Molecular Analysis of Sergestid Shrimp *Acetes* spp. from Coastal Water of Sarawak, Malaysian Borneo Using CO1 Sequence

Ruhana Hassan[1,2]* and Muhammad Nur Arif Othman[2]

[1]Centre for Pre University Studies, Universiti Malaysia Sarawak, Sarawak, Malaysia
[2]Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Sarawak, Malaysia

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Abstract

Molecular analysis is an alternative to the conventional method of species identification. Misidentification of sergestid shrimp *Acetes* using morphological assessment data could occur due to its small size and the requirement of skilled microscope personnel. This study aims to evaluate the diversity of *Acetes* in Sarawak coastal water using mitochondrial cytochrome c oxidase 1 (CO1) gene analysis. The samples were collected from three sampling sites namely Miri, Lundu and Telaga Air, Sarawak. Based on CO1 gene analysis, two *Acetes* species were revealed, namely *Acetes erythraeus* and *A. serrulatus* with intraspecific variation of 0.20%-2.40% and 0.20%-1.19%, respectively. Besides, the diversity of this species based on geographical pattern could be observed, with two subclades of *A. erythraeus* (Miri, Sarawak and Lundu, Sarawak) and *A. serrulatus* (Telaga Air, Sarawak and west coast of Malaysia). The phylogenetic trees show that both species are reciprocally monophyletic. This finding implies that COI gene is a reliable genetic marker in species identification of *Acetes*.

Keywords: CO1 gene; *Acetes*; monophyletic; biodiversity
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1. Introduction

Molecular study involves a non-destructive method where only a small quantity of DNA sample is needed when running the analysis. It has been practiced globally to aid identification of a species, study the relationships among and within population of organisms from different regions, assess the level of genetic variability and estimate the gene flow of targeted species [1]. This technique is claimed to provide precise, timesaving, consistent and reliable information on the targeted organisms.

Cytochrome oxidase subunit 1 (CO1) gene is a widely used genetic marker due to its conserved characteristics, making it suitable for DNA barcoding exercise in animal species. Besides, this gene is highly variable among species and it has higher evolutionary rate compared to nuclear genes, making it useful to distinguish closely related species [2]. The universal LCO1490 and HCO2198 primers are commonly used to amplify CO1 region [3]. These primers have shown to be

*Corresponding author: Tel.: (+60) 82582332 Fax: (+60) 82582330
E-mail: hruhana@unimas.my

successful in the amplification of CO1 gene, which helped in the identification of invertebrate species such as Echinodermata, Mollusca, Annelida, Pogonophora, Arthropoda, Nemertinea, Echiura, Sipuncula, Platyhelminthes, Tardigrada and Coelenterata [3-5]. Additionally, the primers also have been successful in species identification of shrimps and prawns [6-9].

Miri is a coastal city in the northeastern Sarawak, Malaysia, sharing the border with Brunei. The city is situated on the alluvial plain of the Miri River which then flows to the South China Sea. Miri coastal zone is made up of coral reef (Sibuti area); seagrass and beach forest ranging from Kuala Bakam to Lutong; mangrove forests found in Baram and Bakam and peat swamp areas in Senadin and Tudan. Miri coastal water is well-known for sergestid shrimp *Acetes* spp., locally known as 'Bubok' [10]. *Acetes* is the main ingredient of local menus, namely 'belacan', a fermented shrimp paste and 'cincalok', a shrimp pickle. Shrimp and its products could be sold as high as RM50 (approximately USD 12) per kilogram. The abundance of the shrimp during peak season, in between February until April every year, has provided additional income to the fishermen and local communities [11].

Recently, five out of 14 species of *Acetes* CO1 sequences are available in the database of GenBank, namely *Acetes serrulatus*, *A. sibogae*, *A. americanus*, *A. japonicus* and *A. indicus*. For *Acetes*, the species identification using morphological approach is very laborious, as it requires certain level of skill in microscope handling and is time consuming. In order to aid identification of *Acetes*, molecular approach is needed, and such data could also help to solve phylogenetic relationships among species. Therefore, the objectives of this study are (i) to develop DNA barcodes to discriminate between *Acetes* species in coastal water of Sarawak using CO1 gene, and (ii) to assess the phylogenetic relationships among *Acetes* species.

