Original Research

Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Journal of Environmental Biology

JEB™ p-ISSN: 0254-8704
e-ISSN: 2394-0379
CODEN: JEBIDP

DOI : http://doi.org/10.22438/jeb/41/5(SI)/MS_36

Phylogenetic analysis revealed first report of *Eleutheronema rhadinum* lineage in the coastal waters of Malaysia

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Paper received: 12.10.2019 *Revised received*: 18.01.2020 *Accepted*: 14.08.2020

Abstract

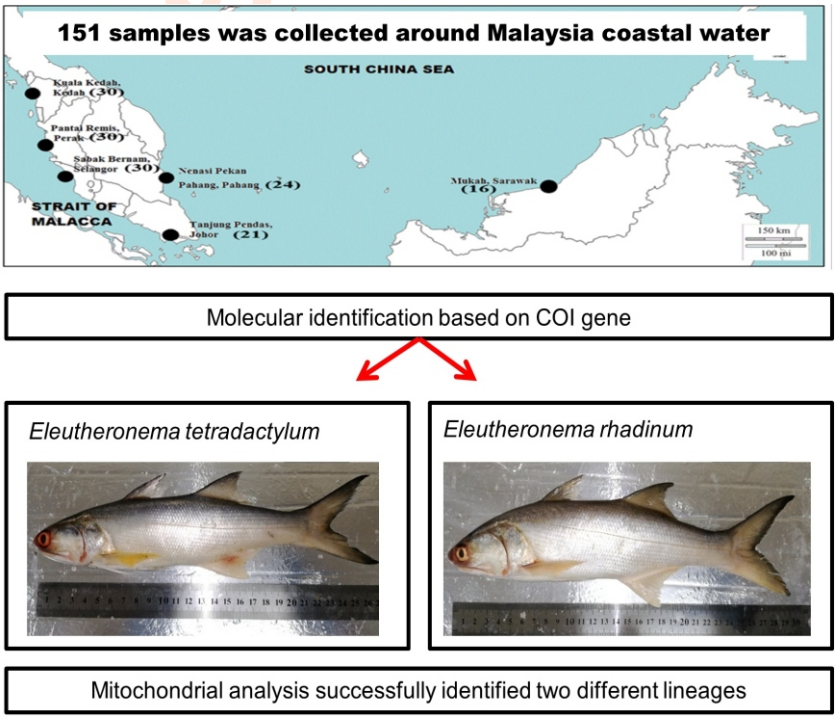
Aim: To genetically identify *Eleutheronema* specimens and to perform phylogenetic analysis of *Eleutheronema* from the coastal waters of Malaysia using sequence analysis of mitochondrial Cytochrome c oxidase I (COI) gene.

Methodology: A total 151 samples of *Eleutheronema* sp. were collected from Malaysian coastal water. DNA was extracted from 25 mg caudal tissue and standard procedures were followed for COI gene amplification and sequencing. The sequences were then proceeded for the analysis of mitochondrial COI sequence by using MEGA7.

Results: A total of 20 haplotypes were found. Phylogenetic analysis identified two genealogical lineages; *E. tetradactylum* and *E. rhadinum,* respectively, harbouring 10 haplotypes each. The genetic distance range was 16%- 17%, thus supported the two lineages as distinct taxa. *E. rhadinum* lineage has been reported for the first time from Malaysian coastal water.

Interpretation: Mitochondrial analysis successfully distinguished two reciprocally monophyletic taxa using COI dataset. The results also managed to provide first genetic evidence of *E. rhadinum* existence in Malaysian coastal waters.

Key words: COI mtDNA, *E. rhadinum*, *Eleutheronema*, Genetic differentiation, Phylogeny



How to cite : Atikah, N.W., Y.B. Esa, M. Rozihan, M.F.S. Ismail and I.S. Kamaruddin: Phylogenetic analysis revealed first report of *Eleutheronema rhadinum* lineage in the coastal waters of Malaysia. *J. Environ. Biol.*, **41**, 1424-1431 (2020).

