



Phenotypic and genotypic analysis of Asian clam, *Corbicula fluminea* Megerle von Mühlfeld, 1811 (Venerida, Cyrenidae) in Kelantan, Malaysia

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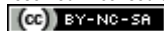
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Abstract: The existence of *Corbicula* in Malaysia has little attention, which was scarcely being reported. Recent research presents the phylogenetics analysis of *Corbicula fluminea* using phenotypic and genotypic in Kelantan, Malaysia. The phenotypic analysis of *C.fluminea* was examined based on the morphotypes and morphometric characters. The mitochondrial DNA cytochrome b (cytb) gene analysed the genotypes. Results, a distinct morphotypes round (R) was found for all evaluated samples. In addition, five morphometric characters such as shell height (SH), shell width (SW), umbo length (UL), anterior length (AL), and posterior length (PL) were significantly different ($p < 0.05$) between the *C.fluminea* populations. Discriminant analysis shows a significant difference in the characters among evaluated populations. The analysis of genotypes validates the evaluated *Corbicula* as *C.fluminea* through employing the mtDNA cyt b sequences. The phylogenetic tree of mtDNA cyt b formed three genotypes. Phylogenetic trees revealed polymorphism and low genetic variations among geographically isolated populations, which were consistent with the phenotypic characterisation. Thus, these findings elucidate the variation of *C.fluminea* and suggest that *C.fluminea* in Kelantan is descended from a common ancestor.

1. INTRODUCTION

Asian clam, genus *Corbicula* distributed worldwide, natively inhabits Asia freshwaters (Renard *et al.*, 2000; Schmidlin, 2011; Morden, 2015). This clam plays different roles in freshwater bodies in different regions, predominantly supporting the sustainability of the ecosystem in general. The existence of *Corbicula* in western countries is considered an invasive pest that negatively affects the economy and ecology (Mackie & Claudi, 2009; Peñarrubia *et al.*, 2016). Contrariwise, the *Corbicula* is significantly consumed in Asia's countries such as Taiwan, Japan, and China (Wang *et al.*, 2014). In previous reports, the *Corbicula* originated from the South East Asia countries such as Thailand, Cambodia, and Malaysia, and it has high economic importance (Ramli *et al.*, 2020). Particularly, in Malaysia, the *Corbicula* is highly consumed by peoples in Kelantan state, and currently, the foreign clams from Thailand and Cambodia were brought in to satisfy the local demands (Lee *et al.*, 2018). In addition, this imported *Corbicula* was

also found to be released in the local water body. Hence, this activity may introduce foreign species into local freshwater or raise the genetic introgressions incidence in *Corbicula* populations.

To date, the *Corbicula* in Malaysia is poorly known since few records were found. Previously, the *Corbicula* in Malaysia was found to have been documented in the early 1930s known as a local name 'etok' without a distinct taxa name and deposited in Mollusk Museum, Mahidol University, Bangkok, Thailand. Then, *C. tweediei* was found documented in Perak in 1940 deposited in Raffles Museum, Singapore, and the name of *C. malaccensis* populations was probably obtained from Malacca, Malaysia (Rajagopal et al., 2000; Tan et al., 2013). Unfortunately, this uncertain name occurred due to misnaming taxa solely based on morphology and location. More recently, the name of *C. fluminea* was commonly used in literature, referring to genus *Corbicula* in Malaysia without published genetic evidence (Yusof et al., 2020). Though, the taxonomy of *Corbicula* in Malaysia remains uncertain due to a vast shell polymorphic and scarcely being reported in the literature.

Polymorphism in species can be determined through the phenotypes differences contributed by the genetic materials, environmental factors, geographical location, or interaction between these factors (Heino, 2014). Shell characteristics and size of the species have been a prior phenotypic determination in bivalves, although they may have different shell features due to the high plasticity (Renard et al., 2000; Hamli et al., 2015). This conventional practice is significant in phenotypic characterisation, which acquires no cost and is easier and faster than genetic approaches (Hamli et al., 2015). Schmidlin (2011) coined that the morphometric characterisation approach is reliable in interspecific but intricate at the intraspecific level since the morphotypes variance may contain similar genotypes. Previously, 200 pseudo-*Corbicula* species from around the world were identified using morphometric characteristics-based taxonomy (Pigneur et al., 2011; Gomes et al., 2016). However, this number had reduced to two eminent species, *C. fluminea* and *C. fluminalis*, which are currently agreed upon by the researchers (Renard et al., 2000; Morden 2015). These *Corbicula* have equivalve, intergropaleate, near symmetrical, profound, and broad shells, whereas *C. fluminalis* was darker, taller, and more triangular in shell morphology than *C. fluminea* (Pigneur et al., 2011). The varied *Corbicula* species morphology seen in this study was sufficient to establish that they are distinct species. Despite this, in some cases, the classification of *Corbicula* was imprecise due to high polymorphism and peculiar reproduction modes, hermaphroditism, and androgenesis (Komaru et al., 2013).

