Mitochondrial COI Gene Sequence Variation and Taxonomic Status of Three Macrobrachium Species

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Abstract: Freshwater prawns (Decapoda: Caridea: Macrobrachium) play an important role in domestic fishery resources. Culturing M. rosenbergii and M. nipponense brings great economic benefits, as the two species were widely farmed in China. M. qilianensis, a native species with natural distribution limited in Gansu province, was classified into genus Macrobrachium based on external morphological characters. In order to understand the molecular genetic differences among the three species of Macrobrachium, i.e., M. rosenbergii, M. nipponense, and M. qilianensis, we analyzed sequences of mitochondrial cytochrome oxidase subunit I (COI) of them. It would provide theoretical basis of exploiting and utilizing Macrobrachium resources rationally. A total of 30 individuals (10 individuals of each species) were collected from Gansu and Zhejiang province. Samples of M. qilianensis were wild, however, that of M. rosenbergii and M. nipponense were cultured. Their mitochondrial COI gene segment sequences were obtained by using the method of PCR amplification and sequencing. After alignment, 649 bp consensus sequences of COI were obtained. One hundred and sixty-nine variable sites were detected in all 30 individuals, accounting for 26.04% of total sequence. A total of seven haplotypes were also detected. Nucleotide diversity was 0.411% within M. rosenbergii, 0.092% within M. nipponense, and 0.031% within M. qilianensis. Genetic diversity of wild M. qilianensis was much lower than that of cultured M. rosenbergii and M. nipponense. Genetic distances between different haplotypes of the three prawns ranged from 19.87% to 23.84%. It suggested that the three species were valid species, because genetic distances among them were quite great.

To further determine the taxonomic status of the three prawns in family Palaemonidae, we downloaded the corresponding COI sequences of Palaemonidae prawns from Genbank and analyzed the phylogenetic relationships of them. Phylogenetic tree (NJ) showed that M. nipponense, M. rosenbergii and other Macrobrachium species constituted one monophyletic group. However, M. qilianensis, Exopalaemon carinicauda, and Palaemon deblis formed the other clade. Thus, results of COI sequences did not support that M. qilianensis belonged to genus Macrobrachium. The taxonomic status of M. qilianensis should be reevaluated with more comprehensive evidences.

Key words: Macrobrachium; Genetic differences; Taxonomy; COI gene; Mitochondrial DNA

三种沼虾的 COI 基因序列变异及其分类地位探讨

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摘要: 罗氏沼虾 (Macrobrachium rosenbergii) 和日本沼虾 (M. nipponense) 已经在我国得到广泛的养殖, 产生巨大的经济效益。祁连沼虾 (M. qilianensis) 是自然分布在我国甘肃省的土著虾种, 因其外部形态符合沼虾属的特征, 而被前人归入沼虾属。为了从分子生物学的角度理解罗氏沼虾、日本沼虾与祁连沼虾的遗传差异, 为合理开发和利用沼虾资源提供理论基础, 作者对这 3 种沼虾的线粒体 COI 基因序列进行研究。从甘肃、浙江等地分别采集这 3 种沼虾的样本各 10 尾, 共 30 尾, 其中祁连沼虾是野生样本, 而罗氏沼虾和日本沼虾都是养殖样本。通过 PCR 方法扩增线粒体 COI 基因, 并测序。通过比对, 获得一致序列 649 bp。在 30 个样本中共检测到 169 个变异位点,
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占总变异的 26.04%；共检测到 7 种单倍型。3 种沼虾的核苷酸多态性分别为：罗氏沼虾 0.411%、日本沼虾 0.092%、祁连沼虾 0.031%。野生的祁连沼虾遗传多样性远远低于养殖的罗氏沼虾和日本沼虾。三种沼虾单倍型之间的 Kimura 双参数遗传距离在 19.87%~23.84%，三者之间的遗传距离较大，提示三者均为有效种。为进一步确定这三种沼虾在长臂虾科的分类地位，我们从 NCBI 数据库中下载了长臂虾科的其他种类的 COI 序列进行系统发生分析。用 NJ 法构建的分子系统树显示：日本沼虾和罗氏沼虾与沼虾属的其他种类聚成一枝，而祁连沼虾与同亚科的脊尾白虾（Exopalaemon carinicauda）和长角长臂虾（Palaemon debilis）较沼虾属另 10 种虾的遗传距离近，即祁连沼虾与白虾属及长臂虾属聚成另一枝。因此，COI 序列的结果不支持祁连沼虾归入沼虾属。但其分类地位应该综合多方面证据重新进行分析确定。

