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Usage Mitochondrial *16S rRNA* Gene as Molecular Marker in Taxonomy of Cyprinin Fish Species (Cyprinidae: Teleostei)

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Abstract. To confirm the taxonomical status of seven cyprinin fish species in Iraqi inland waters: *Barbus xanthopterus, B. kersin, B. barbulus, B. grypus, B. sharpeyi, B. luteus and Cyprinus carpio*, the mitochondrial *16S rRNA* gene fragment was used as a molecular marker. The primer was modified to include limited 120 bp fragment. PCR product tested on 2% agarose gel electrophoresis. The size of bands was estimated using the 100 bp ladder, while the correlation between the size of 100 bp ladder bands and the migrated distance was high since the $R^2 > 0.99$. Molecular profile of mtDNA *16S rRNA* gene fragment appeared that the six *Barbus* species and *Cyprinus carpio* had responded similarly to the modified primer. Whereas the second profile showed that the muglid *Liza abu* and *Liza klunzingeri* (used as out-group family) and leuciscin *A. vorax* (out-group sub-family) did not respond to the modified primer but the cyprinin *Carassius auratus* responded and the marker band of 120 bp length proved that this species also belongs to sub-family Cyprininae.

Introduction

Traditionally, the biometry was used in Iraq to classify fish species by international ichthyologists such as Heckle 1843; Gunther 1874; Berg 1949; Karaman, 1971), Iraqi researchers (Khalaf, 1961; Mahdi, 1962; Al-Daham 1977), while ostiological characters were used later by (Muhammad, 1987). Nevertheless protein electrophoresis also was used to the same purpose (Al-Hassan, 1985, 1988). Whereas after the

discovery of Polymerase Chain Reaction (PCR) technique the genetic makeup used to differentiate the fish species. In the past two decades the mitochondrial *16S rRNA* gene has been widely used to explore the Phylogenetic relationships of fishes at various taxonomic levels. Therefore, the mt*16S rRNA* gene has great potential for the inference of divergence among the cyprinid lineages and resolution of relationships within Cyprininae (Li *et al*., 2008).

There is an important aspect of ribosomal RNA gene that has conserved secondary structures that are moderately well conserved among distantly related taxa (Caetano-Anolles, 2002). While Simons and Mayden (1998) used *12S* and *16S rRNA* sequences to clarify the phylogenetic relationship of Western North American genus *phoxinus* (Cyprinidae) with the western clad and concluded that they likely have Asian or European relatives.

Similarly, Gilles *et al*. (1998) used these mitochondrial *16S rRNA* and *Cyt b* genes to resolve the phylogenetic relationships among Cyprinidae subfamilies.

Therefore, *16S rRNA* genes sequences were used to clarify the taxonomy of species in family Cyprinidae (Li *et al*., 2008), family Sisoridae (Guo *et al*., 2005), genetic divergence between the species of family Serranidae in Mediterranean Sea (Carreras-Carbonell *et al*., 2008).

On the other hand Nguyen *et al*. (2006) utilized the mt*16S rRNA* gene sequence data to investigate the DNA diversity of broodstock of two indigenous mahseer cyprinid species, *Tor tambroides* and *T. douronensis*.

In addition Rastogi *et al*. (2007) reported that mt*16S rRNA* and *ND4* gene sequence variation is suitable for addressing general questions on interspecific diversity.

Materials and Methods

A. Sampling sites

Three sites at Shatt Al-Arab were chosen to collect experimental fish species as following:

1. Qurnah (Northern of Shatt Al-Arab River).
2. Garmat Ali River.

3. Abul-Khaseeb (Southern of Shatt Al-Arab).

The sampling locations are illustrated on the Iraqi map (Fig. 1).

