การยืนยันการจำแนกชนิดของปูแสมสกุล *Metopograpsus* H. Milne Edwards, 1853 (Crustacea: Grapsidae) จากจังหวัดชลบุรี โดยใช้ลำดับนิวคลีโอไทด์บางส่วนของยีนบนไมโทคอนเดรียบริเวณ 16S rRNA, 12S rRNA และ Cytochrome c Oxidase Subunit I (COI)

Verification on Morphological Identification of Grapsid Crabs Genus *Metopograpsus* H. Milne Edwards, 1853 from Chon Buri Province Using Partial Sequences of Mitochondrial 16S rRNA, 12S rRNA and Cytochrome c Oxidase Subunit I (COI) Genes

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บทคัดย่อ

การศึกษานี้ได้วิเคราะห์ลำดับนิวคลีโอไทด์บางส่วนของบริเวณยีน 16S rRNA, 12S rRNA และ Cytochrome c oxidase subunit I (COI) เพื่อจะแยกความแตกต่างระหว่างชนิดของปูแสมที่มีสัณฐานคล้ายกัน สกุล *Metopograpsus* 3 ชนิดจากจังหวัดชลบุรี คือ *Metopograpsus oceanicus* (Hombron and Jacquinot, 1846), *M. frontalis* Miers, 1880 และ *M. latifrons* (White, 1847) ผลการศึกษาบ่งชี้ว่าเครื่องหมายพันธุกรรมทุกประเภทสามารถบ่งชี้ความแตกต่างระหว่างชนิดได้ชัดเจน โดยความแตกต่างของลำดับนิวคลีโอไทด์ ระหว่างปูทั้งสามชนิด มีค่าระหว่าง 5.8% ถึง 7.9% (ยีน 16S rRNA และ 12S rRNA genes) และระหว่าง 8.7% ถึง 12.3% (COI) ส่วนความแตกต่างของลำดับนิวคลีโอไทด์ภายในชนิดมีค่า 0.0% ถึง 1.9% การวิเคราะห์ความสัมพันธ์ทางวิวัฒนาการที่ได้จากทุกยีน บ่งชี้ความแตกต่างระหว่างชนิดได้อย่างชัดเจน การวิเคราะห์ยีน 16S rRNA และ 12S rRNA โดยวิธี Maximum Likelihood, ML; Neighbor Joining, NJ; Maximum Parsimony, MP แสดงความใกล้ชิดของ *M. frontalis* และ *M. latifrons* อย่างไรก็ตาม ผลการวิเคราะห์ยีน COI แสดงความสัมพันธ์ใกล้เคียงกันของ *M. frontalis* และ *M. oceanicus*

คำสำคัญ : ยีน 16S rDNA ยีน 12S rDNA ยีน Cytochrome c oxidase subunit I การจำแนกชนิด *Metopograpsus*

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Abstract

We analyzed the partial sequences of mitochondrial 16S rRNA, 12S rRNA and Cytochrome c oxidase subunit I (COI) genes to discriminate three morphological species of grapsid crabs, *Metopograpsus oceanicus* (Hombron and Jacquinot, 1846), *M. frontalis* Miers, 1880 and *M. latifrons* (White, 1847). All genetic markers were informative for species identification. Interspecific nucleotide divergence among these species ranged from 5.8% to 7.9% (16S rRNA and 12S rRNA genes) and from 8.7% to 12.3% (COI gene). Intraspecific divergence levels ranged from 0.0% to 1.9%. Phylogenetic analyses based on all approaches revealed clear differentiation among the three *Metopograpsus species* (bootstrap values = 50 to 100). Mitochondrial 16S rRNA and 12S rRNA gene sequences suggested *M. frontalis* and *M. latifrons* were closely related by Maximum Likelihood, ML; Neighbor Joining, NJ; Maximum Parsimony, MP analyses. However, COI gene sequence suggested that *M. frontalis* and *M. oceanicus* were more closely related.

