การพัฒนาการสกัดดีเอ็นเอจากฟองน้ำทะเลสีน้ำเงิน *Xestospongia* sp.

นางสาว มนัชยา ชวนชื่น

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ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

DEVELOPMENT OF DNA EXTRACTION FROM THE BLUE SPONGE *XESTOSPONGIA* SP.

Miss Manatchaya Chuanchen

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacognosy
Department of Pharmacognosy and Pharmaceutical Botany
Faculty of Pharmaceutical Sciences
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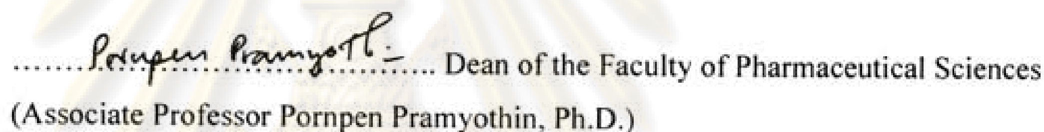
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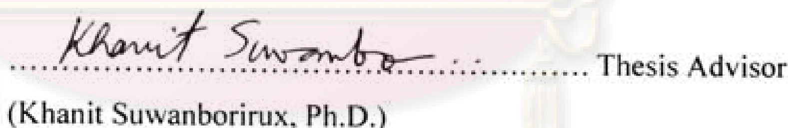
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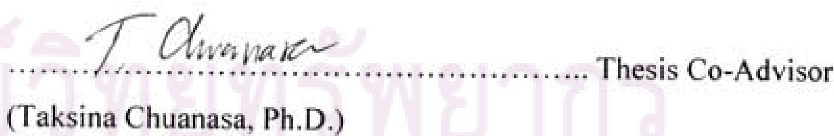
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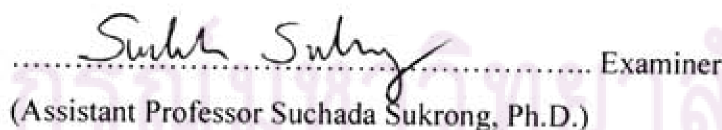
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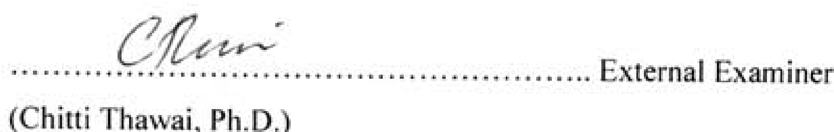
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มนัชยา ชวนชื่น: การพัฒนาการสกัดดีเอ็นเอจากฟองน้ำทะเลสีน้ำเงิน *Xestospongia* sp. (DEVELOPMENT OF DNA EXTRACTION FROM THE BLUE SPONGE *XESTOSPONGIA* SP.) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร. คณิต สุวรรณบริรักษ์ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร. ทักษิณา ชวนอาษา, ๑๐๔ หน้า

ฟองน้ำทะเลสีน้ำเงิน *Xestospongia* sp. เป็นแหล่งผลิตที่ให้สารกลุ่มเรเนียรามัยซินในปริมาณสูงมาก ซึ่งสารกลุ่มนี้มีศักยภาพที่สามารถพัฒนาเป็นสารต้านมะเร็งชนิดใหม่ได้ จึงมีความสนใจที่จะศึกษาวิถีชีวสังเคราะห์ของสารเรเนียรามัยซินในฟองน้ำชนิดนี้ การตอบคำถามเบื้องต้นที่เกี่ยวกับชีวสังเคราะห์ของสารกลุ่มนี้จำเป็นต้องใช้สารพันธุกรรมที่มีทั้งปริมาณและคุณภาพที่ดีสำหรับงานในขั้นต่อไป งานวิจัยนี้เริ่มด้วยการหาวิธีที่เหมาะสมในการสกัดสารพันธุกรรมชนิดดีเอ็นเอจากฟองน้ำทะเลสีน้ำเงิน *Xestospongia* sp. โดยได้เปรียบเทียบการสกัดดีเอ็นเอด้วยชุดสกัดสำเร็จรูป ๑ ชุด กับวิธีการที่เคยมีรายงานวิจัยมาก่อนอีก ๖ วิธี ตรวจสอบ ดีเอ็นเอที่สกัดได้ด้วยเจลอิเล็คโตรโฟเรซิส วัดคุณภาพดีเอ็นเอโดยใช้ค่าอัตราส่วนการดูดกลืนแสงที่ความยาวคลื่น ๒๖๐/๒๘๐ นาโนเมตร และวัดปริมาณดีเอ็นเอโดยใช้ค่าการดูดกลืนแสงความยาวคลื่นที่ ๒๖๐ นาโนเมตร ผลการวิจัยพบว่า วิธีที่เหมาะสมที่สุดคือ วิธี NaOAc salting-out protocol จากนั้นได้พัฒนาการสกัดดีเอ็นเอโดยใช้วิธี NaOAc salting-out protocol เป็นต้นแบบ แล้วเปลี่ยนแปลงปัจจัยที่มีผลต่อการสกัดดีเอ็นเอ ได้แก่ ปริมาณส่วนประกอบของสารละลายที่ใช้สลายเซลล์ ชนิดและความเข้มข้นของสารละลายเกลือที่ใช้ในการตกตะกอนโปรตีนฯและวิธีการตกตะกอนดีเอ็นเอ รวมถึงเพิ่มการใช้เอนไซม์ RNase A ในวิธีการสกัด พบว่าวิธีการที่พัฒนาขึ้นใหม่นี้เหมาะสมที่จะใช้สกัดดีเอ็นเอจากฟองน้ำทะเลสีน้ำเงิน *Xestospongia* sp. มากกว่าสิ่งมีชีวิตชนิดอื่น จึงตั้งชื่อวิธีนี้ว่า the blue sponge *Xestospongia* sp. DNA extraction protocol จากนั้นได้ทำการทดสอบเพื่อยืนยันถึงคุณภาพของดีเอ็นเอที่สกัดได้ โดยการทำปฏิกิริยาลูกโซ่พอลีเมอเรสของยีน 28s rRNA และ COX I พบว่าให้ผลผลิตพีซีอาร์ตรงกับขนาดที่คาดหมายไว้ เมื่อทำการวิเคราะห์ลำดับนิวคลีโอไทด์ของผลผลิตพีซีอาร์ที่ได้จากการเพิ่มจำนวนยีน COX I ที่ใช้ดีเอ็นเอที่สกัดได้เป็นต้นแบบ พบว่าเหมือนกับลำดับนิวคลีโอไทด์ของยีน COX I จากฟองน้ำ *Xestospongia* อื่นที่มีในฐานข้อมูลของ Genbank ทำให้สามารถแน่ใจได้ว่า ดีเอ็นเอที่สกัดได้จากวิธีนี้มีคุณภาพดีพอที่จะนำไปใช้ในการศึกษาทางชีววิทยาระดับโมเลกุลต่อไป

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

ภาควิชาเภสัชเวทและเภสัชพฤกษศาสตร์ ลายมือชื่อนิสิต..
สาขาวิชา เภสัชเวท ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก..
ปีการศึกษา 2551 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม..

4976588233: MAJOR PHARMACOGNOSY
KEY WORD: DNA EXTRACTION, BLUE SPONGE, *XESTOSPONGIA* SP.

MANATCHAYA CHUANCHEN: DEVELOPMENT OF DNA EXTRACTION FROM THE BLUE SPONGE *XESTOSPONGIA* SP. THESIS PRINCIPAL ADVISOR: KHANIT SUWANBORIRUX, Ph.D., THESIS CO-ADVISOR: TAKSINA CHUANASA, Ph.D., 104 pp.

The blue sponge *Xestospongia* sp. was found to produce the potent cytotoxic renieramycin alkaloids. It is motivating to study renieramycin biosynthesis to produce these compounds by biotechnology. To primarily answer the biosynthesis question, genetic DNA materials with good in both quantity and quality are required. A commercial kit for DNA extraction from the sponge samples was compared with other six protocols reported in literatures. The quality and the total yield of obtained DNA were estimated by measuring the absorbance ratio (OD_{260}/OD_{280}) and absorbance at 260 nm (OD_{260}), respectively. The most appropriate protocol for DNA extraction from the marine blue sponge *Xestospongia* sp. is the NaOAc salting-out protocol. The NaOAc salting-out protocol was selected for further modification to improve DNA extraction from the sponge *Xestospongia* sp. by optimizing lysis buffer solution compositions, salt solution for protein precipitation, DNA precipitation method, and addition of RNase enzyme. The DNA quantity and purity from the modified protocol were significantly better than those from the original protocol. The modified protocol was remarkably suitable for DNA extraction from the blue sponge *Xestospongia* sp. more than other organisms and was specifically named as the blue sponge *Xestospongia* sp. DNA extraction protocol. Finally, to investigate DNA quality in molecular study aspect, PCR amplifications were performed. The amplicons amplified by 28s rRNA and COX I genes of *Xestospongia* genome were in expected size. Further DNA sequence analysis showed that the amplicons of COX I gene using the obtained DNA extracts as templates were identical to the published *Xestospongia* COX I gene in the database. All the results demonstrated that the blue sponge *Xestospongia* sp. DNA extraction protocol was a preferable procedure to extract *Xestospongia* sp. DNA for further molecular biology study.

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Field of study: Pharmacognosy Principal Advisor's signature..........................
Academic year: 2008 Co-Advisor's signature..........................

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LIST OF ABBREVIATIONS

%	=	percent or part per hundred
µg	=	microgram
µl	=	microliter
µM	=	micromolar
conc.	=	concentration
COX I	=	cytochrome oxidase subunit I
DNA	=	deoxyribonucleic acid
EDTA	=	ethylenediaminetetraacetic acid
g	=	gram or earth's gravitational field
hr	=	hour
M	=	molar
mg	=	milligram
min	=	minute
ml	=	milliliter
mM	=	millimolar
ng	=	nanogram
nm	=	nanometer
PCR	=	polymerase chain reaction
rRNA	=	ribosomal ribonucleic acid
SD	=	standard deviation
SDS	=	sodium dodecyl sulphate
sp.	=	species
TAE buffer	=	Tris-acetate and EDTA buffer
UV	=	ultraviolet

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CHAPTER I

INTRODUCTION

Many marine invertibrates have been proven to be the excellent sources of diverse secondary metabolites (Bewley and Faulkner, 1998; Faulkner, 1998). Some of these secondary metabolites showed significant cytotoxicity and anticancer activity which are valuable in the discovery and development of novel anticancer drugs. In this research, we pay attention to study the marine blue sponge *Xestospongia* sp. that has been found in Sichang Island, Cholburi Province, the Gulf of Thailand. The isolation of the bistetrahydroisoquinoline renieramycins in high yield were reported from the sponge *Xestospongia* sp. pretreated with potassium cyanide (Suwanborirux *et al*., 2003). The renieramycins showed potent cytotoxic activity against several cancer cell lines (Suwanborirux *et al.,* 2003; Amnuoypol *et al.,* 2004; Saito *et al.,* 2004). Development of the renieramycins as new anticancer agents from natural sources is currently being investigated in our group.

Early on, there have been noticed that invertebrate-derived natural products sometimes resemble known microbial-symbiont compounds. Morever, some studies reported that invertebrate-derived natural products might originate from symbiotic bacteria living within the host tissues rather than from animals themselves (Moore, 1999; Piel, 2004; Richelle-Maurer *et al*., 2003). The true sources of marine natural products have been required to be clarified by localizing the compounds in the invertebrate tissues or separating microorganisms from host tissues by a variety of techniques for example, a flow cytometry using auto fluorescent properties of some symbionts as sorting criterion and a density gradient centrifugation (Bewley and Faulkner, 1998).

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For the blue sponge *Xestospongia* sp., sponge and bacterial cells were separated by Ficoll density gradient centrifugation. And the amount of renieramycins from both cell types were detected by HPLC analysis. The result showed that renieramycin M, a major bistetrahydroisoquinoline alkaloid, was associated with the sponge fractions but not with the bacterial fraction (Boonsiriluck, 2006). This information led to the hypothesis that the blue sponge *Xestospongia* sp. is probably the true producer of renieramycins. Therefore, renieramycin biosynthesis is essentially required to understand the production of this interesting alkaloid in nature. The understanding of biosynthetic pathway and regulations involved will provide the possibility to make new renieramycin derivatives by biotechnology. However, genomic DNA from the marine blue sponge *Xestospongia* sp. has not yet been investigated. In this research, development of a suitable methodology will be investigated to extract DNA in good quality from the marine blue sponge *Xestospongia* sp. for further molecular biology studies in genes involved the biosynthesis of the renieramycins.

CHAPTER II

LITERATURE REVIEW

1. The blue sponge *Xestospongia* sp.

The sponge genus *Xestospongia* belongs to phylum Porifera, subphylum Cellularia, class Demospongiae, order Haplosclerida, and family Petrosiidae. According to "Sponguide" (Hooper, 2000), the character of the genus *Xestospongia* de Laubenfels 1932 (syn. *Neopetrosia* de Laubenfels 1949; *Prianos* Gray 1867) was described as following:

"Ectosomal skeleton indistinct; choanosomal skeleton confused isotropic reticulation of multispicular tracts, generally lacking spongin and sometimes with single spicules scattered throughout mesohyl between major spicule tracts, stony texture; oxeote spicules in one size category only".

The marine blue sponge *Xestospongia* sp. was collected from Sichang Island, Cholburi Province, Thailand. The sponge was identified by Dr. John N. A. Hooper as *Xestospongia* sp. #2133 (family *Petrosiidae*). The voucher specimens have been deposited at the Queensland Museum, South Brisbane, Australia (sample code QMG306998) and at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. The sponge *Xestospongia* sp. exhibits thick, encrusted, lobate growth. Its texture is hard, brittle, and easily crumbled. It is light bluish-gray when alive and pinkish in ethanol. Oscules are numerous and of moderate size and are found, on apexes of surface lobes, with slightly raised lips. The surface has prominent bulbous surface lobes with some that are nearly digitate in size. The surface is translucent, membranous, optically smooth, macroscopically bulbous, and microscopically even, with choanosomal drainage canals that are slightly visible below the surface. Ectosomal skeleton membranes have no specialized speculation or structure. Choanosomal skeleton with isotropic reticulation of paucispicular tracts of to moderately sized subdermal cavities are observed throughout skeleton. There are no visible fibers, and only small amounts of collagen in the mesohyl. The oxeas are

robust, straight or slightly curved at center, sharply pointed, hastate (190-210 x 12-18 μm). This species is probably a new species as shown in Figure 2.1 (Hooper, 2000).

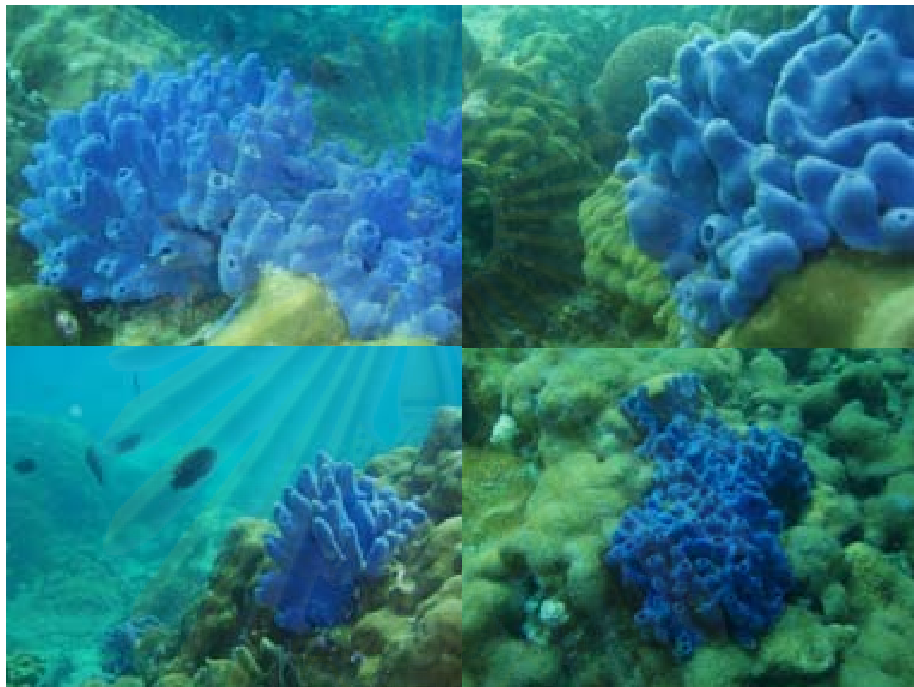

Figure 2.1 Pictures of the marine blue sponge *Xestospongi*a sp. (photographs by Khanit Suwanborirux).

2. Renieramycin alkaloids

The renieramycins are bistetrahydroisoquinoline alkaloids and are classified into two groups, the bisquinones and the quinone-hydroquinones, depending on whether ring E is a quinone or a hydroquinone (Amnuoypol *et al*., 2004; Saito *et al*., 2004). Most of renieramycins containing an angelate ester side chain at C-22 were reported from various marine sponges as shown in Figure 2.2. Since the first discovery of renieramycins A-D **[1a-d]** from the Maxican sponge *Reniera* sp. in 1982 (Frincke and Faulkner, 1982), ten additional renieramycin marine natural products have been isolated from the marine sponges belonging to genera *Reniera* (Faulkner, 1982)*, Xestospongia* (Ireland, 1987)*, Haliclona,* and *Cribrochalina* (Pettit, 1992). In 1989, He and Faulkner isolated renieramycins E **[1e]** and F **[1f]** from the marine sponge *Reniera* sp. and the compounds contain a hydroxyl group in stead of a carbonyl group at C-21 of ring C (He and Faulkner, 1989). The unstability of

renieramycin E **[1e]** was suggested by oxidative cleavage to give the "monomeric" isoquinolines; mimosamycin and renierone as shown in Figure 2.3 (Frincke and Faulkner, 1982; He and Faulkner, 1989; Edrada *et al.,* 1996; Petit *et al.,* 2000; Rashid *et al.,* 2001; Saito *et al*., 2004; Sung *et al.,* 2006). In 1992, Davidson isolated renieramycins G **[1g]** from the marine sponge *Xestospongia caycedoi* (Davidson, 1992), which is different from renieramycin E **[1e]** by containing a carbonyl group instead of a hydroxyl group at C-21 of ring C. In 2003, the Thai blue sponge *Xestospongia* sp. from the vicinity of Sichang Island, in the Gulf of Thailand were extracted and isolated the active compounds. The more polar fraction was purified to yield dimeric isoquinoline quinones ; renieramycin J **[1j]**, K **[1k]**, and L **[1l]**, the first example of alkylated analogues of renieramycins at C-21 position, along with two known monomeric isoquinolinequinones (Suwanborirux *et al*., 2003). Currently, Suwanborirux and coworkers have succeeded in gram-scale preparation of renieramycins from the Thai blue sponge *Xestospongia* sp. by pretreatment with potassium cyanide (Suwanborirux *et al*., 2003; Amnuoypol *et al*., 2004; Saito *et al*., 2004). The Thai blue sponge *Xestospongia* sp. was pretreated with potassium cyanide to increase the mass-production of renieramycin M **[1m]**, a major renieramycin, and four minor compounds including renieramycins O **(1o)**, Q **(1q)**, R **(1r)** and S **(1s)**. Addition of potassium cyanide converted the unstable renieramycin E **[1e]** into the more stable renieramycin M **[1m]**. This strategy has provided more renieramycins from marine sources for further chemical and biological investigations (Suwanborirux *et al*., 2003; Amnuoypol *et al.,* 2004).

