THE MOLECULAR APPROACH REVEALS THE RELATIONSHIP AMONG VENUS CLAMS (*Meretrix* spp.) COMMUNITY IN MALAYSIA

MOHD HANAFI IDRIS[1], ABU HENA MUSTAFA KAMAL[1], HADI HAMLI[2]*, AMY HALIMAH RAJAEE[2,3] AND ABDULLA-AL-ASIF[2]

[1]*Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, 21300 Kuala Nerus, Terengganu, Malaysia*
[2]*Department of Animal Science and Fishery, Faculty of Agricultural and Forestry Sciences, Universiti Putra Malaysia, Bintulu Sarawak Campus, Bintulu, Sarawak, Malaysia*
[3]*Institut Ekosains Borneo, Universiti Putra Malaysia, Bintulu Sarawak Campus, Bintulu, Sarawak, Malaysia*

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ABSTRACT

Molecular study is important to detect variations and similarities among species from the same genus, in case if they do not encompass any morphological or physiological differences. The study was conducted to differentiate among species of *Meretrix* spp. (*Meretrix lyrata*, *M. meretrix*, and *M. lusoria*) obtained from two locations in Malaysia through the phylogenetic tree. The adductor muscle tissues were used to extract DNA and to perform other procedures; the samples were subjected to analyses using PCR and gel electrophoresis. The multiple sequence comparison was conducted by MUSCLE and the phylogenetic relationships were established using Maximum Likelihood (ML) statistical methods with MEGA 6.0 statistical software. *M. lyrata* samples showed 99% similarity to the three accessions sequence, where *M. lyrata* indicated 87% similarities, and *M. meretrix* showed not more than 89% similarities from the deposited sequence. The nucleotide base composition sequences consisted of the mean of Thiamine (T) 37.9%, Cytosine (C) 15.4%, Adenine (A) 27.4%, and Guanine (G) 19.4%. Maximum Likelihood (ML) analysis was conducted using the Tamura 3-parameter model to establish five major clades on *Meretrix* spp. and two out-groups clades significantly different from the *Meretrix* spp. These major clades were closely related to each other at the 50% evidence of bootstrap, which grouped as genus *Meretrix*. The present study on *Meretrix* spp. from the Sarawak locality was able to differentiate COI sequences between *M. lyrata*, *M. meretrix*, and *M. lusoria*. *M. lusoria* was close related to *M. meretrix* with strong bootstrap supporting evidence at 96% scoring. Moreover, *M. lyrata* was inferred as the ancestor to *M. meretrix*, and *M. lusoria* from Sarawak, Malaysia.

Keywords: bivalve, BLAST, Borneo, mtDNA, PCR, phylogenetic analysis

INTRODUCTION

Genetic variation can be characterized into two groups which are intraspecific and interspecific (Arruda *et al.* 2009; Ehlers *et al.* 2016; Layton *et al.* 2016; Thia *et al.* 2016). Intraspecific is the variation within individuals from similar species such as subspecies (Stevens *et al.* 2010). On the other hand, interspecific is the variation within individuals above the species level. Genetic variation of natural populations is thought to be governed by the mutual effects of irregular genetic drift, restricted gene flow, and variance of selection pressures. Intense natural selection is an important factor influencing variation among, and within groups (Endler 1986). The natural selection can be stabilized the species characters such that genetic variation within the population might be reduced. Disruptive selection may lead to genetic divergence, and therefore, may eventually increase genetic variation among population (Backeljau *et al.* 2001).

Mitochondrial DNA (mtDNA) is one of the DNA markers that are popular for DNA identification. This marker is the maternal inheritance that supplies information absent in nuclear markers (Okumuú & Çiftci 2003).

*Corresponding author, email: hadihamli@upm.edu.my

Therefore, selecting tissue from the female individual is essential to ensure information regarding the species ancestor. However, the maternal inheritance by mtDNA can be restricted by the geographical with no mixing of mtDNA haplogroups from the different geological regions (Luttikhuizen *et al.* 2003; Mishmar *et al.* 2003; Layton *et al.* 2016). Furthermore, inheritance characteristics on a particular defect gene could be recognized using an mtDNA marker (Wallace *et al.* 1999). This is significant, particularly in aquaculture, to ensure cultured species are free from any inherited diseases from the parents. Moreover, mtDNA could be applied for species identification and phylogeny studies due to the particular inherited gene variance.

The typical mitochondria gene used for the phylogenetic study is cytochrome C oxidase subunit I (COI). This mtDNA marker has been applied for genetic relationship and phylogenetic analysis for *Meretrix* spp. from Japan, Korea, and China (Yamakawa *et al.* 2008, Chen *et al.* 2009; Torii *et al.* 2010; Kim & Yoon 2014; Sato *et al.* 2016). Parentage assignment from a similar population but different parents was also successfully implemented for *Meretrix meretrix* culture (Lu *et al.* 2011). Due to the intensive mtDNA marker application, the universal primer of COI had been created based on the conserve gene sequence across the species to facilitate COI analyzing region on different metazoan species (Folmer *et al.* 1994).

The study of Mollusca started from the study of Morris & Purchon (1981), their approach was to enlist and taxonomic study of available species; however, the story of mollusk study goes on, and Malaysian scientists and academician studied a different aspect of Mollusca; from taxonomy to diversity, along with the genetic aspect of this community (Hamli *et al.* 2012a, b; 2013; 2015; 2017; 2019; 2020a, b, c; Foon *et al.* 2017; Al-Asif *et al.* 2020; Al-Asif *et al.* 2021; Idris *et al.* 2017a, b). Genetic and molecular study of class Bivalvia and Gastropoda is not new in Malaysian territory, including East Malaysia (Sabah and Sarawak Provinces). Some previous study revealed the genetic diversity of Asian green mussel (*Perna viridis*) in the Waters of Sabah (Lau *et al.* 2018), DNA study of Neritid (Chee & Mohd Nor 2016), genetic diversity of razor clam in Kuala Selangor, Malaysia (Hassan & Kanakaraju 2016). There is no genetic approach of Venus clam (*Meretrix* spp.) was found in Malaysia, and the genetic relationship among the available Venus clam species is still unknown. Therefore, the present study aims to differentiate among *Meretrix* spp. from different through the phylogenetic tree.

