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DESCRIPTION OF *Chironex indrasaksajiae* Sucharitakul sp. nov. (CNIDARIA, CUBOZOA, CHIRODROPIDA): A NEW SPECIES OF BOX JELLYFISH FROM THE GULF OF THAILAND

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ABSTRACT: In this paper a new species of box jellyfish is described. The species, *Chironex indrasaksajiae* Sucharitakul sp. nov. (Cnidaria: Cubozoa, Chirodropida, Chirodropidae), from the Gulf of Thailand has been identified using morphological structure analysis together with mitochondrial and ribosomal (COI, 16S and 18S) gene data. Results of morphological, molecular and phylogenetic analysis demonstrate that *Chironex indrasaksajiae* is a species distinct from its congeners. The pedalial canal bend is a character that can be readily used to distinguish this species from its congeners. Clarifying the identity of this box jellyfish is important for understanding box jellyfish biodiversity and has implications for public safety in the Gulf of Thailand.

Keywords: Chirodropidae, phylogeography, molecular systematics, venomous

INTRODUCTION

The genus *Chironex* was first described from Australia in 1956 (Southcott, 1956). A key character of the genus is the shape of the gastric saccules which can be used to distinguish *Chironex* from the other members of the order Chirodropida (Barnes, 1965; Kinsey, 1986; Gershwin, 2006). For members of *Chironex*, the distinctive gastric saccules have been described as cock's comb-like or grape-cluster-like (Gershwin, 2005; Lewis and Bentlage, 2009). Species within the genus share a number of other morphological characteristics including claw-like pedalia, lack of warts on exumbrella, V-shaped gastric phacellae, smooth perradial lappets with four frenulae and highly branched velarial canals, dome-shaped rhopalial niche ostia and four rhopalia, each with a set of 6 eyes (Southcott, 1956; Lewis and Bentlage, 2009).

Until 2009, there was only a single species in the genus, *Chironex fleckeri*. In 2009, Lewis and Bentlage described a second *Chironex* species from Japan using both morphological and molecular methods and named the specimen as *Chironex yamaguchii* (Lewis and Bentlage, 2009). According to Lewis and Bentlage (2009), the most reliable characters for distinguishing *C. fleckeri* from *C. yamaguchii* are the shape of the pedalial canal knee bend and the numbers of tentacles per pedalium. *C. fleckeri* has an upswept corniculum, rose thorn-shaped pedalial canal knee bend (Kinsey, 1986: p. 6) and 12 to 15 tentacles while *C. yamaguchii* shows a volcano-shaped pedalial canal knee bend and 7 to 9 tentacles (Lewis and Bentlage, 2009). Apart from traditional taxonomic methods, Lewis and Bentlage (2009) investigated a fragment of the mitochondrial COI gene and found distinctive genetic differentiation (16.7%) between the two species.

Remarkably, even though identification of *Chironex* species is vital, given that deaths related to box jellyfish envenomation have been reported in the Thai media (see also Lewis and Bentlage, 2009; Everingham, 2015; Marine and Coastal Research and Development Institute, 2015; Thaikrue and Siriariyaporn, 2016), no species of this genus

has been formally described in Thailand. The aims of this study are to describe the species of *Chironex* from the Gulf of Thailand. By identifying and describing the culprit of dangerous and potentially fatal box jellyfish envenomation in the Gulf of Thailand, we hope to educate beach-goers and first responders and so mitigate the damaging effects of box jellyfish in Thai waters.

MATERIAL AND METHODS

Morphological study

Specimens were previously collected by a shrimp trammel net and a dip net, fixed and preserved in 3% formalin and stored at Phuket Marine Biological Center (PMBC), Marine and Coastal Resources Research and Development Center, the Eastern Gulf of Thailand (MCRRDE) and Marine and Coastal Resources Research and Development Center, the Central Gulf of Thailand. All samples were collected from the Gulf of Thailand. Morphological observations were conducted using stereomicroscope.