Keywords : 16S rRNA gene, 12S rRNA gene, Cytochrome c oxidase subunit I, species identification, *Metopograpsus*

Introduction

Analyses of mitochondrial gene sequences can greatly assist species identification and phylogenetic inference because mitochondrial genes do not undergo recombination and they are inherited maternally. Animal mitochondrial DNA (mtDNA) contains two ribosomal RNAs, 13 protein-coding genes, 22 transfer RNAs and a control region or a site for replication and transcription initiation. Most mtDNA regions can be amplified by available or slightly modified universal primers (Freeland, 2005). The length of sequences useful for phylogenetic analyses varies among genes. The lengths of the sequences available in the GenBank database range from approximately 400 basepairs (bp; 12S rRNA gene) to 600 bp (Cytochrome c oxidase subunit I gene, COI) of the total length of the mitochondrial genome, about 14 to 17 kb (Wolstenholme, 1992 cited in Hwang & Kim, 1999). In addition, molecular information can help determine whether morphological variation observed is due to genetics or the environment.

Identification of species based on morphology can be problematic for species with similar morphology (e.g., lobster Genus *Munida*, Macpherson & Machordom, 2005). For grapsid crabs, the identification of this group has also been somewhat problematic as there has been no global standardization. Worldwide, the genus *Metopograpsus* consists of six species, *M. frontalis* Miers, 1880, *M. oceanicus* (Hombron and Jacquinot, 1846), *M. latifrons* (White, 1847), *M. quadridentatus* Stimpson1858, *M. messor* (Forskål, 1775), and *M. thukuhar* (Owen, 1839) (Ng *et al*., 2008). In Thailand, there are at least four species (Matchajib, 1973; Naiyanetr, 1998) and species listings are still inconclusive. Matchajib (1973) described identification keys for four species found in Thai waters, *M. frontalis, M. oceanicus, M. latifrons* and *M. quadridentatus*. However, a recent survey reported all six species (e.g., Naiyanetr, 1998). Due to small differences in diagnostic morphological characteristics among species (Table 1), the identification of some species is still confusing (e.g., *M. messor* and *M. thukuhar*). We, therefore, attempt to use nucleotide sequences to verify three morphological species of grapsid crabs, *M. frontalis, M. oceanicus* and *M. latifrons* of the Family Grapsidae MacLeay, 1838. These species are common in the Gulf of Thailand and they are morphologically similar. The only key distinctive characters are the presence and absence of anterolateral spines (between *M. oceanicus* and the remaining species), the shape of telson (between *M. frontalis* and *M. latifrons*) and the shape of males' first gonopod (Ai-Yun and Si-Liang, 1991; Paulay, 2007). Moreover, their native ranges greatly overlap in the Indo-Pacific region, including South China Sea, Hawaii, Guam, Kenya and Tanzania (Marine Species Identificaiton portal, http://species-identification.org/). The report of the distribution range has been clouded by taxonomic confusion.

This study evaluated and compared the utility of three mitochondrial genes to detect genetic divergence and phylogenetic relationships among *Metopograpsus* species. 16S rRNA, 12S rRNA and COI genes have been useful to identify taxa in crustaceans (Schubart *et al*., 2001; Robles *et al*., 2007; Malay and Paulay, 2009), amphibians (Vences *et al*., 2005) and birds (Hebert *et al*., 2004). However, the varying mutation rates among different genes make them appropriate for different applications (Lefébure *et al*., 2006; Hwang and Kim, 1999). The least conservative COI tends to be useful for the detection of a recently diverged species while a more conservative 16S rRNA gene tends to be more useful for older lineages. In our case the comparisons among the three genes may offer insights on their usefulness in discriminating species within this genus as well as closely related morphological species.

Materials and methods

Taxonomic Sampling

We analyzed approximately 500 to 600 basepair fragments of mitochondrial 16S rDNA, 12S rDNA and COI

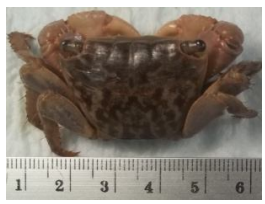
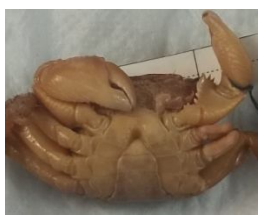
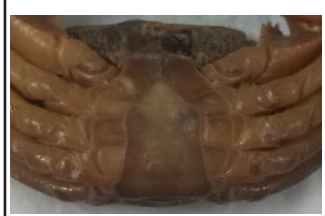
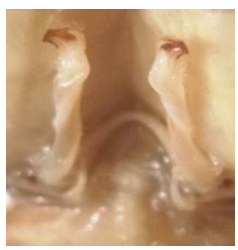
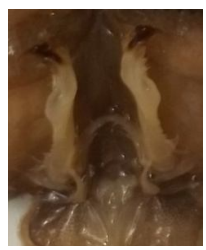
sequences of three grapsid crab species, *Metopograpsus frontalis, M. oceanicus* and *M. latifrons* collected from Chon Buri Province, Thailand during 2008 to 2010. These samples were collected in rocky areas along the seashore or shallow water in the Samaesarn Islands protected area using nets and pincers as well as in open water in the Angsila area using local fishing gear by fishermen. We analyzed one to four individuals per species (Table 2). All specimens were identified to species level based on Ai-Yun and Si-Liang (1991) and Paulay (2007) (Table 1). All specimens were preserved in 95% Ethanol.

