

DNA Barcoding of Mountain Crabs (Potamidae) from Phetchabun Mountains of Thailand: The First Report of Endemic and Endangered Species

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Citation: Suthamrit W, Thaewnon-Ngiw B (2021) DNA Barcoding of Mountain Crabs (Potamidae) from Phetchabun Mountains of Thailand: The First Report of Endemic and Endangered Species. *Biochem Mol Biol* Vol.7 No.2:6

Abstract

This study aims to examine the efficiency of mt-DNA (cytochrome c oxidase I) for the identification of freshwater crabs. One hundred thirty-eight fresh crabs were collected from 15 locations in five provinces in the Phetchabun Mountains (Loei, Phetchabun, Nongkhai, Udonthani, and Chaiyaphum) of Thailand. DNA extraction (claw tissue) of individual crab was amplified using PCR by *COI* primers. The PCR products were examined for nucleotide sequence before the genetic analysis. The result indicated there are 32 haplotypes. The phylogenetic tree showed a clear separation of three main clades. Clade A was classified to be *Indochinamon bhumibol*, *Indochinamon ahkense*, and *Indochinamon mieni*. Clade B was classified to be *Iomon nan* and *Vietopotamon phluangense*. Clade C was classified of *Larnaudia chaiyaphumi*. This study is the first report of the genetic sequences of all 6 mountain crabs species.

Keywords: DNA barcoding; Potamidae; *COI* gene; Mountain crabs; Phetchabun Mountains

Received: December 29, 2020; **Accepted:** February 11, 2021; **Published:** February 18, 2021

Introduction

The mountain crabs are big group of freshwater crabs which have habitat in fresh water streams on mountains in Indochina region [1]. Their population play important role in food chain the mountain stream ecosystem. The freshwater crabs will survive in large enough natural forest areas to maintain the good water quality of the original streams [2]. Previously, almost mountain crabs were identified in the genus *Potamon* because of the similarity of external characters. After that they were divided into many genera later by the reason that there are much different types of habitats and continents [3]. Presently, the study of freshwater crabs (Potamidae) in Thailand found 94 species in 25 genera. Phetchabun Mountains have a high biodiversity, and various endemic creatures especially mountain crabs [4]. Most of their habitat is distributed in the tropical and sub-tropical on the mainland [5]. The mountain crabs play an important role in the source of food and income for the local people. The development of anthropogenic activities is rapidly increasing such as tourism activities, deforestation, agricultural chemicals, Overconsumption and others. On the other hand,

The number of mountain crabs is rapidly declining. In addition, local communities and organizations still lack in biodiversity and genetics information and clear management resources strategies.

Now-a-days, mitochondrial DNA (mt-DNA) is widely accepted that it is a highly accurate technique of identifying organism. There are many of partial mt-DNA genes that are used to be taxonomical to animals (*COI*), plants (*matK*, *rbcL*, *psbA-trnH*, *ITS*), bacteria (*COI*, *rpoB*, *16S*, *RIF*, *gnd*), fungus (*ITS*, *RPB* and *18S*), and protists (*ITS*, *COI*, *rbcL*, *18S* and *28S*) [6]. *COI* gene is especially popular in classify various animals around the world such as shrimp *Macrobrachium lanchesteri* [7], freshwater prawn [8] sea cucumbers [9], worms (*Pomphorhynchus tereticollis*) [10], insect [11], gastropod [12], mud crab (*Scylla serrata*) [13] etc. This study intends to use morphology and applies the *COI* gene to identify mountain crabs family Potamidae. Studying genetics to accurately identify of mountain crabs species can be very helpful in the selection of mountain crab breeders. Breeding to selling will support local income. It also help reduce catching of crabs out of the wild. Finally, it is supporting of sustainable conservation of mountain crabs to stay together in the natural forest.

Materials and Methods

Collecting specimen and identification

The fresh specimens were collected from 15 locations in five Provinces in Phetchabun Mountains of Thailand: (I) Loei Province (Phuruea=PRI, PRII, Dansai=DS, Thali=TL, Phuluang=PLI, and PLII, Nahaew=NH, Nonghin=NHI, Pakchom=PCH, Wangsaphung=WP, Phukradueng=PD); (II) Phetchabun Province (Lomkao=LK); (III) Nongkhai (SangKhom=SK); (IV) Udonthani (Nayoong=NY), and (V) Chaiyaphum Province (Phakdichumphon=PHD) (**Figure 1**). The specimens were frozen at -20°C at the Molecular Biology Laboratory, Faculty of Science Mahasarakham University until used.

