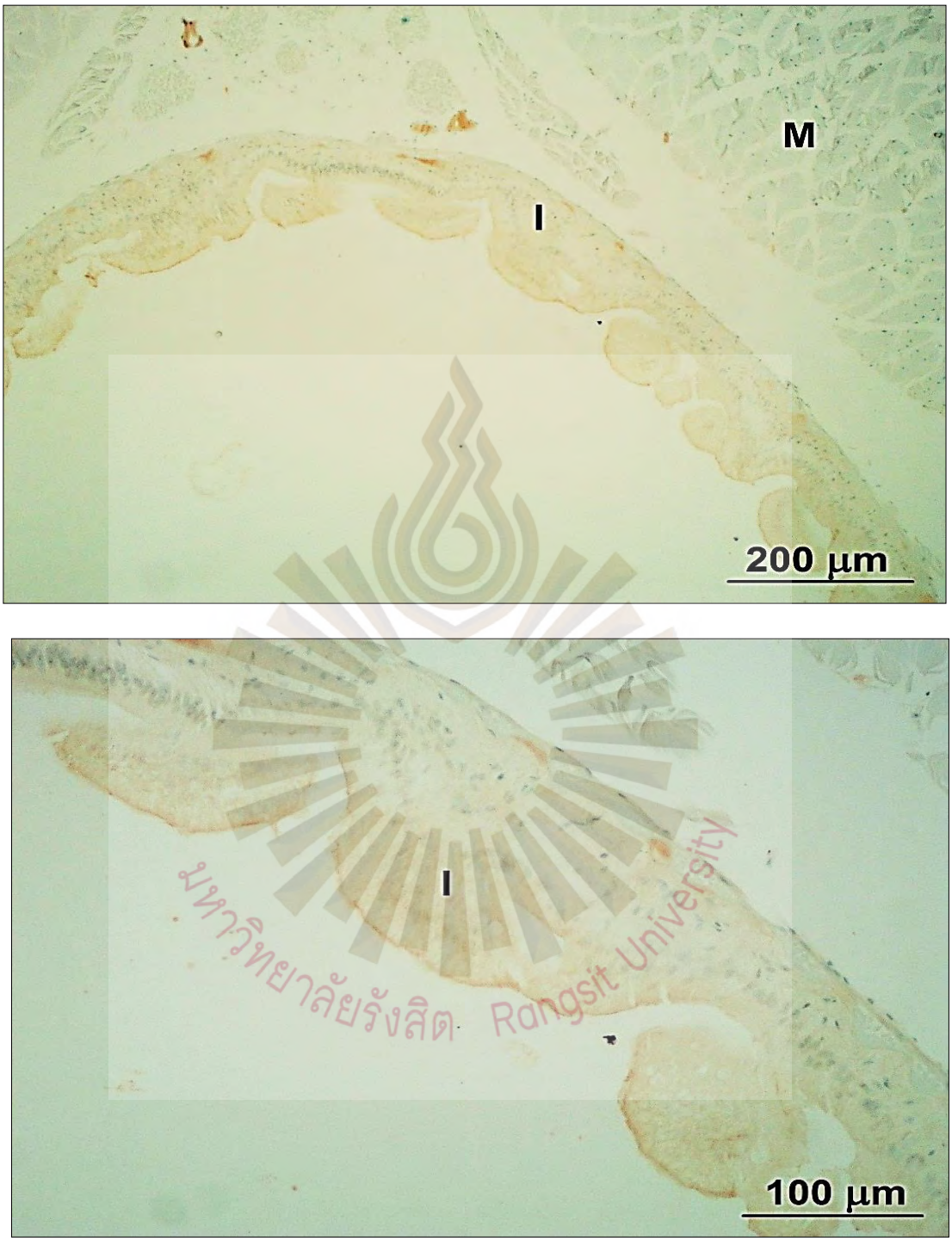

Figure 4.22 Photomicrographs of immunoperoxidase labeling for ER-beta deposition in female broodstock reveal positive reaction at the mucosal cell layer of intestine (I).