Introduction

Eleutheronema belongs to Polynemidae family that consists of three valid species; three fingers threadfin, *Eleutheronema tridactylum* (Bleeker, 1849), four fingers threadfin, *Eleutheronema tetradactylum* (Shaw, 1804) and East Asian four fingers threadfin*, Eleutheronema rhadinum* (Jordan and Evermann, 1902*). E. tetradactylum* was the first *Eleutheronema* erected as a valid species by Bleeker (1862) and subsequently supported by other researchers (Menon, 1974; Menon and Babu Rao, 1984; Rainboth, 1996; Motomura *et al*., 2002) and was considered as the most common and most important commercial widespread threadfin in the Southeast Asia and East Asia regions (Motomura *et al.,* 2002). The rare *E. tridactylum* was also considered as a valid species (Weber and de Beaufort, 1922; Kottelat *et al*., 1993; Motomura *et al*., 2002). Nevertheless, the taxonomic status of *E. rhadinum* was much more complicated because it was previously (old name *Polynemus rhadinus*) treated as a junior synonym to *E. tetradactylum* (Weber and de Beaufort, 1922; Kagwade, 1970) until Motomura *et al*. (2002) revised the taxonomic status of genus *Eleutheronema* and recognized *E. rhadinum* as a valid species endemic only to the East Asia (China and Japan) region.

Eleutheronema tridactylum can be easily distinguished from the other two *Eleutheronema* species through several morphological characters such as having vomer without tooth plates, lower count of second dorsal fin soft rays, pectoral fins and gill rakers (Motomura *et al*., 2002). However, the morphological discrimination between *E. tetradactylum* and *E. rhadinum* are much more difficult due to their external morphological similarities which can led to much confusion (Motomura, 2004), especially for preserved or degraded specimen. Accordingly, the differences between *E. tetradactylum* and *E. rhadinum* are the number of pored lateral line scales and colour of pectoral fin membrane, which would easily been degraded or damaged after being caught and/or during storage in ice or freezer. The number of *E. tetradactylum* pored lateral line scales was 71 to 80 while for *E. rhadinum* was 82 to 95. The colour of pectoral fin membranes was vivid yellow when fresh for *E. tetradactylum* (except in large specimens over about 350 mm standard length) and black for *E. rhadinum* (Motomura *et al*., 2002).

In Malaysia, *E. tetradactylum* was known as one of the highly commercial and important marine capture species and also regarded as having high potential for aquaculture (Wang *et al*., 2016). On the other hand, *E. rhadinum* has never been recorded from the Malaysian coastal waters although Motomura (2003) reported the occurrence of the species in the Vietnam coastal area. Nevertheless, detailed catch statistics by the Department of Fisheries, Malaysia was not reported for Polynemidae including fishes of the genus *Eleutheronema*, and fish landing was normally summarized as a vernacular name *i.e.,* local name, Senangin, which might actually correspond to more than one species.

Therefore, due to taxonomic and geographical distribution uncertainties, particular for *E. rhadinum*, the application of molecular markers such as mitochondrial DNA sequencing would be useful tool for a more accurate species and broodstock identification (Hebert *et al*., 2003) among *Eleutheronema* as well as resolving phylogenetic and cryptic lineage issues (Mat Jaafar *et al*., 2012). Mitochondrial genes are extensively used for elucidating population genetic structure, phylogeography, and phylogenetic relationships at various taxonomic levels (Avise, 1992) due to the advantages associated with maternal inheritance, lack of recombination and rapid evolutionary rate (Giles *et al*., 1980). Moreover, the high degree of polymorphism detected in mitochondrial gene is also applicable for intraspecific analysis (Azmir *et al*., 2017).

Several studies on the phylogenetic relationships and population genetic structure of threadfin fishes, including *Eleutheronema* species have been conducted (Horne *et al*., 2011; Horne *et al*., 2013; Sun *et al*., 2013; Wang *et al*., 2014; *Thirumaraiselvi and Thangaraj, 2015*) for specimens from Australia, East Asia and South Asia, but study on specimen of *Eleutheronema* from Malaysian waters has never been conducted. Hence, the present study aims to genetically identify Eleutheronema specimens collected from various locations in Malaysian coastal waters and further analyzed their phylogenetic relationships using sequence analysis of mitochondrial Cytochrome c oxidase I (COI) gene.