2. Materials and Methods

Acetes samples were collected from three sampling sites; Miri (LC1, N 4°30'16.7" E 113°59'13.6"), Telaga Air (LC2, N 1°45'49.5" E 109°52'00.3") and Lundu (LC3, N 1°40'38.0" E 110°12'37.9) (Figure 1), with help from local fishermen. The shrimp samples were preserved in 70% ethanol in the field, specifically for molecular analysis work, and brought back to the laboratory in the Faculty of Resource Science and Technology, Universiti Malaysia Sarawak. Prior to genetic analysis work, *Acetes* samples were identified using identification keys provided by Fischer and Bianchi [12], Amin *et al*. [13], Omori [14], Pathansali [15] and Vereshchaka *et al*. [16]. Two species were morphologically identified as *A. erythraeus* and *A. serrulatus*. A total of 14 individuals (10 samples for *A. erythraeus* and 4 samples for *A. serrulatus*) were used in molecular study (Table 1).

Total genomic DNA extraction of *Acetes* was carried out following the modified Cetyl-trimethyl Ammonium Bromide (CTAB) protocol proposed by Doyle and Doyle [17]. Amplification of cytochrome oxidase subunit 1 (CO1) gene fragment was conducted using primer LCO1490 and HCO2198 designed by Folmer *et al*. [3].

PCR protocol was carried out following the method proposed by Costa *et al*. [18] in 25 µl reaction mixture containing 17.2 µl ultrapure water, 2.5 µl 10X buffer, 1.5 µl $MgCl_2$ (50mM), 0.13 µl dNTPs (25mM), 0.75 µl primer forward LCO1490 (10µM), 0.75 µl primer reverse HCO2198 (10µM), 0.2 µl Taq DNA polymerase (5U) and 2.0 µl DNA template. One negative control was included in every PCR batch. The cycle parameters consist of pre-denaturation step at 94°C for 1 min, 5 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 1 min 30 s, extension at 72°C for 1 min, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 51°C for 1 min 30 s, extension at 72°C for 1 min and final extension at 72°C for 5 min. The PCR products were sent to Apical Scientific Sdn. Bhd. Selangor Malaysia for PCR product purification followed by single pass DNA sequencing, forward and reverse strands.

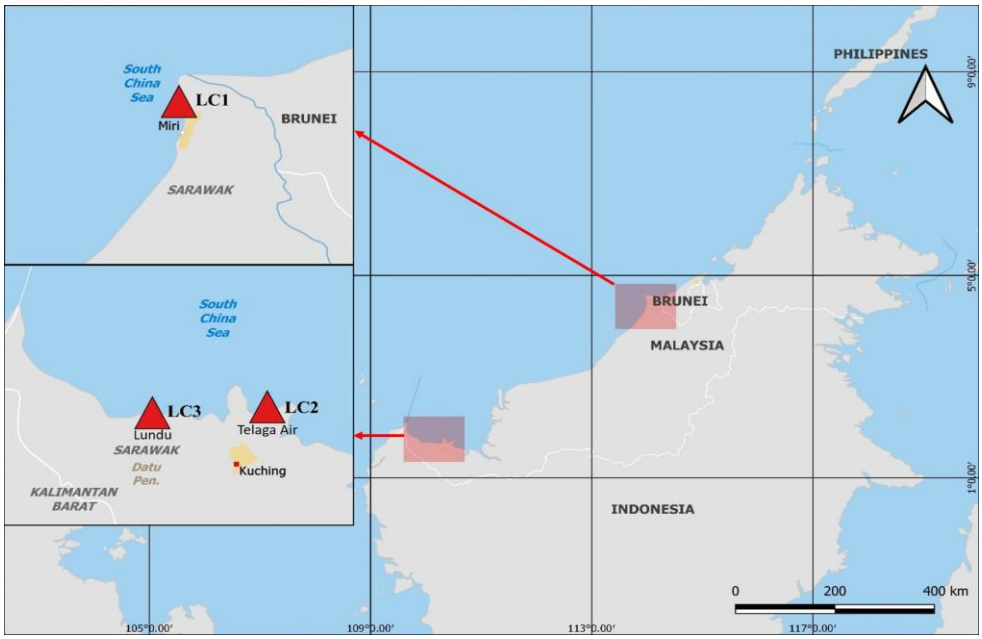

Figure 1. Locations of sampling sites in Sarawak coastal water as noted in red triangles; Miri (LC1): Telaga Air (LC2), Lundu (LC3)