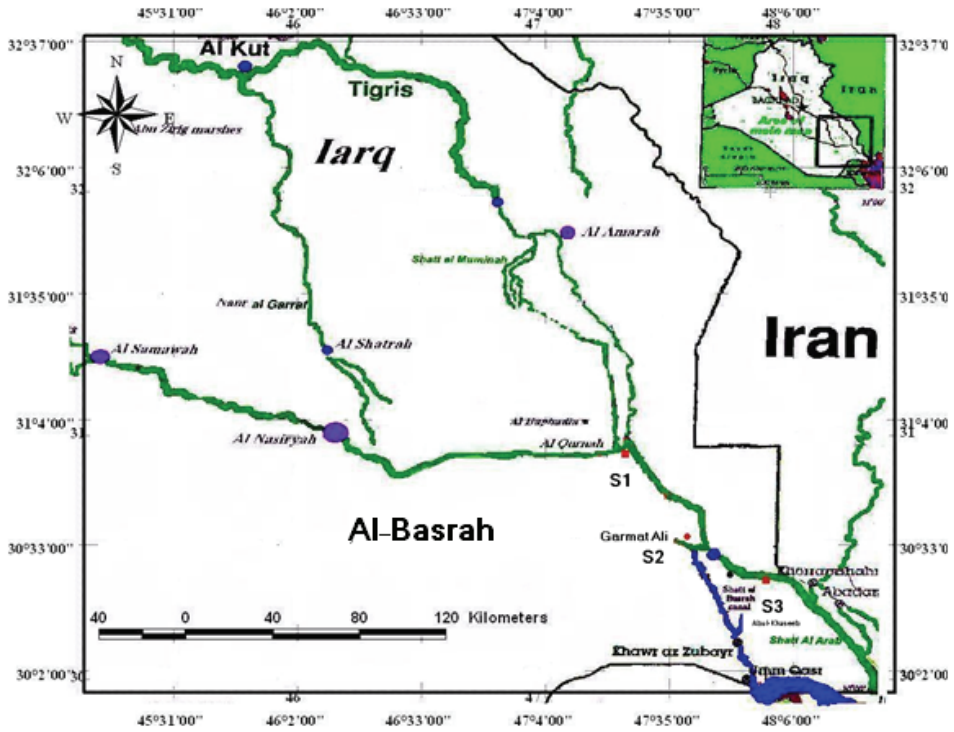

Fig. 1. Sampling sites on Iraqi map.

B. Specimens Collection Details

Seine net was used to collect 120 specimens, distributed as: Six species belong to the genus *Barbus*: *Barbus. xanthopterus* Heckel, 1848, *B. kersin* Heckel, 1843, *B. barbulus* Heckel, 1849, *B. grypus* Heckel, 1843, *B. sharpeyi* Günther, 1874, *B. luteus* (Heckel, 1843), and only one species of *Aspius*(*A. vorax* Heckel, 1843) representative to sub-family Luciscinae, representatives to native fresh water ichthyofauna in Iraqi inland waters. While *Cyprinus carpio* Linnaeus, 1758 used as introduced cyprinin species (Fig. 10). Another cyprinin species *Carassius auratus* Linnaeus, 1758 and two muglid species *Liza abu* (Heckel, 1843) and *Liza klunzingeri* (Day, 1888) as out-group family member used for comparison in mitochondrial *16S rRNA* test. The numbers of specimens and collection sites were tabulated as in Table 1.

Table 1. Collection Sites and number of specimens of fish samples.

No.	Fish species	Total number	Qurnah	Garmat Ali	Abul-Khaseeb
1	*B. xanthopterus*	18	7	8	3
2	*B. kersin*	5	3	2	---
3	*B. barbulus*	7	2	5	---
4	*B. grypus*	5	3	2	---
5	*B. sharpeyi*	15	11	4	---
6	*B. luteus*	15	10	3	2
7	*A. vorax*	18	12	3	3
8	*C. carpio*	18	10	4	4
9	*C. auratus*	10	10	---	---
10	*Liza abu*	3	3	---	---
11	*Liza klunzingeri*	3	3	---	---

The genomic DNA extracted from the fin tissues of seven species samples following the Sambrook and Russel (2001) and tested for DNA integration by 0.8% agarose gel.