DNA extraction, amplification and sequencing

DNA extraction (Adapted from Sucharitakul *et al*., 2016)

Subsamples were fixed in absolute ethanol. Approximately 5 mm of tissues from exumbrella and tentacles from 5 specimens were excised 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 60ºC 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 was transferred to a new microcentrifuge tube followed by an equal amount of phenol. The total volume was then mixed by vortexing before samples were centrifuged at 13,500 rpm for 15 minutes. Then, 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. After that, supernatant solution was transferred to a new microcentrifuge tube filled with 30 µl of 6 M NaCl and mixed by inverting gently. Following this procedure, 2.5 volume of extremely cold absolute ethanol was added and kept overnight in –20ºC 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, the purified nucleic acids were eluted in 30 µl of TE buffer.

Primers

PCRs with three primer sets for three genes, COI (Geller *et al*., 2013), 18S gene (Medlin *et al*., 1988) and 16S, as indicated in Table 1, were performed in this study. 16S primers for cubozoans were designed using 8 sequences of 16S sequences from members of Chirodropidae from the 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 sequences were aligned to search for conserved regions. After that, forward primer and reverse primer were designed by Primer-BLAST

Polymerase Chain Reaction (PCR)

DNA amplification of partial 16S and 18S rRNA gene were performed using a thermal cycler. Primers utilized in each reaction were shown in Table 1. The reaction mixture for PCR consisted of a total volume of 25 µl which contained 2.5 µl of 10X buffer, 0.2 *Taq* DNA polymerase, 0.2 mMdNTP, 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 minutes followed by 35 cycles of 95ºC for 30 seconds, 64ºC for 30 seconds and 72ºC for 1 minute. Finally, final extension at 72ºC for 5 minutes was carried out. Likewise, amplification of COI locus was performed using primer as shown in Table 1. The PCR reaction mixture consisted of a total volume of 25 µl which contained 2.5 µl of 10X buffer, 0.2 *Taq* DNA polymerase, 0.2 mMdNTP, 0.2 µM forward primer, 0.2 µM reverse primer and 1 µl of 25 ng DNA. PCR protocol was

Table 1. Primers used in this study together with their Tm (Melting temperature).

Gene			Calculated Tm (°C)	Working Tm (°C)	Reference
16S	Forward primer	P16sf (5'AAGGGCCGCGGTAACTCTG 3')	62.31	64	This study
	Reverse primer	S16sr (5'ACCCTGTTATCCCCGTGGT 3')	60.23		
18S	Forward primer	18SAf (5' CCG AAT TCG TCG ACA ACC TGG TTG ATC CTG CCA GT3')	73.53	64	Medlin *et al*., 1988
	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		

conducted in a PCR tube using an initial denaturing step at 95ºC for 5 minutes followed by 35 cycles of 95ºC for 30 seconds, 50ºC for 30 seconds and 72ºC for 1 minute, and a final extension at 72ºC for 5 minutes was carried out. The PCR products were visualized using 1.5% agarose gel electrophoresis and 1,000 bp ladder. Corresponding DNA sequence data were obtained following sequencing by 1st Base Company, Selangor, Malaysia.

Phylogenetic tree construction

Multiple alignments were performed using CLUSTAL W and a phylogenetic tree was constructed using Maximum Likelihood under Bootstrap method and Kimura 2-parameter model by Molecular Evolutionary Genetics Analysis 6 (MEGA6) program. 16S rRNA, 18S rRNA and mt-COI sequences of Cubozoa and Scyphozoa from NCBI (National Center for Biotechnology Information) were used as the out-groups for this research.

RESULTS

Systematic account

Phylum Cnidaria Verrill, 1865
Subphylum Medusozoa Peterson, 1979
Class Cubozoa Werner, 1973
Order Chirodropida Haeckel, 1880
Family Chirodropidae Haeckel, 1880
Genus *Chironex* Southcott, 1956

Chironex indrasaksajiae Sucharitakul sp. nov.
(Figs. 1–5; Table 2).

Abbreviations. Bell Height: BH, measured from the tip of the bell to the velarial turnover; Bell Width: BW, measured from the center of one pedalium to the next one; Manubrium Length: ML measured from the tip of the manubrium to the base that connect to gastric saccule; Number of tentacles: NT, number of tentacle on each corner of the bell.

Material examined. *Holotype*: MCRRDE 9, mature, 127 mm BH, 136 mm BW, 68 mm ML, 12 NT, Chao Lao Beach, Chanthaburi Province, Thailand, 28 January 2015.