Additional related compound, jorumycin **[2a]** containing an acetyl ester instead of an angelate ester, was isolated from the skin and the mucus of the nudibranch *Jorurunna funebris*. In 2006, Charupant and coworkers isolated new stabilized renieramycin-type bistetrahydroisoquinolinequinones, jorunnamycins A – C **[2b – 2d]** from the mantles, the visceral organs, and the egg ribbons of the Thai nudibranch *Jorunna funebris* pretreated by potassium cyanide (Charupant *et al.*, 2006). The structures of jorunnamycins A – C **[2b – 2d]** were elucidated from spectroscopic data and by chemical conversion of **1m** into **2d** *via* **2b**. The chemical stability and the oxidative degradation generating simple isoquinoline alkaloids of a carbinolamine analog **[2e]**, which was easily prepared by reacting **2d** with silver nitrate in aqueous acetonitrile. In 1977, saframycin A, is an antibiotic isolated from

Streptomyces lavendulae No.314 (Arai *et al*., 1977) which falls into the category of the N-heterocyclic quinine group. In 1990, the potent anticancer agent ecteinascidin-743 (ET-743) was isolated from the Caribbean tunicate *Ecteinascidia turbinata*. The common structural feature of ET-743 consists of three tetrahydroisoquinoline subunits and an active carbinolamine functional group (Rinehart *et al.*, 1990).

The chemical structure of renieramycins are closely related to other tetrahydroisoquinoline natural products from different sources as shown in Figure 2.4.

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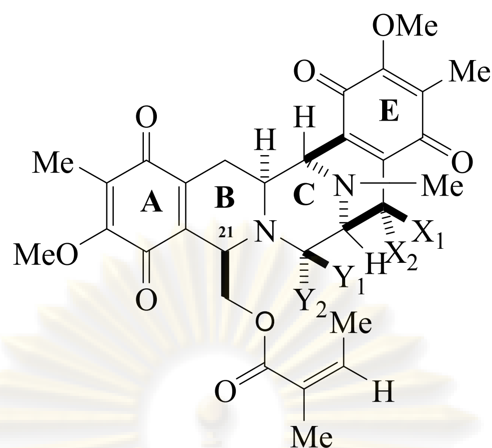

renieramycin

A **[1a]**: X_1 = H, X_2 = OH, Y_1 = Y_2 = H

B **[1b]**: X_1 = H , X_2 = OC_2H_5, Y_1 = Y_2 = H

C **[1c]**: X_1 = H, X_2 = OH, Y_1, Y_2 = O

D **[1d]**: X_1 = H, X_2 = OC_2H_5, Y_1, Y_2 = O

E **[1e]**: X_1 = X_2 = H, Y_1 = H, Y_2 = OH

F **[1f]**: X_1 = H, X_2 = OMe, Y_1 = H, Y_2 = OH

G **[1g]**: X_1 = X_2 = Y_1 = Y_2 = H

J **[1j]**: X_1 = X_2 = Y_1 = H, Y_2 = CH_2COCH_3

K **[1k]**: X_1 = Y_1 = Y_2 = H, X_2 = OCH_3

M **[1m]**: X_1 = X_2 = Y_1 = H, Y_2 = CN

R **[1r]**: X_1 = Y_1 = H, X_2 = OCH_3, Y_2 = CN

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Figure 2.2 The chemical structures of renieramycins from marine sponges.

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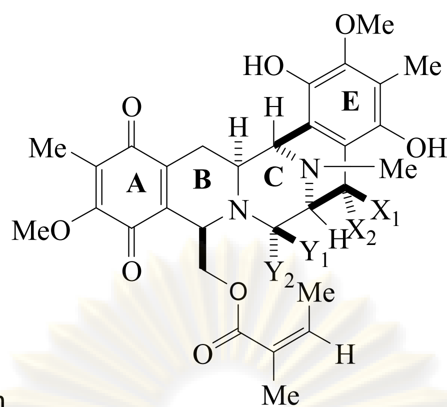


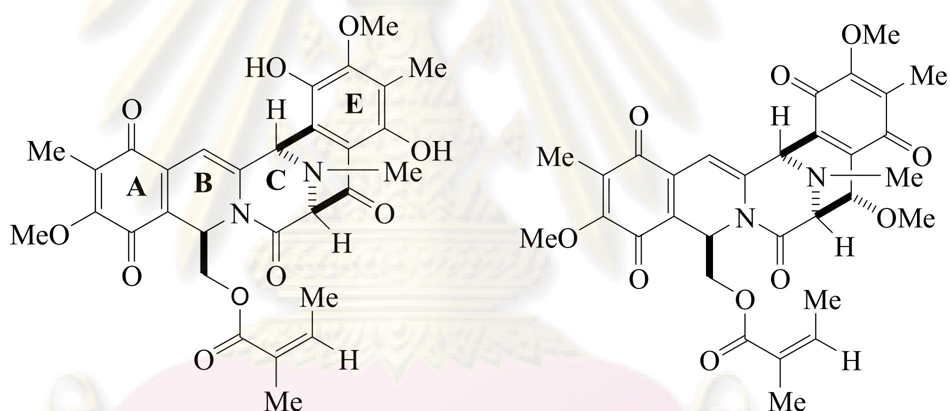
renieramycin

L**[1l]**: $X_1 = X_2 = Y_1 = H$, $Y_2 = CH_2COCH_3$

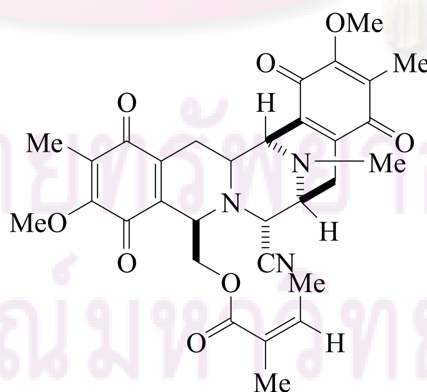
N **[1n]**: $X_1 = Y_1 = H$, $X_2 = OH$, $Y_2 = CN$

P **[1p]**: $X_1 = H$, $X_2 = OH$, $Y_1 = H$, $Y_2 = OH$

Q **[1q]**: X_1, $X_2 = O$, $Y_1 = H$, $Y_2 = CN$



renieramycin H **[1h]** renieramycin I **[i]**



renieramycin S **[1s]**

Figure 2.2 (continued)

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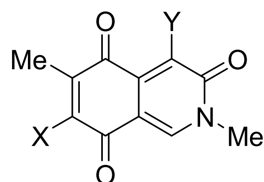
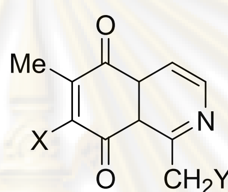
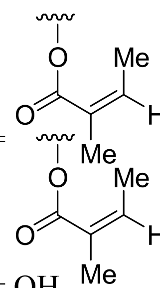


mimosamycin: X = OMe, Y = H

4-amino-mimosamycin: X = OMe, Y = NH_2

7-amino-7-demethoxy mimosamycin: X = NH_2, Y = H





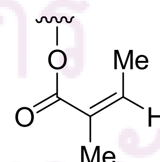
renierone: X = OMe, Y =

O-demethylrenierone: X = OH, Y =

renierol: X = OMe, Y = OH

renierol acetate: X = OMe, Y = $OCOCH_3$

renierol propionate: X = OMe, Y = $OCOCH_2CH_3$



N-formyl-1,2-dihydrorenierone: X =

N-formyl-1,2-dihydrorenierol acetate: X = $OCOCH_3$

N-formyl-1,2-dihydrorenierol propionate: X = $OCOCH_2CH_3$

Figure 2.3 The chemical structures of monomeric isoquinolinequinones.

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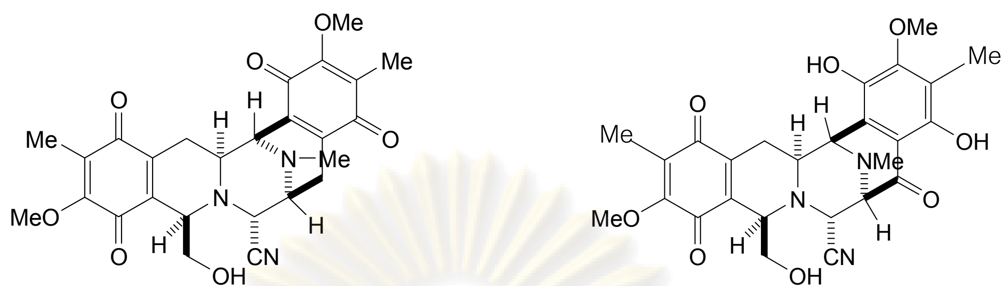
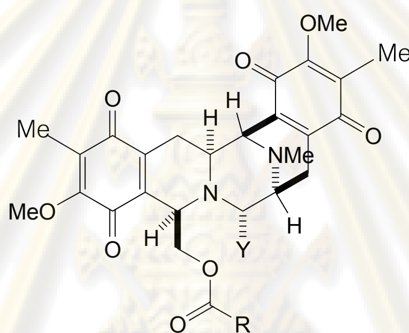


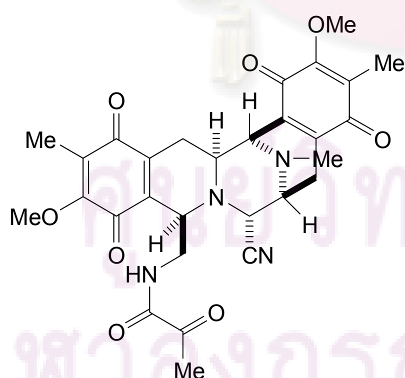
jorunnamycin A **[2b]** (deangeloylrenieramycin M)   jorunnamycin B **[2c]**



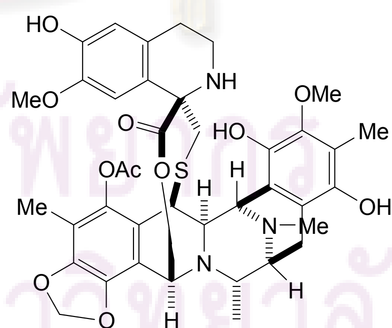
jorumycin **[2a]**: R = CH_3, Y = OH

jorunnamycin C **[2d]**: R = C_2H_5, Y = CN

carbinolamine analog **[2e]**: R = C_2H_5, Y = OH



saframycin A



ecteinascidin 743 (ET-743)

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Figure 2.4 The chemical structures of bistetrahydroisoquinolines from other natural sources.

Renieramycins A-D (Frincke and Faulkner, 1982) and renieramycins H - I (Parameswaran *et al.,* 1998) showed moderate antimicrobial activities. Two related alkaloids, reinerol and mimosamycin were reported from the marine sponge *Xestospongia calycedoi* and exhibited mild antimicrobial activity against gram positive bacteria (Fontana *et al.,* 2000).

In 2001, Rashid *et al.* also described mimosamycin from the cytotoxic fractions of an aqueous extract of the marine sponge *Haliclona* sp., which was the principle cytotoxin with an IC_{50} approximately 10 μg/mL against melanoma and ovarian human tumor cell lines. Several renieramycins (1m – 1o, and 1q – 1s) showed very potent cytotoxic activity against two human cell lines, HCT116 (human colon carcinoma), and QG56 (human lung carcinoma) with IC_{50}s in the range of 5.6 nM – 7.1 nM (Suwanborirux *et al.,* 2003; Amnuoypol *et al.,* 2004; Saito *et al.,* 2004). Renieramycin G **[1g]** was isolated from the sponge *Xestospongia caycedoi* by Davidson in 1992 and exhibited cytotoxicity against KB (human epidermoid carcinoma of nasopharynx) and LoVo cell lines (human colon adenocarcinoma cell) with MIC values of 0.5 and 1.0 μg/mL, respectively. Furthermore, renieramycin G and an analogue, 3-*epi*-renieramycin G exhibited mild cytotoxic activity against both human colon (HCT116) and human lung (A549) cancer cell lines at GI_{50} in nM (Lane *et al.,* 2006).

3. DNA extraction and purification

DNA is composed of nucleotides found in all known living organisms. Some particular nucleotide segments of DNA sequences carry genetic information which is called gene. DNA or gene plays an important role in development and regulation of biological activities of the living cells.

DNA has been utilized in several molecular studies such as species identifications (Houdebine, 2007; Daniell and Dhingra, 2002), forensic DNA analysis (Collins and Morton, 1994; Weir *et al.* 1997), Pharmaceutical therapeutics (Edward, 2001) and biosynthetic study of bioactive natural products (Pospiech *et al.,* 1995; Tsuda *et al*., 2002; Piel *et al*., 2004).

As of primary steps in experiments to our interest, DNA from the blue sponge *Xestospongia* sp. is extensively investigated in an aspect of DNA extraction with high quality and quantity. DNA extracts are subsequently considered as good sources for biosynthesis study of renieramycins produced by this blue sponge.

3.1 DNA extraction is a procedure to remove DNA from the cells in which it locates. In general, there are four basic steps in a DNA extraction.

1. Cell disruption or cell lysis by grinding or sonicating the sample.

2. Removal of membrane lipids by using lysis buffer containing a detergent such as sodiumdodecylsulfate (SDS) or cetyltrimethylammoniumbromide (CTAB) and a chelating agent to sequester divalent cations Mg^{2+} and Ca^{2+}.

3. Removal of cellular and histone proteins to get free DNA by proteinase K.

3.2 DNA purification is optionally an additional step in DNA extraction to clean up DNA from contaminants

3.2.1 <u>Solution-based technique</u>

3.2.1.1 Phenol-chloroform extraction is a liquid-liquid extraction technique and based on phase separation principle. This technique is commonly utilized to remove protein and lipid from the DNA mixture. Generally, phenol and chloroform with equal volumes are mixed with an aqueous sample. Centrifugation is subsequently applied to separate a mixture of the aqueous sample from the organic solvent (mainly chloroform). DNA is dissolved in the aqueous phase, whereas protein

is presented in the organic phase (Chomczynski *et al.*, 2006). Finally, the DNA is precipitated by adding absolute EtOH or isoproranol and centrifuged for DNA pellet collection.

3.2.1.2 Salt solutions for purification is one of the most common technique to remove proteins from the DNA. Indeed, solubility of a protein depends on a number of factors such as type of the salt and concentration of the salt (Mathews *et al.*, 1990). The salts commonly used in DNA purification, including NaOAc, NaCl, and NH_4OAc at various concentrations. The DNA is precipitated by adding absolute EtOH or isoproranol and centrifuged for DNA pellet collection.

3.2.2 Column-based nucleic acid purification is a solid phase extraction method and based on adsorption chromatography principle. Generally, the mixture of DNA and high pH binding solution containing high salt concentration is added to the column. DNA is bound to the column and subsequently eluted by water or an eluting buffer (Marko *et al*., 1982; Boom *et al*., 1990).

4. DNA determination

4.1 DNA quantification

4.1.1 DNA quantification using UVspectrophotometer

DNA contains either purine or pyrimidine base which is able to absorb ultraviolet light. Wavelength of maximal absorption of DNA is at 260 nm. Thus, spectrophotometers are normally utilized to examine the concentration of DNA in a solution. The concentration of double-stranded DNA can be calculated by below equation (Sambrook and Russell, 2001).

$$1\ OD_{260}\ \text{unit} = 50\ \mu g/ml\ \text{double-stranded DNA}$$

4.1.2 DNA quantification using fluorescent dyes

DNA concentration is alternatively determined by measuring the fluorescence intensity of dyes that bind to DNA and selectively fluoresce when bound. This method is used when concentration of DNA is too low to accurately measure with spectrophotometry and contaminants in DNA solution are also able to absorb UV at 260 nm.

4.2 DNA purity determination

DNA samples are commonly contaminated by other molecules (such as protein, phenol, and other organic compounds). These contaminant molecules have their own characteristic absorption spectra and may contribute to an error in DNA concentration estimated at 260 nm due to wide range of absorption spectra.

4.2.1 Protein contamination and the 260:280 ratio

Maximal absorptions of DNA and protein are at 260 and 280 nm, respectively. The ratio of absorptions at 260 nm to 280 nm is commonly used to calculate the purity of DNA with respect to protein contamination, since proteins (in particular, the aromatic amino acids) regularly absorb at 280 nm. Percentages of DNA and protein are predicted by OD_{260}:OD_{280} ratios as shown in Table 2.1 (Sambrook and Russell, 2001).

4.2.2 Other common contaminants

Some chemicals such as phenol are commonly used in DNA purification, leading to a miscalculation of DNA quantities. Phenol absorbs with a peak at 270 nm and the 260:280 ratio around 2. Nucleic acid preparations uncontaminated by phenol should have the 260:270 ratio around 1.2 (Sambrook and Russell, 2001). Contamination by phenol can significantly contribute to overestimation of DNA concentration.

Some contaminants, including phenolate ion, thiocyanates, and other organic compounds absorb at 230 nm. For the pure nucleic acid sample, the 260:230 ratio should be around 2.

The particles contaminating in the solution, causing scattering of light in the visible range absorb at 330 nm and higher. The value in a pure nucleic acid sample should be zero.

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Table 2.1 Absorbance ratio at 260 and 280 nm and percentage of nucleic acids and proteins

% Protein	% Nucleic acid	$OD_{260}:OD_{280}$	% Protein	% Nucleic acid	$OD_{260}:OD_{280}$
100	0	0.57	45	55	1.89
95	5	1.06	40	60	1.91
90	10	1.32	35	65	1.93
85	15	1.48	30	70	1.94
80	20	1.59	25	75	1.95
75	25	1.67	20	80	1.97
70	30	1.73	15	85	1.98
65	35	1.78	10	90	1.98
60	40	1.81	5	95	1.99
55	45	1.84	0	100	2.00
50	50	1.87	-	-	-

Using the predicted values in this table, Glasel (1995) derived an empirical equation to describe $\%N$ for a range of $OD_{260}:OD_{280}$ ratios: $\%N = F([11.16R - 6.32], [2.16 - R])$, where $R = OD_{260}:OD_{280}$. Note that estimates of purity of nucleic acids based on $OD_{260}:OD_{280}$ ratios are accurately only when the preparations are free of phenol. Water saturated with phenol absorbs with a characteristic peak at 270 nm and an $OD_{260}:OD_{280}$ ratio of 2. Nucleic acid precipitations free of phenol should have $OD_{260}:OD_{280}$ ratios of ~ 1.2.

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CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Source of the blue sponge *Xestospongia* sp.

The marine blue sponge *Xestospongia* sp. #2133 was identified by Dr. John N.A. Hooper and the voucher specimens have been deposited at Queensland Museum (serial No. QM G306998), Australia and the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. The sponge *Xestospongia* sp. (Class: *Demospongiae*, Order: *Haplosclerida*, Family: *Petrosiidae*) is a bluish sponge occurring commonly in the coral reef in the Gulf of Thailand.

For this study, the sponge samples were taken from Sichang Island, Cholburi Province, on May 2008 by SCUBA diving at the depth range of 3-5 meters. The living samples were cleaned up the contaminated organisms before being carried to our laboratory and stored at -20°C prior to DNA extraction.

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1.2 Chemicals and reagents

Absolute ethanol (Merck, 99.8%)

Acetic acid (Merck, Germany)

Agarose (Vivantis™, England)

Cetyl trimethylaceticbromide: CTAB (Fluka®)

Chloroform/isoamylalcohol (24:1) (Fluka®, 99.5%)

Deoxynucleoside triphosphates (dNTPs) (Vivantis™, England)

Ethylacetate, AR grade (Lab scan®)

Ethylenediaminetetraacetic acid, Disodium salt dehydrate (Fluka, 98%)

Isopropanol (Lab scan®)

Liquid nitrogen

Lysozyme (Vivantis™, England)

Magnesium chloride (Vivantis™, England)

Phenol/chloroform/isoamylalcohol (25:24:1) (Fluka®)

Primers (Invitrogen™)

Proteinase K (Vivantis™, England)

RNase A (Vivantis™, England)

Sodium acetate (Sigma®)

Sodium chloride (Merck, Germany)

Sodium dodecyl sulphate (Vivantis™, England)

Sodium sulfate, anhydrous (Merck, 98.4%)

Steriled ultrapure water

Taq DNA polymerase (Vivantis™, England)

Tris-Hydrochloride (Vivantis™, England)

10x PCR amplification buffer (Vivantis™, England)

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1.3 Instruments

Dry bath (Labnet Inc., USA)

Gel doc MiniBis Pro (DNR Bioimaging System Ltd., Israel)

MultiGene Thermalcycler (Labnet Inc., USA)

Refrigerated centrifuge (Hettich Zentrifugen, Germany)

UV spectrophotometer (Shimadzu UV 160 A, Japan)

Vortex mixer (Labnet Inc., USA)

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2. Methods

2.1 Comparison of protocols for DNA extraction from the blue sponge *Xestospongia* sp.

The frozen sponge samples were cut into small pieces and accurately weighed for 100 mg. DNAs from the sponge samples were extracted by using the MasterPure® DNA&RNA Extraction Kit (protocol 1, EPICENTRE®) and the following six protocols 2-7 reported in literatures. The procedure of each protocol is described as follows and summarized in Table 3.1. DNA extraction from each protocol was done in triplicate.