In addition, we analyzed 16S rDNA sequences of four *Metopograpsus* species from the GenBank database (Table 2). As an outgroup, we included sequences of three xanthid species, *Leptodius exaratus* collected from Chon Buri Province, *Pilodius flavus* and *Chlorodiella crispipleopa* from the GenBank database (Table 2).

Genetic analysis

We extracted genomic DNA from the third or fourth periopod of specimens using a commercial DNA extraction kit (Invitrogen, USA), following the manufacturer's protocol. We evaluated the quality and quantity of extracted DNA against 0.2 ng of 100 bp DNA standards (RBC Bioscience) on a 1% agarose gel. The quantity of extracted DNA was determined by its intensity under UV light, compared to that of a known quantity

Table 1 Main morphological diagnostic characters of the three *Metopograpsus* species based on descriptions provided in Ai-Yun and Si-Liang (1991) and Paulay (2007).

Traits	*M. oceanicus*	*M. frontalis*	*M. latifrons*
Present/absent anterolateral spines	 (present)	 (absent)	 (absent)
Shape of telson	 (obtusely triangular)	 (obtusely triangular)	 (bluntly triangular)
Shape of male first gonopod (stout-chitinous process)	 (trumpet-like)	 (spoon-shaped lobe)	 (three whorls, petal-like distally)

of a DNA fragment, and the fragment size of extracted DNA indicated the DNA quality. The electrophoresis was performed at 66 volts for 25 minutes. After electrophoresis, the agarose gel (Vivantis) was stained using Ethidium bromide (Etbr) for 10 minutes and then visualized on a UV transluminator (Vilber Lourmat ETX-40M, France).

We amplified the partial 16S rDNA, 12S rDNA and COI fragments using the polymerase chain reaction (PCR, 9700 GeneAmp PCR system, Applied Biosystems, USA) and primer pairs 16S 1472 (5'-AGA TAG AAA CCA ACC TGG-3') and 16S L2 (5'-TGC CTG TTT ATC AAA AAC AT-3') (Mathews and Anker, 2009); 12S H2 (5'-ATG CAC TTT CCA GTA CAT CTA C 3') (Fratini *et al*., 2005) and 12S L4 (5' GTG CCA GCM GCC GCG GTT A '3) (Schubart *et al*., 2006), and COI LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-'3) and COI HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-'3) (Folmer *et al*., 1994). A PCR cocktail (with 30 µl reaction volume) contained 10-20 ng of DNA templates, 3 µl 10X PCR buffer (100 mM Tris-HCl, pH 9.1 at 20°C, 500 mM KCl and 0.1 % Triton™X-100), 0.24mM of each dNTP, 1.5 mM $MgCl_2$, 0.1-0.5 µM each primer, and 0.5-1 U *Taq* polymerase (Mathews and Angker 2009; Folmer *et al*., 1994). The PCR temperature profile consisted of an initial denaturation at 94°C for 10 min, followed by 40 cycles of 94°C for 1 min, 45-48°C for 1 min, 72°C for 1.5 min and final extension 72°C for 10 min (Mathews and Angker, 2009; Folmer *et al*., 1994).

We obtained sequences either from direct sequencing of PCR products with good quality (> 40 ng/µl) or from cloned PCR fragments. The PCR products were purified using HiYield™ Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience) following the manufacturer's protocol and the plasmids were purified using HiYield Plasmid mini kit (RBC Bioscience). For each individual, sequencing was performed in both forward and reverse directions using an automated sequencer (ABI 3730XL, Applied Biosystem) at a commercial laboratory (1st Base Pte. Ltd., Malaysia). Each sequence was manually edited using Sequence Scanner software version 1.0 (Applied Biosystem), and assembled (forward and reverse sequences) using CAP3 sequence assembly program (Huang & Madan, 1999). To verify these sequences, we aligned them with those available in the GenBank database using the BLAST

Table 2 Number of samples collected from Samaesarn Islands and description of sequences available in the GenBank database.