Every species has a unique characteristic that distinguishes them and forms the population structure (Heino, 2014). Molecular approaches through genetic characterisation have discovered the species characteristics, genetic similarity, population structure, and genetic introgressions in *Corbicula* (Renard et al., 2000; Peñarrubia et al., 2017; Zhu et al., 2018). This approach anchored the morphological characterisation where targeted genes were used to infer similar species from deposited data in the gene bank. Mitochondrial DNA (mtDNA) markers were widely and effectively used in characterising the population of *Corbicula* (Gomes et al., 2016; Zhu et al., 2018). The purpose of this study was to examine the phylogeny of *C. fluminea* in populations from Kelantan, Malaysia using morphometric and genetic analysis.

2. MATERIALS AND METHODS

2.1 Study area and sample collection

A total of 250 specimens of *C. fluminea* were randomly collected from five locations: Jeli, Tumpat, Pasir Mas, Pasir Putih, and Bachok, Kelantan, Malaysia. These locations were determined based on the harvested primary sources of *C. fluminea* in Kelantan. The *C. fluminea* samples were collected using a hand dredger and brought to the Aquaculture laboratory, Universiti Malaysia Kelantan. Samples were preserved in alcohol and kept in the refrigerator at -20°C.

Table 1: Sampling location and coordinates

Number	Sampling Locations	(Latitude, Longitude)
1	Lake Pergau, Jeli	5°37'13.4"N 101°42'11.5"E
2	Kampung Kubang Batang, Tumpat	6°13'32.8"N 102°15'21.7"E
3	Kampung Gadong, Pasir Mas	6°04'13.0"N 102°08'41.8"E
4	Kampung Gunong, Bachok	5°59'17.8"N 102°20'12.5"E
5	Kampung Tasek Pauh, Pasir Putih	5°53'41.3"N 102°21'08.8"E

2.2 Phenotypes

All samples taken from five different locations were examined morphologically, including shell colour and shape (morphotypes), as well as compared to deposited specimens (paratypes) at the Mollusk Museum at Mahidol University in Bangkok, Thailand. Following that, morphometric characteristics of clams from five different places were analysed.

2.3 Shell Morphotypes

Shell morphotypes are mainly described the shell colour and shape, which were visually classified based on the European interior-surface morphotypes descriptions: dark-coloured round (R), round light-coloured (Rlc), and saddle (S) (Pigneur *et al.*, 2011; Peñarrubia *et al.*, 2017). Both characteristics were observed in 250 individuals from five locations.

2.4 Morphometric characterisations of Corbicula

Initially, the visceral of the clam was removed using a scalpel and preserved in 95% ethanol for further use. The shell was dried in the oven overnight at 60°C before taking the measurement. This method could improve the visibility of the characters' lines. It was determined and measured fifteen morphometric characters. A digital calliper was used to take the morphometric measures (Absolute Digimatic Caliper/Mitutoyo (UK) Ltd — the accuracy of 1/50 mm). A total of 250 individuals from five locations were measured. Figure 1 below shows the morphometric character's measurements for *C. fluminea* and their description in Table 2.

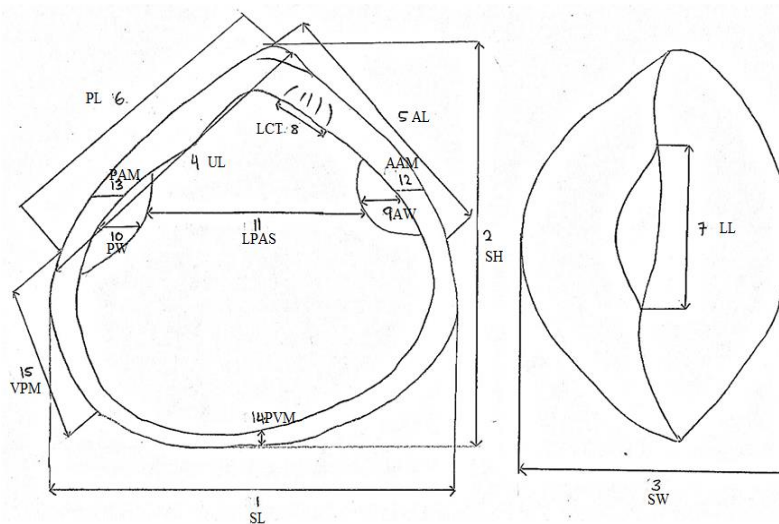


Figure 1: Morphometric characteristics of *Corbicula fluminea*

Table 2: Description of morphometric characters

Number	Morphometric Characters	Acronyms
1	Standard length	SL
2	Shell height	SH
3	Shell width	SW
4	Umbo length	UL
5	Anterior length	AL
6	Posterior length	PL
7	Ligament length	LL
8	Cardinal tooth length	LCT
9	Anterior adductor muscle scar width	AW
10	Posterior adductor muscle scar width	PW
11	Length from anterior adductor muscle scar to posterior adductor muscle scar	LPAS
12	Length from anterior adductor muscle scar to anterior margin	AAAM
13	Length from posterior adductor muscle scar to posterior margin	PAPM
14	Length from ventral margin to the pallial line	PVM
15	Ventral posterior margin length	VPM