2.1.1 Protocol 1: MasterPure® DNA&RNA Extraction Kit (EPICENTRE®)

2.1.1.1 Ground sample 100 mg in liquid nitrogen with mortar and pestle and added Tissue&Cell lysis solution 300 µl

2.1.1.2 Centrifuged at 1,000 g for 5 min and transferred supernatant into a new 1.5 ml microcentrifuge tube

2.1.1.3 Added proteinase K 1 µl, mixed by a vortex mixer, and incubated at 65°C for 15 min

2.1.1.4 Lowered the mixture temperature to 37°C, added RNase A 1 µl, and incubated at 37°C for 30 min

2.1.1.5 Added MPC protein precipitate reagent 150 µl and mixed by a vortex mixer

2.1.1.6 Centrifuged at 10,000 g for 10 min and transferred supernatant into a new 1.5 ml microcentrifuge tube

2.1.1.7 Added isopropanol 500 µl and mixed thoroughly

2.1.1.8 Centrifuged at 10,000 g for 10 min and discarded isopropanol

2.1.1.9 Washed pellet with 75% EtOH, dried pellet, and resuspended in steriled ultrapure water100 µl

2.1.2 Protocol 2: Simple DNA extraction protocol (Edwards, 1991)

2.1.2.1 Ground sample 100 mg in liquid nitrogen with mortar and pestle, added extraction buffer (0.5%SDS, 25mM EDTA, 200mM Tris-HCl, and 250mM NaCl) 400 µl, and mixed by a vortex mixer

2.1.2.2 Incubated at room temperature for 1 hr

2.1.2.3 Centrifuged at 13,000 g for 1 min, transferred supernatant into a new 1.5 ml microcentrifuge tube

2.1.2.4 Added cold isopropanol 300 µl, incubated at room temperature for 2 min

2.1.2.5 Centrifuged at 13,000 g for 5 min, discarded isopropanol, dried pellet, and resuspended in steriled ultrapure water 100 µl

2.1.3 Protocol 3: SDS/phenol protocol (Maria, 2004)

2.1.3.1 Ground sample 100 mg in liquid nitrogen, dispersed in lysis buffer (2%SDS, 50mM EDTA, and 100mM NaCl) 200 µl, and incubated at 60°C for 30 min

2.1.3.2 Centrifuged at 1,000 g for 5 min and transferred supernatant into a new 1.5 ml microcentrifuge tube

2.1.3.3 Added proteinase K 1 µl, incubated at 50°C for 3 hr, added RNase A 1 µl, and incubated at 37°C for 3 hr.

2.1.3.4 Added phenol/chloroform/isoamylalcohol 600 µl

2.1.3.5 Centrifuged at 12,000 g for 10 min and transferred the aqueous solution into a new 1.5 ml microcentrifuge tube

2.1.3.6 Added 3 M NaOAc and centrifuged at 12,000 g for 10 min

2.1.3.7 Transferred the aqueous solution into a new microcentrifuge tube, added absolute EtOH 500 µl, centrifuged at 12,000 g for 10 min, dried pellet, and resuspended in steriled ultrapure water100 µl

2.1.4 Protocol 4: CTAB protocol for plants (Weising, 2005)

2.1.4.1 Preheated CTAB extraction solution

2.1.4.2 Ground sample 100 mg in liquid nitrogen with mortar and pestle, added CTAB extraction solution (2%CTAB, 1.4M NaCl, 20mM EDTA, and 100mM Tris-HCl) 600 µl, incubated at 65°C for 30 min, and mixed twice

2.1.4.3 Centrifuged at 1,000 g for 5 min and transferred supernatant into a new 1.5 ml microcentrifuge tube

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2.1.4.4 Added 24:1 chloroform/isoamylalcohol 600 µl and mixed thoroughly

2.1.4.5 Centrifuged at 13,000 g for 5 min and transferred the aqueous solution into a new 1.5 ml microcentrifuge tube

2.1.4.6 Added 1/10 volume of CTAB/NaCl solution (4.1g NaCl in 10%CTAB), added 1 volume of 24:1 chloroform/isoamylalcohol, and mixed thoroughly

2.1.4.7 Centrifuged at 13,000 g for 5 min and transferred the aqueous solution into a new 1.5 ml microcentrifuge tube

2.1.4.8 Added 1 volume of CTAB precipitation solution (1%CTAB, 10mM EDTA, and 50mM Tris-HCl) and mixed thoroughly

2.1.4.9 Centrifuged at 13,000 g for 5 min and discarded the supernatant

2.1.4.10 Added high-salt TE buffer (1M NaCl, 0.1mM EDTA, and 10mM Tris-HCl) 300 µl and added cold absolute EtOH 200 µl

2.1.4.11 Centrifuged at 13,000 g for 10 min, washed pellet with 80% EtOH 200 µl, and resuspended in steriled ultrapure water100 µl

2.1.5 Protocol 5: CTAB protocol for sponges (Roovere, 2006)

2.1.5.1 Preheated CTAB extraction buffer (2%CTAB, 1.4 M NaCl, 20 mM EDTA, and 100 mM Tris-HCl)

2.1.5.2. Ground sample 100 mg in liquid nitrogen with mortar and pestle

2.1.5.3 Added sterile water 300 µl, CTAB extraction buffer 500 µl, treated with proteinase K 1 µl, and incubated at 65°C for 90 min

2.1.5.4 Centrifuged at 1,000 g for 5 min and transferred supernatant into a new 1.5 ml microcentrifuge tube

2.1.5.5 Added chloroform 500 µl, centrifuged at 13,000 g for 5 min and transferred the aqueous solution into a new 1.5 ml microcentrifuge tube

2.1.5.6 Added CTAB precipitation buffer (0.5%CTAB and 40mM NaCl) 600 µl, incubated at 4°C for 60 min, centrifuged at 13,000 g for 5 min, and discarded supernatant

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2.1.5.7 Added 1.2 M NaCl 350 µl, isopropanol 250 µl, centrifuged at 13,000 g for 5 min, and discarded supernatant

2.1.5.8 Washed pellet with 70% EtOH 200 µl, dried pellet and resuspended in steriled ultrapure water100 µl

2.1.6 Protocol 6: SDS/CTAB/phenol protocol (Aguilera, 2006)

2.1.6.1 Ground sample 100 mg in liquid nitrogen with mortar and pestle

2.1.6.2 Added proteinase K 15 µl, 10%SDS 100 µl, and incubated at 65°C for 1 hr

2.1.6.3 Added 5M NaCl 200 µl, 1%CTAB 150 µl, and incubated at 65°C for 1 hr

2.1.6.4 Added lysosyme 30 µl and incubated at 37°C for 1 hr

2.1.6.5 Added phenol 500 µl, centrifuged at 12,000 g for 5 min, and transferred the aqueous solution into a new 1.5 ml microcentrifuge tube

2.1.6.6 Added 24:1 chloroform/isoamylalcohol 500 µl, centrifuged at 12,000 g for 5 min, and transferred the aqueous solution into a new 1.5 ml microcentrifuge tube

2.1.6.7 Added absulute EtOH 800 µl and kept overnight at-20°C

2.1.6.8 Centrifuged at 8,000 g for 10 min, discarded supernatant, dried pellet, and resuspended in steriled ultrapure water100 µl

2.1.7 Protocol 7: NaOAc salting-out protocol (Ferara, 2006)

2.1.7.1 Ground sample 100 mg in lysis buffer (20%SDS, 5mM EDTA, and 10 mM Tris-HCl) 750 µl, centrifuged at 500 g for 1 min, and transferred supernatant into a new 1.5 ml microcentrifuge tube

2.1.7.2 Added proteinase K 1 µl and incubated at 56°C for 3 hr

2.1.7.3 Centrifuged at 1,000 g for 5 min and transferred supernatant into a new 1.5 ml microcentrifuge tube

2.1.7.4 Added 6.1 M NaOAc 250 µl, centrifuged at 5,000 g for 20 min, and transferred supernatant into a new 1.5 ml microcentrifuge tube

2.1.7.5 Added cold absolute EtOH 1 ml and kept at –20°C for 30 min

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2.1.7.6 Centrifuged at 13,000 g for 30 min, discarded supernatant, dried pellet, and resuspended in steriled ultrapure water100 µl

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Table 3.1 The components of the solutions used in DNA extraction protocols

Steps \ Protocols	Protocol 1 (MasterPure® kit)	Protocol 2 (Simple DNA extraction protocol)	Protocol 3 (SDS/phenol protocol)	Protocol 4 (CTAB protocol for plants)	Protocol 5 (CTAB protocol for sponges)	Protocol 6 (SDS/CTAB/ phenol protocol)	Protocol 7 (NaOAc salting-out protocol)
Cell grinding	ground in liquid nitrogen	ground in liquid nitrogen	ground in liquid nitrogen	ground in liquid nitrogen	ground in liquid nitrogen	ground in liquid nitrogen	ground in buffer
Lysis buffer	Tissue&cell lysis solution	0.5%SDS, 25mM EDTA, 200mM Tris-HCl	2%SDS, 50mM EDTA	2%CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris-HCl	2%CTAB, , 10mM EDTA, 50mM Tris-HCl	10%SDS, 1%CTAB	20%SDS, 5mM EDTA, 10mM Tris-HCl
Enzyme	Proteinase K (50µg/µl)	-	Proteinase K (50µg/µl)	-	Proteinase K (20µg/µl)	Lysozyme, Proteinase K (10µg/µl)	Proteinase K (10µg/µl)
RNase A	10µg/µl	-	10µg/µl	-	-	-	-
Protein precipitation	MPC Protein precipitate reagent	250mM NaCl	- 100mM NaCl, 3M NaOAc - Phenol/$CHCl_3$ /IAA	- 1M NaCl - $CHCl_3$/IAA	- 1.2M NaCl - $CHCl_3$/IAA	- 5M NaCl - Phenol/$CHCl_3$ /IAA	6.1M NaOAc
DNA precipitation	Isopropanol, 70% EtOH	Isopropanol	Absolute EtOH	Isopropanol, 80% EtOH	Isopropanol, 70% EtOH	Absolute EtOH	Absolute EtOH

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2.2 Development of the NaOAc salting-out protocol for DNA extraction

From the previous experiments in 2.1, the NaOAc salting-out protocol gave the greatest amount of DNA and the highest DNA purity when compared to other protocols. This experiment was required to develop the NaOAc salting-out protocol to gain better DNA in quantity and purity by variation of lysis buffer compositions, salt solutions for protein precipitation, and DNA precipitation methods.

2.2.1 Optimization of lysis buffer compositions

The lysis buffer used in the NaOAc salting-out protocol consists of 20%SDS, 5 mM EDTA, and 10 mM Tris-HCl. This experiment was designed to use combinations of various concentrations of SDS (1, 5, 10, 15, 20%), EDTA (5, 10, 15, 20, 25 mM), and Tris-HCl (1, 5, 10, 50, 100 mM). The details of lysis buffer compositions are shown in Appendix A.

Preparation of stock solutions of lysis buffer compositions

Solution A: 40% SDS solution

Accurately weighed 200.00 g of SDS to a 500 ml volumetric flask and adjusted to volume with steriled ultrapure water.

Solution B: 0.1 M EDTA solution

Accurately weighed 9.306 g of EDTA to a 250 ml volumetric flask and adjusted to volume with steriled ultrapure water.

Solution C: 0.5 M Tris-HCl

Accurately weighed 7.822 g of Tris-HCl to a 100 ml volumetric flask and adjusted to volume with steriled ultrapure water.

Preparation of lysis buffers containing various concentrations of compositions

To prepare the series of 10 ml lysis buffers, 0.25, 1.25, 2.50, 3.75, and 5.00 ml of **solution A** were transferred separately into 10 ml volumetric flasks. Thereafter, following the details given in Table A1 (see Appendix A), **solution B** were pipetted into those flasks by the volumes of 0.50, 1.00, 1.50, 2.00, and 2.50 ml, respectively. Finally, as detailed in Table A1 (see Appendix A), the series of 125 lysis buffer solutions were furnished by adding 0.02, 0.10, 0.20, 1.00, and 2.00 ml of **solution C** into those flasks, and adjusted to volume with steriled ultrapure water.

The modified lysis buffers were replaced the lysis buffer to extract DNAs from the sponge *Xestospongia* sp. as described in the NaOAc salting-out protocol.

2.2.2 Optimization of the salt solution in protein precipitation

From the previous experiment (2.2.1), 6.1 M NaOAc solution was used in the NaOAc salting-out protocol to precipitate proteins. This experiment is required to search for the better salt solution with optimal concentration to precipitate proteins. NaOAc and NaCl at various concentrations were selected because of their common use for protein precipitation.

Preparation of NaOAc solutions in various concentrations

To make 1, 3, 5, 6, 7, and 9 M NaOAc solutions, 0.82, 2.46, 4.10, 4.92, 5.74, and 7.38 g NaOAc were accurately weighed, transferred into a separate 10 ml volumetric flask, and adjusted to volume with steriled ultrapure water, respectively.

Preparation of NaCl solutions in various concentrations

To make 1, 3, 5, 6, 7, and 9 M NaCl solution, 0.58, 1.75, 2.92, 3.51 4.09, and 5.26 g NaCl were accurately weighed into a separate 10 ml volumetric flask and adjusted to volume with steriled ultrapure water, respectively.

Extraction of the DNAs from the sponge *Xestospongia* sp. was followed by the NaOAc salting-out protocol using the optimal lysis buffer (from 2.2.1). And then the protein contaminants were precipitated by each serial salt solution.

2.2.3 Optimization of DNA precipitation method

In the NaOAc salting-out protocol, absolute EtOH was used to precipitate DNA, for 30 min. This experiment was designed to search for the better condition to precipitate DNA by comparison between absolute EtOH and isopropanol.

The DNA from the sponge *Xestospongia* sp. was extracted by following the NaOAc salting-out protocol using the optimal lysis buffer solution from 2.2.1 and the optimal salt solution from 2.2.2. And then the DNA was precipitated by either absolute EtOH or isopropanol at 0, 30, 60, 120, 180 min, and 12 hr.

2.3 Comparison of the modified protocol with the NaOAc salting-out protocol

The final modified protocol (2.2.3) was directly compared with the original NaOAc salting-out protocol to extract DNAs from the same colony of the blue sponge *Xestospongia* sp.

2.4 Reliability evaluation of the modified protocol

2.4.1 DNA extraction of the blue sponge *Xestospongia* sp. from various locations

Twelve sponge *Xestospongia* sp. samples were collected from various locations near Sichang Island, Cholburi Province, on January, 2009 by SCUBA diving at the depth range of 3-5 meters. These samples were assigned following the collection sites.

KAM 1 and KAM 2 were collected from Kham Island

SAM 1, SAM 2, and SAM 3 were collected from Sam Pun Yue Island

PRONG 1 and PRONG 2 were collected from Prong Island

DOK 1and DOK 2 were collected from Ran Dok Mai Island

NOK 1, NOK 2, and NOK 3 were collected from Nok Island

The living samples were cleaned up to remove the contaminated organisms before being carried to our laboratory and stored at -20°C prior to DNA extraction.

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2.4.2 DNA extraction of other representative organisms by the modified protocol

2.4.2.1 Seven sponge samples, including *Gelliodes petrosioides*, *Gelliodes sp.*, *Clathria (Thalysias) reinwardti, Pachastrissa nux*, *Cacospongia* sp., *Xestospongia testudinaria*, and the barrel sponge *Xestospongia* sp. (Figure 3.1) were collected from Sichang Island, the Gulf of Thailand at the depth range 3-5 meters on October, 2008 by SCUBA diving. The living samples were cleaned up the contaminated organisms before being carried to our laboratory and stored at -20°C prior to DNA extraction.

2.4.2.2 Two tunicates, including *Clavelina cyclus* and *Ecteinascidia thurstoni* (Figure 3.1) were collected from Phuket Island by SCUBA diving at depth of 1-5 meters. The samples were stored at -20°C before being carried to our laboratory.

2.4.2.3 Two leave samples of *Punica granatum* and *Ocimum basilicum* were collected from the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

The DNAs from these organisms were extracted by the blue sponge *Xestospongia* sp. DNA extraction protocol.

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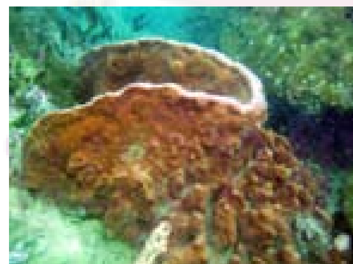
Gelliodes petrosioides

***Gelliodes* sp.**

Clathria (Thalysias) reinwardti

Pachastrissa nux

***Cacospongia* sp.**



Xestospongia testudinaria

The barrel sponge *Xestospongia* sp.

Figure 3.1 Pictures of seven sponge samples from Sichang Island, and two tunicates from Phuket Island
(photographs by Khanit Suwanborirux)

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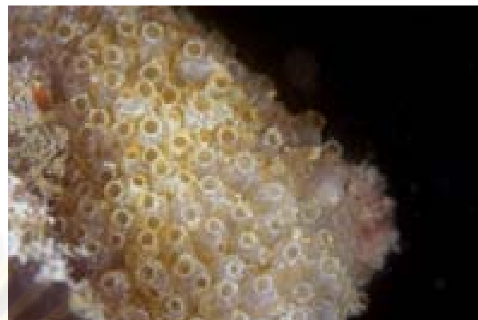
Clavelina cyclus



Ecteinascidia thurstoni

Figure 3.1 (continued)

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2.5 DNA determination

The obtained DNAs were determined by gel electrophoresis and UV spectroscopy.

2.5.1 Gel electrophoresis

Weighed the agarose powder 0.8 g for DNA running and 1.2 g for PCR products running. Agarose powder was mixed with electrophoresis buffer (1X TAE buffer) 100 ml and heated in a microwave oven until completely melted. After cooling the solution to about 60°C, it was poured into a casting tray (50 ml solution for tray size 5.5x6x1 cm and 25 ml solution for tray size 11x6x1 cm) containing a comb (0.5cm-wide slot) and allowed to solidify at room temperature. After the gel has solidified, the comb was removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, was inserted into the electrophoresis chamber and just covered with 1X TAE buffer. Samples containing DNA mixed with loading buffer were then pipetted into the sample wells, the lid and power leads are placed on the apparatus, and run at constant voltages 100 volts for about 20 min. When adequate migration occurred, DNA fragments were stained with ethidium bromide (EtBr 0.5 µg/ml) for 20 min, then removed excess EtBr on gel by steriled ultrapure water and visualized under UV light at 254 nm.

2.5.2 UV spectroscopy

DNA quantity and quality were measured by a UV spectrophotometer (SHIMADZU UV 160A, Japan) to determine absorbance at 260 nm and absorbance ratio at 260/280 nm, respectively. To prepare a sample solution, DNA samples 10 µl was pipetted into a new 1.5 ml microcentrifuge tube, mixed thoroughly with ultrapure water 90 µl, and transferred to a 50 µl disposable cuvette (UVette®, USA).

2.6 PCR amplification on 28s rRNA gene

2.6.1 Primers design

Primer set was designed from the published DNA sequence database (NCBI GenBank) (http://www.ncbi.nlm.nih.gov). Initially, the sequences of 28s rRNA gene of *Xestospongia caminata* (accession number AF441348) and *Xestospongia subtriangularis* (accession number AF441341) were compared using a progressive pairwise alignment program (Multalin) (http://bioinfo.genotoul.fr/

multalin/multalin.html) (Figure B1 in Appendix B). The designed primers were synthesized by Invitrogen™ (Japan).

