Diversity Analysis of Asian Clam Collected from Selected Rivers in Kelantan

Norlaila-Liyana Ab Wahab[1], Mohd-Zharif Ramli[2], Norhazlini MZ[1], Aweng Eh Rak[1,*]

[1]Faculty of Earth Science, Universiti Malaysia Kelantan, Jeli Campus, 17600 Jeli, Kelantan, Malaysia
[2]Faculty of Agro-Based Industry, Universiti Malaysia Kelantan, Jeli Campus, 17600 Jeli, Kelantan, Malaysia.

Abstract. The phylogenetic study was conducted to reveal the types of Corbicula species located on a selected river in Kelantan. The morphotypes and morphometric features were used to analyse the phenotypic analysis, and genotypes were examined exploiting the mitochondrial DNA cytochrome b (cytb) gene. A unique morphotype round (R) was discovered for every examined sample. Furthermore, there were significant differences ($p<0.05$) in the morphometric features (umbo length, UL; shell width, SW; and shell height, SH) between the C. fluminea populations. The mtDNA cytb sequences are used in the genotyping analysis to confirm that the assessed Corbicula is C. fluminea. Three genotypes were identified in the mtDNA cytb phylogenetic tree. In geographically isolated populations, phylogenetic trees showed polymorphism and minimal genetic variability, which aligned with the phenotypic characterisation. The results clarify the differences among C. fluminea and imply that Corbicula sp. in Kelantan originated from a single species. These findings are significant not only for biologists or those involved in ecology and ecosystems but also for the fisheries sector as it serves as the basis for breeding purposes, overcome the extinction, and enhancing the economy of the inland fishermen in Kelantan.

1 Introduction

The genus Corbicula is a significant freshwater clam inhabited in East Asia waters and has lately spread to North America, South America, and Europe. This tiny mussel is often called the Asian or Asiatic clam in the West. However, it is known as golden clams, bloom, or lucky clams in Southeast Asia. Several species were named based on localities, where recent reports synonymised them with Corbicula fluminea, which inhabited the Malaysian ZDWHU ERGLHV LQ .HODQWDQ > @ ,W LV DOVR ZLGHO\ N known as *etak sala*, which is popular in Kelantan and Southern Thailand and contributes to the local economy [2].

The Corbicula is a filter feeder which acts as a biofilter that removes all particles from the water column. This genus is a hermaphroditic organism that gives advantages for them to propagate easily. In addition, other reproductive characteristics, such as androgenesis and

* Corresponding author: aweng@umk.edu.my

self-fertilisation at different ploidy levels, extend their propagation ability [3]. The latest research mentioned that the extinction of *Corbicula* in several areas in Kelantan negatively impacts people, especially the sellers. The extinction of *Corbicula* in Kelantan caused the sellers to not get raw materials for processing, which could ultimately affect their income. Besides that, it also affects *tak salai* as a heritage food in Kelantan. Therefore, determining this diversity is significant as the basis for broodstock selection for breeding purposes to meet demand and conservation purposes.

Corbicula in several areas of Kelantan had a low population, making the people lose their daily income. However, in some areas, freshwater clams might attract tourists interested in activities like clam harvesting, which could generate local income. The extinction of these clams could negatively impact such tourism-related activities. Their extinction could affect the availability of a protein source, forcing communities to find alternative and potentially more expensive food sources. Therefore, *Corbicula* phenotypic and genotypic diversity analysis is vital to obtain information on the best broodstock source for conservation and breeding purposes

2 Materials and methods

2.1 Sampling area

The *Corbicula* samples were collected in several districts in Kelantan where harvesting activities are actively occurring. The coordinate of Kelantan state is 5.1151º N, 101.8892º E. Figure 2.1 shows the sample collection in the selected river in Kelantan. It included five districts: Jeli, Pasir Mas, Tumpat, Pasir Putih, and Bachok.

Fig. 2.1. Five locations of *Corbicula fluminea* specimens collected in Kelantan

2.2 Phenotypic characteristics

A total of 200 *C. flumineas*amples were randomly collected from each of the locations (n=40) by using a dredger. The samples were stored in ice before being transported to UMK for further use. For phenotypic characteristics, shell colour, morphotypes, and morphometric characteristics were assessed [4]. The colour of the interior and external shells was scrutinised to ascertain their shell morphology. The morphotypes of *C. flumi*nea were compared with the previous report, and they were distinguished by dark round (R), saddle (S), and light coloured round (Rlc) [5]. 7 K H F O D P ¶ V Y L V F H U D P X surgically removed and stored in 95% ethanol before being used to measure. Then, the V K H O O V G U L H G D W f & R Y H U Q L J K W L Q W K H R Y H Q W R H A digital calliper (Absolute Digimatic Calliper/Mitutoyo (UK) with an accuracy of 1/50mm) was used to measure the morphometric of *C. flumi*nea (umbo length (UL), shell width (SW), shell height (SH), and standard length (SL)).