Allometric ratios were used to transform morphometric data into allometric ratios, and the standard length (SL) was used as a predictor to minimise demographic influences. Data was analysed for normality using Shapiro-Wilk's and Levene's test for homogeneity of variances for the normality test. The data were then analysed using Principal Component Analysis (PCA) to emphasise variance and indicate a dataset's strongest component. (Pigneur *et al.*, 2011). Following that, differences in *C.fluminea* populations were analysed using a one-way analysis of variance (ANOVA) and the Tukey HSD posthoc test to evaluate whether the difference was statistically significant at the probability level (p), $p < 0.05$. Further analysis, canonical discriminant analysis was carried out using allometric data in classifying the *C.fluminea* populations based on their most significant factors (Renard *et al.*, 2000). A two-dimensional plot of the individuals was composed based on the first two canonical variables. The statistical analyses were executed using SPSS (version 23; SPSS Inc.).

2.5 Genotypes

2.5.1 DNA extraction

The DNA extraction was carried out and followed standard Protocol 6: Rapid Isolation of Mammalian DNA (Sambrook & Russel, 2001). A total of 150 individuals were randomly collected from five sites in Kelantan. Thirty individuals of each location (fresh or preserved in 95% ethanol) were dissected, and the mantle tissue was obtained. A total of 100mg of tissue was pulverised using a micro pestle in a 15ml centrifuge tube containing 3ml of ice-cold cell lysis buffer. Then, 1ml of ammonium acetate solution was added and vigorously vortex for the 30s. A pellet of protein was visible at the bottom of the centrifuge tube after centrifugation at maximum speed (16,000 rpm) for 5 min at 4°C. The supernatant was transferred to a new centrifuge tube containing 3ml isopropanol. The solution was well mixed then the precipitation of DNA was recovered by centrifuging the centrifuge tube at maximum speed for 3 min at room temperature. Next, the supernatant was removed and allowed to aspiration before 3ml of 70% ethanol was added to the DNA pellet. The centrifuge tube was inverted several times and centrifuged at maximum speed for 3 min at room temperature. The supernatant was removed, and the DNA was allowed to dry for 15 min. Finally, the DNA pellet was dissolved in 500µl of TE buffer (pH 7.6) and stored in a freezer (-20°C) for further use.

2.5.2 Quality and quantification of DNA Sample

Analysis of the quality of the extracted DNA samples was carried out using a 1 percent agarose gel electrophoresis followed by quantification using a Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA) at ratio readings of 260nm and 278nm. The purity of nucleic acids was assessed by calculating the 260/280 ratio based on the purity range of 1.8 to 2.0 (Desjardins & Conklin,

2010). Afterwards, each DNA sample was diluted in TE buffer to a concentration of 50-100ng/mL in preparation for polymerase chain reaction (PCR) amplification.

2.5.3 Gene amplification and sequencing

The genetic markers amplify the targeted gene in PCR; partial mitochondrial cytochrome b (cyt b) (481bp). The amplification was performed in a total volume of 50µl containing 25µl PCR master mix (SMOBIO Technology, Inc. Taiwan), 0.5µM forward and reverse primers, 1µl of DNA template (50-100ng), and 22µl of nucleus free water, which make up to 50µl per tube. A 96 well thermal cycler (T-100, BioRad, USA) was used to perform PCR amplification using the respective following program after optimisation was carried out for each primer (Table 3). PCR amplicons were separated on 1% agarose gel electrophoresis and sequenced by accredited laboratory 1st Base.

Table 3: Genetic markers, primer sequences, and their PCR conditions

Gene Markers	Primer Sequences	PCR Conditions	Reference
mtDNA cytb	F:5'GAAGGGTTCATGCTA ACGGAG-3' R:5'GTGGTGTACTTATAG GGTCGGC-3'	Initial denaturing at 94°C for 2 min, followed by 35 cycles of 94°C for 3min, annealing at 54°C for 45s and 72°C for 2 min with a final extension at 72°C for 5min.	Gomes <i>et al.</i> (2016); Zhu <i>et al.</i> (2017); Ramli <i>et al.</i> (2020)

2.6 Data Analysis

Multiple Sequence Alignment was used to align the partial mtDNA sequences of cytb genes in consensus (ClustalW, Kyoto University, Japan). Following that, using the Basic Local Alignment Search Tool (BLAST), the consensus gene sequences were compared to the DNA nucleotide database and corresponding to identify the unknown sequences (Magare *et al.*, 2015). Then, using the software packages Molecular Evolutionary Genetics Analysis (MEGA 6.0), phylogenetic analysis was carried out by generating phylogenetic trees using Maximum Likelihood (ML), Neighbour-Joining (NJ), and Maximum Parsimony (MP) (MEGA 6.0). This analysis was constructed using 1000 bootstrap replicates. Then, phylogeographic analysis for mtDNA cytb sequences was analysed, particularly for *Corbicula* assessment where *Polymesoda caroliniana* (KX713250) was incorporated as an out-group (Ramli *et al.*, 2020).