Morphology

External morphological study from these characters of first gonopod (G1), second gonopod (G2) and others. In this study, using the methods of Brandis et al. [14], Chuensri et al. [15], Yeo et al. [3], Pramual et al. [16], Naiyanetr et al. [17-19], Brandis et al. [14], Supajantra et al. [20], Guinot et al. [21], and Naruse et al. [22].

Genetic analysis

Genomic DNA was extracted using the Genomic DNA kit GF-1 and stores at -20°C. By using UV spectrophotometer of optical density at 260nm (OD 260) and 280nm (OD 280) to checked the DNA concentration. We used polymerase chain reaction (PCR) methods with the primers LCO1490 (F) 5'-GGT-CAA-CAA-ATC-ATA-AAG-ATA-TTG-G-3' and HC02198 (R) 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3', which were developed by Folmer et al. [23]. The

PCR was conducted with 50 μ l: 5 μ l 10X buffer, 2 MgCl₂, 0.05mM dNTPs, both 0.5 mM *COI* primer of forward and reward, 0.3 μ l Taq DNA polymerase and 3.5 μ l DNA samples. The PCR consisted four steps: (I) initial denaturation of 2 min at 94°C, (II) stand at 10 cycles of 30 sec at 94°C, 40 sec at 50°C and 10 min at 72°C (III) stand at 35 cycles of 40 sec at 94°C, 40 sec at 50°C and 1.10 min at 72°C (IV) stand at 7 min of 72°C and stand at -4°C. Then, the PCR product was determined by electrophoresis technique (1% agarose gel). To determine DNA sequence (forward and reward of the *COI* gene), the PCR product was sent to 1st BASE sequencing service (Malaysia these sequences were analyzed and the genetic differentiations (Intra-genetic divergence) were obtained and constructed the phylogenetic tree diagram: Neighbor-Joining (NJ). We use to get phylogenetic tree diagram using the MEGA-X program to get the genetic differentiations and phylogenetic tree [24-26]. Lastly, using the Arlequin 3.5 program, we analyzed the inter-specific genetic divergence [24,27,28].

Results

Collecting specimen and morphology

A total of 138 crabs (103 males and 35 females) were collected from Phetchabun mountain ranges (latitudes 101-102, longitude 16.5-18.30) between 310 and 845 m high above sea level (**Table 1**). Their habitat is digging holes beside muddy streams or hides in between rocky streams. The external morphological study was identified by the first gonopod and other physically characters found that there were six species of mountain crabs: (I) *Indochinamon bhumibol* was dark-purple and dark-brown, (II) *Indochinamon ahkense* was brown-orange, (III) *Indochinamon mieni* was onyx-dark-blue, (IV) *lomon nan* was dark-brown-olive,