关键词：沼虾；遗传差异；分类；COI 基因；线粒体 DNA
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The Palaemonoidae (Rafinesque, 1815) is the largest family of the order Decapoda, comprised of 2 subfamilies and 102 genera (Li et al, 2003). The subfamily Palaemoninae are less diverse phylogenetically, but more diverse ecologically. They distribute widely in world marine-, estuarine- and fresh-waters. Traditional morphology-based classification in this family was doubted, because the prawns appear to be morphologically highly conservative (Holthuis, 1950, 1952; Johnson, 1973). Molecular genetic approaches were used to clarify the systematic relationships of different species (Pereira, 1997). Murphy & Austin (2002), in a study of Australian palaemonid shrimps, using mitochondrial 16S rRNA sequences, found inconsistencies between the current morphologically based classification system and the phylogenetic relationships of five species.

The giant river prawn, Macrobrachium rosenbergii, belongs to genus Macrobrachium (Crustacea: Decapoda: Palaemonidae), is one of major aquaculture species with great economic value in China (Liu & Wan, 1997). The oriental river prawn (M. nipponense) is also a commercially important aquaculture species with temperate distribution and natural reproduction in China (New, 2006). A native prawn species, M. qilianensis, is known locally as the Hexi prawn, and its natural distribution is limited in Shule River, Hei River, Shiyang River, their adjacent lakes and reservoirs in Gansu province, China. The most-common morphological characters used in taxonomy of the genus Macrobrachium is the 2nd pereiopod or the rostrum (Li et al, 2008). The morphological characters of this prawn are consistent with that of Macrobrachium. M. qilianensis is abundant and important in Gansu province from an ecological point of view and as a commercially fishery resource, yet knowledge on its genetic characters is limited. Cheng et al (2007) thought that M. qilianensis and M. nipponense were supposed to be close related species based on sequence analysis of mitochondrial 16S rRNA gene. Previous studies suggested that DNA barcoding of life using a standardized mitochondrial cytochrome oxidase subunit I (COI) sequence was proposed as a species indentification system, and as a method for detecting putative new species (Yang et al, 2007; Tavares & Baker, 2008). We hypothesized that M. qilianensis might be a native species of genus Macrobrachium in Gansu province. Thus, our aim is to report the findings of mtDNA COI use in taxonomy and phylogenetic relationships among genus Macrobrachium. The results of this study would be important and useful making scientific managements for the natural resource protection and genetic breeding of Macrobrachium species.

1 Material and Methods

1.1 Sampling

A total of 30 individuals, i.e., 10 individuals of each Macrobrachium species, were collected from Gansu and Zhejiang provinces. Thereinto, individuals of M. qilianensis were captured from wild stock of Haimaku lake, Jiuquan city, Gansu province, and individuals of M. rosenbergii and M. nipponense were obtained from hatchery stocks of Institute of Freshwater Fisheries, Zhejiang province, and Qiyi Reservoir, Jiuquan city, Gansu province, respectively. All samples were transferred in 95% ethanol to the laboratory and stored at −20 ℃ until used. Henceforth, based on their Chinese names, M. qilianensis, M. rosenbergii and M. nipponense were abbreviated as QL, LS and RB, respectively.

1.2 DNA extraction, PCR amplification and sequencing

Muscle tissues were dissected, then digested by proteinase K overnight, followed by phenol-chloroform extraction and 100% ethanol precipitation (Sambrook et al, 1989). Extracted genomic DNA was checked using 1.5% agarose gel electrophoresis, then diluted to appropriate concentration (about 100 ng/µL) for PCR amplification.
Amplification reaction of the COI gene was in 50 µL volumes. Amplification reaction mixtures consisted of 100 ng DNA template, 0.2 mmol/L dNTPs, 1.0 mmol/L primers each, 4.0 mmol/L MgCl₂, 5.0 µL 10 × reaction buffer, 2U Taq polymerase, with sterilized water added to make up to the final volume to 50 µL. The PCR profile consisted of an initial denaturation at 94℃ for 5 min; then 35 cycles of 94℃ for 30 s, annealing 50℃ for 45 s, and 72℃ for 45 s; then 1 cycle of 72℃ for 7 min. Primer sequences were LCO1490 (5'-GGTCA ACAAA TCATA AAGAT ATTGG-3') and HCO2198(5'-TAAAC TTCAG GGTGA CCAAA AAATC A-3') (Folmer et al, 1994). PCR products were purified in 1.5% agarose (Takara) and sequencing using BigDye Deoxy Terminator Cycle Sequencing Kit with an automated DNA sequencer (ABI 3100) following the manufacturer’s instructions.