MATERIALS AND METHODS

Sample Collection

A total of 30 samples of *Meretrix lyrata*, *M. meretrix,* and *M. lusoria* were collected from two areas, Kuching and Kabong, Sarawak Province, Malaysia (Fig. 1). Adductor muscle tissues from the females were separated from the shell and preserved with 95% of ethanol (Wang *et al.* 2010) for further analysis at the Parasitology Laboratory, Department of Animal Science and Fishery, Universiti Putra Malaysia, at the Bintulu Sarawak Campus.

DNA Extraction

About 10 to 20 mg of adductor muscle tissue from each sample was used for extraction using a tissue DNA extraction kit (Vivantis) following the manufacturer's instructions and stored at -20 °C. The extracted DNA was then quantified through agarose gel electrophoresis.

DNA Quantification and Gel Electrophoresis

Extracted DNA was quantified following Rengarajan *et al.* (2002) to ensure the amount of DNA within 1 µL. Each quantified DNA was adjusted to 100 µL to ensure the total amount of DNA sample used for amplification was equal. Quantification of genomic DNA was compared based on Lambda Hind III marker (23130 bp). Gel agarose concentration used for DNA quantification was 0.8% in 1X TAE (Tris-Acetate-EDTA) buffer and the electrophoresis procedure was conducted following Lee *et al.* (2012). A total of 5 µL of extracted genomic sample and 1 µL of dye (EZ-Vision One dye) were added into the well. After all the samples and markers were loaded, electrophoresis was run for 40 minutes with 120 Volts of electricity. The running of electrophoresis was monitored until the red dye was moved 3/4 of the gel length, and the electrophoresis was stopped.

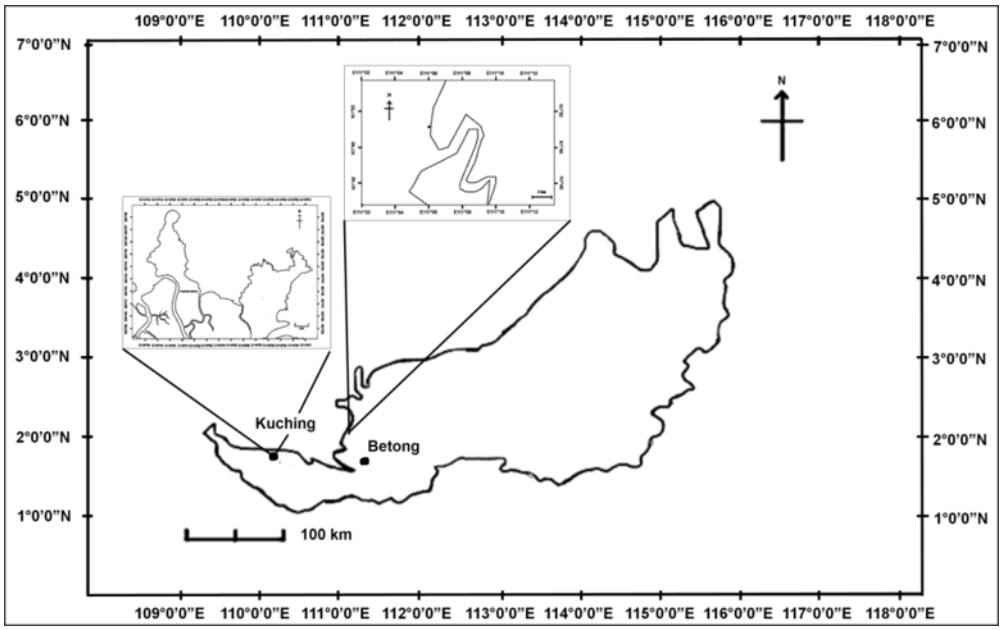

Figure 1 Study area of *Meretrix* spp. for genetic study

The gel was then removed from the tank and was photographed under a UV light gel imager (Red™, Alpha Innotech). Quantification process based on appeared bands on the gel to be viewed with Alpha View software and band intensity from extracted samples was compared with Lambda Hind III marker for 100 ng of DNA.

While 1.5% of gel agarose concentration was used for the Polymerase Chain Reaction (PCR) through 0.3 g of agarose powder into 20 mL of 1X TBE (Tris-Borate-EDTA) buffer, the mixture was swirled and heated in the microwave to ensure agarose powder was dissolved. Heated mixture was monitored to make sure the mixture was not boiled. After agarose powder dissolved in the buffer, the mixture was left to cool and then was poured slowly into the prepared gel tank while ensuring that no bubble was trapped. Immediately, the comb was inserted into the poured mixture, while letting the agarose mixture to be cool and solidify for 30 minutes. After the gel became solid, the comb was removed and the running buffer 1X TBA or 1X TBE was poured into the gel tank until all the gel parts submerged.

Polymerase Chain Reaction (PCR) Amplification

Amplification of DNA was performed in 50 µL of PCR mixture using universal marker cytochrome C oxidase subunit I (COI) with sequence LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer *et al.* 1994). Each PCR reaction consisted of 0.1 mM of reverse and forward primers, 0.2 mM of dNTP, 2.0 mM $MgCl_2$, 1x buffer, 1.25 units of Taq polymerase, 100 ng of template DNA, and ultrapure water to complete 50 µL.

A total of 50 µL of PCR mixture were amplified in XP Thermal Cycler Block (Bioer Technology co. LTD) with pre-denaturation at 95 °C for 5 minutes, then followed by 35 cycles of 1 minute at 95 °C for denaturation, 1 minute for annealing at 40 °C, 1 minute 30 second at 72 °C for extension and 72 °C for 7 minutes for final extension (Folmer *et al.* 1994). The last cycle temperature was maintained at 4 °C. The PCR product then underwent electrophoresis.

Gel Electrophoresis

Gel agarose concentration used was 1.5% in 1X TBE (Tris-Borate-EDTA) buffer. The electrophoresis procedure was carried out following Lee *et al.* (2012). A total of 100 ng of PCR product was added into the well with 1 µL of dye (EZ-Vision One dye) and 1 µL of 100 bp ladder (Promega) with 1 µL of dye (EZ-Vision One dye) at the end of the well. After all samples and markers were loaded, electrophoresis was run for 60 minutes with 80 Volts of electricity. The running of

electrophoresis was monitored until the red dye was moved 3/4 of the gel length, and the electrophoresis was stopped. The gel was then removed from the tank and was photographed under a UV light gel imager (Red™, Alpha Innotech). The appeared band was then compared to the ladder to identify the fragment size.