Materials and Methods

Sample collection and DNA isolation : A total of 151 *Eleutheronema* samples were collected from six sampling sites (Fig. 1) including one from Borneo (Mukah, Sarawak) and five sites in Peninsular Malaysia (Sabak Bernam, Selangor; Tanjung Pendas, Johor; Pantai Remis, Perak; Kuala Kedah, Kedah; and Nenasi Pekan Pahang, Pahang). All samples were collected from fish landing area of commercial fishing activities. The entire fish were morphologically identified based on morphological features according to the description provided by Motomura (2004). Caudal fin tissue samples were preserved in 95% alcohol (ethanol) for DNA extraction. Preserved tissue were extracted by following the standard extraction protocol by ReliaPrep gDNA Tissue Miniprep System (Promega Corp, Madison, USA).

PCR amplification and sequencing of mitochondrial DNA: Polymerase chain reaction (PCR) amplifications of mitochondrial DNA (mtDNA) was conducted using universal primers FishF1 (5'TCAACCAACCA CAAAGACATTGGCAC-3'), and FishR1 (5'–TAGACTTCTGGGTGGCCA AAGAATCA–3') following Ward *et al*. (2005). This primer pair amplified a Cytochrome C Oxidase I (COI) mtDNA gene region that corresponds to positions (5 to 3) 681–1294 of the mitochondrial genome. PCR final volume was

