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Phylogenetic Relationships of the Genus *Meretrix* (Mollusca: Veneridae) Based on Mitochondrial *COI* Gene Sequences

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Abstract: Fifteen sequences from the mitochondrial cytochrome c oxidase subunit I gene (*COI*) were determined for 4 species of the genus *Meretrix,* with the homologous sequences of *M. petechialis* obtained from the GenBank data library. The alignment length of the sequences was 574bp after excluding ambiguous sites, including 93 parsimony informative sites. In the fragments, the percentages of A, T, C and G were 21.15%, 44.71%, 14.05% and 20.09% respectively. There were 12 haplotypes identified: 4 *M. meretrix*, 2 *M. lamarckii*, 3 *M. lusoria*, 1 *M. lyrata* and 2 *M. petechialis*. Furthermore, it was revealed that *M. meretrix*, *M. petechialis* and *M. lusoria* shared some haplotypes. Phylogeny trees were reconstructed by Maximum-parsimony (MP) and Bayesian method using *Cylina sinensis* as the outgroup. Our results indicated that *M. lusoria*, *M. petechialis* and *M. meretrix* are closely related species. This is in accordance with the viewpoint that *M. petechialis* and *M. lusoria* should be treated as a junior synonym of *M. meretrix*.

Key words: *Meretrix*; *M. meretrix*; *COI* gene; Phylogeny

基于线粒体 *COI* 基因序列的文蛤属（软体动物门：帘蛤科）系统发育关系

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摘要：测定了 4 种文蛤属贝类的 15 个个体的 *COI* 基因序列，并从 GenBank 下载了短文蛤 (*M. petechialis*)的相应序列。比对后的序列长度为 574bp，包括 93 个简约信息位点，A、T、C 和 G 的平均含量分别为 21.15%、44.71%、14.05%和 20.09%。通过对序列的分析，共定义了 12 个单倍型：文蛤 (*M. meretrix*) 4 个，斧文蛤 (*M. lamarckii*) 2 个，丽文蛤 (*M. lusoria*) 3 个，琴文蛤 (*M. lyrata*) 1 个，短文蛤 2 个。以青蛤(*Cylina sinensis*)为外群，用 MP 法和贝叶斯法构建系统树。结果显示，短文蛤、丽文蛤和文蛤为亲缘关系较近的物种, 支持短文蛤和丽文蛤为文蛤的同物异名的观点。

关键词：文蛤属；文蛤；*COI* 基因；系统发育
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Members of the genus *Meretrix* are highly appreciated table food for internal and export markets, fetching a high price. Some species have already become regional economic mainstay in coastal areas of China. However, previous research on species of the *Meretrix* mainly focused on aquaculture and fishery and paid little attention on its classification. Thus, the taxon of the *Meretrix* is still in argument over several discrepancies among systematists in the world.

Traditionally hard clams are mainly identified based on visible shell characters. Commonly, taxonomic ambiguities exist due to morphological variability. Modern taxonomic work includes analysis of a host of other traits, including anatomy, physiology, behavior, and genetics. Citations of clam studies include many synonyms, indicating ambiguities in *Meretrix* species

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identification. Accurate species identification is very important in the case of multiple morphology-based classification and determining similar species for aquaculture management, biodiversity studies, and population dynamics.

The mitochondrial cytochrome c oxidase subunit I gene (*COI*) shows distinct divergence and provides valuable information in species identification to complete taxonomic data and validation of systemic position, phylogeny (Machordom et al, 2003; Smith et al, 2004; Donald et al, 2005). In the present study, the *COI* gene is selected to elucidate taxonomy and phylogenetic relationships among 5 *Meretrix* species from the Chinese coast.

1 Materials and Methods

1.1 Samples and DNA extraction

Geographical origins of the *Meretrix* used here are shown in Tab. 1. All the clams were identified morphometrically, and then fifteen of each species' adductor muscle tissue samples were collected and preserved in 100% alcohol.