PCR thermocycler (PCR SPRINT Thermal Cycler) was used for mtDNA *16S rRNA* gene amplification. The volume of reaction mixture was 25 µl in eppendorf tube (0.2 ml volume) contains 12.5 µl master mix solution, 1 µl forward primer, 1 µl reverse primer, 4 µl template DNA and 6.5 µl distilled water. This means component concentration 25 ng of template DNA, 5pmoles of primer, 0.1 mM of each dNTPs, 4 mM $MgCl_2$, 0.5 U of Taq polymerase and master mix buffer solution.

The reaction conditions were one cycle at 95^o C for 5 min., 35 cycle at 95^o C for 1 min., 58^o C for 1 min and 72^o C for I min. and final extension at 72^o C for 6 min.. Primers for mitochondrial *16S rRNA* gene amplification were selected from Li *et al*. (2008) which is specific for sub-family Cyprininae. So the primer became16Sp2F-5'-GAC CTG TAT GAA TGG CTA A-3' and 16Sp1R 5-CTT AAG CTC CAA AGG GTC-3 for amplifying specific fragment to recognize sub-family Cyprininae. The propriety of primer was tested with Primer-Blast (Primer designing tool) (NCBI.org) These pair of primers located from plus 2128 base locus till minus 2247, these numbers were the loci on the mitochondrial DNA sequences of *Cyprinus carpio* (see appendix C), its product was 120 base pair.

The PCR product electrophoresed on 2% agarose gel stained with ethidium bromide dye with working solution TBE under voltage 60V. Then tested on UV light illuminator to be photographed by gel documentation apparatus. The bands on the profile were estimated

according to the ladder bands while the correlation of natural logarithm of the ladder bands size *vs*. the distance migrated by these bands were obtained by MS Excel program.

Results and Discussion

Molecular profile (Fig. 2) showed that the six *Barbus* species and *Cyprinus carpio* had responded similarly to the modified primer to amplify 120 bp fragments. The size of bands estimated using 100 bp ladder. While the correlation between the size of ladder bands and the migrated distance was high since the $R^2 > 0.99$.

Whereas the second profile (Fig. 3) showed that the muglid *Liza abu* and *Liza klunzingeri* and leuciscin *A. vorax* did not respond to the modified primer, but the cyprinin *Carassius auratus* responded and the marker band of 120 bp long were amplified.

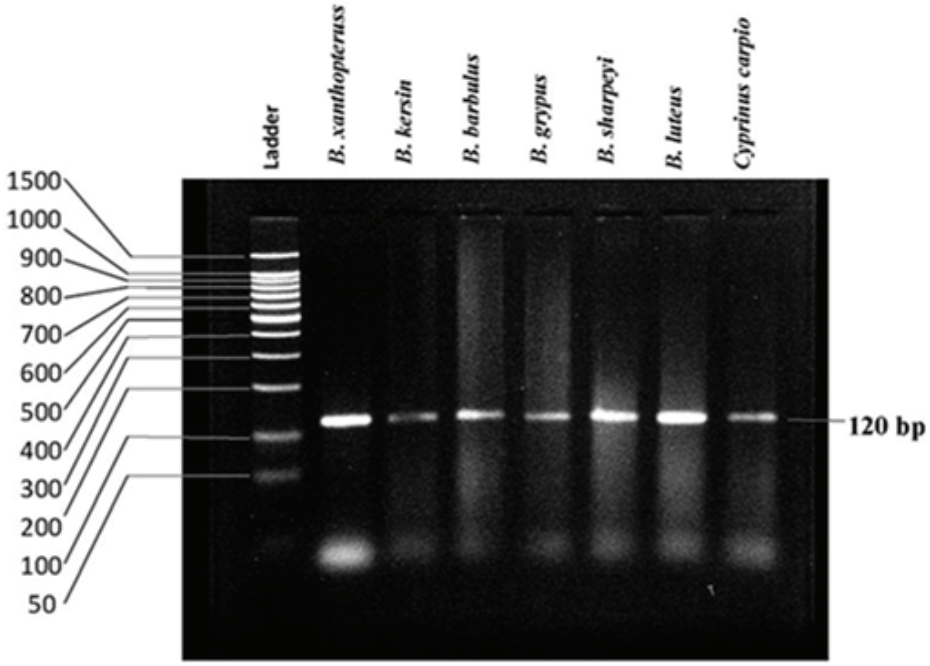

Fig. 2. Mitochondrial *16S rRNA* fragment (120 bp) of six *Barbus* spp. and *Cyprinus carpio* migrated on 2% agarose gel electrophoresis on 60V for 50 min.. The ladder measured by base pair (bp).