1.3 Data analysis

Partial COI sequences of 30 individuals (QL 10, LS 10, RB 10) of Macrobrachium were aligned using Clustal X (Thompson et al, 1997). The sequences were compiled with BioEdit 7.0.9 (Hall, 1999). Base composition and transition/transversion (ti/tv) ratios were examined using Arlequin 3.1.1 (Excoffier, 2005). Pairwise sequence distances of different haplotypes were calculated using Kimura 2-parameter model by MEGA 4.1b (Tamura, 2007). Additional characterization of the population genetic parameters were carried out in the software package DnaSP 5.0 (Librado & Rozas, 2009). Indexes of genetic diversity within species were measured with haplotype diversity (Hd) and nucleotide diversity (π) by MEGA 4.1b (Tamura, 2007).

To clarify the taxonomic status of these three Macrobrachium species, we downloaded 10 COI gene sequences of genus Macrobrachium from Genbank database. As outgroups, from NCBI database, we also downloaded Palaemon debilis and Exopalaemon carinicauda used for subfamily-level comparative species, Periclimenes soror used for family-level comparative species, Halocaridina rubra used for order-level comparative species (Tab. 1).

In order to judge if the data set have already lost phylogenetic information due to substitution saturation, the number of transitions and transversions against the corrected Kimura-2-parameter distances (K-2-p, K80 distance, Kimura, 1980) were plotted for each pair of unique sequences of species. In addition, another index (Iₜₛ) suggested by Xia et al (2003) also being used to measure substitution saturation of these COI sequences of species.

Inter- and intraspecific genetic distances were calculated using the Kimura 2-parameter model with the pairwise deletion option in the MEGA program. The phylogenetic tree was estimated using a Neighboring Joining (NJ) method, and confidence level in the tree generated was obtained using 1 000 bootstraps.

| Tab. 1  Sources of additional prawn mtDNA COI sequences used in this study |
| Order  | Family    | Subfamily | Genus   | Scientific name | English name | Accession number |
|--------|-----------|-----------|---------|----------------|--------------|-----------------|
| Decapoda | Palaemonidae | Palamoninae | Macrobrachium | M. asperulum | Jaro river prawn | AB250520 |
|         |           |           |         | M. edentatum |             | AB250552         |
|         |           |           |         | M. fukiense |             | FM958065         |
|         |           |           |         | M. hainanense |           | FM958068         |
|         |           |           |         | M. gracilirostre P |       | FM958067         |
|         |           |           |         | M. jaroense |             | FM958071         |
|         |           |           |         | M. latimanus P |           | FM958073         |
|         |           |           |         | M. nipponense | Giant river prawn | FM958077 |
|         |           |           |         | M. rosenbergii |             | Ay659990         |
|         |           |           |         | M. pinguis |             | FM958087         |
|         | Palaemon |           |         | P. debilis | Feeble shrimp | FM958086 |
|         | Exopalaemon |           |         | E. carinicauda | Ridgetail white prawn | EF560650 |
|         | Pontoniinae | Periclimenes | P. soror | Seastar shrimp | FJ386278 |
|         |           |           |         | H. rubra | Hawaiian red shrimp | NC008413 |
2 Results

2.1 COI gene sequences of these three Macrobrachium species

The COI gene can all be amplified clearly in these three species. The sizes of the COI gene were about 720 bp. The COI sequences were corrected and aligned, and 649 bp consensus sequences were obtained. Among the 30 sequences of these 3 species, 7 distinct haplotypes were detected (including 3 haplotypes in LS, 2 in RB, and 2 in QL). None of the haplotypes were shared by them. All 7 haplotypes sequences were submitted to Genbank databases (the accession numbers were between FJ958195 and FJ958201).