Paratype: MCRRDE 10, Suan Son Beach, Rayong Province, Thailand, 15 August 2015; MCRRDE 12, mature, 128 mm BH, 126 mm BW, 68 mm ML, 12 NT, Suan Son Beach, Rayong Province, Thailand, 15 June 2015; PMBC 27932, mature, 170 mm BH, 130 BW, 10 NT, Pha Ngan Island, Surat Thani Province, 14 August 2015; PMBC 27933, mature, 150 mm BH, 120 mm BW, 12 NT, 14 August 2015, Pha Ngan Island, Surat Thani Province.

Type locality. Chao Lao Beach, Chanthaburi province, Thailand.

Etymology. The specific name was chosen to honor the Queen Consort Indrasaksaji, who dedicated herself to the welfare of Thai people throughout her life.

Diagnosis. *Chironex indrasaksajiae* Sucharitakul sp. nov. has a maximum known height of 170 mm (notable larger than *C. yamaguchii* and smaller than *C. fleckeri*); with up to 12 tentacles per pedalium (versus up to 9 in *C. yamaguchii* and up to 15 in *C. fleckeri*); bulbous shaped pedalial canal knee bend (in contrast to "volcano-shaped" in *C. yamaguchii* and "upswept corniculum" in *C. fleckeri*) The differences between species belonging to the genus *Chironex* are presented in Table 2.

Description. Live specimen cube shaped with transparent bell (Fig. 1A). Maximum bell height about 170 mm. and maximum bell width about 136 mm, manubrium length about 2/3 of bell height. Pedalia, 4, claw-like with up to twelve tentacles (Fig. 1B). Pedalial canal knee bend bulbous-shaped (Fig. 1C). Tentacles flat in life, rounded in preserved specimens (Fig. 1D). Gastric phacellae, 4, V-shaped in preserved specimens with numerous and unbranched gastric cirri (Fig. 2A). Long manubrium with four lanceolate lips hanging from the stomach (Fig. 2B). Gastric saccules, 4, cock's comb shape (Fig. 2C). Perradial lappets, 4 pairs, smooth lacking nematocyst warts, triangular-shaped with a single frenulum on each side of the bell (Fig. 2D). Velarial canals numerous and highly branched (Fig.3A). Rhopalial niche ostia dome shaped. Four rhopalia, each bearing six eyes (Fig. 3B). Nematocysts on tentacles shaped cucumber microbasic *p*-mastigophores and oval trirhopaloid (Fig. 4A–B).

Table 2. Comparison of the characters of species within the genus *Chironex* (adapted from Gershwin, 2005; Lewis and Bentlage, 2009).

	Tentacles	Pedalial canal bend	Geographic location	Reference
Chironex fleckeri	12–15	Rose thorn-shaped	Australia	Southcott, 1956
Chironex yamaguchii	5–9	Volcano	Japan and The Philippines	Lewis and Bentlage, 2009
Chironex indrasaksajiae sp. nov.	10–12	Bulbous	Thailand	This study

Remarks. Only a few morphological differences between *Chironex indrasaksajiae* and its congener were noticed (Table 2), and the shape of pedalial canal bend tended to be the most reliable distinguishing character as mentioned by Lewis and Bentlage (2009). All specimens have a bulbous shaped pedalial canal (Fig. 5A-D). However, MCRRDE 12 has a concaved pedalial canal on one side of pedalia (Fig. 5E-F).