Tab. 1 Collection locality and GenBank accession numbers of the specimens studied

Genus	Species and haplotype number identifying the sequence	Locality	GenBank accession numbers
Meretrix	*M. meretrix* 1	Jiangsu, China	FJ434675
	M. meretrix 2	Jiangsu, China	FJ434676
	M. meretrix 3	Jiangsu, China	FJ434677
	M. meretrix 4	Jiangsu, China	FJ434678
	M. lusoria 1	Jiangsu, China	FJ434679
	M. lusoria 2	Jiangsu, China	FJ434680
	M. lusoria 3	Jiangsu, China	FJ434681
	M. lyrata	Hainan, China	FJ434682
	M. lamarckii 1	Hainan, China	FJ434683
	M. lamarckii 2	Hainan, China	FJ434684
	M. petechialis 1		AB280785*
	M. petechialis 2		AY874530*
Cylina	*C. sinensis* 1	Jiangsu, China	FJ434685
	C. sinensis 2	Jiangsu, China	FJ434686

* Sequences downloaded from GenBank.

Total genomic DNA was extracted from the stored tissue samples by the standard Proteinase-K/Phenol-Chloroform-ethanol method. Before incubation the samples were soaked in ultra pure water for 2 days. Scissored tissue samples were re-suspended in 400 μL digestive system, which contained Tris (pH 8.0) 0.01 mol/L, EDTA (pH 8.0) 0.1 mol/L, NaCl 0.05 mol/L, SDS 1% and 10 μL Proteinase K, and was incubated at 52℃ for 12 h. The digested samples were phenol-extracted, ethanol-precipitated once more, and redissolved in 10 mmol/L Tris-HCl, pH8.0. The concentration of DNA was estimated using a UV spectrophotometer. The DNA was diluted to a final concentration of 50 ng/μL. To avoid DNA damage caused by multigelation, DNA samples were stored at 4℃.

1.2 PCR amplification

The partial region of the mitochondrial *COI* gene was amplified from 15 individuals of each species using the universal invertebrate primers COI-F 5'-GGTCAA-CAAATCATAAAGATATTGG-3' and COI-R 5'-TAA-ACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al, 1994) synthesized by Shanghai Sangon Company, China.

The polymerase chain reaction (PCR) is employed in this study to amplify the *COI* region. A total 30 μL of each PCR reaction comprised the following: PCR buffer (10×) 3.0 μL, $MgCl_2$ (25 mmol/L) 2.0 μL, dNTPs (2 mmol/L) 2.0 μL, forward primer (10 μmol/L) 1.0 μL, reverse primer (10 μmol/L) 1.0 μL, *Taq* polymerase 0.2 μL, DNA (50 ng/μL) 1.0 μL, ultra pure water 19.8 μL. The Hot-Start PCR was employed with initial denaturation of 5 min at 94℃ followed by 30 cycles of denaturation for 30 s at 94℃, annealing at 52℃ for 30 s and an extension of 72℃ for 45 s. After the completion of 30 cycles, a final extension step of 7 min at 72℃ was performed. The PCR product was then kept at 15℃ until removed from the machine. The amplified product was tested in 2% agarose gel and visualized using the Gel Doc system. Those products with distinct and intense bands were selected after a series of sequencing protocols. The sequencing was done both in the forward and reverse directions by Shanghai Sangon Company.