Table 1. Locality, number of samples and field voucher of *Acetes* for molecular study

Species	Locality	No. of Samples	Field Voucher
A. serrulatus	Telaga Air, Sarawak	4	T01 – T04
A. erythraeus	Miri, Sarawak	6	M01 – M06
	Lundu, Sarawak	4	L01 – L04

CHROMAS software was used to display CO1 sequence results. The sequences were subjected to Basic Local Alignment Search Tool (BLAST) for sequence validation. Multiple alignments of the sequences were constructed using the CLUSTAL X program (version 1.81) and subsequently aligned with other sequences of *Acetes* from the GenBank and *Allosergestes pectinatus* was chosen as outgroup (Table 2).

Table 2. List of *Acetes* and other species analysed in this study

Species	Locality	Accession no.
A. serrulatus	Malaysia	HQ630561, HQ630562
A. sibogae	Malaysia	HQ630587
A. aff. sibogae	India	KX399434
A. americanus americanus	Brazil	KX196595
A. japonicus	Malaysia	HQ630575
A. japonicus	China	KF977240
A. indicus	Malaysia	HQ630497
A. indicus	India	MK784109
Allosergestes pectinatus (outgroup)	USA	MH572651

The phylogenetic relationship of 24 CO1 sequences were analysed using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BPP) using PAUP (version 4.0). The genetic divergence was obtained using Kimura's Two Parameter Model [19]. Bayesian analysis was conducted based on the substitution model and standard phylogenetic parameters of Akaike Information Criterion (AIC) using MrBayes [20]. AIC is a mathematical method for evaluating how well a model fits the data. Modeltest 3.7 together with PAUP (Version 4.0) was used to select the suitable model for dataset [21]. Model GTR+I+G was chosen for ML and BPP analyses.

3. Results and Discussion

Amplification of CO1 gene was successful for six samples of *A. erythraeus* from Miri (M01, M02, M03, M04, M05, M06), four samples of *A. erythraeus* from Lundu (L01, L02, L03, L04) and four samples of *A. serrulatus* from Telaga Air (T01, T02, T03, T04). The BLAST analysis showed that sequences of T01, T02, T03 and T04 were approximately 98% similar to *A. serrulatus* (HQ630561 and HQ630562) from west coast of Malaysia. Sequences of M01, M02, M03, M04, M05, M06, L01, L02, L03 and L04 which are *A. erythraeus* based on morphological analysis show 85% similarity to *A. japonicus* (KF977240) from China (Table 3). The limited similarity of about 15% between *A. serrulatus* samples in this study with *A. japonicus* from the GenBank suggested that both are most likely from different species.

Table 3. Summary of BLAST results for all CO1 sequences obtained in this study

Species	Voucher no.	BLAST top hit	Accession no.	% Similarity
A. serrulatus	T01, T02, T03, T04	*A. serrulatus*	HQ630561, HQ630562	98
A. erythraeus	M01, M02, M03, M04, M05, M06	*A. japonicus*	KF977240	85
	L01, L02, L03, L04	*A. japonicus*	KF977240	85