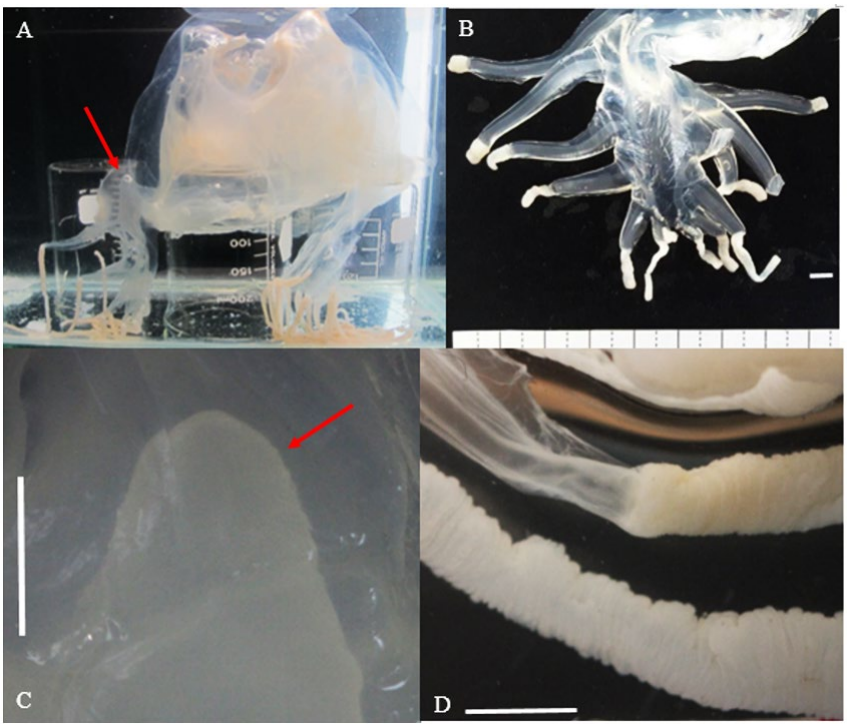

Figure 1. *Chironex indrasaksajiae* Sucharitakul sp. nov. (MCRRDE 9): A. Transparent bell, pedalia canal bend (red arrow), B. Claw-like pedalia with pedalial canal split into two mirror-symmetric cannals, C. Bulbous- shaped pedalial canal knee bend (red arrow), D. Filiform tentacles in preserved specimen. Scale bar represent 0.5 cm.

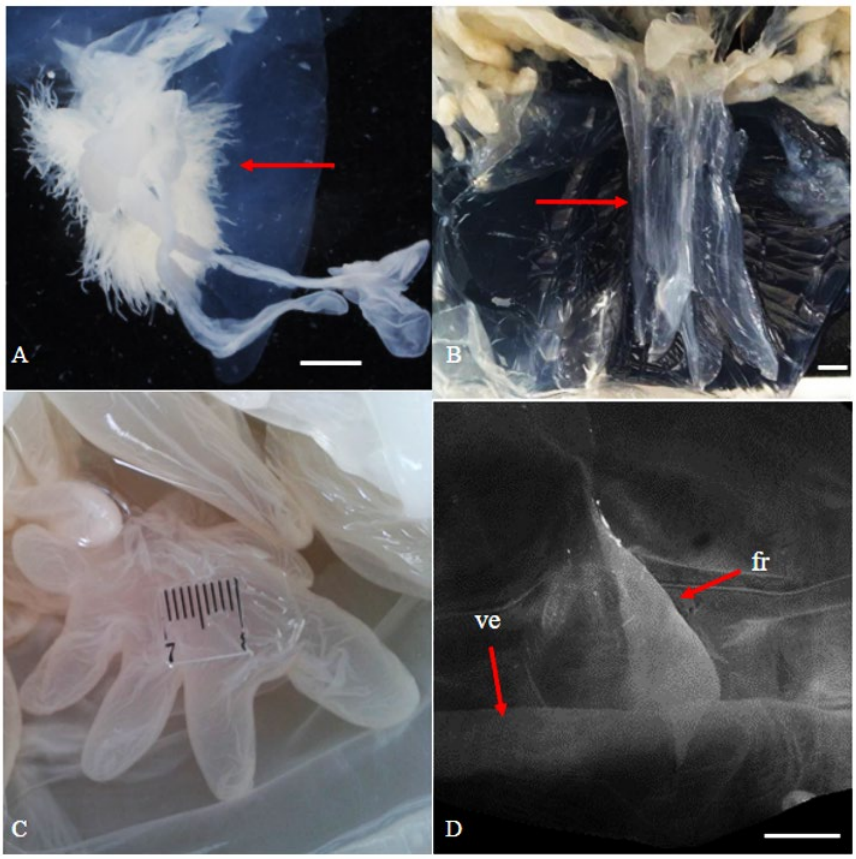

Figure 2. *Chironex indrasaksajiae* Sucharitakul sp. nov. (A–B: PMBC 27933 and C–D: MCRRDE 12): A. Numerous gastric cirri (red arrow), B. Manubrium (red arrow) connecting to gastric saccule at the base, C. Cock's comb shaped gastric saccule, D. A single frenulum (fr) that hold the velarium (ve) on one side of the bell. Scale bars represent 0.5 cm.