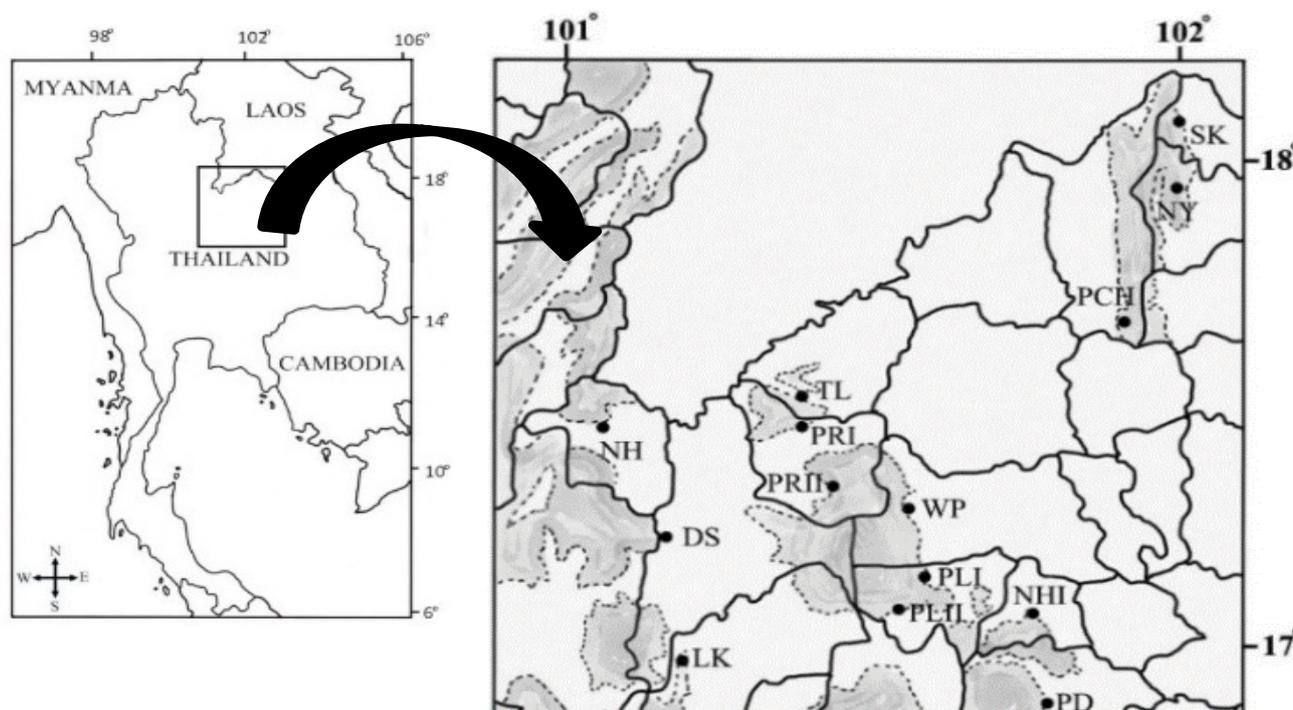


Figure 1 Sampling location of mountain crabs in Phetchabun Mountains of Thailand.

Table 1 Details of the six Potamidae species from collecting location and GenBank accession number are tabulated below.

Species/Locality	GPS co-ordinates	Elevation (m)	Number sequence (N)	Name of sequence
A) <i>Indochinamon bhumibol</i> (Naiyanetr, 2001)			14	
1	Phuruea District Loei Province (PRI)	17 23'09.4" N 101 29'20.1" E	733	3
				RPI1
				PRI2
2	Phuruea District Loei Province (PRII)	17 28'55.88" N 101 23'04.45" E	789	3
				PRII1
				PRII2
3	Dansai District Loei Province (DS)	17 15'01.70" N 101 08'24.99" E	414	3
				DS1
				DS2
4	Thali District Loei Province (TL)	17 32'09" N 101 19'41" E	829	2
				TL1
				TL3
5	Phuluang District Loei Province (PLI)	17 06'04" N 101 33'28" E	845	3
				PLI1
				PLI3
B) <i>Indochinamon ahkense</i> (Naruse, Chia and Zhou, 2018)			3	
6	Lomkao District, Phetchabun Province (LK)	17 06'25.94" N 101 19'06.62" E	602	3
				LK3
				LK4
C) <i>Indochinamon mieni</i> (Dang, 1967)			9	
7	Pakchom District Loei Province (PCH)	17 02'50" N 101 50'56" E	720	3
				PCH3
				PCH5
8	Sang-Khom District Nongkhai Province (SK)	18 12'05.01" N 102 06'39.51" E	369	3
				SK1
				SK2
9	Nayoong District Udonthani Province (NY)	18 28'5" N 102 87'7" E	473	3
				SK3
				NY1
D) <i>Iomon nan</i> (Ng and Naiyanetr, 1993)			3	
10	Nahaew district Loei province (NH)	17 18'16.90"N 101 05'49.93"E	795	3
				NH1
				NH2
E) <i>Vietopotamon phluangense</i> (Bott, 1970)			8	
11	Nonghin District Loei Province (NHI)	17 02'50" N 101 45'40" E	594	2
				NHI1
12	Phuluang District Loei Province (Plii)	17 05'29.37" N 101 34'11.31" E	461	1
				NHI5
13	Wangsaphung District Loei Province (WP)	17 16'16" N 101 34'42" E	411	3
				PLII1
				WP1
14	Phukradueng District Loei Province (Pd)	16 50'15.59" N 101 49'42.49"	310	2
				WP2
				WP5
F) <i>Larnaudia chaiyaphumi</i> (Naiyanetr, 1982)			3	
15	Phakdichumphon District Chaiyaphum Province (PHD)	16 08'42.42" N 101 27'49.23" E	462	3
				PHD1
				PHD2
			Total=40	

(V) *Vietopotamon phluangense* was dark-brown or brown-yellow (VI) *Larnaudia chaiyaphumi* was bark-brown-orange [29].