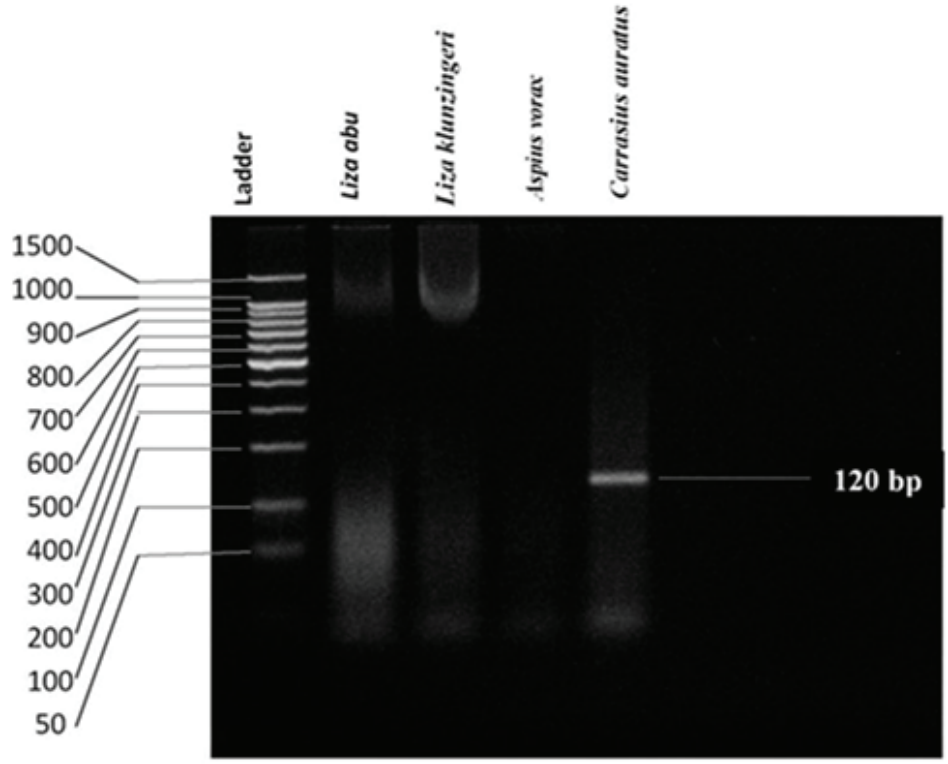

Fig. 3. Mitochondrial *16S rRNA* fragment (120 bp) migrated on 2% agarose gel electrophoresis on 60V for 50 min.. Lane 1: 100 bp ladder, lane 2: muglid *Liza abu*, lane 3: muglid *Liza klunzingeri*, lane 4: leuciscin *Aspius vorax* and lane 5: cyprinin *Carassius auratus*.

To confirm the systematic status of the six *Barbus* species compared to *Cyprinus carpio* that belong to sub-family Cyprininae (Family Cyprinidae) and compared to *Liza abu* and *Liza klunzingeri* as members of family Mugilidae and *Aspius vorax* of sub-family Leuciscinae (Family Cyprinidae) as out-groups and we included *Carassius auratus* as additive member of sub-family Cyprininae.

The *Cyprinus carpio* was previously tested genetically using complete mitochondrial genome (Chang *et al.*, 1994; Mabuchi *et al*., 2006 and Wang *et al.*, 2008) and partial ribosomal *16S rRNA* (Smith and McVeagh, 2005 and Li *et al*., 2008) which were deposited as reference sequences in the gene bank. So the primer pairs 16Sp2F and 16Sp1R was modified from the primer set of Li *et al*. (2008) to amplify short conserve

fragment (120 bp) of *16S rRNA* gene, in order to avoid the unexpected mutations.