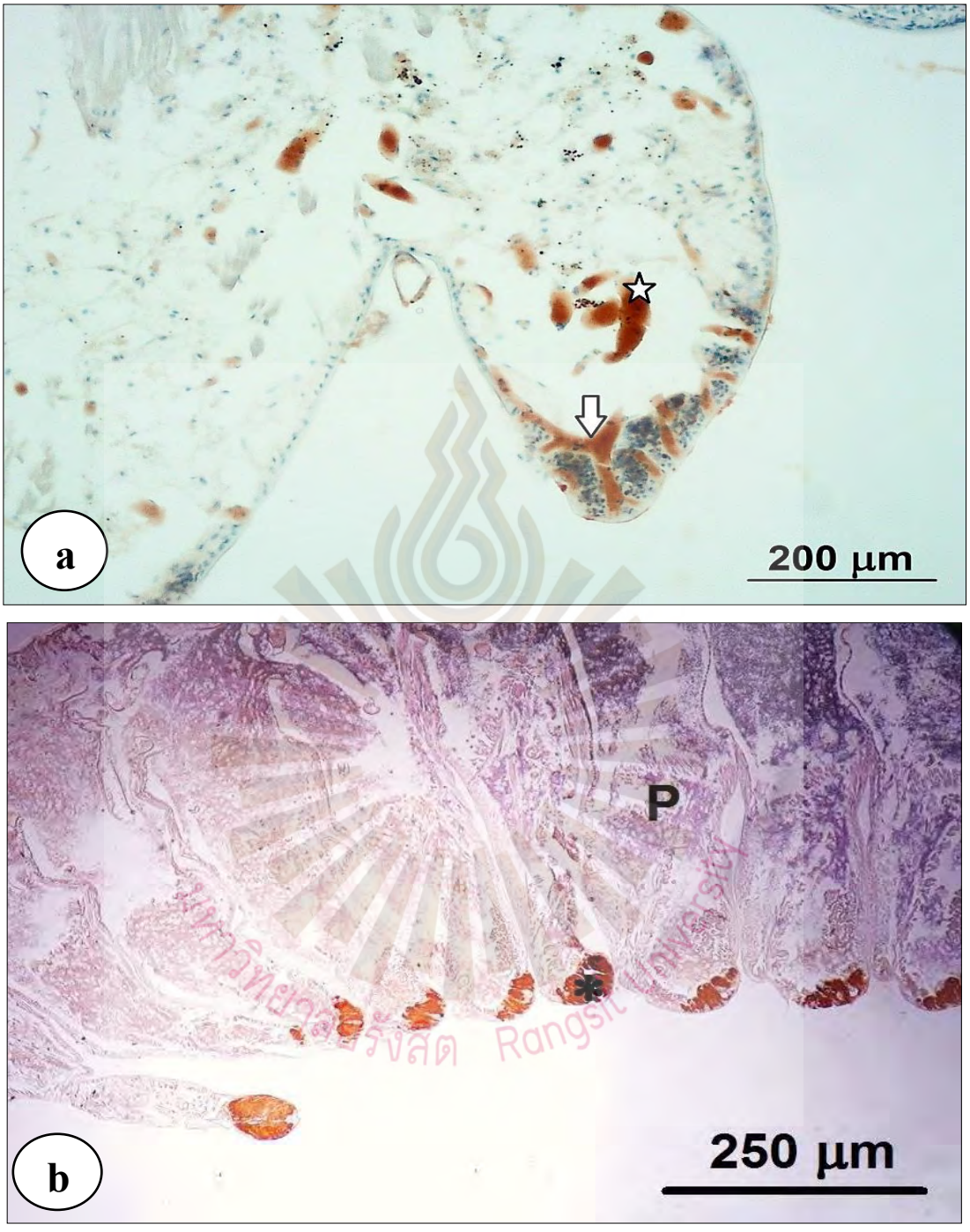

Figure 4.23 Photomicrographs of indirect immunohistochemical labeling of female (a) and male (b) broodstocks exhibit positive reaction of anti-estrogen receptor-beta at the cluster of cells as red-brownish color (arrow, *), at the tip of parapodia. This feature corresponds to the reproductive stage of egg and sperm productions and deposition, found in the coelomic cavity.

CHAPTER 5

DISCUSSION AND CONCLUSION

The presumption of this study is that the reproductive sandworm may generate and use estrogen to control inter-cellular group for reproductive functions as was reported in Korean lugworm, *P. aibuhitensis* and other vertebrate species. Actions of estrogen at its target cells are commonly known since it binds with the ER. Six brooders of sandworm at 5-6 months of age and 5 pre-reproductive animals at 2-3 months old were examinedThe number of the males and females were at ratio of 1:1 (3 females and 3 males). To optimize tissue suitability for light microscopy (LM) and for possible research applications including Immuno -histochemistry studies and validation of genes that regulate the production of estrogen receptor by PCR analysis.