3. RESULTS

3.1 Phenotypes

The paratypes referred to in the Mollusk Museum are predominantly R and Rlc morphotypes for *C. fluminea*, *Corbicula sp.*, and *C. lamarkiana* (Table 4). Several species, however, were designated for S morphotypes that were later synonymised with *C. petiti*, *C. lamarkiana*, *C. moreletiana*, and *C. ostiorum* syntypes. As a result, determining which *Corbicula* (morphotype S) belonged was more difficult. The paratypes of *Corbicula spp.* deposited in various locations around Thailand are listed in Table 4. Therefore, this reference is limited to specimens from Thailand, which are useful for comparison as phenotypic references.

Table 4: Paratypes of genus *Corbicula* with location and morphotypes deposited in Mollusk Museum, Mahidol University, Bangkok, Thailand.

Paratypes	Location	Morphotypes
<i>Corbicula fluminea</i> , TMMU43	Tha Sa Ra, Kanchanaburi	R
<i>Corbicula sp.</i> , DN81100-20	Ban Ko Village, Chiang Rai	R
<i>Corbicula fluminea</i> , SMRL 2710	River Chao Praya, Chainat	R
<i>Corbicula lamarkiana</i> , SMRL 2705	River Chao Praya, Chainat	R
<i>Corbicula fluminea</i> , SMRL 6821	River Ping, Chiang Mai	R
<i>Corbicula fluminea</i> , TMMB14	River Ping, Kamphaeng Phet	Rlc
<i>Corbicula petiti</i> , SMRL 2701	Nong Han Lake, Sakhon Nakon	S
<i>Corbicula lamarkiana</i> , SMRL 2750	Petburi River, Petburi	S
<i>Corbicula moreletiana</i> , SMRL 2765	Irrigation canal, Ubon Ratchathani	S
<i>Corbicula ostiorum</i> , SMRL 2722	Ta Pi River, Surat Thani	S

Analysis of variance (ANOVA) showed five characteristics (SH=Shell height, SW= standard width, UL= Umbo length, AL=Anterior Length, PL= Posterior Length) were significantly different ($p<0.05$) between *C. fluminea* in seven localities. Shell height (SH) for *C. fluminea* in Cambodia, Thailand, and Tumpat were found larger in the allometric ratio ($p<0.05$) compared to the other localities where the ratio was above 0.90 (Table 5). Then, *C. fluminea* in Jeli showed significantly smaller (0.845 ± 0.03) while other localities were not significantly different (Table 5). Standard width (SW) for Thailand and Cambodia was found significantly larger than those from Kelantan, where the ratio was documented at 0.698 ± 0.08 and 0.734 ± 0.06 , respectively. Meanwhile, *C. fluminea* in Tumpat, Jeli, and Pasir Mas has an insignificantly SW ratio ($p<0.05$). The SW for Bachok seems to share similar characteristics with the *C. fluminea* populations from Pasir Putih and Pasir Mas (Table 5). The longest umbo length (UL) and anterior length (AL) among the *C. fluminea* were found in Jeli and Cambodia. These characters were significantly differed compared with other locations (Table 5). Finally, the posterior length (PL) was varied between *C. fluminea* in all locations where the length ratio was recorded as less than 0.60.

Table 5: Analysis of variance (ANOVA) of five morphometric characteristics corresponding to the locations

Locality / Morphometric Characteristics	Shell Height (SH/SL)	Shell Width (SW/SL)	Umbo Length (UL)	Anterior Length (AL)	Posterior Length (PL)
Jeli	$0.931\pm 0.05ab$	$0.575\pm 0.06c$	$0.752\pm 0.04a$	$0.559\pm 0.07b$	$0.598\pm 0.05a$
Tumpat	$0.845\pm 0.03d$	$0.577\pm 0.03c$	$0.653\pm 0.06b$	$0.495\pm 0.03c$	$0.585\pm 0.04ab$
Bachok	$0.878\pm 0.07c$	$0.585\pm 0.04bc$	$0.676\pm 0.13b$	$0.523\pm 0.04c$	$0.553\pm 0.05ab$
Pasir Putih	$0.900\pm 0.05bc$	$0.637\pm 0.04b$	$0.655\pm 0.03b$	$0.506\pm 0.02c$	$0.577\pm 0.02ab$
Pasir Mas	$0.891\pm 0.02cd$	$0.611\pm 0.03c$	$0.650\pm 0.02b$	$0.501\pm 0.03bc$	$0.569\pm 0.02bc$

* Each value represents the group's mean ratio, and values in the same row with different letters are significantly different ($p<0.05$).