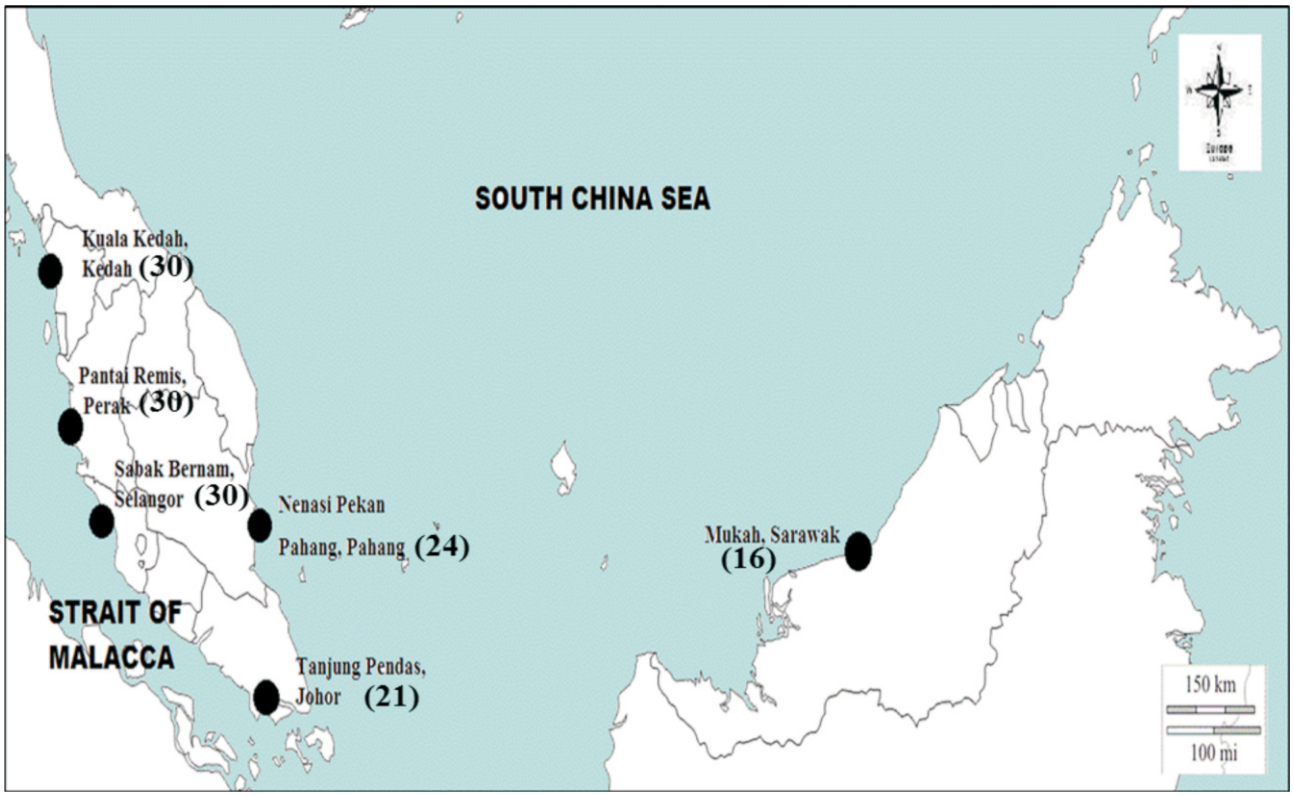


Fig. 1 : Map showing the sampling area for this study

25µl total reaction mixture, containing 14.3 µl of sterile distilled water, 5 µl Taq buffer 5×, 2.0 µl of 25mM MgCl, 0.5 µl of 10mM dNTP, 0.5 µl of 10 µM of each primer, 0.2 µl of 5µ μl^{-1} of Taq DNA polymerase and 2 µl of DNA template. PCR reactions were performed by using a Mastercycler Gradient PCR system (Eppendorf, Hamburg, Germany). PCR reaction was conducted as follow: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing of primer at 64°C for 1 min, primer extension at 72°C for 1 min and 30 sec and a final extension at 72°C for 2 min.

Purification process and DNA sequencing : PCR product was electrophoresed using 1% of agarose gel matrix (Fisher Scientific, NJ, USA), stained with 1 µl of GelRed (Thermo Fisher Scientific, USA) and visualized and documented under UV light using Alphalmager HP (Biotechne, USA). The BenchTop 100bp DNA Ladder (Promega Corp, Madison, USA) was used as a molecular weight (MW) standard. PCR product was later purified with Wizard® SV Gel and PCR Clean-Up System (Promega Corp, Madison, USA) and was sent for Sanger sequencing to a private laboratory (First BASE Laboratories Sdn. Bhd., Selangor, Malaysia).

Sequences alignment and data analysis : The Chromas 2.6.2 (Technelysium) software was used to check and view the COI sequences and chromatograms results. All successful sequences were subsequently aligned and edited using ClustalX (Thompson *et al*., 1997). The aligned sequences were subsequently translated into protein to ensure accurate alignment and detection of stop codons, if present. Nucleotide composition and variables site were analyzed using MEGA 7 (Kumar *et al*., 2016). Genetic relationships among haplotypes were then created using the median joining method implemented in Network 4.1 (Bandelt *et al*., 1999). Phylogenetic tree was reconstructed based on the Maximum Likelihood (ML) method in MEGA 7 (Kumar *et al.,* 2016) with a confidence level of 1000 bootstrap replications. Prior to this, the Model Test was conducted to determine the best model for tree construction also using MEGA 7 (Kumar *et al*., 2016). Hasegawa-Kishino-Yano model (Hasegawa *et al*., 1985) was identified as the best evolutionary model and was used in the phylogenetic tree reconstruction and pairwise genetic distance estimates through 1000 bootstrap replications. An assessment of pairwise Fst values was performed in Arlequin 3.5.2 (Excoffier *et al.,* 2005) with 1000 permutations of the data set.

Results and Discussion

The sequence analysis for COI gene generated a 650 base pairs region from 151 *Eleutheronema* specimens obtained from 6 different populations along the coastal water of Malaysia. A total of 69 (10.95%) were variables/polymorphic sites while 561