Purification and Sequencing

PCR products were purified before sequencing. According to the manufacturer's instruction, purification has been done to remove excess dinucleotide triphosphate (dNTP) using Themo Scientific GeneJet PCR Purification Kit. Afterward, purified samples were sent to the First Base Laboratory, Malaysia, for DNA sequencing. Both strands of PCR products and primers were sequenced using the Applied Biosystems BigDye Terminator v3.1 cycle sequencing kit.

Statistical Analysis

The obtained sequences were inspected and assembled using BioEdit version 7.0. The assembled sequence of *M. lyrata, M. meretrix,* and *M. lusoria* were then compared with all species of *Meretrix* spp. in GenBank of National Center for Biotechnology Information (NCBI) using Basic Local Alignment System Tool (BLAST) 2.2.31 (National Center for Biotechnology Information, Bethesda, MD, USA [http://www.ncbi.nlm.nih.gov/BLAST/]). The highest similarity sequence from the GenBank with *Meretrix* spp. was selected for phylogenetic analysis in the present study.

The present *Meretrix* spp. sequence, 16 accessions sequence mtDNA, COI sequence, and 2 species with COI sequence as an outgroup which *Mercenaria mercenaria* and *Paphia gallus* (Table 1) in Genbank NCBI were aligned based on multiple sequences comparison by log-expectation using MUSCLE (Edgar 2004). Phylogenetic relationships were developed using Maximum Likelihood (ML) statistical methods with MEGA 6.0 statistical software (Tamura *et al.* 2013).

The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura 1992). The non-uniformity of evolutionary rates among sites was modeled using discrete Gamma distribution (+G) with 5 rate categories. The bootstrap agreement tree inferred from 500 replicates (Felsenstein 1985) was taken to represent the evolutionary history of the analyzed taxa (Felsenstein 1985). Branches equivalent to partitions reproduced in more than 50% bootstrap replicates were illustrated. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was shown next to the branches (Felsenstein 1985). The heuristic search's initial tree was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with a superior log-likelihood value. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.* 2013).

Table 1 List of *Meretrix* accession sequences from the GeneBank (NCBI)

Meretrix accessions	Geographical locations	GenBank Acession Number	Citation
Meretrix lyrate	Sarawak, Malaysia	Present study	Present study
M. lusoria	Sarawak, Malaysia	Present study	Present study
M. meretrix	Sarawak, Malaysia	Present study	Present study
M. lyrata	China	KC832317.1	Wu *et al.* (2014)
M. lyrata	China	J N898944.1	Cheng *et al.* (2013)
M. lyrata	China	HM124580.1	Chen *et* al. (2011b)
M. lusoria	China	GQ903339.1	Wang *et al.* (2010)
M. lusoria	Japan	AB853870.1	Yamakawa & Imai (2013)
M. lusoria	Japan	AB853865.1	Yamakawa & Imai (2013)
M. meretrix	China	GQ463598.1	He *et al.* (2011)
M. meretrix	China	HM124578.1	Chen *et al.* (2011b)
M. meretrix	China	JN043623.1	Wang *et al.* (2011a)
M. petechialis	China	EU145977.1	Ren *et al.* (2009)
M. petechialis	China	HM124582.1	Chen *et al.* (2011b)

Table 1 (Continued)

M. petechialis	China	HQ703177.1	Chen *et al.* (2011a)
M. lamarckii	China	GU071281.1	Wang *et al.* (2011b)
M. lamarckii	China	HM124579.1	Chen *et al.* (2011b)
M. lamarckii	Japan	AB059420.1	Hamaguchi *et al.* (2001)
M. casta	India	JQ773441.1	Ranjith *et al.* (2012)
Mercenaria mercenaria *	Canada	HM884239.1	Layton *et al.* (2014)
Paphia gallus *	China	HQ703232.1	Chen *et al.* (2011a)

Note: * = Outgroup.

RESULTS AND DISCUSSION

The COI marker produces a fragment size at 710 bp for *M. lyrata*, *M. meretrix*, and *M. lusoria* (Fig. 2).

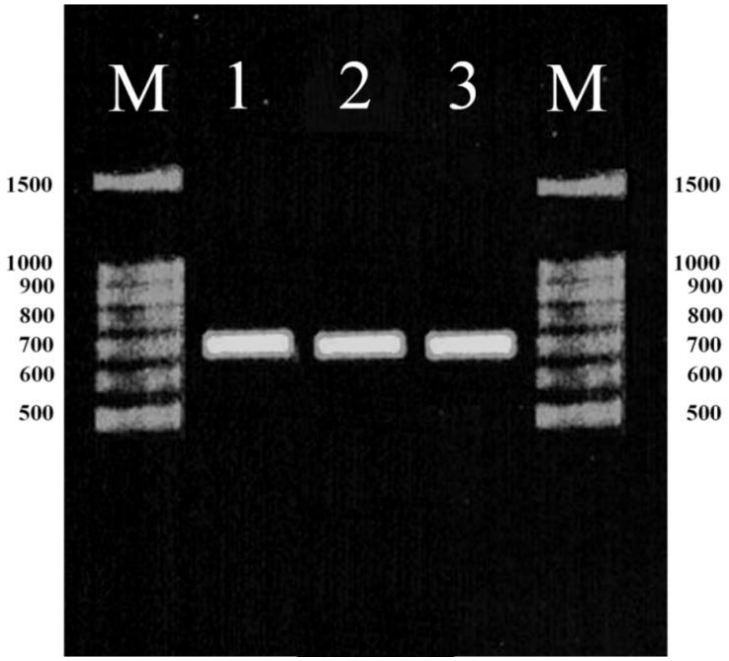

Figure 2 Fragment size produced by *Meretrix* spp. using COI marker
Notes: 1 = *M. lyrata*; 2 = *M. lusoria*; 3 = *M. meretrix*.

Basic Local Alignment System Tool (BLAST) analysis indicated that the *M. lyrata* samples from the present study had 99% similarity to the three accessions sequence (KC832317.1, JN898944.1, HM124580.1) of *M. lyrata* compared to other accessions sequence (Table 2). Different accession sequences were only showed lower than 87% of similarities with *M. lyrata* from the Sarawak locality. The present study on *M. lusoria* showed higher similarities with *M. lyrata* with accessions sequence KC832317.1 and HM124580.1, and *M. meretrix* with accessions sequence HM124578.1 and JN043623.1. Other accessions sequence were less than 89% of similarities with the present study on *M. lusoria.* There was no similarity of more than 89% within *M. meretrix* from Sarawak Province with GeneBank NCBI accessions sequence of *M. meretrix*. The higher similarity sequence was represented by the accession sequence HM124580.1 of *M. lyrata* with 89% similarity.