2.6.2 Reaction and condition for PCR amplification

PCR amplification of 28s rRNA gene was performed using 50-100 ng of DNA as a template in 50 µl of a reaction mixture consisting of 10X amplification buffer A (Vivantis Technologies, England), 50 mM $MgCl_2$ (Vivantis Technologies, England), 25 mM of each dNTPs (Vivantis Technologies, England), 1.5 U *Taq* DNA polymerase (Vivantis Technologies, England), and 20 µM of each primer, forward primer, XSF001 (5′-GTTTCCCTCAGGATAGCTGGA-3′) and reverse primer, XSR002 (5′-CATCGCCGGTTCTGCTTACCA-3′).

Amplification was carried out in a PCR thermocycler (MultiGene Thermal Cycler, Labnet International, Inc., U.S.A.). The PCR cycling program started with an initial denaturation step at 94°C for 5 minutes to ensure complete separation of the DNA strands, and followed by strand denaturation at 94°C for 30 seconds, primer annealing at 47°C for 30 seconds, and primer extension at 72°C for 30 seconds for 30 cycles, and final extension step at 72°C for 7 minutes to ensure that all amplicons were fully extended, then held at 4°C. PCR products were analyzed by 1.2% agarose gel electrophoresis in 1X TAE buffer, visualized under UV light and photographed using Gel Quant software, Geldoc MiniBis Pro (DNR Bio-Imaging System Ltd., Israel). A 100bp plus DNA ladder (Vivantis™ Technologies, England) was used as standard molecular size.

2.7 PCR amplification on cytochrome oxidase subunit I (COX I) Gene

2.7.1 Primers design

Primer set was designed from published DNA sequence database (NCBI GenBank) (http://www.ncbi.nlm.nih.gov). Initially, the sequences of cytochrome oxidase subunit I (COX I) gene of *Xestospongia muta* (accession number EF519697, EF519698, EF519699, EF519700, EF519701) and *Xestospongia proxima* (accession number AM076980) were compared using a progressive pairwise alignment program (Multalin) (http://bioinfo.genotoul.fr/multalin/multalin.html) (Figure B2 in Appendix B). The designed primers were synthesized by Invitrogen™ (Japan).

2.7.2 Reaction and condition for PCR amplification

PCR amplification of COX I gene was performed using 50-100 ng of DNA as a template in 50 µl of a reaction mixture consisting of 10X amplification buffer A (Vivantis Technologies, England), 50 mM $MgCl_2$ (Vivantis Technologies, England), 25 mM of each dNTPs (Vivantis Technologies, England), 1.5 U *Taq* DNA polymerase (Vivantis Technologies, England) and 20 µM of each primer, forward primer, XSFCOXI_41S (5′-AGTTATGCCAGTAATGATAG-3′) and reverse primer, XSRCOXI_161A (5′-ACAGAT CAGACAAATAATGG-3′).

Amplification was carried out in a PCR thermocycler (MultiGene Thermal Cycler, Labnet International, Inc., U.S.A.). The PCR cycling program was started with an initial denaturation step at 94°C for 5 minutes to ensure complete separation of the DNA strands, and followed by strand denaturation at 94°C for 1 minute, primer annealing at 42°C for 2 minute, and primer extension at 72°C for 2 minute for 30 cycles, and final extension step at 72°C for 7 minutes to ensure that all amplicons are fully extended, then held at 4°C. PCR product were analyzed by 1.2% agarose gel electrophoresis in 1X TAE buffer, visualized under UV light and photographed using Gel Quant software, Geldoc MiniBis Pro (DNR Bio-Imaging System Ltd., Israel). A 100bp plus DNA ladder (Vivantis Technologies, England) was used as standard molecular size.

2.8 PCR amplification on 16s rRNA gene

Primer set of universal eubacterial 16s rRNA primer were designed by Weisburg *et al*., 1991. PCR amplification of 16s rRNA gene was performed using 50-100 ng of DNA as a template in 50 µl of reaction mixture consisting of 10X amplification buffer A (Vivantis Technologies, England), 50 mM $MgCl_2$ (Vivantis Technologies, England), 25 mM of each dNTPs (Vivantis Technologies, England), 1.5 U *Taq* DNA polymerase (Vivantis Technologies, England) and 20 µM of each primer, forward primer, fD1 (5′-ccgaattcgtcgacaacAGAGTTTGATCCTGGCTCAG-3′) and reverse primer, rP2 (5′-cccgggatccaagcttACGGCTACCTTGTTACGACTT-3′).

Amplification was carried out in a PCR thermocycler (MultiGene Thermal Cycler, Labnet International, Inc., U.S.A.). The PCR cycling program started with an initial denaturation step at 94°C for 5 minutes to ensure complete

separation of the DNA strands, follow by strand denaturation at 94°C for 1 minute, primer annealing at 45°C for 2 minute, and primer extension at 72°C for 2 minute for 30 cycles, and final extension step at 72°C for 7 minutes to ensure that all amplicons are fully extended, then held at 4°C. PCR product were analyzed by 1.2% agarose gel electrophoresis in 1X TAE buffer (40 mM Tris-HCl, 20 mM acetic acid, and 1 mM EDTA), visualized under UV light and photographed using Gel Quant software, Geldoc MiniBis Pro (DNR Bio-Imaging System Ltd., Israel). A 100bp plus DNA ladder (Vivantis Technologies, England) was used as standard molecular size.

2.9 Nucleotide sequencing

Nucleotide sequences of purified PCR products were determined by Genome Institute (GI), National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand. Primer for sequencing in the forward direction was T7 (5′-TAATACGACT CACTATAGGG-3′) and primer SP6 (5′-ATTTAGGTGACACTATAG-3′) was used for the complementary strand.

CHAPTER IV

RESULTS AND DISCUSSION

1. Comparison of protocols for DNA extraction from the blue sponge *Xestospongia* sp.

The genomic DNAs from the blue sponge *Xestospongia* sp. were extracted by following seven established protocols, including

- MasterPure® DNA Extraction Kit (Protocol 1)
- Simple DNA Extraction Protocol (Protocol 2)
- SDS/phenol Extraction Protocol (Protocol 3)
- CTAB Protocol for Plants (Protocol 4)
- CTAB Protocol for Sponges (Protocol 5)
- SDS/CTAB/phenol Extraction Protocol (Protocol 6)
- NaOAc salting-out Protocol (Protocol 7)

The obtained DNA from each protocol was monitored on 0.8% agarose gel electrophoresis (Figure 4.1). The result of gel electrophoresis showed that all protocols gave similar smear bands of small fragments DNA and RNA containing less than 564 bps. The ideal DNA obtained should give more genomic bands at the high molecular weight area and less bands at the low molecular weight area. Only the simple DNA extraction protocol and the NaOAc salting-out protocol gave the high molecular weight DNAs up to about 23,000 bps.

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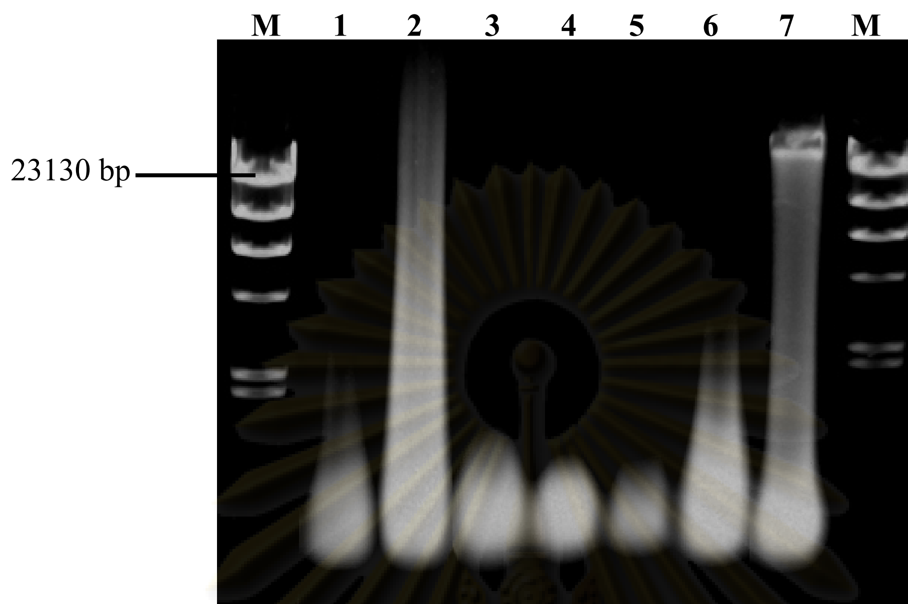

Figure 4.1 0.8% agarose gel electrophoresis of the genomic DNAs from the blue sponge *Xestospongia* sp. by seven DNA extraction protocols

Lane M: VC Lambda DNA/Hind III marker (The sizes are 125, 564, 2027, 2232, 4361, 6557, 9416 and 23130 bp, respectively.)

Lane 1: MasterPure® DNA extraction kit

Lane 2: Simple DNA extraction protocol

Lane 3: SDS/phenol extraction protocol

Lane 4: CTAB protocol for plants

Lane 5: CTAB protocol for sponges

Lane 6: SDS/CTAB/phenol extraction protocol

Lane 7: NaOAc salting-out protocol

All obtained DNAs were further determined their quantity and purity by UV absorptions at 260 and 280 nm as shown in Table 4.1. The DNAs obtained from all seven protocols were significantly different (ANOVA, $p<0.05$) in both quantity and purity with the means of 59 ng/µl and 1.52 ± 0.00, respectively. The NaOAc salting-out protocol gave the highest DNA concentration (98 ng/µl) while the CTAB for

plants protocol gave the lowest DNA concentration (24 ng/µl). Interestingly, only the NaOAc salting-out protocol provided DNAs with UV absorption ratio (OD_{260}/OD_{280}) 1.88 (% nucleic acid = 52%). Other protocols gave the ratios less than 1.8 indicating significant proteins contamination. The ratio value indicated that the DNA purity from the NaOAc salting-out protocol is better than those from other protocols (Sambrook 2001).

Table 4.1 Comparison of DNA quantity and purity from seven DNA extraction protocols

Protocols	**OD_{260} ± SD**	**DNA conc. (ng/µl)**	**OD_{260}/OD_{280} ± SD***	**% Nucleic acid**
MasterPure® DNA extraction kit	0.70 ± 0.04	35	1.59 ± 0.01	20
Simple DNA extraction	1.22 ± 0.08	61	1.06 ± 0.03	5
SDS/phenol extraction	1.16 ± 0.03	58	1.67 ± 0.02	25
CTAB protocol for plants	0.47 ± 0.03	24	1.31 ± 0.01	10
CTAB protocol for sponges	1.09 ± 0.04	55	1.70 ± 0.01	28
SDS/CTAB/phenol extraction	1.60 ± 0.02	80	1.48 ± 0.00	15
NaOAc salting-out	1.96 ± 0.01	98	1.88 ± 0.00	52

*DNA purity was considered from the absorbance ratio (OD_{260}/OD_{280}) and the good purity DNA should be in the range of 1.8-2.0.

To confirm this result, conserve regions within 28s rRNA and COX I genes of the sponge DNA were amplified by PCR technique. The expected products were about 200 bps and 350 bps in length by 28s rRNA and *COX* I specific primers, respectively. The result showed that only DNA from the NaOAc salting-out protocol gave PCR products at expected sizes (Figure 4.2 A-B). Although DNA extracts were

from the same set of sample, PCR products were not found from protocols 1 to 6. This suggested that lower %nucleic acid was possibly a crucial factor for PCR amplification. It is concluded that the NaOAc salting-out protocol is the best among seven protocols used in this study for DNA extraction from the blue sponge *Xestospongia* sp.

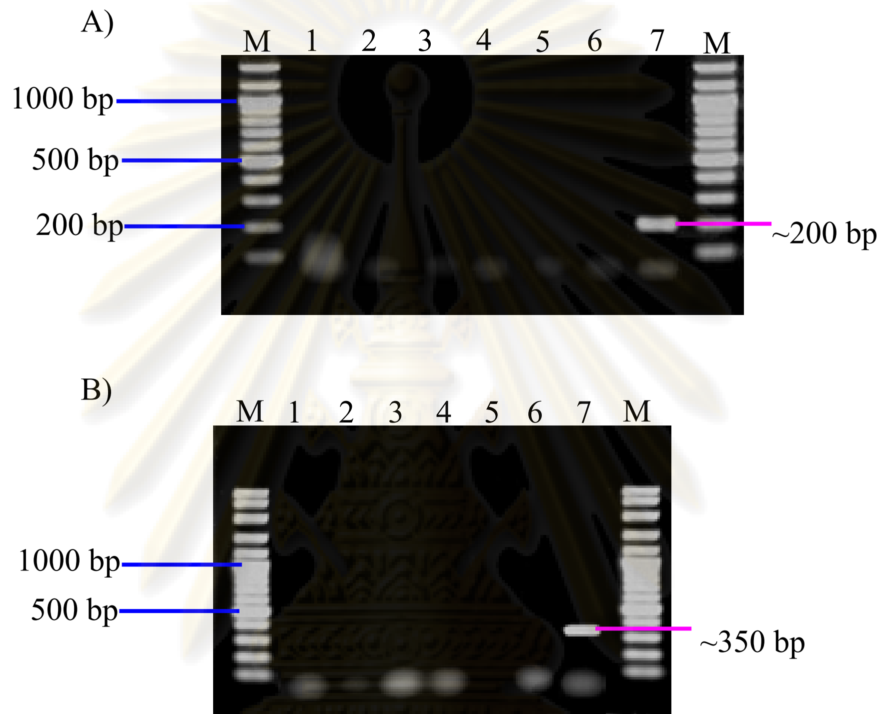

Figure 4.2 1.2% agarose gel electrophoresis of PCR products from the blue sponge *Xestospongia* sp. using seven DNA extraction protocols
A) 28s rRNA gene
B) COX I gene

Lane M: VC 100 bps plus DNA ladder (The sizes are 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000, 2500, and 3000 bps, respectively.)

Lane 1: MasterPure® DNA&RNA extraction kit

Lane 2: Simple DNA extraction protocol

Lane 3: SDS/phenol protocol

Lane 4: CTAB protocol for plants

Lane 5: CTAB protocol for sponges

Lane 6: SDS/CTAB/phenol protocol

Lane 7: NaOAc salting-out protocol

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2. Development of the NaOAc salting-out protocol for improvement DNA extraction

The NaOAc salting-out protocol was selected for further modification to improve DNA extraction from the sponge *Xestospongia* sp. There are essential three consecutive steps to be modified including lysis buffer solution compositions, salt solution for protein precipitation, and DNA precipitation methods.

2.1 Optimization of lysis buffer compositions

The obtained DNAs from the sponge samples were extracted by the NaOAc salting-out protocol with 125 different lysis buffer solutions containing sequential combination of various concentrations of SDS (1, 5, 10, 15, 20%), EDTA (5, 10, 15, 20, 25 mM), and Tris-HCl (1, 5, 10, 50, 100 mM). DNA quantity and purity were measured by a UV absorption at wavelengths 260 and 280 nm as shown in Tables 4.2-4.6. All DNAs obtained from those lysis buffers were significantly different (ANOVA, $p<0.05$) in both quantity and purity with the means of 76 ng/µl and 1.78 ± 0.01, respectively.

In order to separately analyze the effects of SDS, EDTA, and Tsis-HCl, means of DNA concentration and absorbance ratio based on %SDS, EDTA concentration, and Tris-HCl concentration are summarized in Tables 4.7 - 4.9. The series of lysis buffers containing SDS detergent at concentration 10 - 20% and EDTA at 15 - 25 mM yielded means of absorbance ratio about 1.8 while those containing Tris-HCl at all concentrations yielded similar means of absorbance ratio about 1.78. Among these buffers, the series of lysis buffers with 10% SDS gave both the highest means of DNA concentration (82 ng/µl) and DNA purity (OD_{260}/OD_{280} = 1.83).

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Table 4.2 The DNA quantity and purity from the protocol with 1%SDS in lysis buffer and containing various concentrations of other compositions

Lysis buffer	Final concentrations			Results		
	%SDS	EDTA (mM)	Tris-HCl (mM)	$OD_{260} \pm SD$	DNA conc. (ng/µl)	$OD_{260}/OD_{280} \pm SD$
A1	1	5	1	1.06 ± 0.11	53	1.71 ± 0.04
A2	1	5	5	0.95 ± 0.06	48	1.78 ± 0.02
A3	1	5	10	1.11 ± 0.35	56	1.72 ± 0.08
A4	1	5	50	1.93 ± 0.25	97	1.58 ± 0.12
A5	1	5	100	1.74 ± 0.28	87	1.48 ± 0.04
A6	1	10	1	1.78 ± 0.02	89	1.61 ± 0.06
A7	1	10	5	2.08 ± 0.08	104	1.59 ± 0.01
A8	1	10	10	2.06 ± 0.37	103	1.69 ± 0.02
A9	1	10	50	1.91 ± 0.43	96	1.58 ± 0.02
A10	1	10	100	1.61 ± 0.38	81	1.60 ± 0.04
A11	1	15	1	1.75 ± 0.22	88	1.85 ± 0.03
A12	1	15	5	1.03 ± 0.15	52	1.83 ± 0.02
A13	1	15	10	0.98 ± 0.08	49	1.83 ± 0.00
A14	1	15	50	1.15 ± 0.15	58	1.82 ± 0.02
A15	1	15	100	1.04 ± 0.20	52	1.80 ± 0.03
A16	1	20	1	1.39 ± 0.34	70	1.73 ± 0.01
A17	1	20	5	1.57 ± 0.58	79	1.70 ± 0.07
A18	1	20	10	1.14 ± 0.14	57	1.69 ± 0.09
A19	1	20	50	1.48 ± 0.22	74	1.68 ± 0.06
A20	1	20	100	1.09 ± 0.22	55	1.65 ± 0.03
A21	1	25	1	1.61 ± 0.32	81	1.84 ± 0.03
A22	1	25	5	1.10 ± 0.11	55	1.81 ± 0.00
A23	1	25	10	1.14 ± 0.19	57	1.82 ± 0.01
A24	1	25	50	1.35 ± 0.39	68	1.87 ± 0.06
A25	1	25	100	1.50 ± 0.15	75	1.85 ± 0.01

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Table 4.3 The DNA quantity and purity from the protocol with 5%SDS in lysis buffer and containing various concentrations of other compositions

Lysis buffer	Final concentrations			Results		
	%SDS	EDTA (mM)	Tris-HCl (mM)	OD_{260} ± SD	DNA conc. (ng/μl)	OD_{260}/OD_{280} ± SD
B1	5	5	1	1.47 ± 0.24	74	1.74 ± 0.00
B2	5	5	5	1.54 ± 0.19	77	1.73 ± 0.01
B3	5	5	10	1.64 ± 0.04	82	1.72 ± 0.00
B4	5	5	50	1.53 ± 0.01	77	1.78 ± 0.00
B5	5	5	100	1.87 ± 0.02	94	1.80 ± 0.01
B6	5	10	1	2.12 ± 0.02	106	1.80 ± 0.00
B7	5	10	5	1.80 ± 0.03	90	1.78 ± 0.00
B8	5	10	10	1.14 ± 0.01	57	1.77 ± 0.00
B9	5	10	50	1.04 ± 0.00	52	1.77 ± 0.00
B10	5	10	100	1.04 ± 0.02	52	1.70 ± 0.02
B11	5	15	1	1.18 ± 0.01	59	1.69 ± 0.00
B12	5	15	5	1.28 ± 0.02	64	1.77 ± 0.01
B13	5	15	10	1.21 ± 0.00	61	1.75 ± 0.00
B14	5	15	50	1.08 ± 0.00	54	1.76 ± 0.00
B15	5	15	100	0.86 ± 0.00	43	1.75 ± 0.00
B16	5	20	1	1.08 ± 0.05	54	1.72 ± 0.01
B17	5	20	5	1.20 ± 0.01	60	1.77 ± 0.00
B18	5	20	10	1.24 ± 0.00	62	1.76 ± 0.00
B19	5	20	50	1.21 ± 0.01	61	1.78 ± 0.00
B20	5	20	100	1.21 ± 0.00	61	1.82 ± 0.00
B21	5	25	1	1.23 ± 0.01	62	1.76 ± 0.00
B22	5	25	5	1.20 ± 0.01	60	1.71 ± 0.00
B23	5	25	10	1.24 ± 0.01	62	1.76 ± 0.00
B24	5	25	50	1.21 ± 0.00	61	1.75 ± 0.00
B25	5	25	100	1.19 ± 0.00	60	1.76 ± 0.00