1.3 Data analysis

Fifteen *COI* gene sequences of 4 species in the *Meretrix* (except sequences of *M. petechialis*) were

sequenced, and *COI* sequences of *M. petechialis* were downloaded from GenBank. The raw nucleotide sequences obtained were assembled using SeqMan (DNAstar package) and the sequence information checked entirely for consistency from both directions manually. We examined 574 base pairs (bp) and identified 12 haplotypes: 4 of *M. meretrix*, 2 of *M. petechialis*, 3 of *M. lusoria*, 1 of *M. lyrata* and 2 of *M. lamarckii* (Tab. 1). In which, the sequence of *M. lusoria* 3 was identical to the sequence of *M. petechialis* 2, and the sequence of *M. petechialis* 1 was identical to the sequence of *M. meretrix* 2. The collated sequences of 12 haplotypes were aligned using Clustal X1.8 (Thompson et al, 1997) with parameters on default. No insertions/deletions and stop codons were examined. These sequences were translated according to the invertebrate mitochondrial genetic code to the expected 191 amino acids. Nucleotide variation and substitution patterns were examined using the software MEGA3 (Kumar et al, 2004) on the basis of uncorrected pairwise genetic distance (p-distance).

Maximum-parsimony (MP) analyses were performed on PAUP 4.0b10 (Swofford, 2002), using *Cylina sinensis* as an outgroup species. Heuristic MP Search was performed using the simple addition sequence and the tree bisection-reconnection (TBR) branch swapping algorithm. Both weighted and unweighted analyses were performed. Weighting was carried out to equalize contributions from each codon position: positions weighted 3: 11: 1 for first, second, and third positions, respectively. Levels of support for individual relationships were estimated through 1,000 bootstrap replicates.

Bayesian inference was done using MrBayes 3.0b4 (Huelsenbeck & Ronquist, 2001) with the best-fitting model TrN+G estimated by Modeltest 3.7 (Posada, 1998). Trees saved below the burn-in generations were discarded, and a majority-rule consensus of the remains were calculated in MrBayes 3.0b4, providing posterior probabilities for clades. The MrBayes 3.0b4 was run with the following specifications: TrN model with a gamma distribution (invgamma), Markov's chains started from a random tree for 400 000 generations, and sampling of the Markov chains at intervals of 100 generations. Four chains were run simultaneously with the initial 200 cycles discarded as burn-in.

2 Results

2.1 Description of data

After aligning, using Clustal X1.8, the length of COI was 574 bp. The obtained *COI* gene sequences contained 149 variable sites and 93 parsimony-informative sites. The average base composition were 21.15% A, 44.71% T, 14.05% C, and 20.09% G. The A + T contents were higher than those of G+C, which is a pattern that has been repeatedly seen in the mtDNA of Molluscs.

Tab. 2 Nucleotide substitutions and amino acid variability in *COI* fragments for 12 haplotypes

	All sites	1st codon position	2nd codon position	3rd codon position	Amino acds
Total	574	192	191	191	191
Invariant	425	158	184	83	150
Variable	149	34	7	108	41
Parsimony	93	20	6	67	29

Nucleotide variation and substitution patterns were examined using the software package MEGA 3.0. The average values of intraspecific pair-wise sequences divergence was 4.5%. The highest conspecific divergences were among individuals of *M. lamarckii*, which showed uncorrected divergence of 8.54%. Divergence could not be calculated for *M. lyrata* as it was represented by a single haplotype. Sequence variations between different species within the *Meretrix* ranged from about 1.83% to 14.81%. The uncorrected divergences of *M. petechialis* and *M. lusoria* with *M. meretrix* are 1.83%, 4.60%, which are lower the average uncorrected congeneric divergence of 11.08% of the genus *Meretrix*. (Tab. 3, Tab. 4).

Among the 12 haplotypes, the third codon positions were invariant in only 83 out of the 191 sequenced samples in the *Meretrix* (Tab. 2). In contrast, more than four-fifths of the first codon positions (158 of 192) and about 95% of the second codon positions (184 of 191) were monomorphic. The number of parsimony informative sites at each of the codon positions for the whole data set, are also given in Tab. 2. There were approximately eleven times as many parsimony informative sites at the third codon positions (67) than the second codon positions (6), and about three times as many as at first positions (20). For the data set as a whole,

the ratios are 20: 6: 67 for first, second, and third positions, respectively—a ratio of about 3: 1: 11. To offset the preponderance of the third position variation in the reconstruction of phylogenetic trees, weighted analysis using a weighting of sites of 3: 11: 1 was included.

