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

ZHANG Qing-yi[1], CHENG Qi-qun[2,*], GUAN Wei-bing[1]

(1. *The Key Laboratory for Oceanic Fisheries Resources Exploitation of Shanghai Education Commission, College of Marine Sciences, Shanghai Ocean University, Shanghai* 201306, *China*; 2. *The Key Laboratory of Marine and Estuarine Fisheries Resources and Ecology, Ministry of Agriculture, East China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Shanghai* 200090, *China*)

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 Palaemonoidae, we downloaded the corresponding *COI* sequences of Palaemonoidae 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 debilis* 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

张清漪[1]，程起群[2,*]，管卫兵[1]

（1. 上海市教委大洋生物资源开发和利用重点实验室，上海海洋大学 海洋科学学院，上海 201306；
2. 农业部海洋与河口渔业资源及生态重点开放实验室，中国水产科学研究院东海水产研究所，上海 200090）

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三种沼虾的样本各 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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*通讯作者(Corresponding author), Tel: 021-65807698，E-mail: qiquncheng@gmail.com

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它种类的 *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它种类聚成一枝，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 16*S* 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 Haimaquan 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-cholroform 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 $MgCl_2$, 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 (I_{ss}) 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*		AB250520
				M. edentatum		AB250552
				M. fukienense		FM958065
				M. hainanense		FM958068
				M. gracilirostre P		FM958067
				M. jaroense	Jaro river prawn	FM958071
				M. latimanus P		FM958073
				M. nipponense	Oriental river prawn	FM958077
				M. rosenbergii	Giant river prawn	AY659990
				M. pinguis		FM958087
			Palaemon	*P. debilis*	Feeble shrimp	FM958086
			Exopalaemon	*E. carinicauda*	Ridgetail white prawn	EF560650
		Pontoniinae	*Periclimenes*	*P. soror*	Seastar shrimp	FJ386278
	Atyidae		*Halocaridina*	*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 overalltransition/transversion bias was 1.958.

Tab. 2 The mean nucleotide composition of *COI* partial sequences of 3 prawn species

	T	C	A	G
QL	33.60	20.65	27.42	18.34
LS	27.73	26.19	26.50	19.57
RB	30.05	22.47	29.29	18.20
Average	30.46	23.10	27.74	18.70

Within the *Macrobrachium* mtDNA *COI* sequences we got, 169 bases were variable. Of these, 164 sites were phylogenetically informative, accounting for 26.04% of total sequence. Nucleotide diversity and mean number of pairwise differences were low (π=0.031%, k=0.2002) within the *M. qilianensis* wild population (QL), while the *M. rosenbergii* (LS) and *M. nipponense* (RB) hatchery populations exhibited much higher level of variability (π=0.411%, k=2.7079; π=0.092%, k=0.6019, respectively)(Tab. 3).

Tab. 3 Haplotypes and genetic diversity parameters of *COI* partial sequences of three prawn species

Haplotype name	QL		LS			RB	
	QL-hap 1	QL-hap 2	LS-hap 1	LS-hap 2	LS-hap 3	RB-hap 1	RB-hap 2
Haplotype frequency	0.9	0.1	0.3	0.1	0.6	0.1	0.9
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. qilianensis* 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

Tab. 4 Kimura 2-parameter pairwise distance matrix of these three prawn species

	QL-hap1	QL-hap2	LS-hap1	LS-hap2	LS-hap3	RB-hap1	RB-hap2
QL-hap1							
QL-hap2	0.0015						
LS-hap1	0.2320	0.2340					
LS-hap2	0.2364	0.2384	0.0188				
LS-hap3	0.2320	0.2340	0.0015	0.0172			
RB-hap1	0.2020	0.2039	0.2134	0.2047	0.2112		
RB-hap2	0.1999	0.2018	0.2073	0.1987	0.2051	0.0046	

saturation tendency was not shown for both transitions and transversion at about 20% of K-2-P distances (Fig. 1). In addition, the I_{ss} 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_{ss} value (P=0.0000, two-tailed t test). Thus, there was little substitution saturation at these sequences. Both tests suggested that these sequences were useful for phylogenetic reconstruction.

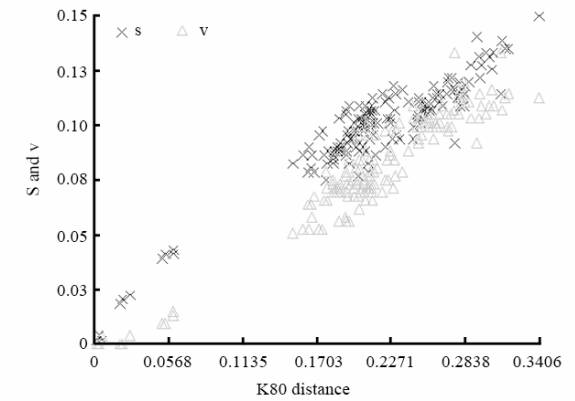

Fig. 1 Saturation test of cytochrome oxidase subunit I (*COI*) against K-2-P distances between pairs of taxa

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*, *Palaemon debilis*, and *Periclimenes soror*) ranged from 22.90% to 24.87%. The distances between the outgroup *Halocaridina rubra*, and all palaemonid species (29.35% – 34.06%), were greater than all pairwise comparisions 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).