The nucleotide sequence for *M. lyrata* in the present study showed potential similarity with the accession nucleotide sequence of *M. lyrata* from the GenBank NCBI with more than 90% similarity compared to *M. meretrix* and *M. lusoria.* Some previous studies conferred the morphology feature on *M. lyrata* as being the easiest to distinguish from *M. meretrix* and *M. lusoria* (Hamli *et al.* 2012b, 2015, 2016, 2017). However, the nucleotide sequence for *M. meretrix* and *M. lusoria* showed lower than 90% similarity with the accession nucleotide sequence from the GenBank NCBI. Hence, the BLAST

analysis could not confirm and distinguish *M. meretrix* and *M. lusoria* from the Sarawak locality through the COI sequence. Current findings were contradictory with results of the morphology and morphometric studies, which distinguished differences between *M. meretrix* and *M. lusoria*. However, Wang & Dunbrack (2004) reported that the application of BLAST only produces short and conserve fragment alignment. Thus, the analysis was frequently unable to identify many relationships, particularly below 40% sequence identity. Low sequence identity of fragment creates uncertainty of gaps and insertion, thus compromising confidence in overall modeling of protein related to genome sequence annotation (Gan *et al.* 2002). *M. lusoria* and *M. meretrix* were hard to identify by rough morphological inspection due to similar features, particularly the outer shell. However, a detailed examination of the inner shell generated morphological variation of the distinct profile of the pallial sinus scar between *M. lusoria* and *M. meretrix*. Yet, using the BLAST application to confirm the differences between the two species appeared to provide uncertain results.

Rather than depending on sequence identity percentage, phylogenetic relationship perhaps provides more justified evidence on the present genome characteristic of *Meretrix* spp. to be evaluated against the accession sequence in the GenBank. The current phylogenetic tree was constructed based on Maximum Likelihood (ML), which applied an algorithm that calculates the probabilistic approaches to build a relationship tree between compared species based upon the nucleotides or amino acid sequences (Guindon & Gascuel 2003). *Meretrix* spp. from the Sarawak locality indicates the close relationship between *M. lusoria* and *M. meretrix*. The variation in the COI sequence of this *Meretrix* spp. can be distinguished, hence, we are able to clarify the intricacy conferred using the BLAST application.

Moreover, *M. lusoria* and *M. meretrix* had similar ancestors represented by *M. lyrata* from a similar locality. Therefore, COI sequence in *M. lyrata* had a low level of sequence identity among *Meretrix* spp. This was in agreement with Chen *et al.* (2009) and Wu *et al.* (2014), which suggested that *M. lyrata* has the level of sequence that portrays as the ancestor to other *Meretrix* spp. by referring to COI and transfer RNA (tRNA), respectively.

Sequence Characteristics

A total of 21 nucleotide sequences were analyzed from three *Meretrix* spp. of Sarawak locality, 16 accessions sequence of *Meretrix* spp. and two outgroup species represented by *Mercenaria mercenaria* and *Paphia gallus*. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 435 positions in the final dataset. The nucleotide base composition sequences were comprised with mean of Thiamine (T) 37.9%, Cytosine (C) 15.4%, Adenine (A) 27.4%, and Guanine (G) 19.4% (Table 3).

Table 2 Similarity percentage of *Meretrix* spp. in the present study with 16 selected *Meretrix* spp. mtDNA accessions sequence from GenBank (NCBI)

Accession sequence GenBank	Species	*M. lyrata* (%)	*M. lusoria* (%)	*M. meretrix* (%)
KC832317.1	*M. lyrate*	99	90	88
JN898944.1	*M. lyrata*	99	< 83	< 84
HM124580.1	*M. lyrata*	99	90	89
GQ463598.1	*M. meretrix*	86	86	85
HM124578.1	*M. meretrix*	87	90	87
JN043623.1	*M. meretrix*	87	90	87
GQ903339.1	*M. lusoria*	< 85	86	86
AB853865.1	*M. lusoria*	85	87	85
AB853870.1	*M. lusoria*	< 85	86	85
EU145977.1	*M. petechialis*	86	85	85
HM124582.1	*M. petechialis*	< 85	85	85
HQ703177.1	*M. petechialis*	< 85	86	85
GU071281.1	*M. lamarckii*	< 85	< 83	86
AB059420.1	*M. lamarckii*	< 85	< 83	87
HM124579.1	*M. lamarckii*	< 85	< 83	86
JQ773441.1	*M. casta*	< 85	88	87

Table 3 Nucleotide base composition for accession sequences

Species	Accession	T %	C %	A %	G %	Total
Meretrix lyrata	Sarawak	24.8	19.5	42.1	13.6	435.0
M. lusoria	Sarawak	24.4	20.2	41.8	13.6	435.0
M. meretrix	Sarawak	23.7	20.7	42.1	13.6	435.0
M. lyrata	KC832317.1	41.8	14.0	23.7	20.5	435.0
M. lyrata	JN898944.1	42.1	13.8	24.1	20.0	435.0
M. lyrata	HM124580.1	42.1	13.8	24.1	20.0	435.0
M. lusoria	GQ903339.1	42.1	13.6	23.0	21.4	435.0
M. lusoria	AB853870.1	23.0	20.5	43.0	13.6	435.0
M. lusoria	AB853865.1	22.3	22.1	43.0	12.6	435.0
M. meretrix	GQ463598.1	42.8	13.1	23.0	21.1	435.0
M. meretrix	HM124578.1	41.6	14.3	22.3	21.8	435.0
M. meretrix	JN043623.1	42.1	13.8	24.1	20.0	435.0
M. petechialis	EU145977.1	42.8	13.1	23.0	21.1	435.0
M. petechialis	HM124582.1	41.8	13.8	23.7	20.7	435.0
M. petechialis	HQ703177.1	42.3	13.3	23.7	20.7	435.0
M. lamarckii	GU071281.1	43.7	13.8	19.8	22.8	435.0
M. lamarckii	HM124579.1	43.7	13.6	20.7	22.1	435.0
M. lamarckii	AB059420.1	42.3	13.8	22.1	21.8	435.0
M. casta	JQ773441.1	42.3	14.7	23.2	19.8	435.0
Mercenaria mercenaria	HM884239.1	40.8	14.7	21.1	23.4	436.0
Paphia gallus	HQ703232.1	44.4	12.0	23.0	20.7	435.0
Average		37.9	15.4	27.4	19.4	435.0