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Table 4.4 The DNA quantity and purity from the protocol with 10%SDS in lysis buffer and containing various concentrations of other compositions

Lysis buffer	Final concentrations			Results		
	%SDS	EDTA (mM)	Tris-HCl (mM)	OD_{260} ± SD	DNA conc. (ng/µl)	OD_{260}/OD_{280} ± SD
C1	10	5	1	0.84 ± 0.01	42	1.74 ± 0.00
C2	10	5	5	1.25 ± 0.01	63	1.70 ± 0.00
C3	10	5	10	1.08 ± 0.00	54	1.78 ± 0.00
C4	10	5	50	1.66 ± 0.01	83	1.70 ± 0.00
C5	10	5	100	1.00 ± 0.01	50	1.73 ± 0.00
C6	10	10	1	1.63 ± 0.02	82	1.80 ± 0.00
C7	10	10	5	1.23 ± 0.02	62	1.78 ± 0.00
C8	10	10	10	1.28 ± 0.01	64	1.79 ± 0.00
C9	10	10	50	1.37 ± 0.01	69	1.77 ± 0.00
C10	10	10	100	1.08 ± 0.01	54	1.81 ± 0.00
C11	10	15	1	2.05 ± 0.01	103	1.85 ± 0.00
C12	10	15	5	2.20 ± 0.00	110	1.91 ± 0.00
C13	10	15	10	2.01 ± 0.01	100	1.88 ± 0.00
C14	10	15	50	1.96 ± 0.13	98	1.85 ± 0.01
C15	10	15	100	1.74 ± 0.19	87	1.83 ± 0.01
C16	10	20	1	1.73 ± 0.03	87	1.84 ± 0.03
C17	10	20	5	1.96 ± 0.01	98	1.92 ± 0.00
C18	10	20	10	1.84 ± 0.00	92	1.88 ± 0.00
C19	10	20	50	1.97 ± 0.02	99	1.92 ± 0.00
C20	10	20	100	1.74 ± 0.02	87	1.92 ± 0.00
C21	10	25	1	1.78 ± 0.02	89	1.80 ± 0.00
C22	**10**	**25**	**5**	**2.14 ± 0.06**	**107**	**1.93 ± 0.00**
C23	10	25	10	2.02 ± 0.01	101	1.88 ± 0.01
C24	10	25	50	1.83 ± 0.01	92	1.91 ± 0.00
C25	10	25	100	1.68 ± 0.02	84	1.86 ± 0.00

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Table 4.5 The DNA quantity and purity from the protocol with 15%SDS in lysis buffer and containing various concentrations of other compositions

Lysis buffer	Final concentrations			Results		
	%SDS	EDTA (mM)	Tris-HCl (mM)	$OD_{260} \pm SD$	DNA conc. (ng/µl)	$OD_{260}/OD_{280} \pm SD$
D1	15	5	1	1.67 ± 0.01	84	1.70 ± 0.00
D2	15	5	5	1.75 ± 0.00	88	1.70 ± 0.00
D3	15	5	10	1.74 ± 0.02	87	1.82 ± 0.00
D4	15	5	50	1.87 ± 0.01	94	1.77 ± 0.00
D5	15	5	100	1.53 ± 0.01	77	1.85 ± 0.00
D6	15	10	1	1.74 ± 0.01	87	1.77 ± 0.00
D7	15	10	5	1.55 ± 0.00	78	1.82 ± 0.00
D8	15	10	10	1.86 ± 0.01	93	1.81 ± 0.00
D9	15	10	50	1.81 ± 0.01	91	1.85 ± 0.00
D10	15	10	100	1.41 ± 0.01	71	1.74 ± 0.00
D11	15	15	1	1.26 ± 0.00	63	1.82 ± 0.00
D12	15	15	5	1.33 ± 0.01	67	1.81 ± 0.00
D13	15	15	10	1.48 ± 0.00	74	1.72 ± 0.00
D14	15	15	50	1.67 ± 0.01	84	1.78 ± 0.00
D15	15	15	100	1.18 ± 0.01	59	1.84 ± 0.00
D16	15	20	1	1.49 ± 0.01	75	1.85 ± 0.01
D17	15	20	5	1.59 ± 0.03	80	1.70 ± 0.01
D18	15	20	10	1.65 ± 0.01	83	1.85 ± 0.00
D19	15	20	50	1.50 ± 0.01	75	1.79 ± 0.00
D20	15	20	100	1.37 ± 0.01	69	1.74 ± 0.00
D21	15	25	1	1.61 ± 0.01	81	1.82 ± 0.00
D22	15	25	5	1.74 ± 0.00	87	1.81 ± 0.00
D23	15	25	10	1.50 ± 0.01	75	1.79 ± 0.00
D24	15	25	50	1.42 ± 0.02	71	1.83 ± 0.01
D25	15	25	100	1.27 ± 0.01	64	1.84 ± 0.00

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Table 4.6 The DNA quantity and purity from the protocol with 20%SDS in lysis buffer and containing various concentrations of other compositions

Lysis buffer	Final concentrations			Results		
	%SDS	EDTA (mM)	Tris-HCl (mM)	$OD_{260} \pm SD$	DNA conc. (ng/µl)	$OD_{260}/OD_{280} \pm SD$
E1	20	5	1	1.68 ± 0.19	84	1.79 ± 0.01
E2	20	5	5	1.70 ± 0.01	85	1.57 ± 0.19
E3	20	5	10	2.11 ± 0.09	95	1.85 ± 0.01
E4	20	5	50	1.58 ± 0.03	79	1.75 ± 0.02
E5	20	5	100	1.82 ± 0.03	91	1.78 ± 0.02
E6	20	10	1	1.44 ± 0.12	72	1.82 ± 0.01
E7	20	10	5	1.68 ± 0.03	84	1.79 ± 0.01
E8	20	10	10	1.64 ± 0.04	82	1.67± 0.02
E9	20	10	50	1.49 ± 0.05	75	1.71 ± 0.01
E10	20	10	100	1.27 ± 0.04	64	1.71 ± 0.01
E11	20	15	1	1.69 ± 0.07	85	1.78 ± 0.01
E12	20	15	5	1.83 ± 0.19	92	1.83 ± 0.07
E13	20	15	10	1.64 ± 0.04	82	1.85 ± 0.01
E14	20	15	50	1.85 ± 0.23	93	1.84 ± 0.07
E15	20	15	100	1.61 ± 0.07	81	1.84 ± 0.01
E16	20	20	1	1.55 ± 0.04	78	1.80 ± 0.00
E17	20	20	5	1.43 ± 0.05	72	1.83 ± 0.01
E18	20	20	10	1.48 ± 0.05	74	1.90 ± 0.01
E19	20	20	50	1.67 ± 0.04	84	1.84 ± 0.01
E20	20	20	100	1.72 ± 0.03	86	1.86 ± 0.01
E21	20	25	1	1.26 ± 0.03	63	1.77 ± 0.01
E22	20	25	5	1.53 ± 0.09	77	1.86 ± 0.02
E23	20	25	10	1.94 ± 0.06	97	1.86 ± 0.02
E24	20	25	50	1.97 ± 0.04	99	1.87 ± 0.01
E25	20	25	100	1.29 ± 0.04	65	1.84 ± 0.01

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Table 4.7 Means of DNA concentration and absorbance ratio based on %SDS

%SDS	Means of DNA conc. (ng/µl)	Means of OD_{260}/OD_{280}
1	71	1.72
5	66	1.76
10	82	1.83
15	78	1.79
20	81	1.80

Table 4.8 Means of DNA concentration and absorbance ratio based on EDTA concentrations

EDTA (mM)	Means of DNA conc. (ng/µl)	Means of OD_{260}/OD_{280}
5	76	1.73
10	78	1.74
15	74	1.81
20	75	1.80
25	76	1.82

Table 4.9 Means of DNA concentration and absorbance ratio based on Tris-HCl concentrations

Tris-HCl (mM)	Means of DNA conc. (ng/µl)	Means of OD_{260}/OD_{280}
1	76	1.78
5	77	1.78
10	76	1.79
50	79	1.78
100	70	1.78

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Finally, the DNA concentration more than 100 ng/µl and the DNA absorbance ratio greater than 1.8 were both used as the criterias for the buffer of choice. However, there were only six lysis buffers passing both criterias, including B06, C11, C12, C13, C22, and C23 (Table 4.10). The lysis buffer C12 giving the greatest amount of DNA (110 µg/ml) contains 10% SDS, 15 mM EDTA, and 5 mM Tris-HCl. The lysis buffer C22 giving the best DNA purity (OD_{260}/OD_{280} = 1.93) contains 10% SDS, 25 mM EDTA, and 5 mM Tris-HCl. Since the DNA purity is more important than the DNA concentration for further usage, the buffer C22 was finally selected as the optimum lysis buffer to extract DNA from the blue sponge *Xestospongia* sp.

Table 4.10 The six lysis buffers passing both criteria

Lysis buffer	%SDS	EDTA (mM)	Tris-HCl (mM)	DNA conc. (ng/µl)	OD_{260}/OD_{280}	%nucleic acid
B06	5	10	1	106	1.8	38
C11	10	15	1	103	1.85	47
C12	10	15	5	110	1.91	60
C13	10	15	10	100	1.88	53
C22	**10**	**25**	**5**	**107**	**1.93**	**65**
C23	10	25	10	101	1.88	53

2.2 Optimization of salt solution in protein precipitation

After the lysis buffer of the NaOAc salting-out protocol was optimized to contain 10%SDS, 25 mM EDTA, and 5 mM Tris-HCl, the protein precipitation was next to be modified. The DNAs from the sponge samples were extracted by the above modified NaOAc salting-out protocol with vary concentrations (1, 3, 5, 6, 7, and 9 M) of either NaOAc or NaCl solutions to optimize protein precipitation method. DNA quantity and purity were measured by UV absorption at 260 and 280 nm as shown in Table 4.11.

Table 4.11 The DNA quantity and purity from the protocol with various salt solutions

Salt solutions	**OD_{260} ± SD**	**DNA conc. (ng/µl)**	**OD_{260}/OD_{280} ± SD**	**%Nucleic acid**
1 M NaOAc	1.39 ± 0.02	70	1.21 ± 0.00	8
3 M NaOAc	1.57 ± 0.01	79	1.47 ± 0.00	15
5 M NaOAc	**1.92 ± 0.02**	**96**	**1.90 ± 0.00**	**57**
6 M NaOAc	1.86 ± 0.02	93	1.87 ± 0.00	50
7 M NaOAc	1.74 ± 0.03	87	1.82 ± 0.00	41
9 M NaOAc	1.65 ± 0.03	83	1.75 ± 0.00	32
1 M NaCl	1.05 ± 0.02	53	1.17 ± 0.00	7
3 M NaCl	1.27 ± 0.02	64	1.36 ± 0.01	11
5 M NaCl	1.46 ± 0.03	73	1.83 ± 0.01	43
6 M NaCl	1.23 ± 0.01	62	1.75 ± 0.00	32
7 M NaCl	1.16 ± 0.02	58	1.72 ± 0.01	29
9 M NaCl	1.12 ± 0.01	56	1.54 ± 0.00	18

The DNAs obtained from both NaOAc and NaCl solutions were significantly different (ANOVA, $p<0.05$) in both quantity and purity with the means of 73 ng/µl and 1.62 ± 0.00, respectively. However, the quality of DNAs obtained from protein precipitation by NaOAc were better than those from NaCl protein precipitation (Table 4.11). The DNA obtained from all concentrations of the NaOAc solutions were significantly different (ANOVA, $p<0.05$) in both quantity and purity with the means of 85 ng/µl and 1.67 ± 0.00, respectively. The DNA obtained from all concentrations of the NaCl solutions were significantly different (ANOVA, $p<0.05$) in both quantity and purity with the means of 61 ng/µl and 1.56 ± 0.01, respectively.

The 5 M NaOAc gave the highest DNA quantity (96 ng/µl) and purity (OD_{260}/OD_{280} = 1.90, % nucleic acid = 52%) while the 1 M NaCl gave the lowest DNA quantity (53 ng/µl) and purity (OD_{260}/OD_{280} = 1.17, % nucleic acid = 8%).

These results represented that the optimal salt solution to precipitate proteins in DNA extraction from the blue sponge *Xestospongia* sp. was 5 M NaOAc solution.

2.3 Optimization of DNA precipitation methods

After the obtained DNAs from the sponge samples were extracted by the modified NaOAc salting-out protocol with optimal lysis buffer by 10% SDS, 25 mM EDTA, 5mM Tris-HCl and the proteins precipitation by 5 M NaOAc, the optimum DNA precipitation was further studied. Then, absolute EtOH and isopropanol, at various precipitation times (0, 30, 60, 120, 180 min, and 12 hr) were selected for DNA precipitation study. DNA quantity and purity were measured by UV absorption at 260 and 280 nm as shown in Tables 4.12 and 4.13.

Table 4.12 The DNA quantity and purity using absolute ethanol for DNA precipitation

DNA precipitation		OD_{260} ± SD	DNA conc. (ng/µl)	OD_{260}/OD_{280} ± SD	%Nucleic acid
Alcohol	**Time to precipitate**				
Absolute ethanol	0 min	1.58 ± 0.00	79	1.79 ± 0.00	37
	30 min	**1.94 ± 0.01**	**97**	**1.93 ± 0.01**	**65**
	1 hr	2.13 ± 0.02	107	1.91 ± 0.00	60
	2 hr	2.16 ± 0.02	108	1.84 ± 0.00	45
	3 hr	2.21 ± 0.01	111	1.82 ± 0.00	41
	12 hr	1.87 ± 0.01	94	1.77 ± 0.00	35

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Table 4.13 The DNA quantity and purity using isopropanol for DNA precipitation

DNA precipitation		**OD_{260} ± SD**	**DNA conc. (ng/µl)**	**OD_{260}/OD_{280} ± SD**	**%Nucleic acid**
Alcohol	**Time to precipitate**				
Isopropanol	0 min	1.32 ± 0.01	66	1.65 ± 0.01	23
	30 min	1.57 ± 0.00	79	1.74 ± 0.01	31
	1 hr	1.69 ± 0.01	85	1.78 ± 0.00	35
	2 hr	1.87 ± 0.00	94	1.72 ± 0.00	29
	3 hr	1.93 ± 0.01	97	1.68 ± 0.00	26
	12 hr	1.72 ± 0.01	86	1.61 ± 0.01	21

The DNA obtained from both alcohol solutions were significantly different (ANOVA, $p<0.05$) in both quantity and purity. The absolute EtOH gave higher means of DNA quantity and purity (99 ng/µl and 1.84 ± 0.0, respectively) than isopropanol (84 ng/µl and 1.70 ± 0.01, respectively).

For the DNA precipitation time, the DNA obtained from all DNA precipitation times by absolute EtOH were significantly different (ANOVA, $p<0.05$). From Table 4.12, the absolute EtOH gave the highest DNA quantity (111 ng/µl) when used the time to precipitate DNA at 3 hr while at 0 min gave the lowest DNA quantity (79 ng/µl). The DNA precipitation time at 30 min gave the greatest DNA purity (OD_{260}/OD_{280} = 1.93, %nucleic acid = 65%) and at overnight gave the lowest DNA purity (OD_{260}/OD_{280} = 1.77, %nucleic acid = 35%). Finally, the DNA precipitation method with absolute EtOH at 30 min was selected as the DNA precipitation method because the best DNA purity and acceptable DNA concentration were obtained.

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3. Comparison of the modified NaOAc salting-out protocol with the original NaOAc salting-out protocol

From the above experiments, the modified NaOAc salting-out protocol was obtained to extract DNA from the blue sponge *Xestospongia* sp. The NaOAc salting-out protocol and the modified NaOAc salting-out protocol were directly compared its efficiency to extract DNA from the sponge. The obtained DNAs were checked on 0.8% agarose gel electrophoresis (Figure 4.3). The result of gel electrophoresis showed that the NaOAc salting-out protocol gave the less intact genomic DNA quantity and greater smear region than those of the modified NaOAc salting-out protocol.

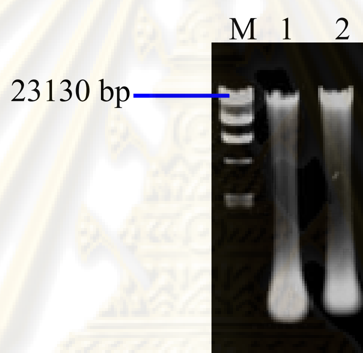

Figure 4.3 0.8% agarose gel electrophoresis of genomic DNA from the NaOAc salting-out protocol (lane 1) and the modified NaOAc salting-out protocol (lane 2)

Lane M: VC Lambda DNA/Hind III marker

Finally, RNase A was added into the modified NaOAc salting-out protocol for removing RNA and degraded RNA contaminants because RNA contaminants might interfere during PCR amplification. The result of gel electrophoresis showed that the modified NaOAc salting-out protocol with RNase A gave the greatest intact genomic DNA with the least smear region (Figure 4.4). The quantity and purity of DNA from the modified NaOAc salting-out protocol with RNase A were measured by UV absorptions at 260 and 280 nm as shown in Table 4.14. This protocol significantly improved the DNA quantity (110 ng/µl) and purity (1.97 ± 0.01, %nucleic acid = 80%) when compared to the modified NaOAc salting-out protocol (quantity = 102

ng/µl) and purity = 1.93 ± 0.00, %nucleic acid = 65%). The last modified protocol was named as **the blue sponge *Xestospongia* sp. DNA extraction protocol**.

Table 4.14 The DNA quantity and purity from the NaOAc salting-out protocol, the modified NaOAc salting-out protocol, and the blue sponge *Xestospongia* sp. DNA extraction protocol

Protocols	OD_{260} ± SD	DNA conc. (ng/µl)	OD_{260}/OD_{280} ± SD	%Nucleic acid
The original NaOAc salting-out protocol	1.94 ± 0.01	97	1.89 ± 0.01	55
The modified NaOAc salting-out protocol	2.03 ± 0.00	102	1.93 ± 0.00	65
The blue sponge *Xestospongia* sp. DNA extraction protocol	2.19 ± 0.01	110	1.97 ± 0.01	80

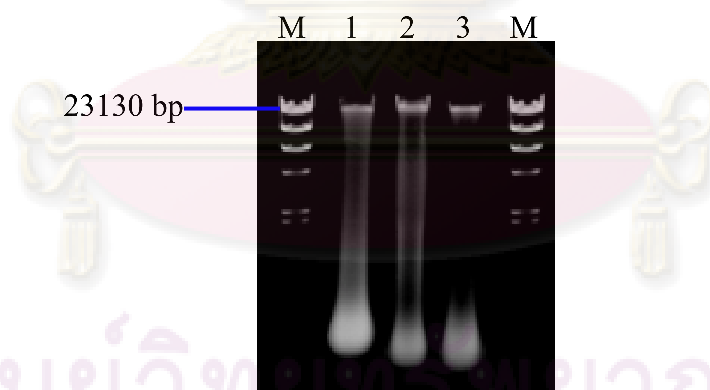

Figure 4.4 0.8% agarose gel electrophoresis of genomic DNA from the NaOAc salting-out protocol (lane 1), the modified NaOAc salting-out protocol (lane 2), and the blue sponge *Xestospongia* sp. DNA extraction protocol (lane 3)

Lane M: VC Lambda DNA/Hind III marker

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To confirm this result, the obtained DNA from each protocol was used as a template, the conserve region within 28s rRNA gene from the sponge genus *Xestospongia* was amplified by PCR amplification technique with a pair of primers from the data of genus *Xestospongia*, XSF001 and XSR002. The PCR products were expected about 200 bps in length as shown in Figure 4.5.