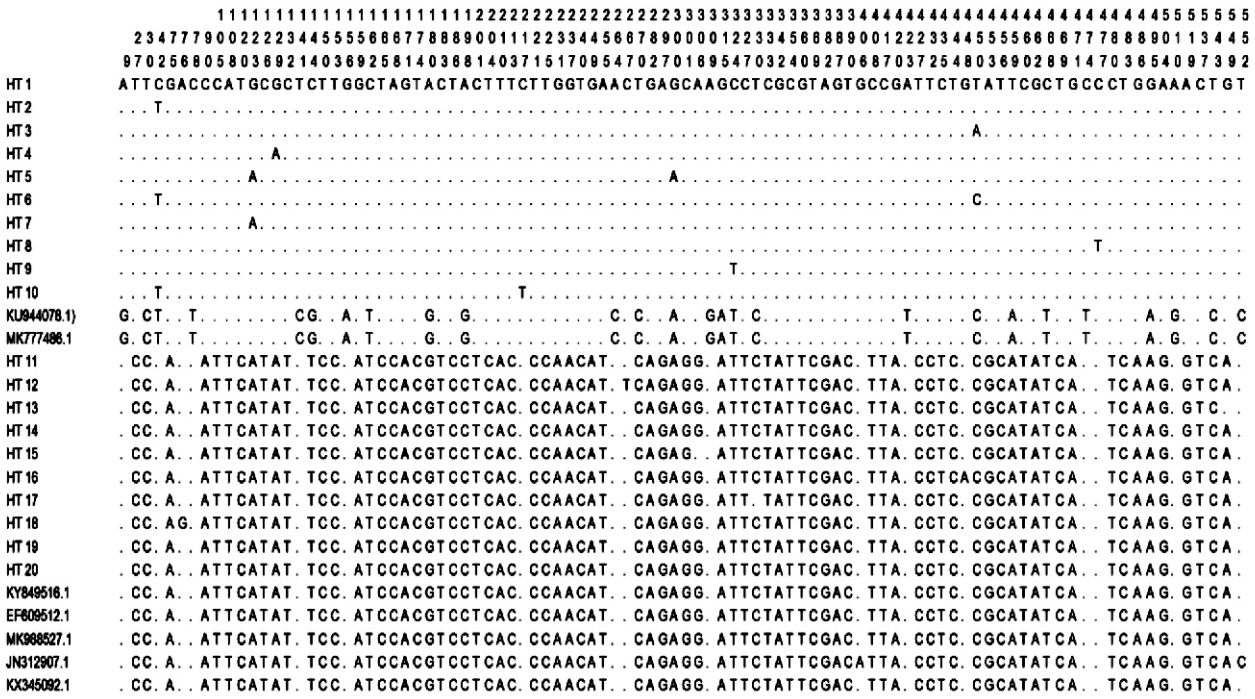


Fig. 2 : Summary of variable sites of 20 observed mitochondrial DNA cytochrome c oxidase I (COI) haplotypes of the *Eleutheronema* sp. (HT 1- HT 10, MK777486, KU944078 were *E. rhadinum*; HT 11-HT 20, KY849516.1, EF609512.1, MK988527.1, JN312907.1, KX345092.1 were *E. tetradactylum*). Dots indicate identity with the HT1 haplotype sequence.

(89.05%) were conserved sites (Fig. 2). The average total nucleotide compositions were 23.14% A, 29.78% T, 28.56% C, and 18.51% G. The 151 COI sequences yielded a total of 20 haplotypes (Fig. 2) which were deposited in GenBank under accession number MG816119-MG816138.

Phylogenetic analysis using Maximum Likelihood (ML) separated 20 haplotypes into two distinct clusters with strong bootstrap support, where each cluster harboured 10 haplotypes each. Inclusion of representative *E. tetradactylum* and *E. rhadinum* sequences from GenBank into the phylogenetic analysis further confirmed that the two clusters corresponded to two different lineages of *E. tetradactylum* and *E. rhadinum*, respectively. (Fig. 3). Median joining networks (Fig. 4) also divided 20 haplotypes into two distinct groups consistent with the maximum likelihood tree result. The average pairwise genetic distances based on Tamura-Nei model between haplotype of two clusters was 16%-17%, which confirmed the taxonomic status between *E. tetradactylum* and *E. rhadinum* as two valid species under both DNA barcoding threshold criterion (Hebert *et al*., 2003) and the Genetic Species Concept criterion (Bradley and Baker, 2001).

The current finding was the first report on the occurrence of *E. rhadinum* in Malaysia coastal water. Previously, the geographical distribution of *E. rhadinum* was thought to be confined only to East Asia (China, Japan and Korea), and was endemic to the region (Motomura *et al*., 2002). Subsequently, Motomura (2003) reported on the first record of *E. rhadinum* in Vietnam water which was found in Long Chau Island near to South China Sea. Nevertheless, it was the last published report on the southern geographical boundary limit of *E. rhadinum* distribution from the region.