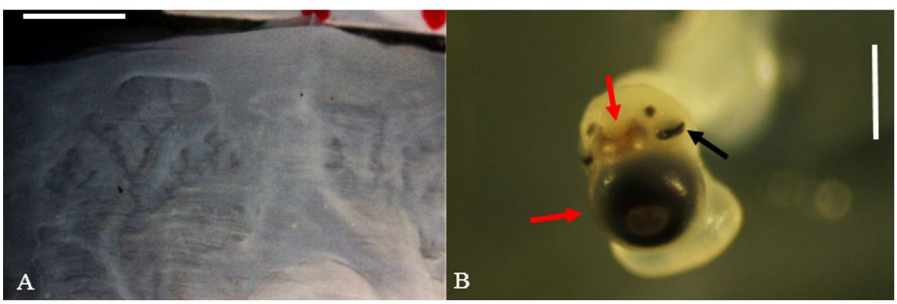

Figure 3. *Chironex indrasaksajiae* Sucharitakul sp. nov. (MCRRDE 10): A. Brunched velarial canal, scale bar represented 0.5 cm, B. Rhopalia comprises of 2 median lensed eyes (red arrows), two pit eyes and 2 slit eyes (black arrow). Scale bar represented 0.3 cm.

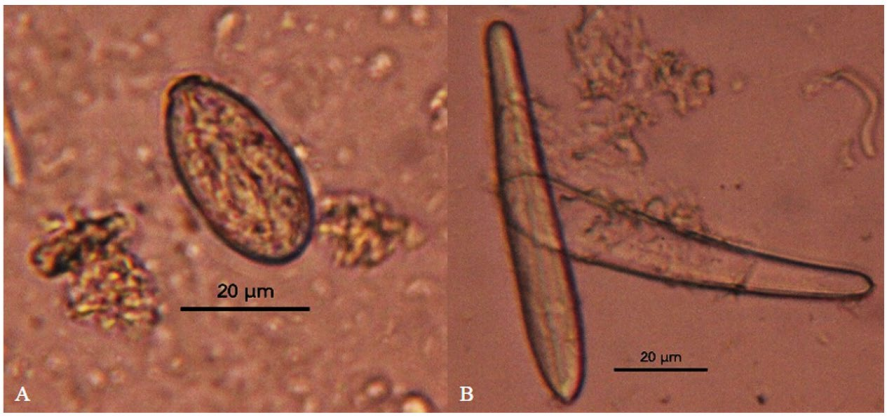

Figure 4. Nematocysts isolated from tentacles of *Chironex indrasaksajiae* Sucharitakul sp. nov. (MCRRDE 12): A. Oval shaped trirhopaloid, B. cucumber-shaped microbasic *p*-mastigophores.