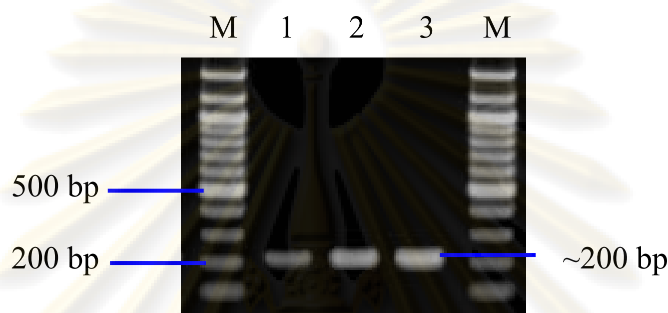

Figure 4.5 1.2% agarose gel electrophoresis of PCR products of 28s rRNA gene from the NaOAc salting-out protocol (lane 1), the modified NaOAc salting-out protocol (lane 2), and the blue sponge *Xestospongia* sp. DNA extraction protocol (lane 3)
Lane M: VC 100 bp plus DNA ladder

The result showed that all DNA extracts from the three protocols gave the same expected size PCR products but difference in intensity under UV light. Intensity of the PCR products noticeably corresponded to both quality and quality of DNA templates. The PCR products using DNA templates from the original NaOAc salting-out protocol showed the least intense band whereas the PCR products using DNA templates from the blue sponge *Xestospongia* sp. DNA extraction protocol gave the most intense band. The stepwise procedure of the blue sponge *Xestospongia* sp. DNA extraction protocol is illustrated in Scheme 4.1.

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<u>The blue sponge *Xestospongia* sp. DNA extraction protocol</u>

Grind sample 100 mg in **lysis buffer** (10%SDS, 25 mM EDTA, and 5 mM Tris-HCl) 750 μl

↓

Centrifuge at 500 g for 1 min and transfer supernatant into a new Eppendorf tube

↓

Add Proteinase K 1 μl and incubate at 56°C for 3 hr

↓

Add RNase A 1μl and incubate at 37°C for 1 hr

↓

Add **5 M NaOAc** 250 μl, centrifuge at 5,000 g for 20 min, and transfer supernatant into a new 1.5 ml microcentrifuge tube

↓

Add cold **absolute EtOH** 1 ml and keep at –20°C for **30 min**

↓

Centrifuge at 13,000 g for 30 min and discard supernatant,

↓

dry pellet and resuspend in steriled ultrapure water 100 μl

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Scheme 4.1 Procedure of the blue sponge *Xestospongia* sp. DNA extraction protocol

4. DNA extraction of the sponge *Xestospongia* sp. from various locations

The objectives of this experiment were to test the reliability of the blue sponge *Xestospongia* sp. DNA extraction protocol and to study the similarity and difference of the genetic material of the blue sponge *Xestospongia* sp. collected from various locations nearby Sichang Island. The extracted DNAs of 12 different colonies of the blue sponge *Xestospongia* sp. were checked on 0.8% agarose gel electrophoresis (Figure 4.6). The result of gel electrophoresis showed that all samples gave the intact genomic DNA at high molecular weight area about 23,000 bps. Smear bands produced by small fragments of DNA and RNA were also found to range from medium to low molecular weight areas in all samples.

Figure 4.6 0.8% agarose gel electrophoresis of genomic DNA of the blue sponge *Xestospongia* sp. from various locations

Lane M: VC Lambda DNA/Hind III marker

Lane 1:	KAM 1	Lane 2:	KAM 2
Lane 3:	KAM 3	Lane 4:	SAM 1
Lane 5:	SAM 2	Lane 6:	PRONG 1
Lane 7:	PRONG 2	Lane 8:	DOK 1
Lane 9:	DOK 2	Lane 10:	NOK 1
Lane 11:	NOK 2	Lane 12:	NOK 3

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Table 4.15 DNA quantity and purity from the sponge *Xestospongia* sp. in various locations

Samples	OD_{260} ± SD	DNA conc. (ng/µl)	OD_{260}/OD_{280} ± SD	% Nucleic acid
KAM1	1.94 ± 0.02	97	1.90 ± 0.00	57
KAM2	1.97 ± 0.01	99	1.89 ± 0.00	55
KAM3	1.97 ± 0.01	99	1.96 ± 0.00	78
SAM1	2.01 ± 0.01	101	1.90 ± 0.01	57
SAM2	1.98 ± 0.01	99	1.89 ± 0.01	55
PRONG1	1.95 ± 0.04	98	1.90 ± 0.00	57
PRONG2	1.93 ± 0.02	97	1.87 ± 0.01	50
DOK1	1.88 ± 0.03	94	1.89 ± 0.00	55
DOK2	1.79 ± 0.01	90	1.88 ± 0.00	53
NOK 1	1.88 ± 0.03	94	1.96 ± 0.00	78
NOK 2	1.85 ± 0.04	93	1.97 ± 0.01	80
NOK 3	1.88 ± 0.01	94	1.96 ± 0.00	78

DNA concentration and DNA purity of all 12 sponge samples were then measured by UV absorption at 260 and 280 nm as shown in Table 4.15 and were in the range of 90 – 101 ng/µl (means = 96 ng/µl) and 1.87 – 1.97 (means = 1.91), respectively. The blue sponge *Xestospongia* sp. DNA extraction protocol was able to provide DNA from each sample with concentration and purity consistency (see in Appendix C). However, the slightly different concentration and purity among the sample might be depended on their individual differences. The sample giving the highest amount of DNA (101 ng/µl) was the sponge sample from Sam Pun Yue Island (SAM 1) while the sponge sample from Ran Dok Mai Island (DOK 2) gave the lowest DNA concentration (90 ng/µl). The sample giving the best DNA purity (OD_{260}/OD_{280} = 1.97, %nucleic acid = 80%) was the sponge sample from Nok Island (NOK 2) while the sponge sample from Prong Island (PRONG 2) gave the lowest DNA purity (OD_{260}/OD_{280} = 1.87, %nucleic acid = 50%).

The quality of DNA was further confirmed by using the obtain DNAs as templates to amplify the conserved region within 28s rRNA gene of the *Xestospongia* genus. A pair of primers, XSF001 and XSR002 was used for PCR amplification technique. The PCR products were examined by 1.2% gel electrophoresis and were shown to be about 200 bps in length as expected for all samples (Figure 4.7). These results revealed that this protocol was a workable DNA extraction protocol for extracting DNA from the sponge *Xestospongia* sp.

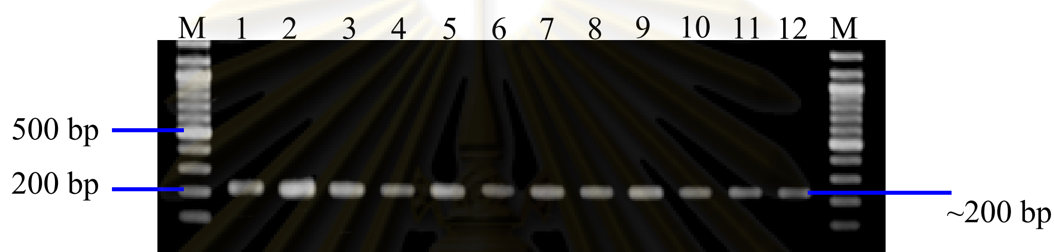

Figure 4.7 1.2% gel electrophoresis of PCR products from the blue sponge *Xestospongia* sp. from various locations

Lane M: VC 100 bp plus DNA ladder

Lane 1:	KAM 1	Lane 2:	KAM 2
Lane 3:	KAM 3	Lane 4:	SAM 1
Lane 5:	SAM 2	Lane 6:	PRONG 1
Lane 7:	PRONG 2	Lane 8:	DOK 1
Lane 9:	DOK 2	Lane 10:	NOK 1
Lane 11:	NOK 2	Lane 12:	NOK 3

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5. Application of the blue sponge *Xestospongia* sp. DNA extraction protocol to other organisms

The objective of this experiment was to test the application of the blue sponge *Xestospongia* sp. DNA extraction protocol to other organisms. Seven other sponge samples were collected, including *Gelliodes petrosioides*, *Gelliodes* sp., *Clathria* (Thalysias) *reinwardti*, *Pachastrissa nux,* *Cacospongia* sp., *Xestospongia testudinaria*, and the barrel sponge *Xestospongia* sp. Two tunicates, including *Clavelina cyclus* and *Ecteinascidia thurstoni*, and two plants, including *Punica granatum* and *Ocimum basilicum* were included. The obtained DNAs from these samples were extracted by the blue sponge *Xestospongia* sp. DNA extraction protocol and were checked on 0.8% agarose gel electrophoresis.

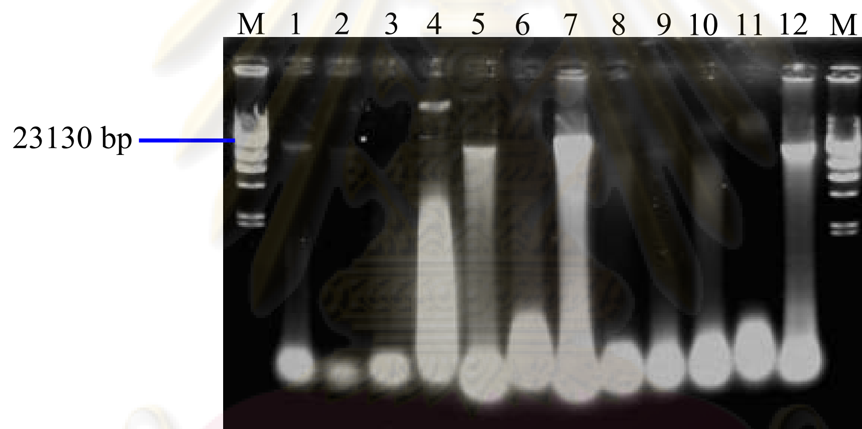

Figure 4.8 0.8% agarose gel electrophoresis of genomic DNA from *Gelliodes petrosioides* (lane 1), *Gelliodes* sp. (lane 2), *Clathria (Thalysias) reinwardti* (lane 3), *Pachastrissa nux* (lane 4), *Cacospongia* sp. (lane 5), *Xestospongia testudinaria* (lane 6), The barrel sponge *Xestospongia* sp. (lane 7), *Clavelina cyclus* (lane 8), *Ecteinascidia thurstoni* (lane 9), *Punica granatum* (lane 10), *Ocimum basilicum* (lane 11), and The blue *Xestospongia* sp. (lane 12)
Lane M: VC Lambda DNA/Hind III marker

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The results of gel electrophoresis showed that all sponge samples showed clear bands of intact genomic DNA at high molecular weight area except two sponges including, *Clathria (Thalysias) reinwardti* (lane 3) and *Xestospongia testudinaria*

(lane 6). Interestingly, clear bands of intact genomic DNA of the two tunicates (lane 8 and 9) and two plants samples (lane 10 and 11) were not detected (Figure 4.8).

DNA quantity and purity were measured by UV absorption at 260 and 280 nm as shown in Table 4.16.

Table 4.16 The DNA quantity and purity from other organisms using the blue sponge *Xestospongia* sp. DNA extraction protocol

Samples	$OD_{260} \pm SD$	DNA conc. (ng/µl)	$OD_{260}/OD_{280} \pm SD$	% Nucleic acid
Gelliodes petrosioides	1.43 ± 0.03	71	1.79± 0.00	37
***Gelliodes* sp.**	1.17 ± 0.07	58	1.64 ± 0.02	23
Clathria (Thalysias) reinwardti	1.37 ± 0.03	68.	1.70 ± 0.00	28
Pachastrissa nux	1.81 ± 0.05	91	1.25 ± 0.00	8
***Cacospongia* sp.**	2.06 ± 0.04	103	1.74 ± 0.01	31
Xestospongia testudinaria	1.94 ± 0.05	97	1.48 ± 0.00	15
The barrel sponge *Xestospongia* sp.	2.17 ± 0.04	109	1.59 ± 0.00	20
The blue sponge *Xestospongia* sp.	**2.00 ± 0.01**	**100**	**1.95 ± 0.00**	**75**
Clavelina cyclus	1.16 ± 0.03	58	1.35 ± 0.00	11
Ecteinascidia thurstoni	1.17 ± 0.01	58	1.37 ± 0.00	11
Punica granatum	1.02 ± 0.01	51	1.23 ± 0.00	8
Ocimum basilicum	1.03 ± 0.00	51	1.26 ± 0.00	9

From the result, the obtained DNAs quantity and purity showed significantly different (ANOVA, $p<0.05$) with the mean of 86 ng/µl and 1.40 ± 0.01, respectively. In general, the DNA concentration from all sponge organisms (except the sponge

Clathria (Thalysias) reinwardti and *Xestospongia testudinaria*) were higher than that from two tunicates and two plant samples. When the criteria about DNA concentration and purity in 2.1 were recognized, the DNA from genus *Xestospongia* gave the DNA concentration nearby 100 ng/µl. Interestingly, all samples gave absorbance ratio much less than 1.8 except the DNA from the blue sponge *Xestospongia* sp. (OD_{260}/OD_{280} = 1.95).

Using the 3 obtained DNAs of the sponge genus *Xestospongia* as template, conserve region within 28s rRNA gene was amplified by PCR amplification technique with a pair of primer, XSF001 and XSR002. The expected size of PCR products were about 200 bps in length. The result showed the intensity of the PCR product produced by each sample seems to correspond to both DNA concentration and quality (Figure 4.9).

Figure 4.9 1.2% agarose gel electrophoresis of genomic DNA from *Xestospongia testudinaria* (lane 1), the barrel sponge *Xestospongia* sp. (lane 2), and the blue sponge *Xestospongia* sp. (lane 3)

Lane M: VC 100 bp plus DNA ladder

These results represented that the blue sponge *Xestospongia* sp. DNA extraction protocol is a suitable protocol for the blue sponge *Xestospongia* sp. rather than other tested organisms and gave the DNA in good quality for further molecular experiment.

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6. Verification of *Xestospongia* DNA extracts from the blue sponge *Xestospongia* sp. DNA extraction protocol

The objective of this experiment is to investigate whether the obtained DNA extracts contain *Xestospongia* DNA. Based on sequence information available on the Genbank, two *Xestospongia* genes including 28s rRNA and *COX* I are appropriately considered. Conserved regions in the 28s rRNA gene among eukaryotes are highly similar. Thus, the COX I gene was selected for sequence verification due to its species-specific character (Figure 4.10). In this study, the DNA extracts from two *Xestospongia* sponges including the blue sponge *Xestospongia* sp. and the barrel sponge *Xestospongia* sp. were used as templates for PCR amplification. The PCR products amplified by a pair of *Xestospongia COX* I primers (forward primer, XSFCOXI_41S; reverse primer, XSRCOXI_161A) were submitted to BIOTEC (Thailand) for DNA sequencing. The sequencing results showed that the PCR products of *COX* I DNA sequences from the blue sponge were 100% identity to the *Xestospongia* COX I gene deposited in the Genbank database. These results suggest that the DNA samples from the blue and the barrel sponges contain the *Xestospongia* DNA (Figure 4.11-4.12).

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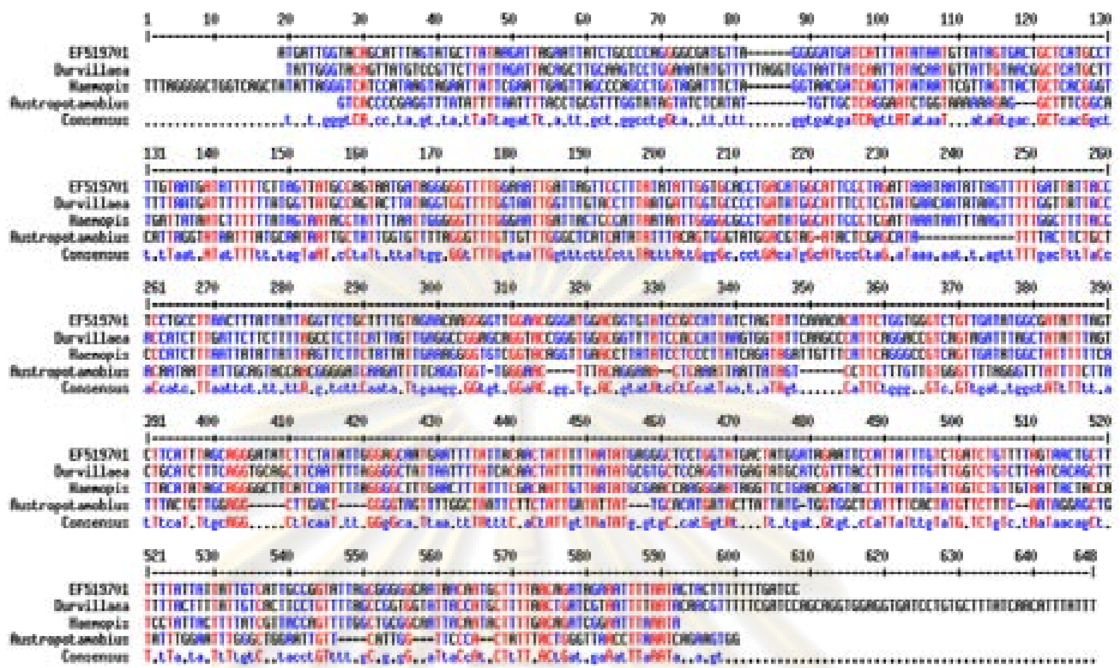

Figure 4.10 The sequence alignment of COX I gene of the sponge genus *Xestospongia* (EF519701) and other organisms. Gaps (-) are introduced for the best alignment. The red andf blue nucleotides are the high and low consensus sequences, respectively.

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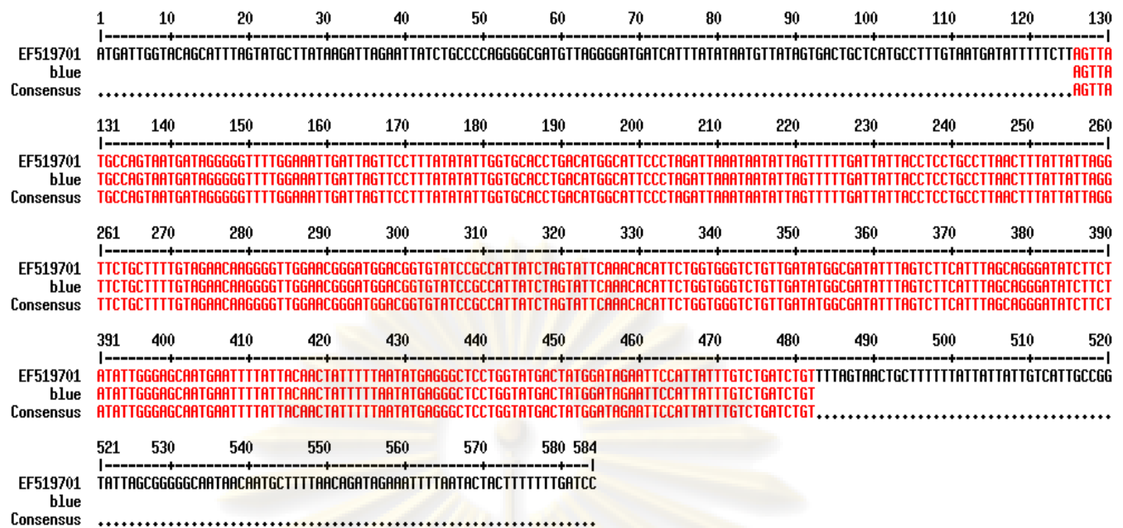

Figure 4.11 The sequence alignment of COX I gene of the sponge genus *Xestospongia* (EF519701) and the blue sponge *Xestospongia* sp. The partial COX I gene region corresponds to positions 126-482. The red nucleotides are the high consensus sequences.