	Samples collected from Chon Buri province			Sequences available in GenBank	
	12S rRNA	16S rRNA	COI	Accession Number	Marker
Metopograpsus frontalis	4	3	3	FR871300.1	16S rRNA
M. oceanicus	4	2	3	-	-
M. latifrons	3	1	4	AJ784028.2	16S rRNA
M. quadridentatus	-	-	-	DQ062732	16S rRNA
M. thukuhar	-	-	-	AJ784027.2	16S rRNA
Outgroup					
Leptodius exaratus	2	2		-	-
Pilodius flavus				JN107968.1	COI
Chlorodiella crispipleopa				JN107925.1	COI

program (NCBI). An appropriate sequence should have more than 90% similarity to the same DNA region of closely related species. All sequences were aligned using the algorithm Clustal W (progressive alignment) (Thompson *et al*., 1994) implemented in the BioEdit sequence alignment editor version 7 (Hall 1999).

Genetic divergence estimation and phylogenetic analyses

We calculated nucleotide divergence among aligned sequences based on the model Kimura two Parameters (K2P), a common model used to estimate divergence among DNA barcodes. To infer phylogeny, we constructed phylogenetic trees using three approaches, namely Maximum Likelihood (ML), Neighbor Joining (NJ) and Maximum Parsimony (MP) and the best evolutionary model (except for MP). All analyses were performed using the software MEGA (Molecular Evolutionary Genetic Analysis) version 5 (Tamura *et al*., 2011). The best model was suggested by MEGA and indicated by the lowest Bayesian Information Criterion (BIC) score. The tree search strategy was close-neighbor-interchange. Gaps were excluded from all analyses. To evaluate the robustness of the constructed phylogenetic trees using ML, NJ and MP analyses, we performed bootstrap analyses with 1,000 replications. The consensus tree only shows the bootstrap values ≥ 50.

Results and discussion

The total length of aligned mitochondrial 16S rDNA, 12S rDNA and COI of examined crabs ranged from 509 (16SrDNA) to 653 (COI) basepairs (excluding the primer regions) (Table 3). The number of variable sites accounted for 10.1% to 18.1% of the total fragment length. The best evolutionary model that described the sequence variation among taxa for 16S rDNA, 12S rDNA and COI sequences was Tamura 3 Parameter with Invariable sites (T92+I; I=0.66), Tamura-Nei 93 with Gamma distribution (TN93+G; G=0.22) and Tamura-Nei 93 with Gamma distribution, (TN93+G; G=0.18), respectively.

Interspecific genetic divergence (K2P distance) among the three *Metopograpsus* species obtained from partial sequences of mitochondrial 16S rRNA, 12S rRNA and COI genes ranged from 5.8% to 7.9% (16S and 12S rRNA genes) and 8.7% to 12.3% for COI gene (Table 4). Interspecific divergence values among the three *Metopograpsus* species obtained from the mitochondrial COI gene were the highest compared to those from 12S and 16S rRNA genes. Interspecific divergence between

Table 3 Variation of partial sequences of mitochondrial 16S rRNA, 12S rRNA and COI genes of *Metopograpsus* spp. included in this study.

Variables	Nucleotide Variation		
	16S rDNA	12S rDNA	COI
Sequence length (bp)	509	592	653
Gap/missing data	23	33	-
Variable sites	92 (18.1%, 5 taxa); 51 (10.1%, 3 taxa)	62 (10.5%)	96 (14.7%)
Number of sequences	10	11	10
Number of taxa	5	3	3
Nucleotide composition (A, C, G, T)	34.6; 18.2; 11.1; 36.1	40.1; 9.1; 16.0; 34.9	37.0; 16.6; 19.0; 27.4
Best evolutionary model	T92+I; I=0.66	TN93+G; G=0.22	TN93+G; G=0.18

Table 4 Pairwise nucleotide divergence of mitochondria (a) 16S rDNA, (b) 12S rDNA and (c) COI sequences among *Metopograpsus* species and the outgroup using K2P distance (%).