2.3 DNA extraction

The protocol for this methodology was described in [4]. Sterilised forceps and a scalpel blade were used to open the shell to collect *C. flumi*nea tissue for DNA analysis. After that, approximately 200 mg of gill tissue was put in 1.5 mL microcentrifuge tube, containing 200 µL of CTAB buffer. The tissue was mashed; and the remaining 400 µL of CTAB buffer was added. The tissue is then incubated in water bath for two hours at 65º C. After that, the sample is frozen at -20ºC for 30 minutes. The sample was then thawed at 65º C for 15 minutes. The tissue was repeatedly withdrawn through a wide-bore one ml pipette tip to disrupt the tissue. The sample was incubated for two more hours at 65º C. Next, three extractions with 500 µL chloroform/isoamyl alcohol are prepared. For each extraction, 500 µL of chloroform/isoamyl alcohol is added, vortexing the sample for a few minutes, letting it settle down for a couple of minutes, and then spun in a microcentrifuge for 3 minutes at 10000 rpm. Then, the upper layer was withdrawn while not trying to get the viscous stuff at the interface and then transferred into a new microcentrifuge tube. The sample was then precipitated with 300 µL cold isopropanol and put on ice for 15 minutes. The tissue sample was spun in a microcentrifuge for 30 seconds, and then the liquid was removed and discarded. The tissue sample was washed with 1 mL of cold 70% ethanol, twice. For each wash, the ethanol was added, the tube was gently inverted a few times, and the tube was spun in a microcentrifuge for 15 seconds. The liquid was removed and discarded without disturbing the pellet. The tissue was air-dried for one to several days. Lastly, 100 µL of TE buffer was added, and the DNA pellet was left to sit in the refrigerator overnight or longer, and it was sucked through a pipette tip several times to resuspend the DNA.

2.4 Genetic analysis

After DNA extraction was obtained, the quantification and qualification analysis were performed before further work. The quality and integrity of DNA are crucial as it can significantly impact the outcomes of any subsequent scientific research [6]. Two approaches were used to determine the quality and quantity of genomic DNA: 1% agarose gel electrophoresis and nanodrop assay using a NanoDrop 2000 UV-Vis Spectrophotometer. PCR amplification and sequencing were conducted targeting the cytochrome b (cytb) gene X W L O L Q J W K H I R U Z D U G D Q G U H Y H GAAGGGTTCATGCTAACGGAG- ¶ 5 -GT¶GGTGTACTTATAGGGTCGGC- ¶ with Genbank accession number ABB551548 [4]. Each PCR reaction was carried out in a total volume of 50µL, consisting of 5µL of 10x reaction buffer, 0.08U/µL exTEN DNA

polymerase, 1.6µL of dNTP (2.5mmol/L), 2.5µL of each forward and reverse primer (10µM), 1µL of template DNA, and 36.6µL of nuclease-free water. The PCR cycling parameters comprised an initial denaturation at 94°C for 5 minutes, followed by 38 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds, extension at 72°C for 1 minute, and a final extension step at 72°C for 20 minutes [4]. Subsequently, PCR products underwent separation via 1% agarose gel electrophoresis before being sent to the 1st Base Laboratory for purification and sequencing. Alignment of the obtained mtDNA sequences was performed using Multiple Sequence Alignment CLUSTALW. Phylogenetic trees were constructed utilising Maximum Likelihood (ML), Neighbour-Joining (NJ), and Maximum Parsimony (MP) methods. Bootstrap analysis with 1000 replicates was employed to assess the robustness of the tree topologies [4].

3 Results and discussion

3.1 Phenotypes analysis

Analysis of variance (ANOVA) showed the three characteristics (SH = Shell height, SW = shell width, and UL = Umbo length) was significantly different ($p<0.05$) between *C. fluminea* in five localities. Subsequently, Table 3.1 indicates that *C. fluminea* in Jeli is considerably bigger (0.945±0.05) than in other sites. Conversely, the SW ratio of *C. fluminea* in Pasir Mas, Tumpat, and Jeli found to be insignificant ($p<0.05$). *C. fluminea* populations from Pasir Mas and Pasir Putih showed similar features to those at Bachok SW.