This gene frequently was used to detect the taxonomy and systematic status of the species in both prokaryotes and eukaryotes due to its conservative properties, but its utilization in fish molecular studies in wide range (Gilles *et a*l., 2001 and Li *et al*., 2008). So the results showed that the modified primer amplified the expected 120 bp fragment in all the six *Barbus* species and *Cyprinus carpio* as shown in the profile of Fig. 2, that means Iraqi six *Barbus* species investigated in this study belong to sub-family Cyprininae as exactly as *Cyprinus carpio*.

To assure that this modified primer amplify this 120 bp fragment exclusively on the cyprinin members, two members of Muglidae *Liza abu* and *Liza klunzingeri* as different family and Leuciscinae *Aspius vorax* as different sub-family member compared to another Cyprininae member *Carassius auratus*.

The result showed that no amplification in all three out-groups species but the 120 bp fragment marker was amplified in *Carassius auratus* in lane 5 shown in Fig. 3, these results above assured that the six *Barbus* species genetically belong to sub-family Cyprininae which belong to family Cyprinidae. On the other hand a molecular marker can prove the tests of all sub-family Cyprininae species.

European cyprinid including few *Barbus* species were tested to resolve the phylogeny using the mitochondrial *16S rRNA* and *Cyt b* genes. They found that what previously thought the sub-family Leuciscinae was the primitive and the Cyprininae is the advance group was not accurate, but the Leuciscinae occupied the extreme point of the phylogenetic tree, and the Cyprininae was monophyletic (Gilles *et al*., 1998).

MtDNA *16S rRNA* gene was utilized to investigate the genetic diversity between two Cyprinid *Tor* species; found that this gene was suitable for testing the genetic variation among *Tor* broodstocks (Nguen *et al*., 2006).

The study recommends using this genetic method to classify and discriminate other fish species in Iraqi inland waters.

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استخدام جين 16S rRNA المايتوكونديري كمؤشر جزيئي في تصنيف أنواع أسماك الشبوطيات من العائلة الثانوية Cyprininae

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المستخلص. لتأكيد الحالة التصنيفية لسبعة أنواع من أسماك الشبوطيات من العائلة الثانوية Cyprininae وهي: سمكة الكطان *Barbus xanthopterus,* وسمكة الجصان *B. kersin,* وسمكة النباش *B. barbulus,* وسمكة الشبوط *B, grypus,* وسمكة البني *B. sharpeyi,* وسمكة الحمري *B. luteus* وسمكة الكارب الشائع *Cyprinus carpio*. استخدمت قطعة جين *16S rRNA* المايتوكونديري كمؤشر جزيئي وتم تحوير بادئ ليتضمن قطعة محددة ب ١٢٠ زوج قاعدي. فحص ناتج تضخيم الجين بتقنية PCR بترحيله كهربائيا على هلام الأكروز ٢,٠٪ وقدر حجم الحزم الظاهرة في الصورة باستخدام المسطرة الجزيئية ١٠٠ زوج قاعدي وقد كان الارتباط عاليا حيث كانت قيمة معامل الأرتباط ٠,٩٩ < R2. استجابت أنواع جنس *Barbus* الستة فضلا عن الكارب الشائع للبادئ وأنتجت عملية التضخيم قطعة جين ١٢٠ زوج قاعدي. بينما تبين فى هلام الأكروز بأن سمكتي الخشني *Liza abu* وسمكة البياح الأخضر *Liza klunzingeri* اللتان تعودان إلى عائلة Mugilidae واللذان استخدما كمجموعة خارجية من عائلة أخرى، وسمكة الشلك *Aspius vorax* التي تعود إلى عائلة الشبوطيات

ذاتها للعائلة الثانوية Leuciscinae كمجموعة خارجية من عائلة ثانوية أخرى لم تستجب للبادئ، في الوقت الذي استجابت فيه السمكة الذهبية *Carassius auratus* التي تعود إلى ذات العائلة الثانوية Cyprininae. استنتجت الدراسة بأن الأنواع الستة من جنس *Barbus* كما هو الحال في الكارب الشائع عائدة الى العائلة الثانوية Cyprininae.