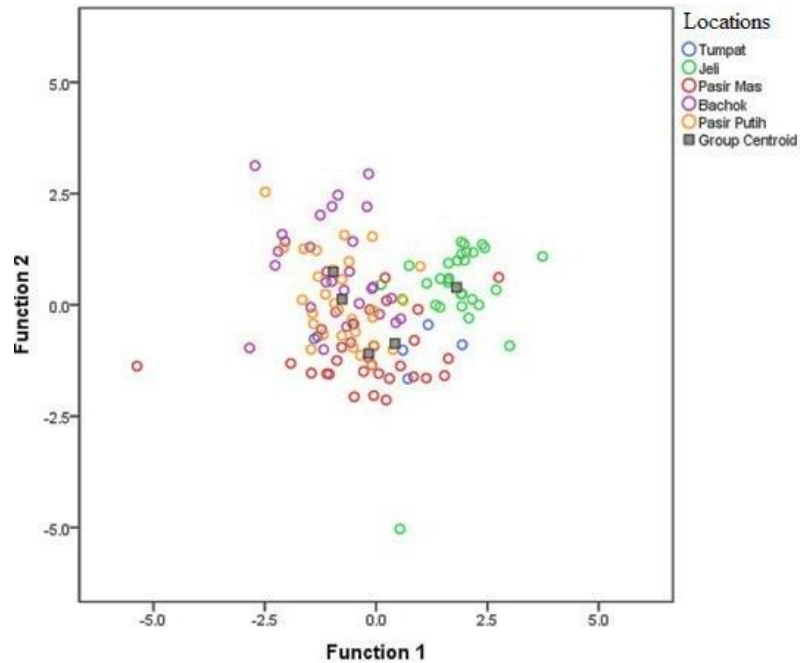


Figure 2: Morphometric analysis using discriminant analysis-group graph for five locations in Kelantan

The canonical discriminant plots of functions 1 and 2 for the morphometric characteristics (Figure 2). In the canonical discriminant functions, the highest Eigenvalue was attained for two functions (2.865 and 0.707). Function 1 accounts for 69.7% of the variability, whereas Function 2 accounts for 17.2%. Based on the Eigenvalue, these functions described the variance in the dependent variable. Figure 2 shows overlay separation between *Corbicula* populations in five locations in Kelantan and two populations from Thailand and Cambodia. Based on the discriminant analysis, function one was positively correlated between the ratio SW/SL and AL/SL. Meanwhile, a negative correlation was found between SH/SL, UL/SL, and PL/SL. These unequal signs of the correlation values explained the shell shape of the *Corbicula*. The second function (Function 2) indicates a measurement of the overall size of the *Corbicula* shell. In addition, the correlation of these morphometric characters was nearly positive in the second function. The discriminant functions are orthogonal, indicating that the shape of the shell (Function 1) is unrelated to the shell size (Function 2). Based on the morphological analysis, Figure 2 shows that all populations from Kelantan were located adjoining each other. Particularly, the population from Jeli was found distant to those are from Pasir Mas, Tumpat, Bachok, and Pasir Putih. Hence, Figure 2 shows an impossible to separate or characterised *C. fluminea* based on morphometric characteristics only.

3.2 Genotypes

The tree's construction was incorporated *Polymesoda caroliniana* (KX713250) sequence as an out-group and based on Kimura 2-parameter genetic distance. The topology depicted in Figure 3 was inferred using the maximum-likelihood (ML) bootstrap value represented by nodes and used to create phylogenetic relationships between *C. fluminea* populations in Kelantan. Similar topology had resulted from Maximum Parsimony (MP) and Neighbouring Joining (NJ), which could be merged as shown in Figure 3. The bootstrap values correspond to ML, MP, and NJ at the branches. The topology displayed robust dichotomous splitting of two clades by disregarding the out-group.

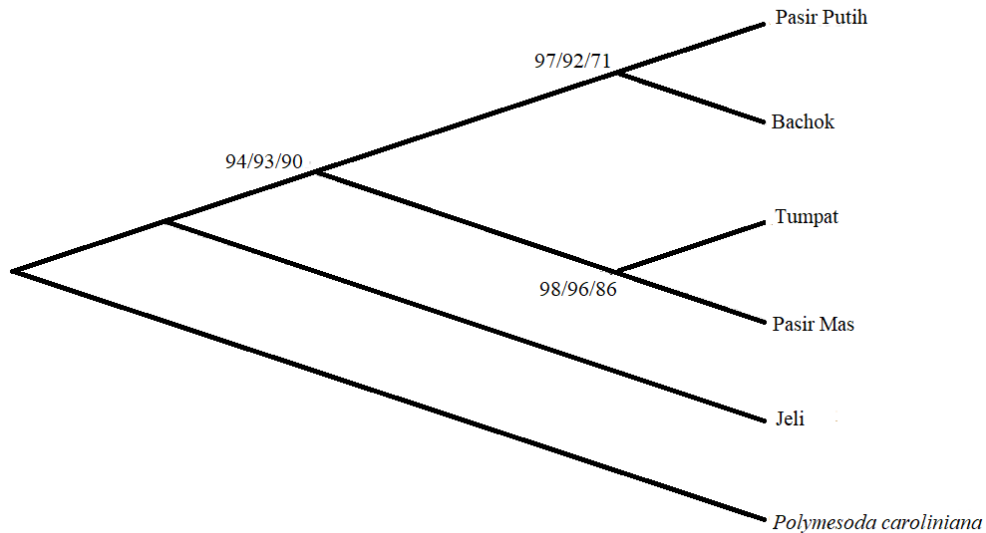


Figure 3: Phylogenetic tree of five *Corbicula fluminea* populations constructed by the Maximum Likelihood (ML), Maximum Parsimony (MP), and Neighbouring Joining (NJ) method using a 481-bp partial fragment of the mitochondrial gene cytochrome b (cytb).