Phylogenetic Analysis

A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 2.0104)). Maximum Likelihood (ML) analysis conducted using the Tamura 3-parameter model has constructed five major clades on *Meretrix* spp. in the present study with designed out-groups *Mercenaria mercenaria* and *Paphia gallus*. These major clades were closely related to each other at the 50% evidence of bootstrap, which grouped as *Meretrix* genus (Fig. 3). Two monophyly groups were formed, represented by Clade 3 and 4, while Clade 1, 2, and 5 were formed, paraphyly groups. *Meretrix* spp. from the Sarawak locality was clustered in Clade 1 alongside two *M. lusoria* of Japan locality with accession sequence number AB853865.1 and AB853870.1. Clade 2 comprised two *M. meretrix* from the China locality with accession sequence number HM124578.1 and JN898944.1. Clade 3 consisted of *M. lyrata* from the China locality with accession sequence number KC832317.1, HM124580.1, JN898944.1, and *M. casta* from the India locality with accession sequence JQ773441.1. *Meretrix lamarckii* from the Japan locality (AB059420.1) was clustered with *M. lamarckii* from the China locality (GU071281.1; HM124579.1) in Clade 4. All *M. petechialis* (HM124582.1, HQ703177.1, EU145977.1) from the China locality were clustered with *M. meretrix* (GQ463598.1) and *M. lusoria* (EU145977.1) from the China locality, respectively, in Clade 5.

Phylogenetic analysis on three *Meretrix* from the Sarawak locality is closely related to the *M. lusoria* from the Japan locality. However, the result contrasts with *M. lyrata* from BLAST analysis which inferred a high similarity sequence with *M. lyrata* accession sequence. The application of ML analysis for the phylogenetic tree was calculated using the substitution rate in the sample sequence and accession sequence, which produced different inferences than BLAST analysis (Guindon & Gascuel 2003). Furthermore, *Meretrix* spp. from the Sarawak locality and *M. lusoria* from the Japan locality had a high percentage of Cytosine and Adenine than that of other *Meretrix* spp. from different localities. *Meretrix* spp. from the Sarawak locality only had a relationship with other *Meretrix* spp. localities at more than 50% of bootstrap scoring, which is inferred as under *Meretrix* genus. Inconsistent cladistic group between *M. meretrix* (GQ463598.1) *M. lusoria* (GQ903339.1), *M. casta* (JQ773441.1), and *M. petechialis* (HM124582.1, HQ703177.1, EU145977.1) from the present study indicated that the three species had a close relationship following Wu *et al.* (2014) and Chen *et al.* (2009). Hence, Chen *et al.* (2009) suggested that *M. lusoria* and *M. petechialis* as a junior synonym to *M. meretrix*.

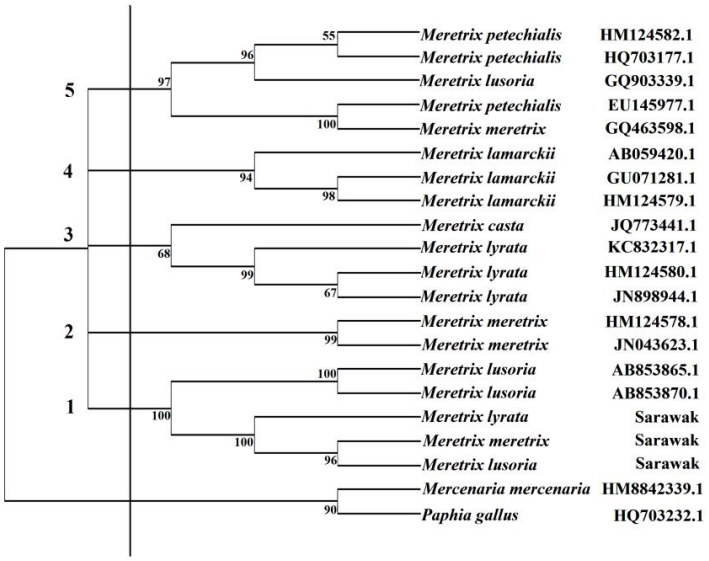

Figure 3 Phylogenetic tree of *Meretrix* accession sequence inferred from the Maximum Likelihood analysis using Tamura three-parameter model
Note: Only bootstrap score greater than 50% are shown.

The present study on *Meretrix* spp. from the Sarawak locality was able to differentiate COI sequences among *M. lyrata*, *M. meretrix,* and *M. lusoria*. *M. lusoria* was closely related to *M. meretrix* with strong bootstrap supporting evidence at 96% scoring. *M. lyrata* became the ancestor to *M. lusoria* and *M. meretrix* with strong bootstrap evidence at 100% of the scoring. *Meretrix* spp. from the Sarawak locality is closely related to the *M. lusoria* of the Japan locality with 100% scoring on bootstrap supporting evidence to form Clade 1. *Meretrix* spp. from the Sarawak locality was closely associated with other accession sequences for *Meretrix* spp. at bootstrap supporting evidence at 50% scoring.

Based on ML phylogenetic analysis, all *M. lyrata* with accession sequence KC832317.1, JN898944.1, and HM124580.1 formed the cladistic group with strong bootstrap evidence at 99%. All *M. lamarckii* with accession sequence GU071281.1, AB059420.1, and HM124579.1 also formed cladistic at 94% of bootstrap evidence. *M. casta* (JQ773441.1) formed an individual clade which indicated this species distance from other species except for *M. lyrata*. Other *Meretrix* spp. accession sequence, such as *M. meretrix* (GQ463598.1) *M. lusoria* (GQ903339.1), and *M. petechialis* (HM124582.1, HQ703177.1, EU145977.1) formed inconsistent cladistic groups, while *M. meretrix* with accession sequence HM124578.1 and JN043623.1 formed individual clade apart from other *M. meretrix* (GQ463598.1) at 99% evidence of bootstrap scoring.