Table 3.1. Analysis of variance (ANOVA) of three morphometric characteristics corresponding to the locations. Different superscripts indicate a significant difference at level ($p<0.05$).

	Morphometric Characteristics		
Locality	Standard Length (SH/SL)	Shell Width (SW/SL)	Shell height (UL.)
Jeli	0.912 ± 0.05^{ab}	0.339 ± 0.06^{c}	0.395 ± 0.04^{a}
Tumpat	0.136 ± 0.03^{d}	0.346 ± 0.03^{c}	0.141 ± 0.06^{b}
Bachok	0.189 ± 0.07^{c}	0.076 ± 0.04^{bc}	0.017 ± 0.13^{b}
Pasir Putih	0.028 ± 0.07^{c}	0.191 ± 0.04^{b}	0.498 ± 0.03^{b}
Pasir Mas	0.028 ± 0.05^{bc}	0.063 ± 0.03^{c}	0.397 ± 0.02^{b}

European Standard [7] was used to differentiate between *Corbicula* morphotypes polymorphism, which correspond to a round shape and broad form with deep ridges (round form, R) and a narrow form with closely spaced ridges (saddle form, S). The *Corbicula* species for morphotypes R and S, which were identified as *C. fluminea* and *C. fluorescentlis*, respectively, were unclearly determined by researchers in earlier literature [1, 8]. Given that distinct characteristics may be used to differentiate the polymorphism of the morphotypes, they might be classified as distinct species [9]. Moreover, genetic and environmental variables caused polymorphism, which resulted in many taxa having incorrect names [10]. Because of its plasticity, the *Corbicula* V K H O O ¶ V S R O \ P R U S K susceptible to environmental changes, geographic distribution, and evolutionary factors, including imperfect meiosis [11, 12]. Recently, the *Corbicula* population, irrespective of kind, has been dominated by the morphotype R. Additionally, their number may be insignificant. The current investigation also discovered *Corbicula* of morphotype R and light colour (Rlc). Physiological and ecological adaptability may have arisen from the presence of morphotype Rlc. Therefore, individuals with morphotype Rlc were grouped and

included in the morphotype R community in this research. This result aligns with [7], who suggested that morphotype Rlc was comparable to morphotype R and that morphotype S only belonged to *C. fluorescentlis*, whereas both morphotypes related to *C. fluminea*. Thus, FXUUHQW UHVHDUFK VXLWDEO\ FRXOG VXJJHVW WKH divergence. Nonetheless, without molecular data, it is inaccurate to deduce their taxonomy. Commonly, the morphotype R of *C. fluminea* exhibits various inner and exterior hues, from light to dark in colour. It opposes morphotype S, limited to dark-coloured shells, such as dark brown to black on the outside and dark purple on the inside. Like colour, this trait is mainly influenced by its form and environment. For instance, it was discovered the outer shell of the *C. fluminea* collected from lakes, such as Lake Pergau in Jeli, Kelantan, was dark-brown to black in colour.

On the other hand, *C. fluminea* from different parts of Kelantan showed to have an exterior shell colour that was yellowish-brown, which was indicative of the environment. This discovery explains the difference in shell colour caused by microevolution events leading to local adaption since the ecosystem consists of low organic matter (OM) and sandy beds. Regardless of their form, *C. fluminea* typically ranges in colour from dark brown to black and inhabits an environment with a higher OM content. On the other hand, [13] clarified that the content of sulphide levels influenced the outer shell to ultimately turning white and brittle. One example of this phenomenon is the huge size of *C. fluminea* in the lakes. New research compares *C. fluminea* from Lake Pergau, where the two species differ in their shell morphologies but are similar in colour (dark). According to earlier UHVHDUFK WKH 3DWWDQL DQG 6DLEXUL ULYHUV ¶ VDQG the circular, yellow-and-black-colored C. fluminea [14]. The species collected from Tumpat, Pasir Mas, Bachok, and Pasir Putih show a similar result, where the bed composition has previously been documented. The varying shell form possibly caused by the following factors: water current, organic matter, food availability, and the settlement bed composition (sandy loam/ rocky/ silt/ or their combination) [7]. Consequently, rather than genetic factors, the *Corbicula* ¶V DGDSWDWLRQ WR WKHLU QDWXUDO for their polymorphisms in shell form.