Tab. 5 Genetic distances of the *COI* mitochondrial gene between QL and other prawns with the lower triangle showing the Kimura 2-parameter distances and the upper triangle showing the *p*-distances

	M. qilianensis (QL)	Genus *Macrobrachium*	*Exopalaemon carinicauda*	*Palaemon debilis*	*Periclimenes soror*	*Halocaridina rubra*
M. qilianensis (QL)		0.1999	0.1365	0.1942	0.2096	0.2442
Genus *Macrobrachium*	0.2348		0.2017	0.2285	0.2212	0.2406
Exopalaemon carinicauda	0.1524	0.2368		0.1923	0.2096	0.2558
Palaemon debilis	0.2290	0.2756	0.2245		0.2346	0.2692
Periclimenes soror	0.2487	0.2649	0.2485	0.2837		0.2404
Halocaridina rubra	0.3003	0.2935	0.3175	0.3406	0.2944	

3 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.*

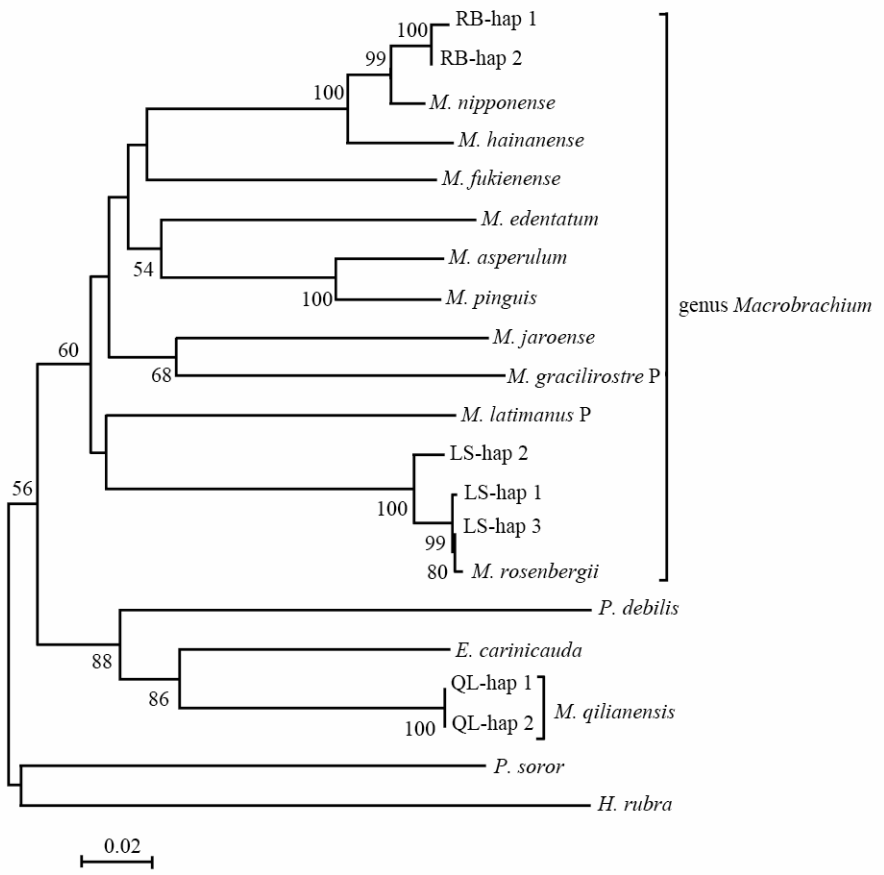

Fig. 2 Molecular phylogenetic tree of neighbor-joining analysis (1000 replications) of prawns generated from partial *COI* mitochondrial sequences based on Kimura 2-parameter substitution model

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

qilianensis might be a native species of genus *Macrobrachium* in Gansu province is clearly unsupported. *M. qilianensis*, *E. carinicauda*, and *P. debilis* form a well-supported clade distinct from the ten other *Macrobrachium* species. Cheng et al (2007) suggested that *M. qilianensis* might be a subspecies of *M. nipponense* which lives in low temperature and saline-alkali waters in Hexi corridor. *COI* gene was thought to be less conserved than the 16S rRNA gene, and it has been frequently used in evolutionary studies (Clary & Wolstenholme, 1985; Beard et al, 1993). Molecular study of *COI* mitochondrial sequences does not support the close relationship of *M. qilianensis* and the ten other species of *Macrobrachium*. The sequence divergence between *M. qilianensis* population and genus *Macrobrachium* is greater than that of *M. qilianensis* population and *E. carinicauda* or *P. debilis*.

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 (eg: cytochrome b markers) can be developed to investigate the taxonomic status of the native prawn species *M. qilianensis*.

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