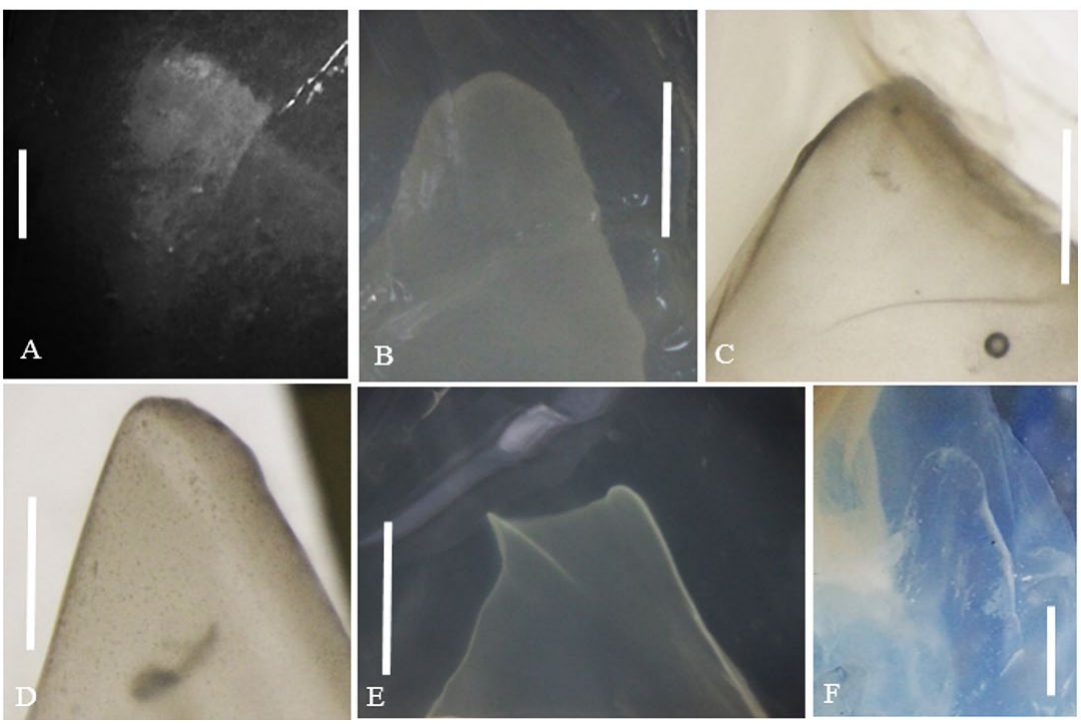

Figure 5. Pedalial canal of *Chironex indrasaksajiae* Sucharitakul sp. nov.: A–D: Bulbous shaped pedalia canal in A. MCRRDE 9, B. MCRRDE 10, C. PMBC 27933 and D. MCRDDE 12; E-F: Concave and bulbous shape on pedalial canal knee bends of MCRDDE 12. Scale bars represent 0.5 cm.

Molecular analysis

Sequences were checked against nucleotide sequences in the NCBI database using BLAST (Basic Local Alignment Search Tool). The result from the COI gene showed 85% identity to *Chironex yamaguchii*, accession number: FJ665180.1. The genetic difference between *C. yamaguchii* (Accession number FJ665180.1) and *C. indrasaksajiae* Sucharitakul sp. nov. (Accession number KT223648.1) was 14.0% with genetic distance of 0.18. Also, the genetic variation between *C. fleckeri* (Accession number FJ665181.1) and *C. indrasaksajiae* (Accession number KT223648.1) was 15.3% with genetic distance of 0.21. Likewise, the genetic variation between *C. yamaguchii* (Accession number FJ665180.1) and *C. fleckeri* (Accession number FJ665181.1) was 15.3% and genetic distance was 0.19. For the18S rRNA, sequences data from *C. yamaguchii* (GQ849076.1), C. fleckeri (GQ849074.1) and *C. indrasaksajiae* (KU097000.1) were calculated. The variation among congeners as calculated by MEGA 6 was less than 1%. For 16S sequences, there is no sequence data of *C. yamaguchii* in NCBI. As a result, only sequences from *C. fleckeri* (GQ849103.1) and *C. indrasaksajiae* (KX065499) were computed by MEGA 6. The genetic difference between *C. fleckeri* and *C. indrasaksajiae* was 17% while the variation within *C. indrasaksajiae* was relatively low (0.0%). The variation among congeners was sufficiently high enough to distinguish *C. yamaguchii* from *C. fleckeri* in a previous study by Lewis and Bentlage (2009).

Phylogenetic trees based on 16S, 18S rRNA and mt-COI (Figs. 6) were reconstructed using maximum likelihood assuming the Kimura 2-parameter model of nucleotide evolution. All *C. indrasaksajiae* samples fell into a monophyletic group, and branched with other chirodropids. According to the trees, *C. indrasaksajiae* was separated from *C. fleckeri* and *C. yamaguchii.* In a previous study, Bentlage and colleagues investigated a phylogeography of *C. yamaguchii* from Japan and *C. fleckeri* from Australia using 16S, 18S and 28S gene. The results indicated that the Japanese clade separated from the Australian clade (Bentlage *et al*., 2010). Interestingly, when comparing the phylogenetic results with the geographical distribution of the species, the three species. *C. indrasaksajiae*, *C. yamaguchii* and *C. fleckeri*, are distributed in different geographical areas (Bentlage *et al*., 2009).