Table 6: Estimates of Evolutionary Divergence between *Corbicula fluminea* populations in Kelantan

Locations						
Pasir Putih		0.006	0.000	0.006	0.006	0.053
Bachok	0.013		0.006	0.003	0.007	0.053
Tumpat	0.016	0.003		0.006	0.006	0.053
Pasir Mas	0.016	0.013	0.016		0.007	0.053
Jeli	0.018	0.019	0.016	0.022		0.050

Between sequences, the number of base substitutions per site is presented. Above the diagonal, standard error estimates were calculated using a bootstrap approach (1000 replicates). MEGA 6 was used to perform analyses involving the Kimura two-parameter model (Kimura, 1980).

4. DISCUSSION

Corbicula species could be comprehensively characterised through the analysis of phenotypes and genotypes. This study reveals that these methods complement each other to propose the evaluated *Corbicula*'s taxonomy. Erstwhile, *C. fluminea* was obtained from different sources of the freshwaters, such as tributaries of the main rivers and lakes. Present research using European standard as described in Pigneur *et al.* (2011) to distinguish the *Corbicula* morphotypes polymorphism, which was corresponded to round shape and broad form with deep ridges (round form, R) and a narrow form with closely spaced ridges (saddle form, S). Previous literature had equivocal among researchers in determining *Corbicula* species for morphotypes R and S which were recognised as *C. fluminea* and *C. fluminalis*, respectively (Renard *et al.*, 2000; Pfenninger *et al.*, 2002; Sousa *et al.*, 2008). The polymorphism of the morphotypes could be defined as different species since unique characters possibly distinguish them (Heino, 2014). Furthermore, polymorphism occurred due to genetic and environmental factors, leading to frequently misnamed taxa (Kamburska *et al.*, 2013). The polymorphism of the *Corbicula* shell due to the high plasticity is prone to environmental influences, geographical range, and evolutionary elements such as incomplete meiosis (Renard *et al.* 2000; Bodis *et al.* 2011). The morphotype R had dominated the *Corbicula* population regardless of their colours in general recently. Besides, the *Corbicula* with morphotype R and light colour (Rlc) also was found in the current study, but their numbers could be negligible. The existence of morphotype Rlc could have resulted in physiological and ecological plasticity. Hence, in this study, individuals with morphotype Rlc were synonymised and clustered in the morphotype R population. This finding was agreed with Pigneur *et al.* (2011), which proposed that the morphotype Rlc was similar to morphotype R, where both morphotypes correspond to *C. fluminea* while morphotype S solely belongs *C. fluminalis*. Therefore, recent findings appropriately could propose the morphotype Rlc

is the divergence of the morphotype R; nevertheless, it is inexact to conclude their taxa without molecular evidence.

The *C. fluminea* with morphotype R commonly has a large range of inner and outer colours varying from light to dark. It contradicts the morphotype S, circumscribed to the dark colour shell such as dark brown to black (outer) and dark-purple (inner). This characteristic, like colour, mainly depends on their habitat and shape. For example, the *C. fluminea* were obtained from the lake, such as in Lake Pergau, Jeli, Kelantan was found to be dark-brown to black (outer shell). In comparison, *C. fluminea* from other locations in Kelantan was found yellowish-brown in outer shell colour, reflecting the habitat conditions. This observation explains the shell colour variation due to the microevolution events towards local adaptation since the habitat comprises sandy beds and low organic matters (OM). In this case, *C. fluminea* lives in a habitat with higher OM content is commonly found from dark brown to black regardless of their shape. On the other hand, Chang *et al.* (2016) had explained that the higher sulphide levels had thinned and blacker the outer shell, which eventually became white colour and brittle. This phenomenon could be seen in the large size of *C. fluminea*, and those are from the lakes. In another comparison, the recent study investigates *C. fluminea* from Lake Pergau, where they were similar in shell colour (dark) but differed in shell forms. The *C. fluminea* (round with yellow and black shell colour) was previously shown to occupy sandy loam and loamy sand substrates in the Pattani and Saiburi rivers, respectively (Zaween *et al.*, 2017). A similar finding can be seen in *Corbicula* populations from Tumpat, Pasir Mas, Bachok, and Pasir Putih, where the river bed composition is described earlier. The shell shape variation is possibly due to the settlement bed state: sandy loam, rocky, silt or their combination, water current, OM, and food availability (Pigneur *et al.*, 2011). Therefore, the *Corbicula* adaptation towards the ecological conditions contributed to their shell shape and colour polymorphisms instead of genetic contributions.