7KH FXUUHQW VWXG\ ¶V GLVFRYHULHV KDYH LPSOLFDWLRQV RQ WKH S knowledge of speciation. These morphotype variations are sympatrically and allopatrically dispersed. For instance, it was thought that the *C. fluminea* in Lake Pergau originally from the Kelantan River, which accidently been spilt into the lake during its construction [15]. Their types differed with those of *C. fluminea* found in Lake Pergau and tributaries of the Kelantan River, which might help to explain the allopatric speciation. They did, however, have comparable morphotypes R. Additionally, the current samples were compared with the specimens kept at the Mollusk Museum of Mahidol University in Bangkok, Thailand. It shows all paratypes of *C. fluminea* (TMMU43, SMRL 2710, and SMRL 6821), *C. lamarkiana* (SMRL 2705), and *Corbicula* sp. (DN811002-20) were synonymised. The *C. fluminea* morphotypes R and Rlc were identified sympatrically in Ping River, Thailand [4]. The geographic span where various ecosystems defined their morphotypes explains this sympatric occurrence. Since the Ping River ran from Chiang Mai to Kamphaeng Phet, where the morphotype Rlc arose from the divergence of morphotype R, these paratypes are most likely from comparable lineages. Furthermore, a similar finding was made in Kelantan, where morphotype R and Rlc coexisted in small quantities. Thus, based on examining the phenotypes, recent studies accurately indicated the presence of *C. fluminea* in morphotypes R and Rlc. It was discovered that the *Corbicula* from Lake Pergau is of morphotype R. For additional understanding, the paratypes of *C. lamarkiana* were discovered in morphotypes R and S, where they lived in two separate Thai rivers. The same holds for morphotype because *C. fluminea* was synonymised with *C. lamarkiana* with morphotype R. These results led to dichotomous conclusions, suggesting that *C. fluminea*

with S morphotype possibly be collected in a brackish water environment close to the river mouth or deep habitats like lakes [1]. Consequently, with hazy taxonomy assumptions, morphotype classification using three traits (R, S, and Rlc) is vital to classify Corbicula across the group.

Ecologically speaking, in the meanwhile, they lived near lakes that a significant number RI WKHP PLJKW H[SHFW %HFDXVH RI WKLV VSHFLHV¶ distribution, which obliquely support population growth, harvesting efforts are limited to a certain region inside these lakes. For instance, Lake Pergau, Jeli, Kelantan, is home to a large population of C. fluminea, with a mean density of 262.8 individuals/m2 [16]. However, the C. fluminea was smaller (e.g., SL in the 0.631–0.912 mm) in Tumpat, Pasir Mas, Pasir Putih, and Bachok. The distribution is declining in these locations, and these tributaries make up the sandy loam riverbed. The current situation results from intense local harvesting to meet demand. Furthermore, sand mining along the Kelantan River impacted the stream. The habitat of C. fluminea was dug, and this action disrupts the benthic environment and shallows the riverbed, and causing the juveniles to be wiped off in the sands. This activity has put the population of Corbicula in danger in the freshwaters of Kelantan, led to changes in demographics, where abundance of tiny shells were found.

They were taken from the tributaries of the Kelantan River, which is different from other C. fluminea populations in Kelantan. The size is slightly smaller than those populated lakes. Moreover, the dispersal mechanism of C. fluminea during its juvenile stage from the main river accounts for its presence in specific tributaries. [12] described this type of life plan. C. fluminea dominates benthic communities due to its high fertility, growth rates, ecological tolerance, and strong dispersion capacities. Unfortunately, due to geographical limitations and the lack of this species, samples of C. fluminea from the main river could not be obtained for this study. As a result, the population of the Kelantan River was presumed to be represented by the C. fluminea from the stretch tributaries in this study. However, the canonical discriminant analysis demonstrates that phenotypic characterisation was the basis for the association between Corbicula populations. This distribution may represent genetic and geographical since phenotypes are the outcome of genotypic expressions.

The Kelantan River was geographically expanded to the Tumpat, Bachok, Pasir Putih, and Pasir Mas tributaries. The C. fluminea from Jeli was discovered to be clustered overall, indicating their distribution throughout several geographic areas. The Jeli people, for example, lived upstream (near the lake), clearly apart from other regions. In Tumpat, the GLVSDULW\ LV DOVR YLVLEOH 'XH WR WKH VDPSOH FD previous theories suggested that Tumpat C. fluminea originated in Thailand. On the other hand, this C. fluminea showed phenotypic characteristics that were more closely connected to the Kelantan lineages according to the canonical discriminant distribution.