2.2 Phylogenetic relationships

Figs. 1-3 present the phylogenies recovered under parsimony and Bayesian analysis, respectively. The values of interior branch test for some of the nodes in the parsimony trees and the posterior probability values for some nodes in the Bayesian tree were low. However, the topologies of the trees were very similar in most clusters. In this analysis, haplotypes of *M. petechialis* and *M. lusoria* are nested within *M. meretrix* forming clade Ⅰ. The difference among all the trees is the position of *M. lyrata*. In parsimony tree (weighted), *M. lyrata* clustered with clade I, but is sister to the assemblage (clade Ⅰ + *M. lamarckii*) in another parsimonious tree and Bayesian tree.

3 Discussion

3.1 Taxonomic position of *M. petechialis* and *M. lusoria*

M. lusoria and *M. petechialis* were previously considered as independent species, different from *M.*

Tab. 3 Intra specific nucleotide *P* distances for the *Meretrix* species

Species	Mean *P* distance
1. *M. meretrix*	0.0325±0.0050
2. *M. petechialis*	0.0052±0.0030
3. *M. lusoria*	0.0697±0.0082
4. *M. lyrata*	n/c
5. *M. lamarckii*	0.0854±0.0113
Average intraspecific *P* distance in the genus = 0.045	

Tab. 4 Inter specific nucleotide *P* distances among the *Meretrix* species

Species	1	2	3	4	5
1. *M. meretrix*					
2. *M. petechialis*	0.0183				
3. *M. lusoria*	0.0460	0.0438			
4. *M. lyrata*	0.1407	0.1385	0.1440		
5. *M. lamarckii*	0.1448	0.1420	0.1414	0.1481	
Average interspecific *P* distance in the genus =0.1108					

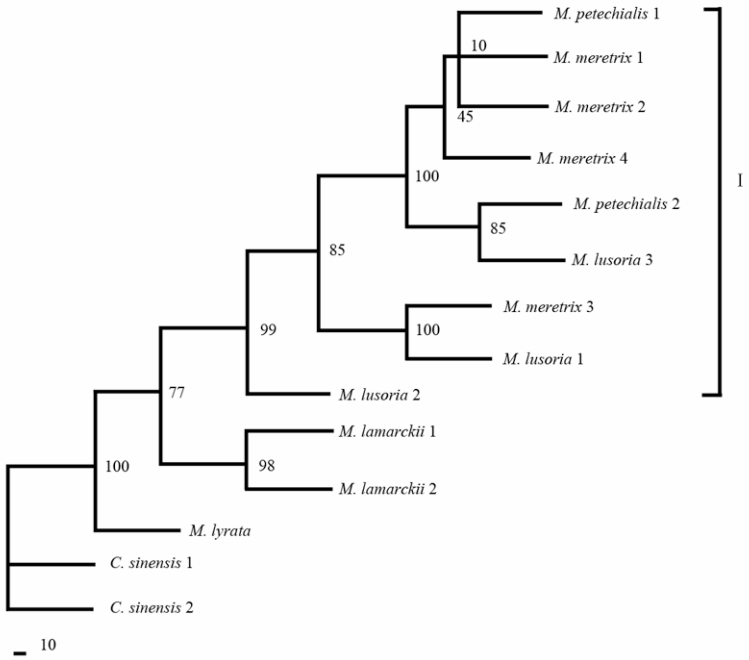

Fig. 1 MP tree resulting from the analysis of the *COI* gene sequences of 12 haplotypes

Numbers on nodes correspond to percentage bootstrap values for 1000 replicates.