Genetic analysis

The 40 specimens from 138 specimens were selected for genetic

study. The 40 aligned sequences of Potamidae was analyzed by 590 bp *COI* gene and blasted with Genbank DNA sequence. The result of the nucleotide blast did show not any match with Genbank. There was not a single similar DNA sample. We then analyse the haplotype (H) frequency by Arlequin 3.5 which

showed 32 unique haplotypes and there were four sharing haplotypes. The compositions were 18.53% C, 38.07% T, 25.82% A and 17.59% G. The highest of GC-content was *In. bhumibol* at 37.01%. The lowest of GC- content was *L. chaiyaphumi* at 33.56%. The mean of variation in GC-content was 36.43% (33.56- 37.01%). The highest of the intra-genetic distance was *V. phuluangense* which had a mean of 4.17% (0.53-8.27%), followed by mean of *In. bhumibol* of 2.03% (0-3.79%). There was almost no genetic differentiation within species of *Io. nan* and *L. chaiyaphumi* (Table 2). The topologies of the phylogenetic tree were constructed by the NJ method. The tree was divided into three main clades: clade A, B and C. The clade A shows three sister taxon of genus *Indochinamon* consisting taxa A= *In. bhumibol*, B= *In. ahkense* and C= *In. mieni*. The average of genus *Indochinamon*'s Intra-genetic divergence was 6.50%. The inter-genetic distance within the genus showed *In. bhumibol* relate closely with *In. ahkense* as 82.02% Fst value (Table 3). The clade B (taxa D and E) were classified to be *Io. nan* and *V. phuluangense* respectively. The clade C, was classified to be *L. chaiyaphumi*. (Figure 2). From the tree, found genetic differences within the species that were split into multiple groups base on sampling location such as *In. bhumibol*, *In. mieni* and *V. phuluangense*. The DNA barcoding of partial *COI* genes of six mountain crab species were submitted on The National Center for Biotechnology Information (NCBI). The detail of GenBank Accession No. was showed in Table 1.

Discussion

In the last centuries, Asean countries have done very few studies of freshwater crabs especially the mountain crabs which almost all of them are endemic species. The study of crab in the Indochina region is based on external characters. For this reason, it causes the misidentification of the species. In addition, there may be various species that are still unknown. That means that we still

lack biological knowledge to manage the mountain crabs. The current biological diversity study has improved the morphology together with the genetic information of the mountain crabs. DNA barcoding is a high effective technique to help to confirm species in a short time [6]. The comparison of Genbank by nucleotide blast does not show any match with any species. There is only a few genetic data (16S gene) on the genus of the study such as *Indochinamon kimboiense*, *Indochinamon ou*, *Indochinamon tannanti*, *Larnaudia beusekomae* [30]. In this study, we provide the first sequence of the *COI* genes of these six mountain crabs in Indochina habitat (Phetchabun Mountains, Thailand). The 32 unique haplotypes show high genetic diversity. The mean of intra-genetic distance shows high value in *V. phuluangense* (4.17%), and *In. bhumibol* (2.03%) and *In. mieni* (0.91%). The phylogenetic tree shows that within the same species, there is branching to multiple groups based on the samples' locations. This can be explained by the geographic factors. The geographical barriers like streams, large rocks may have a significant role in supporting of inbreeding by preventing the mobility of the crabs, and thus the genes' movements as well [31,32]. Most of their activities are finding food, mating, spawning, and digging holes for its nests beside the streams. The reproductive isolate was the obstacle to the gene flow mechanism. This is supported by the studies of various animals show the importance of reproductive isolation force and sympatric divergence such as river crabs (*Potamonautidae*) [33], birds and mammals [34], gastropod (*Littorina saxatilis*) [35], insect *Rhagoletis pomonella* [36]. The clade A shows three sister taxon of genus *Indochinamon* (taxa A, B, and C) and monophyletic grouping that was supported by high-value NJ bootstrap 99%. Three species of Genus *Indochinamon* are very similar in morphology, and slightly in different colors. *In. bhumibol* and *In. ahkense* have close relationships supported by NJ bootstrap 54%. The clade B (taxa D and E) was classified to be

Table 2 Details of the six Potamidae species from genetic samples (K2P) and nucleotide frequencies are tabulated below.