Therefore, the apparent genetic subdivision (4.9%-5.5%: Table 2) observed between *E. rhadinum* sequences from Malaysia and East Asia (Vietnam and Taiwan) strongly indicated that the divergence process was not a recent event and might has been occurred historically through a vicariant event during the Pleistocene glaciation era, however, the evolutionary process could not be estimated based on the current data alone (Avise, 1992; Voris, 2000). Accordingly, during the Pleistocene glaciation periods, the lowering of sea levels of historical Sundaland (currently Southeast Asia) to over 120 m might had led to geographical isolation and genetic structuring of many marine organisms, including between *E. rhadinum* refugia populations from the Sundaland and those from the East Asia region (including Vietnam waters). Hence, any recent events such as fish translocation between region or migration of fish larvae following ocean current movements are highly unlikely and would not fit the current mitochondrial results. Furthermore, similar patterns of genetic subdivision between the two regions has been

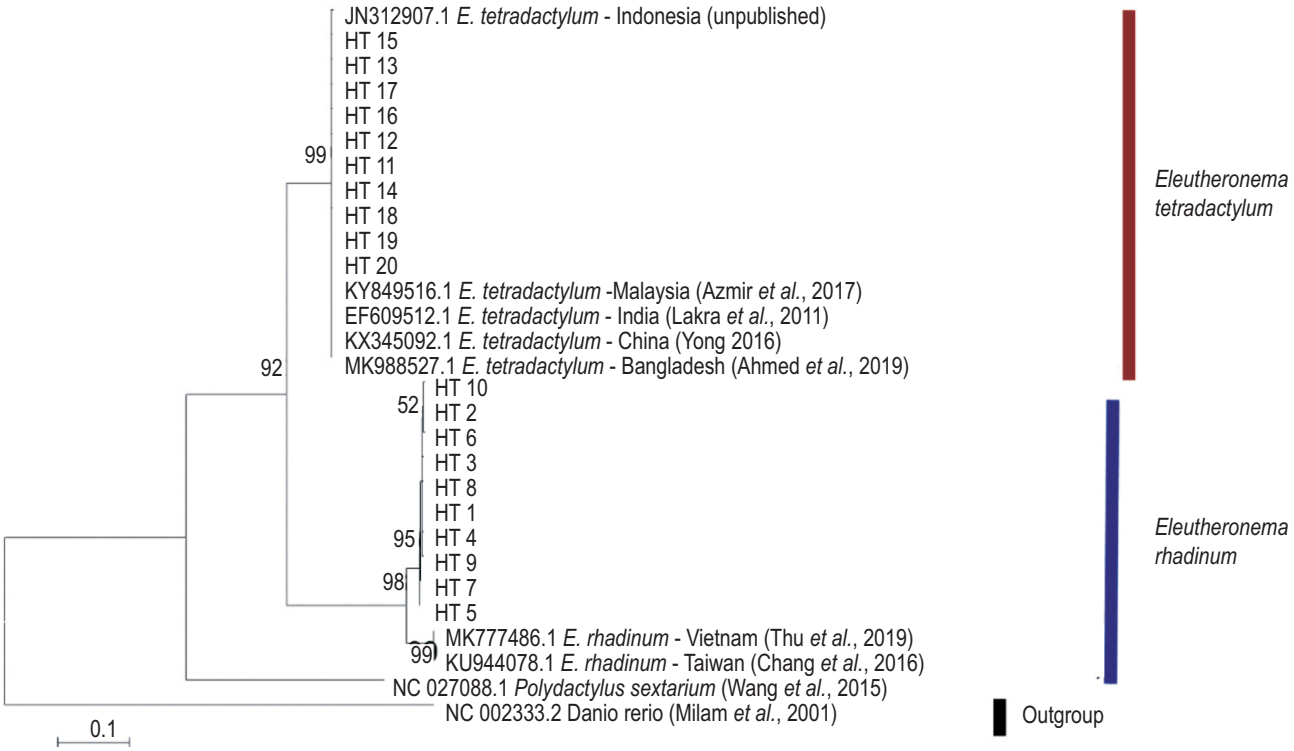


Fig. 3 : Maximum Likelihood (ML) tree (original tree) of cytochrome oxidase I haplotypes constructed based on Hasegawa-Kishino-Yano model for *Eleutheronema* sp.. The number at each node represent the bootstrap value (%) based on 1000 replicate.