Mitochondria DNA is known to have higher transition mutation rates, particularly synonymous sites in the nucleotide sequence that affected the rates of evolution in organisms (Brown *et al.* 1982; Caterino *et al.* 2000; Overton & Rhoads 2004). This fast rate of evolution is one of the features suitable for phylogenetic study, particularly in higher taxa. Moreover, higher taxa less affected by the recombination (Guo *et al.* 2006), uniparental inheritance (Passamonti *et al.* 2003; Passamonti & Plazzi 2020), heteroplasmy with paternal leakage (Bromham *et al.* 2003; Wolff *et al.* 2013; Mastrantonio *et al.* 2019), and selective sweeps (Ballard & Rand 2005; James *et al.* 2016; Hill 2019). Despite proper application on higher taxa, this is the contrast when nucleotide sequences at the species level take into account those which are more defected. The evolution rate is influenced by thermal adaptation, mitochondrial, nuclear interaction, and infection with *Wolbachia* spp. (Ballard & Rand 2005). Hence, *Meretrix* spp. from the Sarawak locality possibly has a different evolution rate in mtDNA, which caused divergence from the *Meretrix* spp. from other localities, particularly from the China regions.

The current phylogenetic analysis is difficult to verify that species *Meretrix* spp. belong to the Sarawak locality. Bivalves had high variability in size and gene group of mtDNA, which vary extensively between species from a similar genus (Xu *et al.* 2012). *Meretrix* spp. from the Sarawak locality are separated in terms of geographical distance. The geographical distance affects the mtDNA feature as described by Mishmar *et al.* (2003) that maternal inheritance can be restricted by the geographical distance with no mixing of mtDNA haplogroups from the different geological region. Therefore, other environmental conditions in each region influence the organisms' mutation rate (Massey & Buckling 2002). Ambiguous *Meretrix* spp. identification from the Sarawak locality might be possibly related to other several features, such as sample used.

The mtDNA marker feature is the maternal inheritance which supplies the missing information in nuclear markers (Okumuú & Çiftci 2003). Therefore, the female individual's tissue selection is crucial during the genetic study to ensure reliable information regarding the species ancestor. However, female selections for the genetic research have not been mentioned in each previous study for the GenBank accession sequence, causing the doubtness of the reliability of accession sequence in the GenBank, leading to the divergence of *Meretrix* spp. from the Sarawak locality compared to the previous species sequence. Moreover, the accession sequence is well doubted due to the non-intensive morphological identification for each species which only depending on the rough observation. The present study suggested that appliance on a single marker is insufficient to support species identification and species-level phylogenetic analysis. Therefore, additional variance types of molecular markers such as allozyme and nuclear markers are required to create substantial evidence on species verification to ensure the intensive morphological study's genetic approachability. Depending on the genetic approach, the lack of intensive morphological evidence would create ambiguous species naming in taxonomy.

CONCLUSION

The molecular approach application was able to distinguish three *Meretrix* spp. from Sarawak Province, Malaysia. Moreover, *M. lyrata* is inferred to as the ancestor of *M. meretrix* and *M. lusoria* from Sarawak. Regardless of ancestor identification and species distinctions, the COI gene in the present study was unable to verify which species *Meretrix* spp. belonged to the Sarawak locality. Several disadvantages to the COI sequence that influence the phylogenetic analysis involve the consistent accession sequence from GenBank as the main reference for the current study. The present study suggested that additional DNA markers for species identification and phylogenetic analysis are recommended in supporting the intensive morphological identification.

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REFERENCES

Al-Asif A, Hamli H, Abu Hena MK, Idris MH, Gerusu GJ, Ismail JB, Karim NU. 2020. Benthic macrofaunal assemblage in seagrass-mangrove complex and adjacent ecosystems of Punang-Sari Estuary, Lawas, Sarawak, Malaysia. Biodiversitas 21(10): 4606-15.

Al-Asif A, Hamli H, Abu Hena MK, Idris MH, Gerusu GJ, Ismail J, Abualreesh MH 2021. Bivalves (Bivalvia) in Malaysian Borneo: status and threats. J Threat Taxa 13(11): 19553-65.

Arruda CCB, Beasley CR, Vallinoto M, Marques-Silva NS, Tagliaro CH. 2009. Significant genetic differentiation among populations of *Anomalocardia brasiliana* (Gmelin, 1791): A bivalve with planktonic larval dispersion. Genet Mol Biol 32(2): 423-30.

Backeljau T, Baur A, Baur B. 2001. Population and conservation genetics. In: Barker GM, Editor. The biology of terrestrial molluscs. New York (US): CABI Publishing. p. 1-383.

Ballard JWO, Rand DM. 2005. The population biology of mitochondrial DNA and its phylogenetic implications. Annu Rev Ecol Evol Syst 36(2): 621-42.

Bromham L, Eyre-Walker A, Smith NH, Smith JM. 2003. Mitochondrial Steve: Paternal inheritance of mitochondria in humans. Trends Ecol Evol 18: 2-4.

Brown WM, Prager EM, Wang A, Wilson AC. 1982. Mitochondrial DNA sequences of primates: Tempo and mode of evolution. J Mol Evol 18(4): 225-39.

Caterino MS, Cho S, Sperling FAH. 2000. The current state of insect molecular systematics: A thriving tower of babel. Annu Rev Entomol 45: 1-54.

Chee SY, Mohd Nor SA. 2016. DNA barcoding reveals neritid diversity (Mollusca: Gastropoda) diversity in Malaysian Waters. Mitochondrial DNA Part A, DNA mapping, Seq Anal 27(3): 2282-4.

Chen A, Li Z, Feng G. 2009. Phylogenetic relationships of the genus *Meretrix* (Mollusca: Veneridae) based on mitochondrial COI gene sequences. Zool Res 30(3): 233-9.

Chen J, Li Q, Kong L, Yu H. 2011a. How DNA barcodes complement taxonomy and explore species diversity: The case study of a poorly understood marine fauna. PLoS One 6: e21326.

Chen J, Li Q, Kong L, Zheng X. 2011b. Molecular phylogeny of Venus Clams (Mollusca, Bivalvia, Veneridae) with emphasis on the systematic position of taxa along the coast of mainland China. Zool Scr 40(3): 260-71.

Cheng H, Peng Y, Dong Z, Yi L, Meng X, Shen X, ..., Chen D. 2013. Phylogenetic analysis of Veneridae (Mollusca: Bivalvia) based on the mitochondrial cytochrome c oxidase subunit I gene fragment. Acta Ecol Sin 33(9): 2744-53.