(a) 16S rDNA

Species		1	2	3	4	5	6	7	8	9	10	11
1	*Metopograpsus frontalis*1											
2	*M. frontalis*2	0.0										
3	*M. frontalis*3	0.0	0.0									
4	*M. frontalis* FR871300.1	1.9	1.9	1.9								
5	*M. latifrons* AJ784028.2	6.3	6.3	6.3	5.8							
6	*M. latifrons*1	6.3	6.3	6.3	5.8	0.0						
7	*M. oceanicus*1	6.5	6.5	6.5	6.8	7.4	7.4					
8	*M. oceanicus*2	6.3	6.3	6.3	6.8	7.2	7.2	0.0				
9	*M. quadridentatus* DQ062732	8.8	8.8	8.8	9.1	9.0	9.0	10.2	10.0			
10	*M. thukuhar* AJ784027.2	12.8	12.8	12.8	12.2	11.5	11.5	10.9	10.7	12.4		
11	Leptodius exaratus1	25.5	25.5	25.5	25.8	25.3	25.3	25.0	24.7	26.2	29.9	
12	*L. exaratus*2	25.2	25.2	25.2	25.5	25.0	25.0	24.7	24.4	25.9	29.5	0.0

(b) 12S rDNA

Species		1	2	3	4	5	6	7	8	9	10	11	12
1	*Metopograpsus frontalis*1												
2	*M. frontalis*2	0.4											
3	*M. frontalis*3	0.2	0.5										
4	*M. frontalis*4	0.0	0.4	0.2									
5	*M. latifrons*1	6.6	7.0	6.8	6.6								
6	*M. latifrons*2	6.2	6.6	6.4	6.2	0.4							
7	*M. latifrons*3	6.2	6.6	6.4	6.2	0.4	0.0						
8	*M. oceanicus*1	7.2	7.7	7.2	7.2	7.8	7.9	7.9					
9	*M. oceanicus*2	7.2	7.7	7.2	7.2	7.8	7.9	7.9	0.0				
10	*M. oceanicus*3	7.2	7.7	7.2	7.2	7.8	7.9	7.9	0.0	0.0			
11	*M. oceanicus*4	7.2	7.7	7.2	7.2	7.8	7.9	7.9	0.0	0.0	0.0		
12	*Leptodius exaratus*1	28.3	28.8	28.5	28.3	28.6	28.3	28.3	27.8	27.8	27.8	27.8	
13	*L. exaratus*2	28.3	28.8	28.5	28.3	28.6	28.3	28.3	27.8	27.8	27.8	27.8	0.0

Table 4 Pairwise nucleotide divergence of mitochondria (a) 16S rDNA, (b) 12S rDNA and (c) COI sequences among *Metopograpsus* species and the outgroup using K2P distance (%).

(c) COI

Species		1	2	3	4	5	6	7	8	9	10	11
1	*Metopograpsus frontalis*1											
2	*M. frontalis*2	0.2										
3	*M. frontalis*3	0.5	0.3									
4	*M. latifrons*1	11.6	11.8	11.9								
5	*M. latifrons*2	11.9	12.1	12.3	0.6							
6	*M. latifrons*3	11.8	11.9	12.1	0.5	0.2						
7	*M. latifrons*4	11.9	12.1	12.3	0.6	0.3	0.2					
8	*M. oceanicus*1	8.9	9.1	9.3	10.8	10.8	11.0	11.0				
9	*M. oceanicus*2	8.7	8.9	9.1	10.7	10.7	10.8	10.8	0.2			
10	*M. oceanicus*3	8.9	9.1	9.3	10.8	10.8	11.0	11.0	0.3	0.2		
11	*Pilodius flavus* JN107968.1	20.8	20.8	21.0	18.6	19.0	18.8	18.8	19.0	18.8	19.0	12
12	*Chlorodiella crispipleopa* JN107925.1	19.5	19.5	19.7	20.0	21.1	20.0	21.5	22.0	21.8	22.0	19.3

Metopograpsus spp. and the outgroup ranged from 19.0% to 29.9%. In addition, based on 16SrDNA sequences, the divergence between the three *Metopograpsus* species and *M. quadridentatus* and *M. thukuhar* deposited in the GenBank ranged from 8.8% to 12.8%. Intraspecific genetic divergence revealed by all genes ranged from 0.0% to 1.9%.

All three markers proved to be useful for differentiating this set of *Metopograpsus* species with COI sequences by revealing their highest levels of divergence. Our study suggested a similar resolution of 16S and 12S rDNA sequences for species discrimination. In addition, 16SrDNA sequences suggest the three *Metopograpsus* species found in Chon Buri are more closely related to each other than to *M. thukuhar* from Inhaca Island, Mozambique and *M. quadridentatus* from China. We also observed nucleotide differences in our collection of *M. frontalis* and the *M. frontalis* sequence in the GenBank (collected from Australia; Schubart, 2011). These differences probably reflect the difference between populations.