The polymorphism in *C. fluminea* found in the recent study has enhanced the understanding of the speciation. These variances of morphotypes are distributed allopatric and sympatric. For example, the *C. fluminea* in Lake Pergau was hypothesised to originate from the Kelantan River, which was unintentionally introduced into the lake through dumped sands during the lake's construction. Their colours contrasted between *C. fluminea* from Kelantan River tributaries and Lake Pergau, perhaps clearly explaining the allopatric speciation. However, they shared similar morphotypes R. Moreover, the evaluated *C. fluminea* morphotypes are compared with the specimens deposited in Mollusk Museum, Mahidol University, Bangkok, Thailand. Recent samples were compared to the specimens where all Kelantan's *C. fluminea* (morphotype R) were synonymised to the paratypes of *C. fluminea* (TMMU43, SMRL 2710, and SMRL 6821), *C. lamarkiana* (SMRL 2705), and *Corbicula sp.* (DN81100-20). Additionally, despite their identical morphotypes, *C. fluminea* and *C. lamarkiana* were discovered to inhabit similar habitats in the Chao Praya River. There is no additional information on how their names were chosen, yet it is reasonable to suggest that these *Corbicula* paratypes in this study were misnamed. In addition, previous reports were synonymised the *C. lamarkiana* to *C. fluminea* based on their morphotypes and genotypes (Glaubrecht *et al.*, 2006; Hedtke *et al.*, 2008; Bódis *et al.*, 2011). Whereas, *Corbicula sp.* remains indistinct, which is usually referring to *C. fluminea* as well. The morphotypes R and Rlc were found sympatrically in *C. fluminea*, which inhabited Ping River, Thailand. This sympatric occurrence was explained by the geographical range where different habitats determined their morphotypes. For example, these paratypes probably from similar lineages since the Ping River flowed from Chiang Mai to Kamphaeng Phet, where the morphotype Rlc resulted from the divergence of morphotype R. In addition, a similar observation can be seen in Kelantan where a few numbers of morphotype Rlc was sympatrically existed with morphotype R. Thus, based on the analysis of the phenotypes, recent findings appropriately identified the *C. fluminea* existed in morphotypes R and Rlc.

The *Corbicula* from Lake Pergau was found it belongs to morphotype R. This variation correspondingly discussed in previous research where the authors had claimed the *C. fluminalis* and *C. fluminea* have sympatrically existed in the largest Italian freshwater lake, Garda Lake, based on these two distinct morphotypes (Ciutti & Cappelletti, 2009). Hence, the findings have generated a conclusion that morphotype S does not belong to *C. fluminalis* since it could be found as *C. fluminea* as well. In addition, the paratypes of *C. lamarkiana* were found in morphotypes R and S, where they were inhabited different rivers in Thailand. Since the *C. lamarkiana* with morphotype R was synonymised to *C. fluminea*, then it also applies to the morphotype S. Based on the previous study, the estuary *Corbicula* samples from Kuala Terengganu, Terengganu, Malaysia, identified as *C. fluminea*, although they possessed a

morphotype S (Ramli *et al.*, 2020). Therefore, these findings had dichotomous inferences where *C. fluminea* with S morphotype could be found in the deep habitat like a lake or found in brackish water habitat near to the mouth of the river. Therefore, discrimination of morphotype through these characteristics (R, S, and Rlc) suffices to distinguish the *Corbicula* across the topography but with uncertain taxonomy assumptions.

Fifteen morphometric characters determined further morphotypes polymorphism of *C. fluminea* populations in this study. However, these characters were stratified through PCA. Only five morphometric characters, such as the proportion of SH/SL, SW/SL, UL/SL, AL/SL, and PL/SL, were evaluated since they were significantly influenced the morphotypes variation. Recent work is similar to the previous study carried out by Pigneur *et al.* (2011), which had evaluated three morphometric characters proportion (SH/SL, SH/SW, and SL/SW) with an addition of 12 characters in order to distinguish *C. fluminea* populations according to their morphotypes (R and S). In general, the ANOVA analysis has depicted the polymorphism of the morphometric characters, and predicting the shell size could be done such as in the recent study. The significant morphometric characters between *C. fluminea* populations indicate shell variation only and could not express their taxa. This finding may elucidate their age and ecological status. A similar observation was found in *C. fluminea* in Jeli, Kelantan. Both results had reflected their status where the large size (e.g., SL in the range 0.931-0.970 mm) reflected an old age.

Meanwhile, in terms of ecology, they inhabited lakes that enormous numbers of them could anticipate. In reality, harvesting activities in these lakes are restricted in a certain area due to the geographical range and benthic structure, which indirectly allow this species to propagate and maintain their populations. For example, *C. fluminea* is abundantly found in Lake Pergau, Jeli, Kelantan, where the mean density was recorded at 113.63 individuals/m² (Eh Rak *et al.*, 2021). On the other hand, the *C. fluminea* in Pasir Mas, Bachok, Pasir Putih, and Tumpat was smaller (e.g., SL in the range of 0.845-0.900 mm). These tributaries comprise the sandy loam river bed, and the distribution is declined in these areas. This status quo is a consequence of severe harvesting activities by locals to fulfil the market. Moreover, the stream was affected by sand mining along the Kelantan River. This activity shallows the river bed and destroys the benthic ecosystem where the *C. fluminea* habitat was excavated. This activity continuously wipes out the juveniles in the sands. Hence, this activity jeopardies the numbers of *Corbicula* in the Kelantan's freshwaters which resulted in the demographic changes as shown in recent research where small shells could be seen abundantly.