Result from PCR products have multiple sequence alignment when apply PCR product to cut the gel into the desired band at 200 and 400 bp. Analyze the sequence of nucleotides. DNA sequence are 296,154,152 and156 bp.*Shown ,that the non-specific primer sets of* estrogen receptor in marine sandworm *Perinereis nuntia* brood stocks. May be it has *mis-amplification* or it have unexpected events *in method.*While, it is not possible to generate ER in marine sandworm *Perinereis nuntia* brood stocks. Should the study be continued

Monitoring of tissue specific location for synthesis and accumulation of estrogen receptor-beta (ER-beta) protein in a marine polychaete, *P. nuntia* using immunohistochemistry described in this article revealed definitive tissues involved in the reproductive system such as cytoplasm of the developing eggs in the coelomic cavity of female broodstock, the intestinal mucosal cells and the cluster of germ cells at the tip of parapodia of both female and male broodstocks. Because *P. nuntia* is a fully segmented marine worms with body segments, its whole body cavities are

containers with gamete production during the breeding season (Cho et al., 2015). Polychaetes are described as pseudohermaphroditic worm without permanent gonad (Rebscher et al., 2007). For instance in the localization of primary gonad of this worm, a molecular germline marker (*Pn-vasa*) sequence was applied to localize the primitive gonad and found that germ cells originate from the mesodermal posterior growth zone (MPGZ) at the tail region of the juveniles and then migrate into the anterior segments to form a transverse cluster of cells at distal end of parapodia of each body segments of the adult age (Maceren-Pates et al., 2015). This feature corresponded to the positive immuno-localization of ERβ in the *P. nuntia* broodstock. Unlike another well-studied annelid species, *Platynereis dumerilii* with incomplete intersegmental-septum, the gamete production mechanism was suggested that germ cells settled from the MPGZ at the tail region of the juvenile and later translocate into the coelomic cavity as a primitive gonad to develop oocytes (Rebscher et al., 2007). Result from tissue distribution analysis of *ER* mRNA in adult Korean lugworm, *P. aibuhitensis* showed the *paER* expressions in various tissues, including stomach, esophagus, esophageal gland, body wall, head, and most strongly in the intestines. (Lv et al., 2017).

In family Nereidae, the gonads have never become localized and oocytes grow freely in the female coelomic cavity. Vitellogenin, the precursor of yolk protein, is secreted by specialized coelomic cell called “eleocytes” and the intestinal mucosal cells during oogenesis in the King lugworm, *Nereis virens* (Garcia-Alonso and Rebscher, 2005). Vitellogenesis is the process of yolk formation in the oocyte or female germ cell and involved in reproduction of marine organisms. It starts when the fat bodies stimulates the release of juvenile hormones and then produces vitellogenenin. A major role of the yolk proteins is involved in oocyte-sperm binding, sperm penetration and fertilization of zygote. An exogenous estradiol-17beta was used to demonstration on up regulation of the vitellogenin mRNA in *Xenopus laevis* (Skipper and Hamilton, 1997).

Polychaetes are used extensively for shrimp broodstock maturation diet due to their qualities in enhancing shrimp reproductive performances (Middleditch et al., 1980; Lytle et al., 1990). Such success partly results from their high-saturated fatty

lipids (HUFAs) component, particularly arachidonic acid content as well as some reproductive hormones recently identified in polychaetes such as prostaglandin E2 (Meunpol et al., 2005) and prostaglandin F2α (Poltana, 2005). Other hormones discovered in polychaetes are ecdysteroid, osmoregulary hormones, oxytocin/vasopression hormones, reproductive hormones, sex hormones and sex pheromone. However, there is no evidence of vertebrate-type steroid identification in polychaetes (Meunpol et al., 2007).

Vertebrate-type steroids can be found in invertebrates such as androsterone, progesterone, estradiol, corticosteroids (Darvas et al., 1997; LaFont, 2000). Fingerman et al. (1993) reported that progesterone and estradiol are sex steroids important to the crustacean reproductive system and involve in ovarian development by stimulation of vitellogenesis and increasing oocyte diameter of *Penaeus vannamei*. A trial by using of 17α-hydroxyprogesterone extracts from natural *P. nuntia* and synthetic progesterone to induce development of the penaeid oocytes exhibited a significant increment of percentage of vitellogenic oocytes and reduce latent period of spawning (Meunpol et al., 2007).