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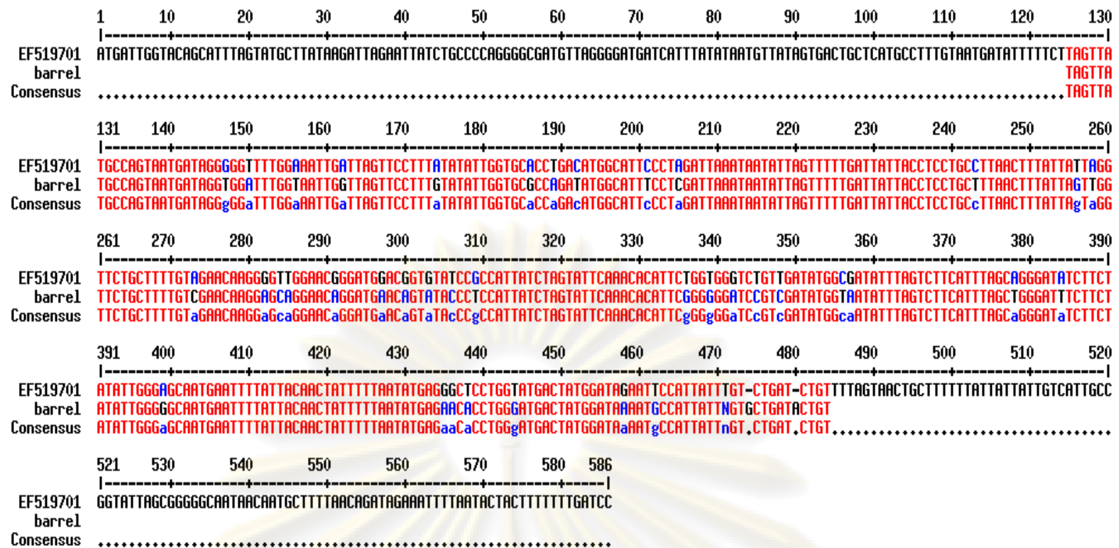

Figure 4.12 The sequence alignment of COX I gene of the sponge genus *Xestospongia* (EF519701) and the barrel sponge *Xestospongia* sp. The partial COX I gene region corresponds to positions 125-484. Gaps (-) are introduced for the best alignment. The red andf blue nucleotides are the high and low consensus sequences, respectively.

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7. Detection of the DNA from associated bacteria

From previous study showed that bacteria were associated with sponge organisms (Boonsiriluck, 2006). It is interesting to investigate if the Xestospongia DNA extraction protocol is able to extract bacterial DNA concomitantly. The objective of this experiment was to test the bacterial contamination in the obtained DNA from the blue sponge *Xestospongia* sp. using the *Xestospongia* DNA extraction protocol. Using the obtained DNA from the blue sponge *Xestospongia* sp., and *E.coli* as templates, 16s rRNA gene in mitochondrial region was amplified by PCR technique using primers follow to Weiseing *et al.,* (2001). The PCR products were about 1,500 bps in length. From this experiment, the expected size of PCR product from the DNA of the blue sponge *Xestospongia* sp. was not found but found in the DNA from *E.coli* (Figure 4.10). The result represented that the bacterial contaminants in the obtained DNA from the blue sponge *Xestospongia* sp. could not be observed. This might be due to no bacterial contaminant or bacterial contamination below detection limit.

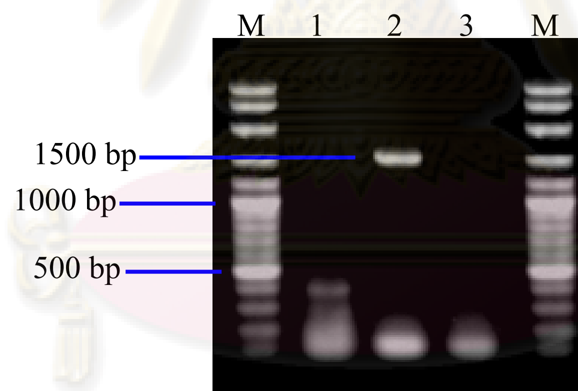

Figure 4.13 1.2% gel electrophoresis of PCR products of the blue sponge *Xestospongia* sp. (lane 1), *E.coli* (lane 2), and no DNA template (lane 3)

Lane M: VC 100 bp plus DNA ladder

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CHAPTER V

CONCLUSION

In this study, we searched for the suitable DNA extraction protocol to extract the genomic DNA from the blue sponge *Xestospongia* sp. The DNAs from the sponge samples were first extracted by using a commercial kit and six protocols reported in literatures. The quality and the total yield of obtained DNA were estimated by using a spectrophotometer to measure the absorbance ratio (OD_{260}/OD_{280}) and the absorbance at 260 nm (OD_{260}), respectively. The most appropriate protocol for DNA extraction from the marine blue sponge *Xestospongia* sp. is the NaOAc salting-out protocol when compared to other protocols. This protocol gave the highest yield of DNA at 98 ng/µl and the best DNA quality at absorbance ratio 1.88. From this result, the NaOAc salting-out protocol was selected for further modification to improve DNA extraction from the sponge *Xestospongia* sp. by modifying lysis buffer solution compositions, salt solution for protein precipitation, and DNA precipitation methods. The DNA quantity and purity from the modified protocol (102 ng/µl, OD_{260}/OD_{280} = 1.93 (%nucleic acid = 65%)) were found to be greater than those from the original protocol (97 ng/µl, OD_{260}/OD_{280} = 1.89 (%nucleic acid = 55%)), representing the better procedure of the modified protocol. Then RNase A was added into the modified NaOAc salting-out protocol for removing RNA and degraded RNA. This protocol significantly improved the DNA quantity (110 ng/µl) and purity (1.97 ± 0.01 (%nucleic acid = 80%)) when compared to the modified NaOAc salting-out protocol. The final modified protocol was named as **the blue sponge *Xestospongia* sp. DNA extraction protocol**. DNAs of the blue sponge *Xestospongia* sp. from various locations and other organism samples were comparatively extracted with the *Xestospongia* DNA extraction protocol to test protocol reliability. The results showed that the *Xestospongia* DNA extraction protocol was suitable protocol for DNA extraction from the blue sponge *Xestospongia* sp. more than other organisms.

When the bacterial contamination was considered, the 16s rRNA gene were used to investigate the bacterial contamination in the genomic DNA from the sponge *Xestospongia* sp. The result showed that the bacterial contaminants in the obtained DNA might be below the detecting limit. To investigate DNA quality in molecular study aspect, using the obtained DNA as a template, the conserve regions within 28s rRNA gene, and COX I gene were amplified by PCR technique. The amplicons from PCR amplification were in expected sizes. The results revealed that the blue sponge *Xestospongia* sp. DNA extraction protocol was a preferable procedure for further molecular biology study.

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APPENDICES

ศูนย์วิทยทรัพยากร
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APPENDIX A

Table A Concentrations of SDS, EDTA, and Tris-HCl in lysis buffers

Lysis buffer	Final concentrations		
	%SDS	EDTA (mM)	Tris-HCl (mM)
A1	1	5	1
A2	1	5	5
A3	1	5	10
A4	1	5	50
A5	1	5	100
A6	1	10	1
A7	1	10	5
A8	1	10	10
A9	1	10	50
A10	1	10	100
A11	1	15	1
A12	1	15	5
A13	1	15	10
A14	1	15	50
A15	1	15	100
A16	1	20	1
A17	1	20	5
A18	1	20	10
A19	1	20	50
A20	1	20	100
A21	1	25	1
A22	1	25	5
A23	1	25	10
A24	1	25	50
A25	1	25	100

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Table A (continued)

Lysis buffer	Final concentrations		
	%SDS	EDTA (mM)	Tris-HCl (mM)
B1	5	5	1
B2	5	5	5
B3	5	5	10
B4	5	5	50
B5	5	5	100
B6	5	10	1
B7	5	10	5
B8	5	10	10
B9	5	10	50
B10	5	10	100
B11	5	15	1
B12	5	15	5
B13	5	15	10
B14	5	15	50
B15	5	15	100
B16	5	20	1
B17	5	20	5
B18	5	20	10
B19	5	20	50
B20	5	20	100
B21	5	25	1
B22	5	25	5
B23	5	25	10
B24	5	25	50
B25	5	25	100

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Table A (continued)

Lysis buffer	Final concentrations		
	%SDS	EDTA (mM)	Tris-HCl (mM)
C1	10	5	1
C2	10	5	5
C3	10	5	10
C4	10	5	50
C5	10	5	100
C6	10	10	1
C7	10	10	5
C8	10	10	10
C9	10	10	50
C10	10	10	100
C11	10	15	1
C12	10	15	5
C13	10	15	10
C14	10	15	50
C15	10	15	100
C16	10	20	1
C17	10	20	5
C18	10	20	10
C19	10	20	50
C20	10	20	100
C21	10	25	1
C22	10	25	5
C23	10	25	10
C24	10	25	50
C25	10	25	100

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Table A (continued)

Lysis buffer	Final concentrations		
	%SDS	EDTA (mM)	Tris-HCl (mM)
D1	15	5	1
D2	15	5	5
D3	15	5	10
D4	15	5	50
D5	15	5	100
D6	15	10	1
D7	15	10	5
D8	15	10	10
D9	15	10	50
D10	15	10	100
D11	15	15	1
D12	15	15	5
D13	15	15	10
D14	15	15	50
D15	15	15	100
D16	15	20	1
D17	15	20	5
D18	15	20	10
D19	15	20	50
D20	15	20	100
D21	15	25	1
D22	15	25	5
D23	15	25	10
D24	15	25	50
D25	15	25	100

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Table A (continued)

Lysis buffer	Final concentrations		
	%SDS	EDTA (mM)	Tris-HCl (mM)
E1	20	5	1
E2	20	5	5
E3	20	5	10
E4	20	5	50
E5	20	5	100
E6	20	10	1
E7	20	10	5
E8	20	10	10
E9	20	10	50
E10	20	10	100
E11	20	15	1
E12	20	15	5
E13	20	15	10
E14	20	15	50
E15	20	15	100
E16	20	20	1
E17	20	20	5
E18	20	20	10
E19	20	20	50
E20	20	20	100
E21	20	25	1
E22	20	25	5
E23	20	25	10
E24	20	25	50
E25	20	25	100

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APPENDIX B

Data of DNA sequences which were submitted to Genbank database

1. *Xestospongia muta* voucher S1 cytochrome oxidase subunit I (*COX* I) gene, partial cds; mitochondrial

LOCUS EF519701 584 bp DNA linear INV 19-FEB-2008

DEFINITION *Xestospongia muta* voucher S1 cytochrome oxidase subunit I (*COX* I) gene, partial cds; mitochondrial.

ACCESSION EF519701

VERSION EF519701.1 GI:155675307

KEYWORDS .

SOURCE mitochondrion *Xestospongia muta* (giant barrel sponge)

ORGANISM *Xestospongia muta*

Eukaryota; Metazoa; Porifera; Demospongiae; Ceractinomorpha; Haplosclerida; Petrosiidae; Xestospongia.

FEATURES Location/Qualifiers

source 1..584

/organism = "*Xestospongia muta*"

/organelle = "mitochondrion"

/mol_type = "genomic DNA"

/specimen_voucher = "S1"

/db_xref = "taxon:178552"

gene <1..>584

/gene = "*COX* I"

CDS <1..>584

/gene = "*COX* I"

/codon_start = 1

/transl_table = 4

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/protein_id = "ABU24989.1"

/db_xref = "GI:155675308"

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DRIPLFVWSVLVTAFLLLLSLPVLAGAITMLLTDRNFNTTFFDP"

ORIGIN

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301 gtgtatccgc cattatctag tattcaaaca cattctggtg ggtctgttga tatggcgata
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421 atttttaata tgagggctcc tggtatgact atggatagaa ttccattatt tgtctgatct
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2. *Xestospongia muta* voucher K65 cytochrome oxidase subunit I (*COX* I) gene, partial cds; mitochondrial

LOCUS EF519700 584 bp DNA linear INV 19-FEB-2008

DEFINITION *Xestospongia muta* voucher K65 cytochrome oxidase subunit I (*COX* I) gene, partial cds; mitochondrial.

ACCESSION EF519700

VERSION EF519700.1 GI:155675305

KEYWORDS .

SOURCE mitochondrion *Xestospongia muta* (giant barrel sponge)

ORGANISM *Xestospongia muta*

Eukaryota; Metazoa; Porifera; Demospongiae; Ceractinomorpha; Haplosclerida; Petrosiidae; Xestospongia.

FEATURES Location/Qualifiers

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/organelle = "mitochondrion"

/mol_type = "genomic DNA"

/specimen_voucher = "K65"

/db_xref = "taxon:178552"

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/gene = "*COX* I"

CDS <1..>584

/gene = "*COX* I"

/codon_start = 1

/transl_table = 4

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/db_xref = "GI:155675306"

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DRIPLFVWSVLVTAFLLLLSLPVLAGAITMLLTDRNFNTTFFDP"

misc_feature 1..>584

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/gene = "*COX* I"

/note = "Heme-copper oxidase subunit I. Heme-copper oxidases are transmembrane protein complexes in the respiratory chains of prokaryotes and mitochondria which catalyze the reduction of O2 and simultaneously pump protons across the membrane. The superfamily...; Region: Heme_Cu_Oxidase_I; cl00275"

/db_xref = "CDD:119684"

misc_feature order(157..159,190..192,211..213,220..222,385..390, 406..408,418..420)

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/note = "D-pathway; other site"

/db_xref = "CDD:29930"

ORIGIN

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241 gccttaactt tattattagg ttctgctttt gtagaacaag ggctggaac gggatggacg
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481 gttttagtaa ctgctttttt attattattg tcattgccgg tattagcggg ggcaataaca
541 atgcttttaa cagatagaaa ttttaatact acttttttg accc

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3. *Xestospongia muta* voucher K20 cytochrome oxidase subunit I (COX I) gene, partial cds; mitochondrial

LOCUS EF519699 584 bp DNA linear INV 19-FEB-2008

DEFINITION *Xestospongia muta* voucher K20 cytochrome oxidase subunit I (COX I)gene, partial cds; mitochondrial.

ACCESSION EF519699

VERSION EF519699.1 GI:155675303

KEYWORDS .

SOURCE mitochondrion *Xestospongia muta* (giant barrel sponge)

ORGANISM *Xestospongia muta*

Eukaryota; Metazoa; Porifera; Demospongiae; Ceractinomorpha; Haplosclerida; Petrosiidae; Xestospongia.

FEATURES Location/Qualifiers

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/organelle= "mitochondrion"

/mol_type = "genomic DNA"

/specimen_voucher = "K20"

/db_xref = "taxon:178552"

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DRIPLFVWSVLVTAFLLLLSLPVLAGAITMLLTDRNFNTTFFDP"

misc_feature 1..>584

/gene = " *COX* I"
/note = "Heme-copper oxidase subunit I. Heme-copper oxidases are transmembrane protein complexes in the respiratory chains of prokaryotes and mitochondria which catalyze the reduction of O2 and simultaneously pump protons across the membrane. The superfamily...; Region: Heme_Cu_Oxidase_I; cl00275"
/db_xref = "CDD:119684"

misc_feature order(157..159,190..192,211..213,220..222,385..390,
406..408,418..420)
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/note = "D-pathway; other site"
/db_xref = "CDD:29930"

ORIGIN

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121 ttcttagtta tgccagtaat gataggggggt tttggaaatt gattagttcc tttatatatt
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481 gttttagtaa ctgctttttt attattattg tcattgccgg tattagcggg ggcaataaca
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```

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4. ***Xestospongia muta*** **voucher K15 cytochrome oxidase subunit I (COX I) gene, partial cds; mitochondrial**

LOCUS EF519698 584 bp DNA linear INV 19-FEB-2008

DEFINITION *Xestospongia muta* voucher K15 cytochrome oxidase subunit I (COX I)gene, partial cds; mitochondrial.

ACCESSION EF519698

VERSION EF519698.1 GI:155675301

KEYWORDS .

SOURCE mitochondrion *Xestospongia muta* (giant barrel sponge)

ORGANISM *Xestospongia muta*

Eukaryota; Metazoa; Porifera; Demospongiae; Ceractinomorpha; Haplosclerida; Petrosiidae; Xestospongia.

FEATURES Location/Qualifiers

source 1..584

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/organelle = "mitochondrion"

/mol_type = "genomic DNA"

/specimen_voucher = "K15"

/db_xref = "taxon:178552"

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/gene = " *COX* I"

CDS <1..>584

/gene = " *COX* I"

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ORIGIN

1 atgattggta cagcatttag tatgcttata agattagaat tatctgcccc aggggcgatg

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121 ttcttagtta tgccagtaat gataggggggt tttggaaatt gattagttcc tttatatatt

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421 atttttaata tgagggctcc tggtatgact atggatagaa ttccattatt tgtctgatct

481 gttttagtaa ctgctttttt attattattg tcattgccgg tattagcggg ggcaataaca

541 atgcttttaa cagatagaaa ttttaatact actttttttg atcc

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5. *Xestospongia muta* voucher BH8 cytochrome oxidase subunit I (COX I) gene, partial cds; mitochondrial

LOCUS EF519697 584 bp DNA linear INV 19-FEB-2008

DEFINITION *Xestospongia muta* voucherBH8 cytochrome oxidase subunit I (COX I) gene, partial cds; mitochondrial.

ACCESSION EF519697

VERSION EF519697.1 GI:155675299

KEYWORDS .

SOURCE mitochondrion *Xestospongia muta* (giant barrel sponge)

ORGANISM *Xestospongia muta*

Eukaryota; Metazoa; Porifera; Demospongiae; Ceractinomorpha; Haplosclerida; Petrosiidae; Xestospongia.

FEATURES Location/Qualifiers

source 1..584

/organism = "*Xestospongia muta*"

/organelle = "mitochondrion"

/mol_type = "genomic DNA"

/specimen_voucher = "BH8"

/db_xref = "taxon:178552"

gene <1..>584

/gene = " *COX* I"

CDS <1..>584

/gene = " *COX* I"

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/db_xref = "GI:155675300"

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DRIPLFVWSVLVTAFLLLLSLPVLAGAITMLLTDRNFNTTFFDP"

misc_feature order(157..159,190..192,211..213,220..222,385..390,

406..408,418..420)

/gene = " *COX* I"

/note = "D-pathway; other site"

/db_xref = "CDD:29930"

ORIGIN

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301 gtgtatccgc cattatctag tattcaaaca cattctggtg ggtctgttga tatggcgata
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481 gttttagtaa ctgctttttt attattattg tcattgccgg tattagcggg ggcaataaca
541 atgcttttaa cagatagaaa ttttaatact acttttttg atcc

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6. *Xestospongia proxima* mitochondrial partial COX I gene for cytochrome oxidase subunit I, specimen voucher SP25199

LOCUS AM076980 1206 bp DNA linear INV 15-SEP-2006

DEFINITION *Xestospongia proxima* mitochondrial partial COX I gene for cytochrome c oxidase subunit I, specimen voucher SP25199.

ACCESSION AM076980

VERSION AM076980.1 GI:114649486

KEYWORDS COX I gene; cytochrome c oxidase subunit I.

SOURCE mitochondrion *Xestospongia proxima*

ORGANISM *Xestospongia proxima*

Eukaryota; Metazoa; Porifera; Demospongiae; Ceractinomorpha; Haplosclerida; Petrosiidae; Xestospongia.

FEATURES Location/Qualifiers

source 1..1206

/organism = "*Xestospongia proxima*"

/organelle = "mitochondrion"

/mol_type = "genomic DNA"

/isolation_source = "Rum Cay 3 m"

/specimen_voucher = "SP25199"

/db_xref = "taxon:344319"

/country = "Bahamas"

gene <1..>1206

/gene = " *COX* I"

CDS <1..>1206

/gene = " *COX* I"

/EC_number = "1.6.3.1"

/codon_start = 1

/transl_table = 4

/product = "cytochrome c oxidase subunit I"

/protein_id = "CAJ29528.1"

/db_xref = "GI:114649487"

90

/translation="TLYLLFGAFGGMIGTAFSMLIRLELSAPGAMLGDDHLYNVIVTA
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ORIGIN

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961 ccaatgcttt gagccgtagg gtttgttttt ttatttacaa taggaggatt aactgggatt
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1201 atgttt

7. *Xestospongia caminata* 28s rRNA gene, partial sequence

LOCUS AF441348 662 bp DNA linear INV 09-APR-2004

DEFINITION *Xestospongia caminata* 28s rRNA gene, partial sequence.