Edgar RC. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32(5): 1792-7.

Ehlers BK, Damgaard CF, Laroche F. 2016. Intraspecific genetic variation and species coexistence in plant communities. Biol Lett 12: 20150853.

Endler J. 1986. Natural selection in the wild. New Jersey, Princeton (US): Princeton University Press.

Felsenstein J. 1985. Confidence limits on phylogenies : An approach using the bootstrap. Evolution 39(4): 783-91.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3(5): 294-9.

Foon JK, Clements GR, Liew TS. 2017. Diversity and biogeography of land snails (Mollusca, Gastropoda) in the limestone hills of Perak, Peninsular Malaysia. Zookeys 2017(682): 1-94.

Gan HH, Perlow RA, Roy S, Ko J, Wu M, Huang J, ..., Schlick T. 2002. Analysis of protein sequence/ structure similarity relationships. Biophys J 83(5): 2781-91.

Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52(5): 696-704.

Guo X, Liu S, Liu Y. 2006. Evidence for recombination of mitochondrial DNA in triploid crucian carp. Genetics 172(3): 1745-9.

Hamaguchi M, Sasaki M, Higano J. 2001. Development of species specific monoclonal antibody to planctonic larvae of *Meretrix lamarckii* for field survey. NCBI (Direct Submission).

Hamli H, Hamed NA, Azmai SHS, Idris MH. 2020a. Conchology variations in species identification of Pachychilidae (Mollusca, Gastropoda, Cerithiodea) through multivariate analysis. Trop Life Sci Res 31(2): 145-58.

Hamli H, Hashim N, Al-Asif A. 2020b. Isolation and potential culture of phytoplankton live feed for freshwater mussels *Sinanodonta woodiana* (Lea, 1834). Asian J Anim Sci 14(4): 127-36.

Hamli H, Azmai SHS, Hamed SA, Al-Asif A. 2020c. Diversity and habitat characteristics of local freshwater Gastropoda (Caenogastropoda) from Sarawak, Malaysia. Singapore J Sci Res 10: 23-7.

Hamli H, Idris M, Abu Hena M, Rajaee AH, Arshad A. 2016. Inner shell as variation key of local hard clam *Meretrix* spp. J Environ Biol 37: 641-6.

Hamli H, Idris MH, Abu Hena MK, Wong SK. 2012a. Diversity of edible mollusc (Gastropoda and Bivalvia) at selected divison of Sarawak, Malaysia. Int J Adv Sci Eng Inf Technol 2(4): 5-7.

Hamli H, Idris MH, Abu Hena MK, Wong SK. 2012b. Taxonomic study of edible bivalve from selected division of Sarawak, Malaysia. Int J Zool Res 8: 52-8.

Hamli H, Idris MH, Abu Hena MK, Wong SK, Arshad A. 2013. Checklist and habitat descriptions of edible gastropods from Sarawak, Malaysia. J Fish Aquat Sci 8(2): 412-418.

Hamli H, Idris MH, Abu Hena MKA, Rajaee AH. 2019. Fisheries assessment, gametogenesis and culture practice of local bivalve: A review. Pertanika J Trop Agric Sci 42: 103-24.

Hamli H, Idris MH, Rajaee AH, Abu Hena MK. 2015a. Reproductive cycle of hard clam, *Meretrix lyrata* Sowerby, 1851 (Bivalvia: Veneridae) from Sarawak, Malaysia. Trop Life Sci Res 26(2): 59-72.

Hamli H, Idris MH, Rajaee AH, Abu Hena MK, Hoque MN. 2017. Condition index of *Meretrix lyrata* (Sowerby 1851) and its relationship with water parameter in Sarawak. Sains Malaysiana 46(4): 545-51.

Hamli H, Rahim AA, Idris MH, Abu Hena MK, King WS. 2015b. Morphometric variation among three local mangrove clam species of Corbiculidae. Songklanakarin J Sci Technol 37: 15-20.

Idris MH, Hamli H, Kamal AHM, Rajaee AH. 2017a. Distribution of mineral contents in the selected tissues of *Meretrix lyrata*. J Fish Aquat Sci 12: 149-56.

Idris MH, Rahim AA, Hamli H, Nesarul MH, Abu Hena MK. 2017b. Determination of gonad development of mangrove clam *Polymesoda expansa* (Mousson 1849) by histological classification. J Fish Aquat Sci 12: 168-76.

Hassan R, Kanakaraju D. 2016. Razor clams (Class Bivalvia) of Kuala Selangor, Malaysia: Morphology, genetic diversity and heavy metal concentration. Borneo J Resour Sci Technol 2(2): 19-27.

He CB, Wang J, Gao XG, Song WT, Li HJ, Li YF, ..., Su H. 2011. The complete mitochondrial genome of the hard clam *Meretrix meretrix*. Mol Biol Rep 38(5): 3401-9.

Hill GE. 2019. Reconciling the mitonuclear compatibility species concept with rampant mitochondrial introgression. Integr Comp Biol 59(4): 912-24.

James JE, Piganeau G, Eyre-Walker A. 2016. The rate of adaptive evolution in animal mitochondria. Mol Ecol 25: 67-78.

Kim D-H, Yoon J-M. 2014. Genetic distances of three white clam (*Meretrix lusoria*) populations investigated by PCR analysis. Dev Reprod 18(2): 89-98.

Lau J-S, Ransangan J, Rodrigues KF. 2018. Genetic diversity and population structure of the Asian green mussel (*Perna viridis*) in the waters of Sabah, Malaysia based on mitochondrial DNA D-Loop sequences. Turkish J Fish Aquat Sci 18: 81-90.

Layton KKS, Martel AL, Hebert PDN. 2016. Geographic patterns of genetic diversity in two species complexes of Canadian marine bivalves. J Molluscan Stud 82(2): 282-91.

Layton KKS, Martel AL, Hebert PDN. 2014. Patterns of DNA barcode variation in Canadian marine molluscs. PLoS One 9: 1-9.

Lee PY, Costumbrado J, Hsu CY, Kim YH. 2012. Agarose gel electrophoresis for the separation of DNA fragments. J Vis Exp 1(62): e3923.

Luttikhuizen PC, Drent J, Van Delden W, Piersma T. 2003. Spatially structured genetic variation in a broadcast spawning bivalve: Quantitative vs. molecular traits. J Evol Biol 16(2): 260-72.