The possibility of polychaetes possessing similar reproductive hormones to crustaceans especially vertebrate-type steroids is raised since polychaetes are acknowledged as being the best diet for shrimp maturation (Lytle et al., 1990, Browdy, 1992, Marsden et al., 1997; Naessen et al., 1997). So, the identification of reproductive hormones as well as the receptors of these hormones in polychaetes can help in developing a reproductive maturation diet by combining these hormones into pellet food. In addition, a phylogenetic analysis of full-range *paER* gene exhibits orthologue of ERs among the annelids, crustaceans and other invertebrate species (Lv et al., 2017).

In summary, the present results describe the first tissue-specific ERβ expression in *P. nuntia* broodstock and indicate that the ERβ was exclusively present in the developing oocytes, intestinal mucosa and cluster of cells at the tip of parapodia of the reproductive worms, which equivalents to the location of primary gonads. On

the contrary, ERβ localization was not observed in the juvenile specimens. These positive tissues of ERβ deposition probably mediate some of the effects of estrogen action in the regulation of growth and development of the gametes. Comparing to the results of immunohistochemical analysis of two ERs subtypes, ERα and ERβ in rat ovary, both ER subtypes are found in the oocytes, germinal epithelium and the granulosa cells of growing follicles, but no staining is detected in the primordial follicles as well as in the ovary of neonatal rat. However, the level of ERβ immunoreaction was higher in comparison with ERα.The exclusive presence of the ERβ in developing oocytes and granulosa cells in ovary indicates that the ERs mediates some effects of estrogen action in the regulation of growth and maturation of ovarian follicles and gametes during reproductive age (Sar and Welsch, 1999).

Polychaetes may need sex steroids at the specific time for reproductive purpose. To confirm this hypothesis, further study needs to examine the possibility of inducing oocyte/ sperm maturation and quantity through hormonal feed, as well as the time-course of ER expression during metamorphosis and growth of *P. nuntia*.

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APPENDICES

APPENDIX A
REAGENT PREPARATION

1. Prepare a stock solution of 10x Tris –borate-EDTA (TBE) buffer

Tris base	54	g
Boric acid	27.5	g
Dissolve in 800 ml distilled water (used stirring mix)		
Add 0.5 M Na_2EDTA (pH 8.0)	20	ml
Adjust volume to 1,000 ml		

2. Prepare 1X TAE buffer volume 100 mL.

5X TAE buffer	20	mL
Distilled water	800.0	ml

3. Prepare 1.5% Agarose gel solution

Agarose powder	1.5	g
1X TAE buffer	100	mL

4. Prepare Davidson's fixative (LM fixative)

Ethanol 95%	330	mL
Formalin 100% (Formaldehyde 37-39%)	220	mL
Glacial acetic acid)	115	mL
Distilled water	335	ml

5. Hematoxylin solution

Hematoxyline (pH 5.-7.2)	2.0	g
Distilled water	800.0	ml
Aluminium potassium sulfate	50.0	g
Sodium iodate	0.4	g
Glycerin	200	ml

1. Dissolve the hematoxyline in Distilled water 100 ml (or 10% ethyl alcohol 10 ml)

2. Dissolve the aluminium potassium sulfate in Distilled water 650 ml and using a magnetic stirrer

3. When the alum is completely dissolved, add the hematoxylin crystals. Add distilled water 150 ml and using a magnetic stirrer

4. When all of the hematoxylin has been dissolved,add the sodium iodate.Let stir for approximately 10 minute

5. Add Glycerin 200 ml.Continue stirring until the Glycerin is completely dissolved.

6. The resulting solution,if properly prepared,will be a deep wine color. One ml. of the solution dropped into tepid water will immediately turn blue.

4. Eosin solution

1% stock alcoholic eosin

Eosin y. water soluble	1.0	g
Distilled water	20	cc.
95% Ethyl alcohol	80	cc.

Dissolve the eosin y in Distilled water and using a magnetic stirrer add 95% Ethyl alcohol. Always filter before each use.

Working Eosin solution

1% Stock alcoholic eosin	1 part
85% ethyl alcohol	2 part

Before using add glacial acetic acid 0.5 ml in Eosin solution 100 ml and using a magnetic stirrer

1. Deparaffinize slide and hydrate to distilled water. Dezenkerize, if necessary, before staining.

2. Stain in Hematoxylin solution for 5 minutes.

3. Wash in running tap water for 2-5 minute.

4. Differentiate in 1% ammonia water, 1-2dips.

5. Wash briefly in tap water

6. Counterstain in eosin solution 2 minutes.

7. Dehydate and clear through 2 changes each 95% ethyl alcohol ,absolute ethyl alcohol, and xylene 2 minutes each

8. Mount with resinous medium.

Results

Nucleus	blue
Cytoplasm	pink to red

BIOGRAPHY

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