3.2 Genotypes analysis

The tree was constructed using the Polymesoda carolinian (KX713250) DNA as an out-group and the Kimura-2-parameter genetic distance as the foundation. Using the maximum-likelihood (ML) bootstrap value represented by nodes, the topology shown in Figure 3 was inferred, and phylogenetic connections between C. fluminea populations in Kelantan were established. Neighbouring Joining (NJ) and Maximum Parsimony (MP) have produced similar topologies, which may be combined, as seen in Figure 3. At the branches, the bootstrap values match the ML, MP, and NJ. The topology demonstrated a strong dichotomous separation of two clades.

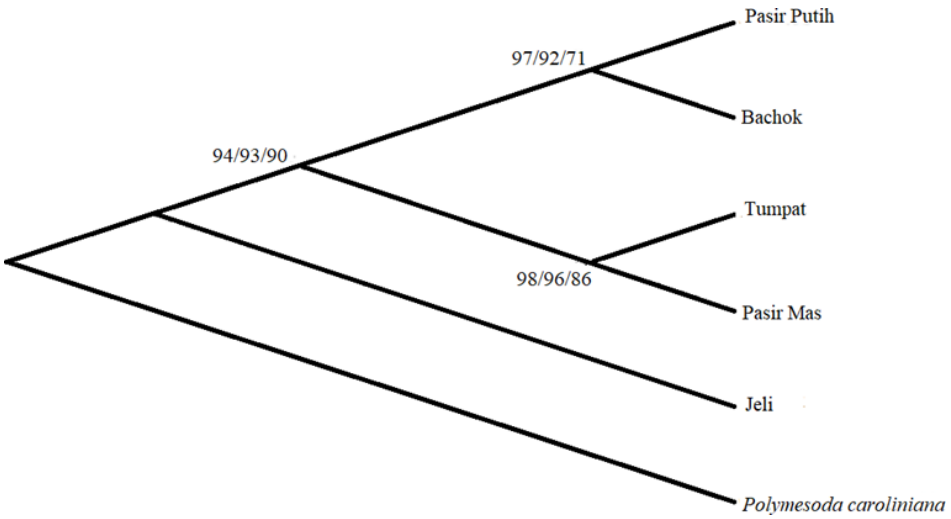

Fig. 3.2. Phylogenetic tree of *Corbicula fluminea* populations constructed by the Maximum Likelihood (ML), Maximum Parsimony (MP), and Neighbouring Joining (NJ) method using a 481 bp partial fragment of the mitochondrial gene cytochrome b (cytb).

The number of base substitutions per location is shown between sequences. A bootstrap technique (1000 repeats) was used to determine standard error estimates above the diagonal. The Kimura two-parameter model [17] was analysed using MEGA 11. *C. fluminea*, phenotypes are influenced by genetic components, environmental influences, or both. The current work focused on the genotypic information of the species to strengthen the phenotypic characterisation. Consequently, the taxonomy names of the assessed *C. fluminea* are clarified and supported by the genetic evaluation used in the current study. Mitochondrial DNA markers (mtDNA), such as incomplete cytb, have been used in recent research. These specific genes were chosen because *Corbicula* genotype analysis presently uses them extensively. According to the most recent discovery, all assessed *Corbicula*, irrespective of their morphotypes and localities, pertain to *C. fluminea* species. Given *Corbicula* ¶ V K L J K G H J U H H R I S R O \ P R U S K L V P W K H V S H F L H V P D \ L U U Z R have distinct morphotypes [1].

This work analysed the distance between nearby *C. fluminea* populations using mtDNA cytb sequences. ML, MP, and NJ produced a similar topology and demonstrated a strong dichotomy separating two clades showing limited genetic evolutions. It is possible to hypothesise that the genetic allopatric evolution of the *C. fluminea* from Jeli sets them apart from other populations of the same species [1]. The Pergau River is the primary river connecting to the Pergau Lake, thus, the population of *C. fluminea* in Lake Pergau, Jeli, may disseminate to the Kelantan River. Therefore, genetic material of *C. fluminea* in Lake Pergau, Jeli may have been introduced into the population of *C. fluminea* in the Kelantan River and other tributaries, such as in Tumpat, Pasir Mas, Bachok and Pasir Putih. The limited variance in evolutionary distance explains the lineages that most likely had a common ancestor. However, because of their hermaphrodite traits, the data showed *C. fluminea* in Kelantan can be hypothesised to have little genetic diversity. There was evidence of this low genetic variation in the population. According to [18], biotic and abiotic adaptation that affects phenotypes can be linked to genetic variation. Accordingly, our work postulates that limited genetic variety among *C. fluminea* in Kelantan might lead to a bottleneck event that needs more study.