Lu X, Wang H, Dai P, Liu B. 2011. Characterization of EST-SSR and genomic-SSR markers in the clam, *Meretrix meretrix*. Conserv Genet Resour 3(4): 655-8.

Massey RC, Buckling A. 2002. Environmental regulation of mutation rates at specific sites. Trends Microbiol 10(12): 580-4.

Mastrantonio V, Latrofa MS, Porretta D, Lia RP, Parisi A, Iatta R, ..., Urbanelli S. 2019. Paternal leakage and mtDNA heteroplasmy in *Rhipicephalus* spp. ticks. Sci Rep 9: 1-8.

Mishmar D, Ruiz-Pesini E, Golik P, Macaulay V, Clark AG, Hosseini S, ..., Wallace DC. 2003. Natural selection shaped regional mtDNA variation in humans. Proc Natl Acad Sci USA 100: 171-6.

Morris S, Purchon RD. 1981. The marine shelled Mollusca of West Malaysia and Singapore Part 3, Bivalvia. J Molluscan Stud 47(2264): 322-7.

Okumuú Ø, Çiftci Y. 2003. Fish population genetics and molecular markers: II-Molecular markers and their applications in fisheries and aquaculture. Turkish J Fish Aquat Sci 3: 51-79.

Overton LC, Rhoads DD. 2004. Molecular phylogenetic relationships based on mitochondrial and nuclear gene sequences for the Todies (*Todus*, Todidae) of the Caribbean. Mol Phylogenet Evol 32(2): 524-38.

Passamonti M, Boore JL, Scali V. 2003. Molecular evolution and recombination in gender-associated mitochondrial DNAs of the manila clam *Tapes philippinarum*. Genetics 164(2): 603-11.

Passamonti M, Plazzi F. 2020. Doubly uniparental inheritance and beyond: The contribution of the Manila clam *Ruditapes philippinarum*. J Zool Syst Evol Res 58(2): 529-40.

Ranjith KR, Vijayan KK, Reynold P, Thomas PC, Gopalakrishnan A, Mohamed KS. 2012. *Meretrix casta* voucher MBTD 09 MCKOL 008 cytochrome oxidase subunit 1 (CO1) gene. NCBI (Direct Submission).

Ren J, Shen X, Sun M, Jiang F, Yu Y, Chi Z, Liu B. 2009. The complete mitochondrial genome of the clam *Meretrix petechialis* (Mollusca: Bivalvia: Veneridae). Mitochondrial DNA 20(4): 78-87.

Rengarajan K, Cristol SM, Mehta M, Nickerson JM. 2002. Quantifying DNA concentrations using fluorometry: A comparison of fluorophores. Mol Vis 8: 416-21.

Sato S, Kaneko Y, Torii H, Hong JS. 2016. Temporal changes in the geographic distribution of two clam species *Meretrix lusoria* and *M. petechialis* along the coast of Japan and South Korea. Ocean Sci J 51(3): 455-63.

Stevens VM, Pavoine S, Baguette M. 2010. Variation within and between closely related species uncovers high intra-specific variability in dispersal. PLoS One 5(6): e11123.

Tamura K. 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. Mol Biol Evol 9(4): 678-87.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30(12): 2725-9.

Thia JA, Hale ML, Chapman HM. 2016. Interspecific comparisons with chloroplast SSR loci reveal limited genetic variation in Nigerian montane forests: A study on *Cordia millenii* (West African Cordia), *Entandrophragma angolense* (Tiama Mahogany), and *Lovoa trichilioides* (African Walnut). Trop Conserv Sci 9: 321-37.

Torii H, Sato S, Hamaguchi M, Henmi Y, Yamashita H. 2010. The comparison of shell morphology and genetic relationship between *Meretrix lusoria* and *M. petechialis* in Japan and Korea. Plankt Benthos Res 5: 231-41.

Wallace DC, Brown MD, Lott MT. 1999. Mitochondrial DNA variation in human evolution and disease. Gene 238: 211-30.

Wang D, Yu S, Chen W, Zhang D, Shi X. 2010a. Enumeration of *Vibrio parahaemolyticus* in oyster tissues following artificial contamination and depuration. Lett Appl Microbiol 51: 104-8.

Wang G, Dunbrack RL. 2004. Scoring profile-to-profile sequence alignments. Protein Sci 13: 1612-26.

Wang H, Zhang S, Li Y, Liu B. 2010b. Complete mtDNA of *Meretrix lusoria* (Bivalvia: Veneridae) reveals the presence of an atp8 gene, length variation and heteroplasmy in the control region. Comp Biochem Physiol - Part D Genomics Proteomics 5(4): 256-64.

Wang H, Zhang S, Liu B. 2011a. Taxonomic study on *Meretrix* (Mollusca, Bivalvia) from China Seas. Unpubl (NCBI), Key Lab Exp Mar Biol Inst Oceanol Chinese Acad Sci 7 Nan Hai Road, Qingdao, Shandong 266071, China.

Wang H, Zhang S, Xiao G, Liu B. 2011b. Complete mtDNA of the *Meretrix lamarckii* (Bivalvia: Veneridae) and molecular identification of suspected M. lamarckii based on the whole mitochondrial genome. Mar Genomics 4(4): 263-71.

Wolff JN, Nafisinia M, Sutovsky P, Ballard JWO. 2013. Paternal transmission of mitochondrial DNA as an integral part of mitochondrial inheritance in metapopulations of *Drosophila simulans*. Heredity (Edinb) 110: 57-62.

Wu X, Xiao S, Li X, Li L, Shi W, Yu Z. 2014. Evolution of the tRNA gene family in mitochondrial genomes of five *Meretrix* clams (Bivalvia, Veneridae). Gene 533: 439-46.

Xu X, Wu X, Yu Z. 2012. Comparative studies of the complete mitochondrial genomes of four *Paphia* clams and reconsideration of subgenus *Neotapes* (Bivalvia: Veneridae). Gene 494: 17-23.

Yamakawa AY, Imai H. 2013. PCR-RFLP typing reveals a new invasion of Taiwanese *Meretrix* (Bivalvia: Veneridae) to Japan. Aquat Invasions 8(4): 407-15.

Yamakawa AY, Yamaguchi M, Imai H. 2008. Genetic relationships among species of *Meretrix* (Mollusca: Veneridae) in the Western Pacific Ocean. Pacific Sci 62(3): 385-94.