The intraspecific variations of four samples of *A. erythraeus* from Lundu (L01, L02, L03, L04) were 0.39% - 0.99% while the intraspecific variation of six samples of *A. erythraeus* from Miri (M18, M22, M23, M26, M43, M44) were 0.00%-0.59%. The intraspecific variation of *A. erythraeus* from Lundu and Miri were 0.20%-2.40%, with variations of 1 to 12 bp out of 509 bp. The comparison of intraspecific value of *A. erythraeus* from Lundu and Miri with samples from other locality could not be done due to unavailability of *A. erythraeus* CO1 information in the GeneBank. *A. serrulatus* from Telaga Air (T01, T03, T05, T09) had intraspecific variation of 0.20%-1.19%. The intraspecific divergence between *A. serrulatus* found in Telaga Air, Sarawak and *A. serrulatus* from Malaysia (HQ630561, HQ630562) ranged from 1.80% to 2.83%. To summarise, *Acetes* sample sequences in this study that has variation between minimum value and 2.83% fall into similar species category. The results in this study are concordant with previous studies of intraspecific CO1 gene variation on crustacean studies [22, 23, 8]. For example, Quan *et al*. [23] had reported intraspecific variation of five different shrimps namely *Metapenaeus affinis, Metapenaues ensis, Penaeus chinensis, Penaeus japonicus* and *Penaeus penicillatus* ranged from 0.20%-1.20%. Moreover, Udayasuriyan *et al*. [8] found that the intraspecific variation of *Macrobrachium rosenbergii* from Brazil and India ranged from 1.39% to 1.61%. Meanwhile, 13 species of *Penaeus* collected from USA, Brazil, Taiwan and Spain had intraspecific variation of 0.00% - 3.00% [22]. In

terms of interspecific variation, both *A. erythraeus* and *A. serrulatus* in this study had variation between 19.17% and 20.20%.

The phylogenetic trees of *Acetes* in Sarawak constructed using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BPP) are shown in Figure 2 with agreement in the tree topologies. The phylogenetic analysis successfully revealed two major clades (clade *A. erythraeus* and clade *A. serrulatus*) and four subclades namely SC A, SC B, SC C and SC D. Both major clades agreed well with species identification of *Acetes* using the morphological approach.

All *A. erythraeus* samples in this study formed a clade with strong bootstrap values of 100% (MP), 100% (ML) and 1.00 (BPP). Detailed examination of *A. erythraeus* clade reveals two subclades in agreement with the geographical distribution of the samples. The first subclade (SC A) consists of *A. erythraeus* from Miri, Sarawak while the second subclade (SC B) is made up of *A. erythraeus* from Lundu, Sarawak. Interestingly, the phylogenetic trees revealed that single individual of *A. erythraeus* from Miri (M02) was grouped together with *A. erythraeus* from Lundu suggesting that there is a relationship between the two populations. Future work should involve more samples in order to resolve the low bootstrap values obtained in this study. In the present study, *A. erythraeus* originated from Lundu probably migrate to Miri during the southwest monsoon because of the direction of wind that causes the current flow towards the north of Sarawak, as Akhir [24] claimed that the water circulation of South China Sea is influenced by monsoon seasons. A similar result was reported by Aziz *et al*. [25] claiming that migration of *A. japonicus* from Malacca and Perak, on the west coast of Peninsular Malaysia, was observed based on RAPD results.

A. serrulatus samples in this study formed a single clade with strong bootstrap values of 100% (MP), 100% (ML) and 1.00 (BPP), therefore *A. serrulatus* is monophyletic. Two subclades could be observed within clade of *A. serrulatus*, based on geographical distributions. The first subclade (SC C) comprises of *A. serrulatus* from Peninsular Malaysia while the second subclade (SC D) contains *A. serrulatus* from Telaga Air, Sarawak.

The phylogenetic trees in this study indicated that CO1 gene is a good marker for the study of the geographical distribution of *Acetes* shrimp. Previous studies have shown the effectiveness of CO1 gene marker in identification of shrimps and prawns [6, 8, 9]. Bilgin *et al*. [6] revealed 12 different species of shrimps in Turkish seas using CO1 gene with the addition of a single cryptic species. Wong *et al*. [9] successfully revealed four different species of *Acetes* along the west coast of Peninsular Malaysia based on CO1 gene information. In addition, Udayasuriyan *et al*. [8] tested the efficiency of using CO1 gene to identify five species of prawns and showed positive results as being a good marker for species identification.