Type value	Number of crabs (N)	number of haplotypes (Nh)	Intraspecific genetic divergence K2P (%) (mean)	C%	T%	A%	G%	CG-content
A) <i>In. bhumibol</i>	14	10	0.00-3.79 (2.03)	19.15%	37.29%	25.70%	17.86%	37.01%
B) <i>In. ahkense</i>	3	2	0.00-0.71 (0.47)	18.70%	38.42%	26.05%	16.84%	35.54%
C) <i>In. mieni</i>	9	8	0.00-1.97 (0.91)	17.59%	38.76%	25.93%	17.72%	35.31%
D) <i>V. phuluangense</i>	8	8	0.53-8.27 (4.17)	18.50%	38.29%	25.41%	17.80%	36.30%
E) <i>Io. nan</i>	3	1	0.00-0.00 (0.00)	19.32%	37.63%	25.42%	17.63%	36.95%
F) <i>L. chaiyaphumi</i>	3	3	0.18-0.53 (0.35)	17.51%	39.15%	27.29%	16.05%	33.56%
Total	40	32	Average	18.53%	38.07%	25.82%	17.59%	36.43%

Table 3 Interspecific genetic divergence of K2P.

species	A	B	C	D	E	F
A) <i>In. bhumibol</i>	-					
B) <i>In. ahkense</i>	82.02%	-				
C) <i>In. mieni</i>	86.01%	89.06%	-			
D) <i>V. phuluangense</i>	84.36%	81.82%	85.63%	-		
E) <i>Io. nan</i>	89.98%	98.40%	94.08%	77.06%	-	
F) <i>L. chaiyaphumi</i>	90.87%	97.21%	94.69%	79.93%	98.58%	-