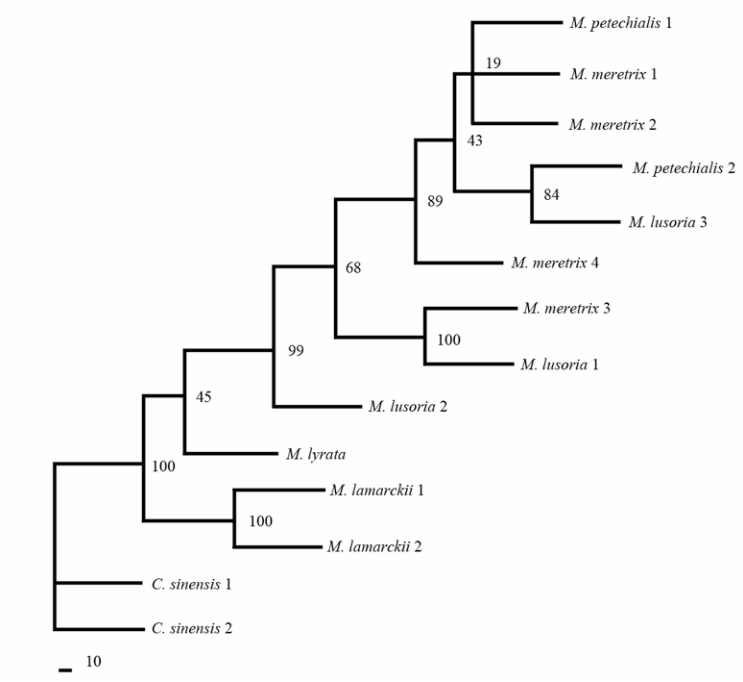

Fig. 2 MP tree resulting from analysis of the *COI* gene sequences of 12 haplotypes

The tree is based on a weighting of codon positions 3:11:1 for first, second, and third codon positions, respectively. Numbers on nodes correspond to percentage bootstrap values for 1000 replicates.

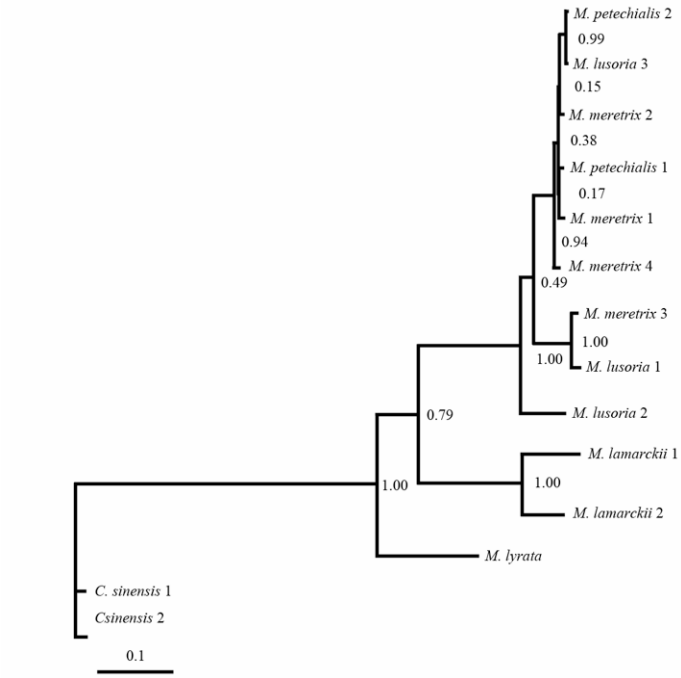

Fig. 3 Bayesian tree resulting from the analysis of the *COI* gene sequences of 12 haplotypes

Numbers on nodes correspond to the value of posterior values.

meretrix, in the genus *Meretrix* (Jukes-Browne, 1914; Habe, 1997; Zhuang, 2001). It was shown that the two species differed from *M. meretrix* by the following shell characteristics: Anterior and ventral margins, posterior end of shell. But the results from *COI* gene sequences analysis strongly disagreed with their viewpoints. According to the phylogenetic trees obtained by this study, *M. petechialis* and *M. lusoria* nested within *M. meretrix*, of them, *M. meretrix* has rounded posterior end, *M. petechialis* and *M. lusoria* are bluntly angled.