3 Conclusion

The phenotype and genotype characterisation of *Corbicula fluminea* has given researchers a thorough understanding of the species taxonomy, polymorphism, and lineage history across its range. Despite their polymorphism leading to phenotypic variety, it is now fair to infer that the *Corbicula* sp. discovered in Kelantan are members from the same species. The phenotypes of *C. fluminea* in the present study have been anchored by genotyping analysis since the results agree. As a result, the combined study of the genotypes and phenotypes of *C. fluminea* produces a consensus taxonomy discovery, clarifies the characteristics of polymorphism, and predicts the life history.

We want to thank the Faculty of Earth Sciences, Universiti Malaysia Kelantan, for the permission to conduct the research and publish the results of this study. We also thank the laboratory assistants who assisted in the laboratory analysis so that the study could be completed in time.

References

1. M.Z. Ramli, V. Ayyapan, A. Yusoff, E.R. Aweng, S.W. Lee, Borneo J. Res. Sci. & Tech. **10,** 1, 24-36 (2020)
2. D.K. Han, S. Appalasamy, M.N. Siti Nor Aini, A. Faizuan, M.N. Maryana, E.R. Aweng, Malaysian J. Fund. Appl. Sci. **16**, 2, 161-165 (2020)
3. T.H. Hsu, A. Komaru, J.C. Gwo, Aqua. Inv. **15,** 4, 633-645 (2020)
4. Z. Ramli, S. Panha, E.R. Aweng, L.S. Wei, Songklanakarin J. Sci. & Tech. **44**, 5, 1272-1278 (2022a)
5. Z. Ramli, E.R. Aweng, S.W. Lee, Agriculture Reports **1**(2), 12-24 (2022)
6. L.J. Cseke, A. Kirakosyan, P.B. Kaufman, M.V. Westfall, Handbook of Molecular and Cellular Methods in Biology and Medicine, Third Edition. United Kingdom. (2011)
7. Z. Ramli, D.K. Han, F. Abdullah, E.R. Aweng, L.S. Wei, Agri. Rep. **1,** 39-44 (2022b)
8. L.M. Pigneur, J. Marescaux, K. Roland, E. Etoundi, J.P. Descy, K. Van Doninck, BMC Evo. Bio. **11**, 147 (2011b)
9. R. Sousa, C. Antunes, L. Guilhermino, Int. J. Limnology **44**, 85-94 (2008)
10. J. Heino, P. Louhi, T. Muotka, Freshwater Biol. **49,** 1230-1239 (2004)
11. L. Kamburska, R. Lauceri, N. Riccardi, Aqua. Inv. **8**, 1, 111-116 (2013)
12. E. Renard, V. Bachmann, M.L. Cariou, J.C. Moreteau, Mol. Ecol. **9**, 2009-2016 (2000)
13. E. Bódis, J. Nosek, N. Oertel, B. Tóth1, E. Hornung, R. Sousa, Comm. Ecol. **12**, 2, 210-219 (2011)
14. P.H. Chang, W.T. Chang, C.H. Shih, D.F. Liu, Y.C. Lee, Aqua. Res. **48,** 3004-3012 (2016)
15. N. Zaween, M. Shamsul, E.R. Aweng, S.O. Sharifah, L.A. Afip, Borneo J. Res. Sci. & Tech. **7**, 76-83 (2017)
16. E.R. Aweng, A.K. Zulhisyam, M.Z. Ramli, C.H. Hasnita, M.S. Suraya, A.A. Hazreen-Nita, A. Faizuan, A.O. Mahmoud Dawood, W. Wendy, S.W. Lee, Saudi J. Bio. Sci. **29**, 4, 2348-2354 (2021)
17. M. Kimura, J. Mol. Evo. **16**, 111-120 (1980)
18. C. Gomes, R. Sousa, T. Mendes, R. Borges, P. Vilares, V. Vasconcelos, L. Guilhermino, A. Antunes, PLoS ONE, **11**, 7: e0158108 (2016)