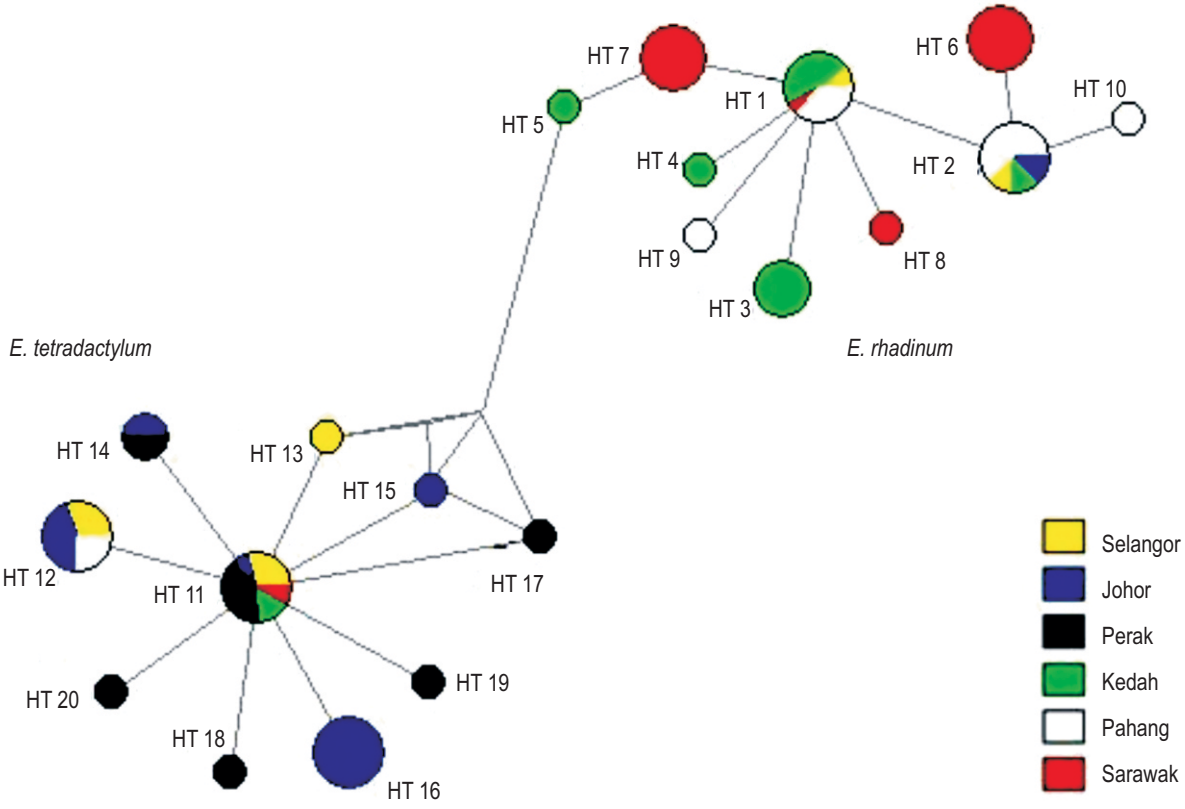


Fig. 4 : Median joining networks constructed for the COI gene haplotypes of *Eleutheronema* sp. from Malaysia population.

Table 1 : Pairwise distance based on Tamura-Nei model among 20 haplotypes of *Eleutheronema* sp. in this study and sequences of *E. tetradactylum* (KY849516 (Malaysia) and KX345092 (China)), *E. rhadinum* (MK777486 (Vietnam) and KU944078 (Taiwan)) from GenBank.