ACCESSION AF441348

VERSION AF441348.1 GI:17064058

KEYWORDS .

SOURCE *Xestospongia caminata*

ORGANISM *Xestospongia caminata*

Eukaryota; Metazoa; Porifera; Demospongiae; Ceractinomorpha;

Haplosclerida; Petrosiidae; Xestospongia.

FEATURES Location/Qualifiers

source 1..662

/organism = "*Xestospongia caminata*"

/mol_type = "genomic DNA"

/db_xref = "taxon:178551"

rRNA <1..>662

/product = "28s rRNA"

ORIGIN

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121 gttgttcgat cgggtgcgag agccccntng aagggcgcat cgcaaaccga gccttcggtc
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421 aaagcgaatg attagaggta tcggggtcga aaggacctcg acctattctc aaactttgaa
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601 aaagtcgggc taaggcgccg gagtcgacgc tgagttagat cccatgaaag gtgttggttg
661 at

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8. *Xestospongia subtriangularis* 28s rRNA gene, partial sequence

LOCUS AF441341 651 bp DNA linear INV 09-APR-2004

DEFINITION *Xestospongia subtriangularis* 28s rRNA gene, partial sequence.

ACCESSION AF441341

VERSION AF441341.1 GI:17064051

KEYWORDS .

SOURCE *Xestospongia subtriangularis*

ORGANISM *Xestospongia subtriangularis*

Eukaryota; Metazoa; Porifera; Demospongiae; Ceractinomorpha;

Haplosclerida; Petrosiidae; Xestospongia.

FEATURES Location/Qualifiers

source 1..651

/organism = "*Xestospongia subtriangularis*"

/mol_type = "genomic DNA"

/db_xref = "taxon:178553"

rRNA <1..>651

/product = "28s rRNA"

ORIGIN

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181 gtatggtcac gctgtggccg gtttcgagtg cgagcgtatc tgttgcgacc cgaaagatgg

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421 tcgggtaaag cgaatgatta gaggtatcgg ggttgaaagg acctcgacct attctcaaac

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601 tatggcgccg gagtcgacgc tgagcagata ccaggaaagg tgttggttga t

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Table B1 PCR amplification primers of 28s rRNA gene used in this study.

Primer name	Primer sequence (5′ to 3′)	Direction
XSF001	GTT TCC CTC AGG ATA GCT GGA	forward
XSR002	CAT CGC CGG TTC TGC TTA CCA	reverse

Table B2 PCR amplification primers of cytochrome oxidase subunit I (*COX* I) gene used in this study.

Primer name	Primer sequence (5' to 3')	Direction
XSFCOXI_41S	AGTTATGCCAGTAATGATAG	forward
XSRCOXI_161A	ACAGATCAGACAAATAATGG	reverse

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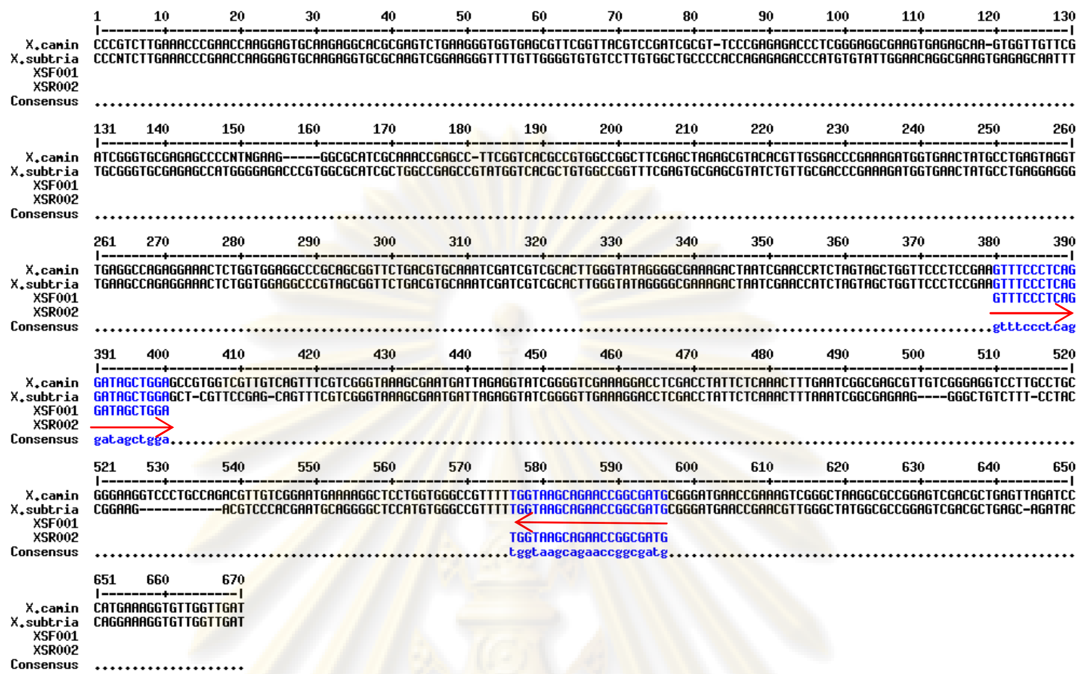

Figure B1 The sequence alignment of 28s rRNA gene from the sponge genus *Xestospongia*

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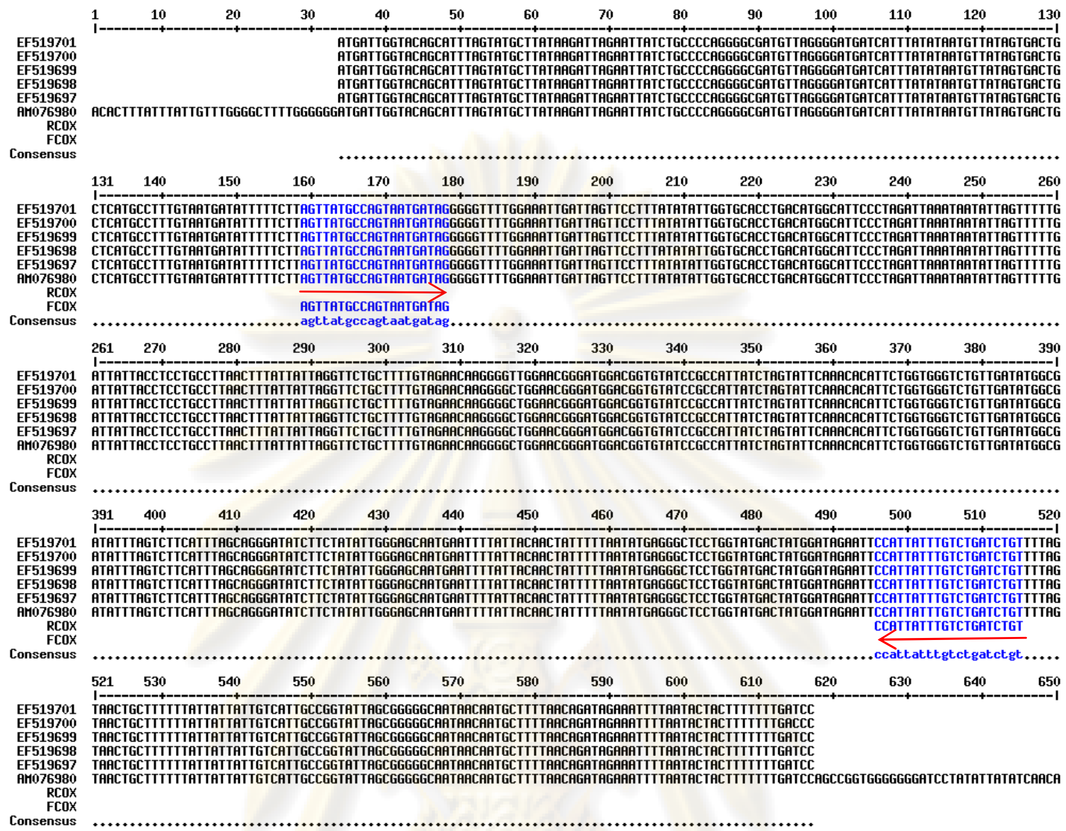

Figure B2 The sequence alignment of COX I gene from the sponge genus *Xestospongia*

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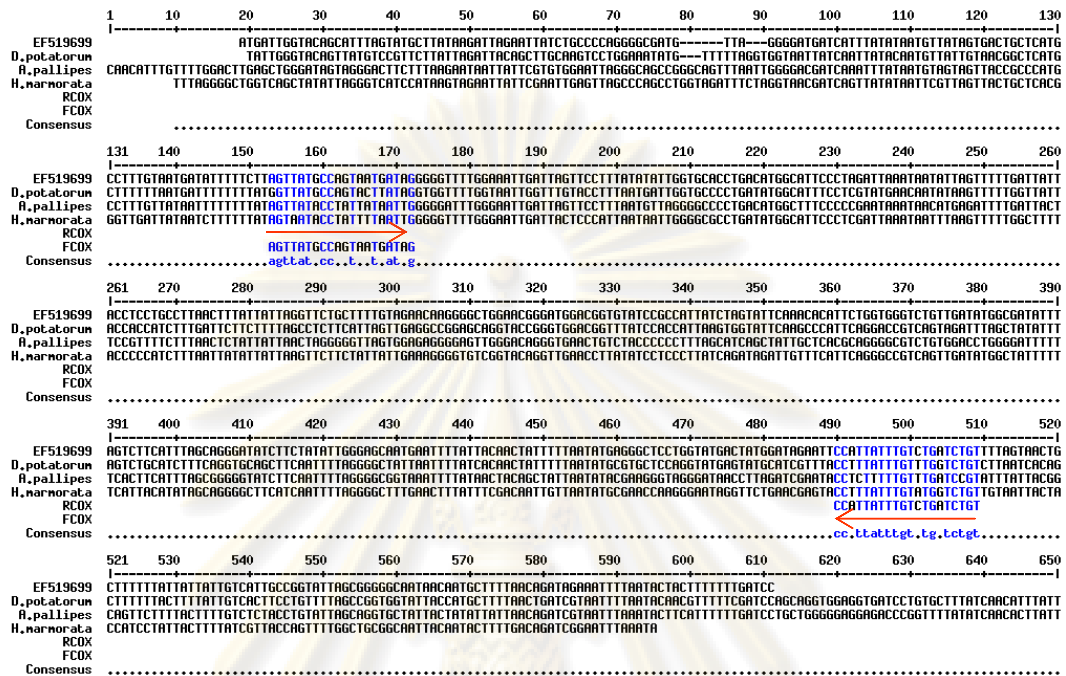

Figure B3 The sequence alignment of COX I gene from the sponge genus *Xestospongia* compared to other organisms

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APPENDIX C

Table C1 Statistical analysis (one-way ANOVA) of DNA concentration (ng/µl) from seven DNA extraction protocols

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11299.143	6	1883.190	1581.880	.000
Within Groups	16.667	14	1.190		
Total	11315.810	20			

Table C2 Multiple comparison of DNA concentration (ng/µl) from seven DNA extraction protocols

Duncan

Protocols	N	Subset for alpha = .05						
		1	2	3	4	5	6	7
CTAB extraction for plants	3	24.33						
MasterPure Kit	3		35.67					
CTAB extraction for sponges	3			55.67				
SDS/phenol extraction	3				58.67			
Simple DNA extraction	3					61.67		
SDS/CTAB/phenol extraction	3						80.33	
NaOAc salting-out	3							98.33
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a) Uses Harmonic Mean Sample Size = 3.000.

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Table C3 Statistical analysis (one-way ANOVA) of absorbance ratio from seven DNA extraction protocols

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.335	6	.223	41291.141	.000
Within Groups	.000	14	.000		
Total	1.335	20			

Table C4 Multiple comparison of absorbance ratio from seven DNA extraction protocols

Duncan

Protocols	N	Subset for alpha = .05						
		1	2	3	4	5	6	7
Simple DNA extraction	3	1.06						
CTAB extraction for plants	3		1.31					
SDS/CTAB/phenol extraction	3			1.48				
MasterPure Kit	3				1.59			
SDS/phenol extraction	3					1.67		
CTAB extraction for sponges	3						1.70	
NaOAc salting-out	3							1.88
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a) Uses Harmonic Mean Sample Size = 3.000.

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Table C5 Statistical analysis (two-way ANOVA) of DNA concentration (ng/µl) in protein precipitate optimization

Dependent Variable: DNA concentration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	17331.273(a)	11	1575.570	1193.928	.000
Intercept	157847.290	1	157847.290	119612.744	.000
Salt	11888.268	1	11888.268	9008.633	.000
Conc	4985.526	5	997.105	755.581	.000
Salt * Conc	457.480	5	91.496	69.333	.000
Error	31.672	24	1.320		
Total	175210.235	36			
Corrected Total	17362.945	35			

(a) R Squared = .998 (Adjusted R Squared = .997)

Table C6 Statistical analysis (two-way ANOVA) of absorbance ratio in protein precipitate optimization

Dependent Variable: DNA purity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	2.585(a)	11	.235	6405.575	.000
Intercept	88.916	1	88.916	2423342.023	.000
Salt	.365	1	.365	9934.594	.000
Conc	2.111	5	.422	11508.154	.000
Salt * Conc	.110	5	.022	597.191	.000
Error	.001	24	3.669E-05		
Total	91.502	36			
Corrected Total	2.586	35			

(a) R Squared = 1.000 (Adjusted R Squared = 1.000)

Table C7 Statistical analysis (two-way ANOVA) of DNA concentration (ng/µl) in DNA precipitate optimization

Dependent Variable: DNA concentration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	5969.152[a]	11	542.650	1670.764	.000
Intercept	302215.900	1	302215.900	930491.546	.000
Alcohol	2024.250	1	2024.250	6232.457	.000
Time	3752.624	5	750.525	2310.788	.000
Alcohol * Time	192.279	5	38.456	118.401	.000
Error	7.795	24	.325		
Total	308192.847	36			
Corrected Total	5976.947	35			

a) R Squared = .999 (Adjusted R Squared = .998)

Table C8 Statistical analysis (two-way ANOVA) of absorbance ratio in DNA precipitate optimization

Dependent Variable: DNA purity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	0.301[a]	11	.027	667.529	.000
Intercept	112.999	1	112.999	2755391.733	.000
Alcohol	0.185	1	0.185	4503.385	.000
Time	0.110	5	0.022	535.990	.000
Alcohol * Time	0.007	5	0.001	31.896	.000
Error	0.001	24	4.101E-05		
Total	113.301	36			
Corrected Total	0.302	35			

a) R Squared = .997 (Adjusted R Squared = .995)

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Table C 9 Statistical analysis (one-way ANOVA) of DNA concentration (ng/µl) in DNA extraction from other samples

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	341.556	11	31.051	27.945	.000
Within Groups	26.667	24	1.111		
Total	368.222	35			

Table C 10 Multiple comparison of DNA concentration (ng/µl) in DNA extraction from other samples

Duncan

Sample	N	Subset for alpha = .05				
		1	2	3	4	5
DOK2	3	90				
NOK2	3		93			
DOK1	3		94			
NOK1	3		94			
NOK3	3		94			
KAM1	3			97		
PRONG2	3			98	98	
PRONG1	3			98	98	
KAM3	3			99	99	
SAM2	3			99	99	
KAM2	3				99	
SAM1	3					101
Sig.		1.000	.299	.094	.177	1.000

Means for groups in homogeneous subsets are displayed.
a) Uses Harmonic Mean Sample Size = 3.000.

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Table C 11 Statistical analysis (one-way ANOVA) of absorbance ratio in DNA extraction from other samples

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.045	11	.004	61.212	.000
Within Groups	.002	24	.000		
Total	.046	35			

Table C 12 Multiple comparison of absorbance ratio in DNA extraction from other samples

Duncan

Sample	N	Subset for alpha = .05			
		1	2	3	4
PRONG2	3	1.87			
DOK2	3		1.88		
SAM2	3		1.89		
KAM2	3		1.89	1.89	
DOK1	3		1.89	1.89	
PRONG1	3		1.90	1.90	
KAM1	3			1.90	
SAM1	3			1.90	
NOK3	3				1.96
KAM3	3				1.96
NOK1	3				1.96
NOK2	3				1.97
Sig.		1.000	.084	.084	.078

Means for groups in homogeneous subsets are displayed.
a) Uses Harmonic Mean Sample Size = 3.000.

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Table C 13 Statistical analysis (one-way ANOVA) of DNA concentration (ng/µl) in DNA extraction of the sponge *Xestospongia* sp. from various locations

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16162.306	11	1469.301	574.944	.000
Within Groups	61.333	24	2.556		
Total	16223.639	35			

Table C 14 Multiple comparison of DNA concentration (ng/µl) in DNA extraction of the sponge *Xestospongia* sp. from various locations

Duncan

Sample	N	Subset for alpha = .05						
		1	2	3	4	5	6	7
Ocimum basilicum	3	51						
Punica granatum	3	51						
Gelliodes sp.	3		58					
Clavelina sp.	3		58					
Ecteinascidia thurstoni	3		58					
Clathria (Thalysias) reinwardti	3			68				
Gelliodes petrosiodes	3			71				
Pachastrissa nux	3				91			
Xestospongia testudinaria	3					97		
The *blue sponge Xestospongia* sp.	3						100	
Cacospongia sp.	3						102	
The *barrel sponge Xestospongia* sp.	3							109
Sig.		.801	.635	.052	1.000	1.000	.086	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

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Table C 15 Statistical analysis (one-way ANOVA) of absorbance ratio in DNA extraction of the sponge *Xestospongia* sp. from various locations

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.905	11	.173	6926.384	.000
Within Groups	.001	24	.000		
Total	1.905	35			

Table C 16 Multiple comparison of absorbance ratio in DNA extraction of the sponge *Xestospongia* sp. from various locations

Duncan

Sample	N	Subset for alpha = .05											
		1	2	3	4	5	6	7	8	9	10	11	12
Punica granatum	3	1.23											
Pachastrissa nux	3		1.25										
Ocimum basilicum	3			1.26									
Clavelina sp.	3				1.35								
Ecteinascidia thurstoni	3					1.37							
Xestospongia testudinaria	3						1.48						
barrel sponge *Xestospongia* sp.	3							1.59					
Gelliodes sp.	3								1.64				
Clathria (Thalysias) reinwardti	3									1.70			
Cacospongia sp.	3										1.74		
Gelliodes petrosiodes	3											1.79	
blue sponge *Xestospongia* sp.	3												1.95
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a) Uses Harmonic Mean Sample Size = 3.000.

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VITA

Miss Manatchaya Chuanchen was born on February 16, 1980 in Kanchanaburi Province, Thailand. She received her Bachelor's degree of Pharmacy in 2003 from the Faculty of Pharmacy, Silpakorn University, Nakonpathom Province, Thailand. Since 2003, she has been working as a pharmacist at the Chachoengsao Provincial Public Health Office, Chachoengsao Province, Thailand.

Poster Presentations

1. Chuanchuen, M., Suwanborirux, K., and Chuanasa, T. Optimal Protocol for DNA Extraction from the Sponge *Xestospongia* sp. Producing Renieramycins. Abstract for The 3rd Medicinal Chemistry Seminar of Asia/Africa Scientific Platform Program. January 13-15, 2009. Meiji Kinenkan, Tokyo, Japan. p. 33.

2. Chuanchuen, M., Suwanborirux, K., and Chuanasa, T. DNA Extraction from the Blue Sponge *Xestospongia* sp. Producing Cytotoxic Renieramycin Alkaloids. The Eight Joint Seminar, Innovative Research in Natural Products for Sustainable Development. February 2-3, 2009. Chulalongkorn University, Bangkok. p. 165-166.

3. Chuanchuen, M., Suwanborirux, K., and Chuanasa, T. Comparison of Protocols for DNA Extraction from the Marine Blue Sponge *Xestospongia* sp. The 12th National Graduate Research Conference. February 12-13, 2009. KhonKhen University, KonKhen. p.1732-1737.

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