The level of interspecific divergence detected in this study is comparable to those observed in other Crustacea. Based on COI sequences, Costa *et al*. (2007) suggested interspecific divergence among species within the Order Decapoda ranging from 4.92% to 23.66% (an average of 17.16%). For 16S rDNA sequences, the interspecific divergence among cryptic species of lobster Genus *Munida* ranged from 1.4% to 9.6% and COI ranged from 8.2% to 14% (Macpherson & Machordom, 2005).

Although Consortium for the Barcode of Life proposed COI gene to be a standard DNA barcode for animal taxa due to several advantages (Hebert *et al*., 2003), this gene may not be informative for some taxa, such as Porifera (Sponges), Ctenopora (Comb Jellies) and Cnidarian (Coral) (Shearer *et al*., 2002; Huang *et al*., 2008). Furthermore, COI may be prone to mutation saturation due to its high substitution rate (about 0.3 substitutions

per site compared to 16S rRNA marker, about 0.2 substitutions per site) (Lefébure *et al*., 2006; Hiller *et al*., 2006). Saturation is commonly found in arthropods and may confound phylogenetic information contained in sequences (Strimmer & Haeseler, 2009). Furthermore, COI-like sequences is common in crustaceans and might cause problems in species identification (Buhay, 2009).

Mitochondrial 16S rRNA and 12S rRNA genes could therefore serve as an alternative or a complementary marker for species identification. In some taxa such as Amphibia (Vences *et al*., 2005) and fish (Steinke *et al*., 2005), 16S rRNA genes may be more informative than COI. In addition, Schubart (2011) showed clear distinction among *Metopograpsus* species included in his phylogenetic study of partial sequences of 16S rRNA gene only. A combination of 16S and 12S rRNA genes are useful for identification of species complex *Callinectes* (Robles *et al*., 2007) and inferring evolution of tree-mangrove climbing Grapsid species (Fratini *et al*., 2005).

Phylogenetic relationships among *Metopograpsus* spp.

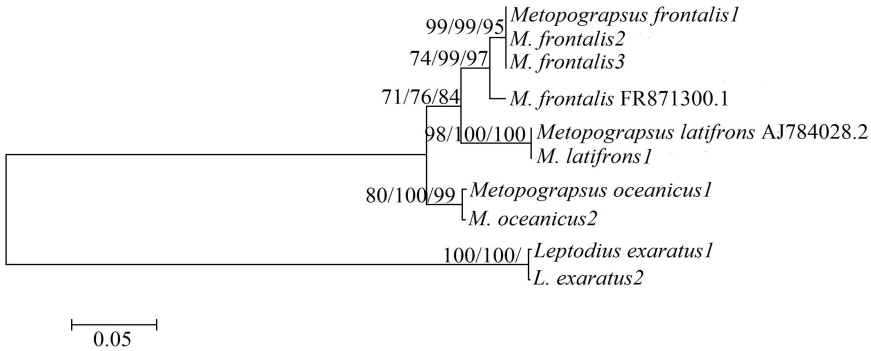
Phylogenetic analyses based on all approaches revealed clear differentiation among the three *Metopograpsus* species (bootstrap values = 50 to 100, Figure 1). However, the relationships among them were inconsistent across genes. Based on all analyses of partial sequences of 16S and 12S rRNA genes, *M. frontalis* and *M. latifrons* were closely related (bootstrap value ≥ 58) while all analyses of COI sequences showed that *M. frontalis* was closely related to *M. oceanicus* compared to *M. latifrons* with bootstrap values ≥ 51.