In comparison to other *C. fluminea* populations in Kelantan, they were collected from the tributaries of the Kelantan River. The size is marginally smaller compared with those are inhabited lakes. Furthermore, the existence of *C. fluminea* in particular tributaries is due to the dispersion mechanism of this species during juveniles from the main river. This kind of life strategy was described by Bódis *et al.* (2011). The *C. fluminea* has good dispersion abilities, ecological tolerance, high fecundity, and growth rates, dominating the benthic communities. In earlier, *C. fluminea* was identified propagating through passive dispersion stream the water current (Mouthon, 2001). In more recent, the distribution of *C. fluminea* was found upstream of the Danube River, Bulgaria tributaries which describe the dispersion path against the water current and may be due to humans intervened (Hubenov *et al.* 2013). Unfortunately, current research could not obtain *C. fluminea* samples from the main river due to geographical factors and the absence of this species. Therefore, this study assumed the *C. fluminea* from the stretch tributaries to represent Kelantan River's population. Nevertheless, the canonical discriminant analysis shows that *Corbicula* populations were associated based on the characterisation of phenotypes (Figure 2). This distribution portrays the geographical separation, and it may reflect the genetic distance since the phenotypes are the results of the genotypic expressions.

A recent finding shows that *C. fluminea* from Tumpat, Pasir Mas, Bachok, and Pasir Putih was similarly predicted in size based on evaluated morphometric characteristics (Table 5). Geographically, the tributaries in Tumpat, Bachok, Pasir Putih, Pasir Mas were extended from the Kelantan River. In general, the *C. fluminea* from Jeli was found out-clustered, demonstrating their position in different geography ranges. For instance, the Jeli population inhabited upstream (lake), distinctly separated from other locations. The divergence can also be seen in Tumpat. Earlier hypotheses had postulated that Tumpat *C. fluminea* was from Thailand lineages since the sampling canal is also geographically connected to Sungai Kolok, Thailand. However, based on the canonical discriminant distribution, this *C. fluminea* exhibited more closely related to the Kelantan lineages in phenotypic characterisations.

Phenotypes in *C. fluminea* are contributed by genetic materials and environmental factors or their interaction. The current study emphasised the genotypic evidence of *C. fluminea* to anchor the phenotypic characterisation. Therefore, genetic assessment applied in a recent study elucidates and confirms the taxa names of the evaluated *C. fluminea*. In a recent study, mitochondrial DNA markers (mtDNA) such as partial cytb were employed. These targeted genes were selected since they were currently widely used in genotypes analysis of *Corbicula*. Based on the recent finding, all evaluated *Corbicula* refer to *C. fluminea* species regardless of their morphotypes and locations. The previous study clarifies that similar species could possess different morphotypes since *Corbicula* has a high degree of polymorphism (Renard *et al.*, 2000).

In this study, mtDNA cytb sequences were used in analysing the distance between local *C. fluminea* populations. Similar topology was generated by employing ML, MP, and NJ, which showed a robust dichotomous separating two clades indicating low genetics evolutions. The *C. fluminea* from Jeli can be hypothesised as the genetics allopatric evolution that separates them from other *C. fluminea* populations. On the other hand, the population of *C. fluminea* in Lake Pergau, Jeli may disperse to Kelantan River since the Pergau River (the only river connected to the Pergau Lake) is one of the main sources for Kelantan River. Hence, the population of *C. fluminea* in Kelantan River and its tributaries in Pasir Mas, Tumpat, Pasir Putih, and Bachok may have an introgression of genetics from *C. fluminea* in Lake Pergau, Jeli. The distance of evolution shows narrow variation, which explains the lineages which probably shared the same ancestral history. On the other hand, the evaluated sequences of *C. fluminea* between populations in Kelantan can be hypothesise a low genetic variability due to hermaphroditic characteristics. This low genetic variation (Table 6) was demonstrated among the population. The genetic variation may be attributed to biotic and abiotic adaptation that influences the phenotypes (Gomes *et al.*, 2016). Thus, this study hypothesises that low genetic variations between *C. fluminea* in Kelantan may result in a bottleneck event requiring extended evidence.

5. CONCLUSION

Characterisation of *C. fluminea* through phenotypes and genotypes had provided a comprehensive perspective in identifying their taxa, polymorphism, and lineages history across the geographic range. At this time, it is appropriate to conclude that the *Corbicula* found in Kelantan belong to a single species, despite their polymorphism having resulted in phenotypic diversity. Then, genotypes analysis has anchored the phenotypes of *C. fluminea* in the current study since the findings coincide. Therefore, the integrated analysis of the phenotypes and genotypes on the *C. fluminea* generates a consensus discovery of taxonomy, elucidates the polymorphism attributes and anticipation of the life history.

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