2.2 Sequence divergence and diversity of these three Macrobrachium species

The base composition differed slightly among species (Tab. 2). The mean A+T content was 58.20%. The rarest base was G (average 18.70%) in the COI gene. These patterns of base composition were consistent with the descriptions of other Macrobrachium prawns, such as *M. asperulum* (Liu, 2007). The overall transition/transversion bias was 1.958.

| Haplotype name | QL | LS | RB |
|----------------|----|----|----|
| Haplotype frequency | 0.9 | 0.3 | 0.1 |
| No. of haplotypes (H) | 2 | 3 | 2 |
| Haplotype diversity (Hd)±S.D. | 0.200±0.1541 | 0.6000±0.1305 | 0.200±0.1541 |
| No. of polymorphic sites (S) | 1 | 12 | 3 |
| Nucleotide diversity (π)% | 0.031 | 0.411 | 0.092 |
| Mean number of pairwise differences (k)±S.D. | 0.2002±0.2693 | 2.7079±1.5691 | 0.6019±0.5203 |

Sequence divergence estimated between the haplotypes of *M. gilianensis* population (QL) and *M. nipponense* population (RB) were 0.15% and 0.46%, respectively, whereas within the haplotypes of *M. rosenbergii* population (LS) ranged 0.15%–1.88% for COI. The sequence divergence ranged from 19.87% (LS-hap2 & RB-hap2) to 23.84% (LS-hap2 & QL-hap2) within the seven haplotypes of the three species (Tab. 4).

2.3 Genetic divergence and phylogenetic analyses of COI gene of all Palamonidae species

For these shrimp sequences, transitions and transversions were plotted by K-2-P distances, and a
saturation tendency was not shown for both transitions and transversion at about 20% of K-2-P distances (Fig. 1). In addition, the \( I_s \) value was 0.2283, the critical \( I_{ss-c} \) value was 0.7149 if the true tree was symmetrical, and 0.4788 if the true tree was asymmetrical, both being highly significantly greater than the observed \( I_s \) value \((P=0.0000, \text{ two-tailed } t \text{ test})\). Thus, there was little substitution saturation at these sequences. Both tests suggested that these sequences were useful for phylogenetic reconstruction.

When we compared our \( M. qilianensis \) sequences with those obtained from NCBI data base, we noted that there were several significant inconsistencies in our COI data set with those species of genus Macrobrachium (maximum identity < 83%). The COI sequence of \( E. carinicauda \) from Shen et al (2009) was the most identical to the sequence of \( M. qilianensis \) (maximum identity=86%). In order to verify the accuracy of the sequences of \( M. qilianensis \), we had re-examined, re-extracted and re-sequenced of our specimens and confirmed the identities of \( M. qilianensis \).

There was 15.24% sequence divergence, based on the K-2-P distance matrix, between the \( M. qilianensis \) population and \( E. carinicauda \), whereas the mean sequence divergence between the \( M. qilianensis \) population and other species in family Palamonidae (genus Macrobrachium, \( P. debilis \), and \( P. soror \)) ranged from 22.90% to 24.87%. The distances between the outgroup \( H. rubra \), and all palaemonid species (29.35% − 34.06%), were greater than all pairwise comparisons between palaemonid species (Tab. 5).

The two haplotypes of \( M. qilianensis \) population, along with \( E. carinicauda \) and \( P. debilis \) formed one monophyletic group and the other ten species of genus Macrobrachium formed another. \( P. soror \) and \( H. rubra \) were isolated as family-level and order-level comparative species. The nodes of most groups were highly supported by bootstrap resampling technique (Fig. 2).

### Discussion

\( M. qilianensis \) is probably endemic to Hexi corridor located in Gansu province. It is a small-sized prawn species. Its gonad matures when the prawn grows to 3 – 4 cm in total length. According to the yield of \( M. qilianensis \) recorded locally, there was more than 30 tons of the native prawn caught in the North Bay Reservoir, Gansu province, China. It is clear that \( M. qilianensis \) is one of the most important fishery species in local area. Genetic diversity of \( M. qilianensis \) wild stock showed lower level than that of \( M. rosenbergii \) and \( M. nipponense \) hatchery stocks. It means that the conservation and sustainable exploitation of \( M. qilianensis \) natural resources should be paid great attention.

The results of this study indicate inconsistencies with the hypothesized taxonomic status based on external morphological characters. The hypothesis that \( M. \)
Fig. 2 Molecular phylogenetic tree of neighbor-joining analysis (1000 replications) of prawns generated from partial COI mitochondrial sequences based on Kimura 2-parameter substitution model.

The numbers are the bootstrap support value of nodes (%).

The present study does not support the current morphologically based classification of Macrobrachium, as well as the Palaemonidae. Although only a limited number of species have been listed, it is under doubt that *M. qilianensis* belongs to genus *Macrobrachium*. *M. qilianensis* shows similar in morphology but distinct in gene. The systematic relationship between *M. qilianensis* and genus *Macrobrachium* should be taken into reconsideration. Furthermore, more molecular markers (e.g., cytochrome b markers) can be developed to investigate the taxonomic status of the native prawn species *M. qilianensis*.

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