CHAPTER III

Materials and Methods

Sample collection

Specimens were collected from the Gulf of Thailand by shrimp trammel net and were preserved in 3% formalin at both Marine and Coastal Resources Research and Development Center, The Eastern Gulf of Thailand (EMCOR) and The Eastern Gulf of Thailand. A part of some specimen was cut and preserved in 70% ethanol for molecular analysis.

Material examination

Specimens from table 1 were examined by both morphological and molecular methods.

Table 1 Voucher and locality of specimens

	Voucher	Locality
Chironex sp. A1	EMCOR 9	Chao Lao beach, Chanthaburi
Chironex sp. A2	EMCOR 10	Suan Son beach, Rayong
Chironex sp. A3	EMCOR 12	Suan Son beach, Rayong
Chironex sp. A4	PMBC 27933	Pha Ngan island, Surat Thani

Morphological identification

A total of 4 specimens were classified followed Gershwin (2005 and 2006). External characters of specimens, including exumbrella, rhopalia niche ostia, tentacles and pedalia

canal, were initially observed.

Molecular methods

DNA extraction (Adapted form Pinto *et al*., 2000)

Excised 5 cm. of a tissue from exumbrella and powdered using liquid nitrogen and placed in a microcentrifuge tube with 400 µl of lysis buffer. A total of 20 mg/ml Proteinase K was added to the final concentration of 1 mg/ml and incubated at 60 ^{o}C for 48 hours. Subsequently 500 µl of phenol was added and mixed before samples were centrifuged at 13,500 rpm for 15 minutes. The supernatant solution were transferred to a new microcentrifuge tube followed by equal amount of phenol. The total volume was then mixed by vortexing before samples were centrifuged at 13,500 rpm for 15 minutes. After that, the supernatant solution was transferred to a new microcentrifuge tube followed by the same amount of chloroform. The mixture was inverted and centrifuged at 13,500 rpm for 15 minutes. Then, transferred the supernatant solution to a new microcentrifuge tube which was filled with 30 µl of 6 M NaCl and mixed by inverting gently. Following this procedure, 750 µl of extremely cold absolute ethanol was added and kept overnight in -20 ^{o}C refrigerator in order to precipitate the DNA. Samples were centrifuged at 13,000 rpm for 10 minutes. The supernatant solution was discarded, Subsequently 1 ml of 70% ethanol was added and the samples were centrifuged at 13,000 rpm for 5 minutes. The supernatant solution was discarded and air-dried. Finally, 30 µl of TE buffer was added.

Primer designation

In this study, three primers from COI from Geller *et al.* (2013), 18S from Gershwin (2005b) and 16S as indicated in table 2 were amplified. Specific 16S primer for cubozoan was designed using 8 sequences of 16S rRNA from chirodropidae members from National Center for Biotechnology Information (NCBI) including *Chironex fleckeri* (accession number: GQ849101.1, GQ849102.1 and GQ849103.1), *Chiropsalmus quadrumanus* (accession number: GQ849109.1, GQ849110.1 and GQ849111.1) and *Chiropsella bronzie* (accession number: GQ849099.1). All sequenced were aligned to search for conserved region within these group. After that, forward primer and reverse primer were designed by Primer-
BLAST.

Table 2 Primers used in this study together with their Tm

Gene			Calculated Tm (°C)	Working Tm (°C)	Reference
16S	Forward primer	P16sf (5'AAG GGC CGC GGT AAC TCT G 3')	62.31	64	-
	Reverse primer	S16sr (5'ACC CTG TTA TCC CCG TGG T 3')	60.23		
18S	Forward primer	18SAf (5' CCG AAT TCG TCG ACA ACC TGG TTG ATC CTG CCA GT3')	73.53	64	Gershwin 2005b
	Reverse primer	int6 (5' GAA TTA CCG CGG CTG CTG 3')	59.29		
COI	Forward primer	jgHCO2198 (5' TAN ACY TCN GGR TGN CCR AAR AAY CA 3')	61.7	50	Geller *et al* 2013
	Reverse primer	jgLCO1490 (5' TNT CNA CNA AYC AYA ARG AYA TTG G 3')	54.9		

Polymerase Chain Reaction (PCR)

DNA amplification of partial 16S and 18S rRNA gene were performed using a thermal cycler. Primers utilized in each reaction are shown in Table 2. The reaction mixture for PCR consisted of a total volume of 25 µl which contained 2.5µl of 10X buffer, 0.2 *Taq* DNA polymerase, 0.2 mMdNTP, 0.2 µM forward primer, 0.2 µM reverse primer (Gershwin, 2005) and 1 µl of 25 ng DNA. PCR protocol was conducted in a PCR tube using an initial denaturing step at 95ºC for 5 min followed by 35 cycles of 95ºC for 30 s, 64ºC for 30 s and 72 ºC for 1 min. Finally, final extension at 72ºC for 5 min was carried out. Likewise, amplification of COI locus was performed using primer as shown in table 2. The PCR reaction mixture consisted of a total volume of 25 µl which contained 2.5µl of 10X buffer, 0.2 *Taq* DNA polymerase, 0.2 mM dNTP, 0.2 µM forward primer, 0.2 µM reverse primer and 1 µl of 25 ng DNA. PCR protocol was conducted in a PCR tube using an initial denaturing step at 95ºC for 5 min followed by 35 cycles of 95ºC for 30 s, 50 ºC for 30 s and 72 ºC for 1 min. Finally, final extension at 72ºC for 5 min was carried out. The PCR products were visualized using 1.5% agarose gel electrophoresis. The sequences were obtained from
DNA sequencing by 1st Base Company, Selangor, Malaysia.

Phylogenetic tree construction

Multiple alignments were performed and the phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis 6 (MEGA6) program. 16S rRNA, 18S rRNA and mt-COI sequences from selected members of cubozoa, and scyphozoa, the out-groups for the purpose of this research.