Therefore, the shape of the shell end is a homoplasious character, and species determination in the *Mereterix* using this character should be investigated further. The results obtained in this study do not support the present taxonomic status of *M. lusoria* and *M. petechialis*. This is in accordance with the ideas from allozyme analysis that *M. lusoria* and *M. petechialis* were the most closely related species within the genus *Meretrix* (Ayako et al, 2008). This viewpoint also supported that *M. lusoria* and *M. meretrix* belong to different geographic subspecies of one species (Pan et al, 2006).

In our study, the *COI* gene fragment of *M. lusoria* 3 is identical to *M. petechialis* 2, and *M. petechialis* 1 is identical to *M. meretrix* 2. Moreover, the unweighted divergences (1.83%, 4.60% and 4.38%) among these three species are much lower than the average interspecific divergence (11.08%). Prashad (1932) pointed out that *M. meretrix* is a species which experienced the greatest variation in the group of bivalves, and because of shades of shells and shell colors, it was wrongly divided into many species. Furthermore, Fischeer-Piette (1941) also suggested that *M. petechialis* and *M. lusoria* should be treated as the same species of *M. meretrix*.

In summary, the views of taxonomic status of *M. lusoria* and *M. petechialis* are ambiguous based on morphology. In our study, the viewpoint that *M. petechialis* and *M. lusoria* should be treated as a junior synonym of *M. meretrix* was supported.

3.2 Classification of the genus *Meretrix*

The *Meretrix* is widely scattered through coastal areas of the Indian Ocean, Southeast Asia, China, Korean Peninsula and Japanese Archipelago. Because of the remarkable variation of shapes and patterns of shells, early researchers divided this genus into many species, such as *M. meretrix*, *M. typical*, *M. petechialis*, *M. castanea*, *M. graphica*, *M. labiosa*, *M. inpudina*, *M. zonaria*, *M. lusoria*, *M. lyrata*, *M. lamarckii*, *M. mophina*, *M. exilis*, etc. When modern scholars (Dautzenberg & Fischer, 1905; Dautzenberg, 1906; Fischer-Piette, 1941, 1976) merged the clams into one genus, but retained the aforementioned names, there was still discrepancies on the species names used. To date, 9 species are generally recognized in *Meretrix* (Ayako et al, 2008). Zhuang (2001) pointed out that *Meretrix* has a little species and only three species, *M. meretrix*, *M. lusoria* and *M. lamarckii*, have been found in China. He admitted *M. lyrata* but didn't incorporate it into Chinese fauna. As *M. lyrata* occurs on the west coast of southern China and *M. petechialis* occurs from the west coast of the Korean Peninsula to southern China and Vietnam, the author considered that they should be included into Chinese fauna. This means that there are 5 species (including subspecies) in the Chinese fauna: *M. meretrix*, *M. lusoria*, *M. lamarckii*, *M. petechialis* and *M. lyrata*.

Former studies show that the *COI* gene sequences are rich in variations and fit for systematic analysis (Machordom, 2003; Donald, 2005). Bayesian tree indicated that the clade *M. lamarckii* is a sister group to the assemblage (*M. lusoria* + *M. petechialis* + *M. meretrix*). This is in accordance with the ideas from 16*S* rDNA and ITS1 that the phylogenetic relationships of four *Meretrix* species: (((*M. lusoria* + *M. meretrix*) + *M. lamarckii*) + *M. lyrata*) (Pan et al, 2006).

A result that is insensible to differential weighting of the data is to be regarded as the most robust hypothesis of phylogeny. As mentioned above, the position of *M. lyrata* in the most parsimonious tree is indeed sensitive to the differential weighting schemes (Figs. 1, 2). However, if one chooses to focus on supported groups (>70%), as we prefer to do, there is no major difference between the weighted supported results. Thus, weighting did not seem to provide any further help in clarifying relationships of the selected taxa.

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