Most phylegenetic studies of Grapsid crabs typically have relied on 16S and 12S rDNA sequences. Our results based on 16S and 12S rDNA sequences were consistent to those observed in Schubart (2011) in that *M. frontalis* and *M. latifrons* were closely related. Using 16S rRNA gene, Schubart (2011) suggested *M. frontalis* and *M. latifrons* were closely related compared to *M. thukuhar* and *M. quadridentatus* (there was no *M. oceanicus*). Based on a combination of 16S and 12S rRNA genes and mangrove tree-climbing behavior, Fratini *et al*. (2005) showed that *M. latifrons* had a distant relationship to *M. thukuhar*. However, our phylogenetic analyses showed inconsistent relationships between *M. thukuhar* and *M. quadridentatus* deposited in the Genbank and the three *Metopograpsus* species found in Chon Buri (data not shown). MP suggested that *M. thukuhar* and *M. quadridentatus* were distantly related to the three *Metopograpsus* species, but NJ and ML analyses suggested that the two species were closely related to *M. latifrons*. Although our study along with Schubart (2011) and Fratini *et al*. (2005) suggested this group was monophyletic, 16S rRNA still cannot resolve relationships among all species within this genus. In contrast to 12S and 16S rRNA genes, the COI results suggested that *M. frontalis* and *M. oceanicus* was more closely related compared to *M. latifrons*.

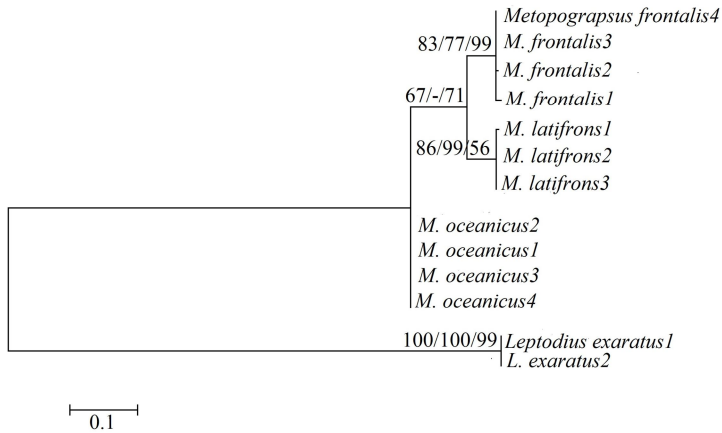
The relationships revealed by 16S and 12S rRNA gene sequences are also in agreement with the morphological similarities between *M. frontalis* and *M. latifrons* (Table 1; Matchajib, 1973). However, our results did not support the morphological similarities between *M. oceanicus* and *M. quadridentatus*. In addition, the inconsistent relationships revealed by 16S-12S rRNA and COI genes are probably due to their different mutation rates. COI gene evolves faster than 16S and 12S rRNA genes (Hebert *et al*., 2003; Lefébure *et al*., 2006). Further investigation with reasonable number of species and specimens across native areas using nuclear H3 genes would provide additional insights on phylogenetic relationships of *Metopograpsus* species as exemplified in Lai *et al*. (2009).

Conclusions

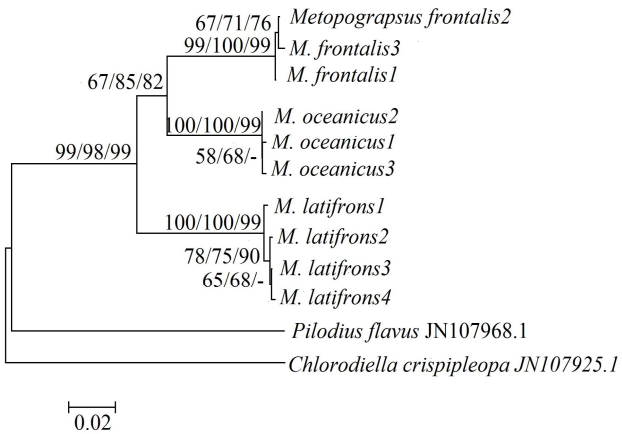
The three mitochondrial genes (16S rRNA, 12S rRNA and COI) investigated in this study were useful for distinguishing morphologically similar *Metopograpsus* spp., with COI gene revealing the highest divergence levels among species (8.7% to 11.9%). Phylogenetic relationships



(a)



(b)



(c)

Figure 1 Phylogenetic trees among three *Metopograpsus* species based on partial sequences of mitochondrial (a) 16S rRNA and TN93+I, (b) 12S rRNA and TN93+G and (c) COI genes and TN93+G models (MP analysis without model). Percentage values supporting each node were generated from bootstrap 1000 replications of ML, NJ and MP, respectively.

of these *Metopograpsus* species remain inconclusive with the three genes however showed inconsistent results. 16S and 12S rRNA genes suggested *M. latifrons* and *M. frontalis* were closely related but COI suggested *M. frontalis* and *M. oceanicus* were a sister taxa. To clarify the relationships, we may need molecular data from nuclear genes.

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