DISCUSSION

Morphological differences among medusae belonging to the genus Chironex and other genera of the family Chirodropidae are shown in Table 3.

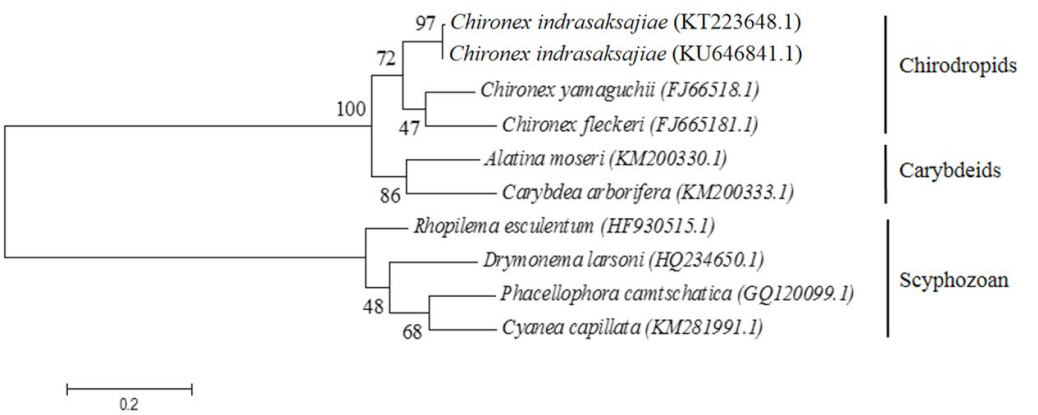

Figure 6. COI cubozoan phylogenetic tree, rooted with members of the class Scyphozoa. 1,000 bootstraps were used. The genus *Chironex* forms a monophyletic group

Table 3. Comparison of the genera within the family Chirodropidae (adapted from Gershwin, 2006 and present study).

Chirodropid genus	Saccule shape	Tentacles per pedalium	Pedalial canal bend
Chirodectes	Absent	9–11	Spike
Chirodropus	Elongate, tapered, with numerous axial processes, or absent	9–21	Spike
Chironex	Cock's comb	5–15	Spike, volcano or bulbous

. The distribution of the genus *Chironex* has been reported to range from Japan to Australia (Bentlage *et al*., 2009). *C. fleckeri* can be found at maximum latitude 24°S from the equator, which includes Indonesia, Papua New Guinea and Australia, whereas *C. yamaguchii* can be found at a maximum latitude of 27°N from the equator, including the Philippines and Japan (Kingsford and Mooney, 2014). However, our *Chironex* specimens were collected in Thailand, between the latitudes 7.5 °N–12 °N.

It was discovered that the genetic diversity among congeners of marine organisms corresponds to their geographical dispersal (Barber *et al*., 2000). For marine organisms with a pelagic stage in their life history, ocean currents are presumed to play an important role in dispersal (Ujiie *et al*., 2012). When comparing the distribution pattern of Thailand *Chironex* with the map of predicted chirodropid geographic distribution (see Bentlage *et al*., 2009), the Sunda Shelf and the Java trench were predicted to serve as a geographical barrier. Moreover, the presence of a marine equivalent of Wallace's line was predicted as it has also been suspected to exist in many other studies (Barber *et al*., 2000; Gershwin, 2001). Furthermore, salinity and temperature also affect the movement of *Chironex*, as hypothesized by Mooney and Kingsford (2012; 2016). The jellyfish may even adjust to environmental fluctuations in salinity and/or temperature, as has been reported for other box jellyfish (Hamner *et al*., 1995; Fossette *et al*., 2015). Thus, it is anticipated that geographical isolation has contributed to the divergence of the Japanese, the Thai and the Australian clades, but confirmation of this speculation awaits future biogeographical studies.

Finally, the envenomation of this genus is known to cause human fatalities in Okinawa and Australia. Although deaths related to box jellyfish envenomation have been documented in the Gulf of Thailand, which may be attributable to *C. indrasaksajiae*, understanding the specific toxicity and biology of this species await the results of further detailed work.

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