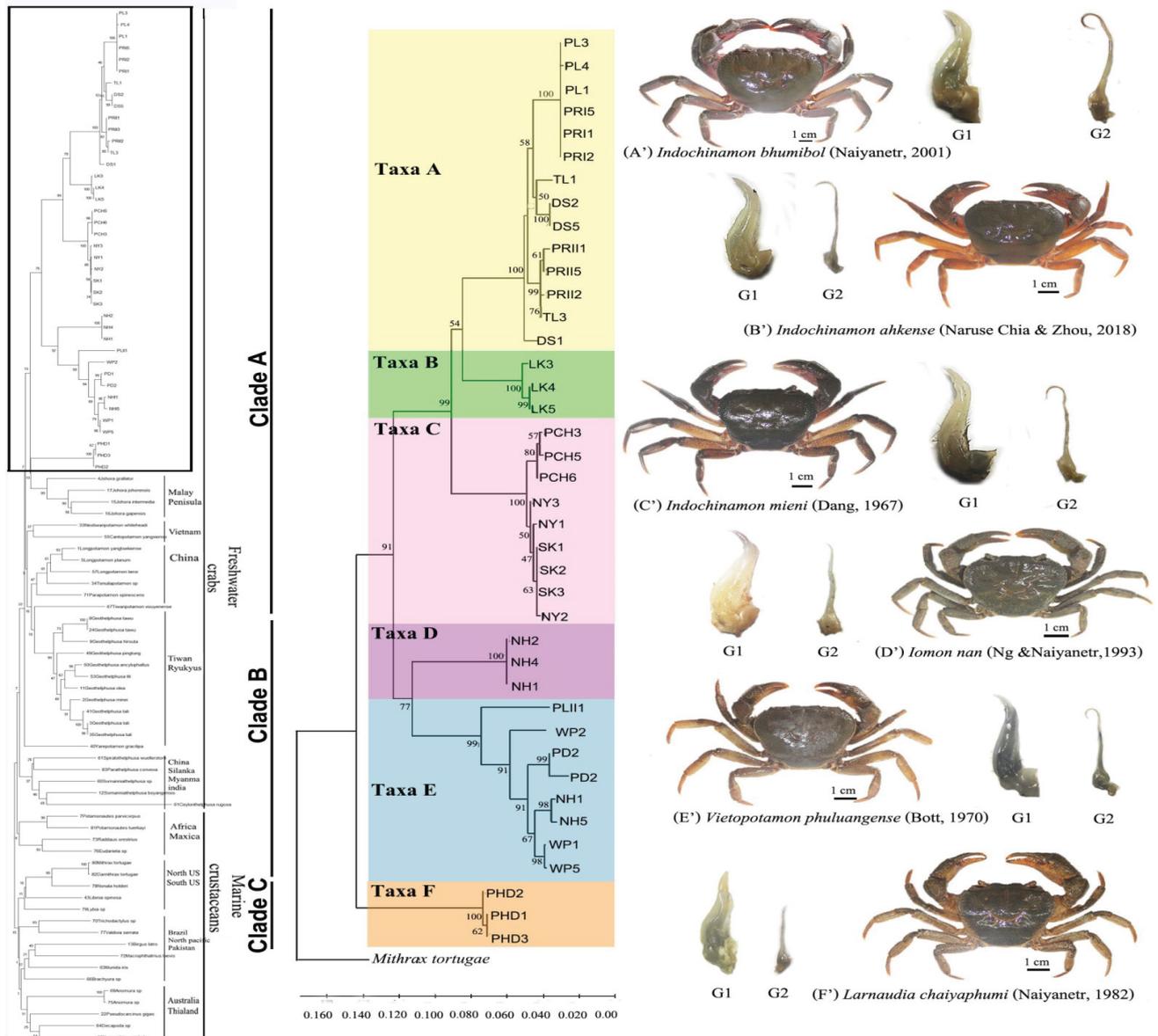


Figure 2 The phylogenetic tree of crabs from Phetchabun Mountains of Thailand represents based on NJ method. It was supported by 1000 bootstraps, K2P model using MEGA-X.

Io. nan and *V. phluangense* supported by NJ bootstrap 77%. The clade C was classified to be *L. chayaphumi*. The Clade A is crosser with the clade B than the clade C (Figure 2).

The average of the GC-content of these six mountain crabs (33.56-37.01%) corresponds to the study of Costa FO [37] that found the mean value of GC-content of *Uca annulipes* and *Uca perlexa* was $39.8 \pm 1.42\%$, and the study of Costa et al. [38] show that the 617 species of crustaceans shows GC-content 29.9% to 49.6%. The phylogenetic tree of the partial COI gene shows accurately the agreeable relationship between morphology and genetic distance of each species, but it difficult to identify without a specialist. These partial COI genes of these crabs studies can be distinguished from the other region freshwater crabs such as genus *Johora* from Malay Peninsula [39], genus *Geothelphusa* from Ryukyus (Taiwan), genus *Neotiwariopotamon* from Vietnam

[40], genus *Longpotamon*, *Tenuilapotamon*, *Parapotamon* from China [41], *Potamonautes* from South Africa [42]. It also clearly distinguished from the marine crabs, genus *Mithrax*, *Libinia* and *Lybia* from USA and European Union [43,44].

From the assessment of IUCN Red List, it was found that the 27% of threatened fauna's extinction is crustaceans. This study also found the report of mountain crabs that is considered to be: 1) Endanger are: *Indochinamon bhumibol* and *Iomon nan*; 2) Vulnerable such as *Indochinamon mieni*; 3) Least Concern such as *Larnaudia chayaphumi*; 4) Data Deficient such as *Vietopotamon phluangense*; 5) No Data such as *Indochinamon akhense* [44]. Therefore to conserve the wild fauna, conservationists and Non-Government Organizations have to apply the biodiversity information and co-operate with the local people to achieve sustainable results [45].

Conclusion

The results of the study's partial COI gene have become a tool of identification (DNA barcoding) of mountain crabs in the Indochina region and also taxonomical tool to be use in the biodiversity information. However, the studying only the partial COI gene might be not enough to infer genetic relationship of the genus. Therefore, many more mt-DNA genes need to be studied to increase the accuracy.

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Acknowledgements

This project received funding from The Promotion of Talented Science and Mathematics Teacher Unit (PSMT) and The Graduate Student Development (master's degree) income budget year 2020. Moreover, we would like to thank Mr. Ewe Choon Lee, BSc (Hons), MBA for proof reading the manuscript.

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