	1	2	3	4	5	6	7	8	9	10	11	12.	13	14	15	16	17	18	19	20	21	22	23	24
HT 1																								
HT 2	0.002																							
HT 3	0.002	0.004																						
HT 4	0.002	0.004	0.004																					
HT 5	0.004	0.005	0.005	0.006																				
HT 6	0.004	0.002	0.004	0.005	0.007																			
HT 7	0.002	0.004	0.004	0.004	0.002	0.005																		
HT 8	0.002	0.004	0.004	0.004	0.005	0.005	0.004																	
HT 9	0.002	0.004	0.004	0.004	0.005	0.005	0.004	0.004																
HT 10	0.004	0.002	0.005	0.005	0.007	0.004	0.005	0.005	0.005															
HT 11	0.163	0.166	0.163	0.161	0.159	0.163	0.161	0.166	0.161	0.168														
HT 12	0.166	0.168	0.165	0.163	0.161	0.166	0.163	0.168	0.163	0.171	0.002													
HT 13	0.161	0.163	0.161	0.159	0.156	0.161	0.159	0.163	0.158	0.166	0.002	0.004												
HT 14	0.163	0.166	0.163	0.161	0.159	0.163	0.161	0.166	0.161	0.168	0.000	0.002	0.002											
HT 15	0.161	0.163	0.161	0.159	0.156	0.161	0.159	0.163	0.158	0.166	0.002	0.004	0.004	0.002										
HT 16	0.166	0.168	0.165	0.163	0.161	0.166	0.163	0.168	0.163	0.171	0.002	0.004	0.004	0.002	0.004									
HT 17	0.161	0.163	0.161	0.158	0.156	0.161	0.158	0.163	0.158	0.166	0.002	0.004	0.004	0.002	0.004	0.004								
HT 18	0.166	0.168	0.165	0.163	0.161	0.166	0.163	0.168	0.163	0.171	0.002	0.004	0.004	0.002	0.004	0.004	0.004							
HT 19	0.163	0.166	0.163	0.161	0.159	0.163	0.161	0.166	0.161	0.168	0.000	0.002	0.002	0.000	0.002	0.002	0.002	0.002						
HT 20	0.163	0.166	0.163	0.161	0.159	0.163	0.161	0.166	0.161	0.168	0.000	0.002	0.002	0.000	0.002	0.002	0.002	0.002	0.000					
KY849516	0.163	0.166	0.163	0.161	0.159	0.163	0.161	0.166	0.161	0.168	0.000	0.002	0.002	0.000	0.002	0.002	0.002	0.002	0.000	0.000				
KX345092	0.163	0.166	0.163	0.161	0.159	0.163	0.161	0.166	0.161	0.168	0.000	0.002	0.002	0.000	0.002	0.002	0.002	0.002	0.000	0.000	0.000			
MK777486	0.049	0.047	0.049	0.051	0.049	0.045	0.051	0.051	0.047	0.049	0.158	0.160	0.155	0.158	0.155	0.160	0.160	0.160	0.158	0.158	0.158	0.158		
KU944078	0.049	0.047	0.049	0.051	0.049	0.045	0.051	0.051	0.047	0.049	0.158	0.160	0.155	0.158	0.155	0.160	0.160	0.160	0.158	0.158	0.158	0.158	0.000	

observed in other taxa like tiger prawn *Peneus monodon* (You *et al.,* 2008), swamp eel *Monopterus albus* (Matsumoto *et al.*, 2010), crimson snapper *Lutjanus erythropterus* (Zhang *et al.*, 2006) and intertidal limpet *Cellana toreuma* (Wang *et al.*, 2016).

The genetic divergence values of 4.9%-5.5% (Table 2) observed between *E. rhadinum* sequences from Malaysia (representing South China Sea) and the representative GenBank accessions from Vietnam and Taiwan (representing East Asian) warranted further examination on their taxonomic status. Accordingly, a genetic divergence values between 2% to 11% might indicate a high probability of conspecific populations or even a valid species and merit additional study concerning their specific status (Hebert *et al.*, 2003; Bradley and Baker, 2001). Future studies should focus on a comprehensive phylogeographic and morphomeristic studies of *E. rhadinum* by expanding the number of samples throughout the East Asian region as well as samples from the South-East Asia region.

In conclusion, the present study has successfully provided novel genetic information on the phylogenetic and species identification of *Eleutheronema* species in Malaysian waters. The high genetic divergence and reciprocally monophyletic status between *E. tetradactylum* and newly reported *E. rhadinum* confirmed their status as distinct taxa. Most interestingly, the first report on the occurrence of *E. rhadinum* lineage in this region has further demonstrated the advantages of molecular marker to identify and resolve issues in taxonomy and geographical distribution of taxa. Additional studies on the population genetic structure and demographic history of both *E. tetradactylum* and *E. rhadinum* based on both mitochondrial and nuclear markers should also be conducted for better estimation on the levels of genetic variations and evolutionary history of the two species. The results of this baseline study should be useful for sustainable management and conservation of *Eleuthorenema* in the region.

Acknowledgments

Authors would like to thank all the staff of the Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia and those who have assisted and contributed either in the field samplings or laboratory works until the completion of the project. The article was part of a Master thesis project of N.W. Wahid.

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