4. Conclusions

In this study, the putative CO1 gene confirmed that the *Acetes* samples from Telaga Air, Sarawak were identified as *A. serrulatus* due to the following reasons: (i) the identification matched *A. serrulatus* from Genebank with accession no. of HQ630561 and HQ630562 (ii) low genetic variation recorded between *A. serrulatus* was obtained in this study with *A. serrulatus* from GeneBank (1.80%-2.83%) and (iii) they were grouped into one clade with *A. serrulatus* from GeneBank with high bootstrap values of 100% (NJ), 100% (MP) and 1.00 (BPP). *Acetes* samples from Miri and Lundu were identified as *A. erythraeus* because they were grouped together into one clade with high bootstrap values of 100% (NJ), 100% (MP) and 1.00 (BPP). Although no comparison could be made between *A. erythraeus* from Miri and Lundu, Sarawak with similar species from GeneBank, the taxonomy of *A. erythraeus* was resolved based on Phylogenetic Species Concept. This study supports the usage of CO1 gene as marker for DNA barcoding of Sergestid shrimp *Acetes*.

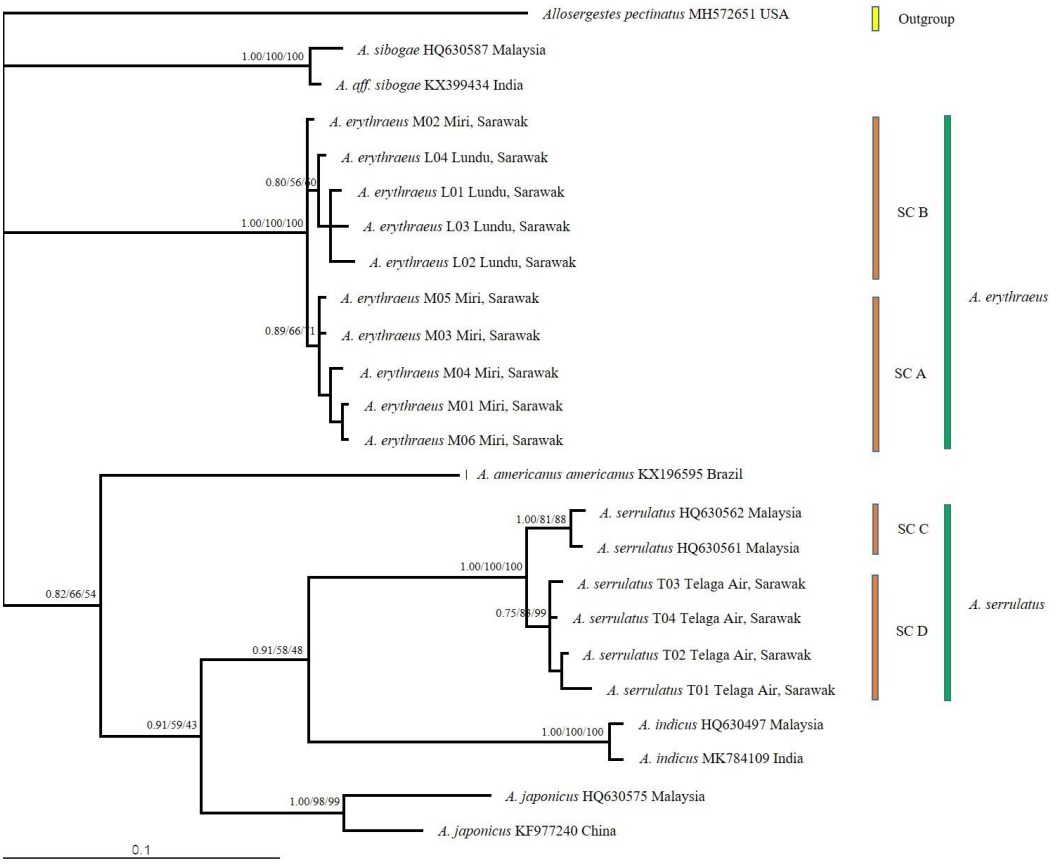

Figure 2. Bootstrap 50% majority rule consensus Bayesian inference tree of *A. erythraeus* and *A. serrulatus* from Miri, Telaga Air and Lundu, Sarawak with species of *Acetes* acquired from GeneBank, *Allosergestes pectinatus* as the outgroup. The values at the node represents BPP, ML (%) and MP (%)

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