Research Article

Environmental DNA for Disclosing of *Luciobarbus barbulus* (Cyprinidae: Teleostei) in the Shatt Al-Arab River, Iraq

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Abstract | Shatt Al-Arab River represents the estuary in the North-West Arabian Gulf. It is affected by many ecological challenges. Conditions of freshwater reduction from upstream and penetration of marine tide of the Arabian Gulf cause a decrease of many intolerant native fish species. Cyprinid *Luciobarbus barbulus* locally called Nabash is one of the important ecological and commercial fish species. *L. barbulus* has been often reported to be absent in fishing product of the Shatt Al-Arab River. Surveying of fish fauna in water bodies by using traditional methods did not often reveal accurate views of underwater. The environmental DNA method (eDNA) was used in this study to detect the cyprinid *L. barbulus*. Four stations in the River were chosen. Samples of water were then collected and filtered. Epithelial cells that detached from living organisms are used to extract genomic DNA. The gene of mitochondrial cytochrome C oxidase (*COI*) used as a diagnostic marker. Therefore, specific primers were designed to amplifying *COI* followed by sequencing. The results proved *L. barbulus* existence in the northern Shatt Al-Arab River. the sequence alignment matched the *L. barbulus* COI gene sequence which had been recorded in the GenBank from Iraqi and neighboring Iranian inland waters. The phylogenetic tree and sequence alignment confirm that the COI gene sequence belongs to *L. barbulus* fish species. Then the COI gene sequence deposited in the GenBank under an Accession number PQ608516. This is the first time in Iraq that used the eDNA to detect fish species and it can conclude that the eDNA method proved its effectiveness and it could be used to detect threatened or exotic fish species in Iraqi waters.

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Introduction

Biodiversity is the master key in the ecological studies. While the living stocks in the aquatic environment are vulnerable due to both natural and anthropogenic factors (Kumar *et al.*, 2024). A large percentage of fish is consumed in the world due to its high quality of protein level (Javaid *et al.*, 1992; Foran *et al.*, 2005; Salam *et al.*, 2005; Naeem and Ishtiaq, 2011). Therefore, fish population decreases rapidly

and need to be conserve by exploring its biodiversity (Clausen and York, 2008). Conventionally, monitoring of fish diversity is depending on fishing or sometimes harvesting the pond or lagoon to come across the target species. At the same time, these traditional methods took a lot of effort, cost, and more work days. Inland water bodies are susceptible environments as a result of adverse impact of humans as well as climate change, habitat loss, food chain and water quality reduction (Revenga *et al.*, 2000; Sala *et al.*, 2000; Dudgeon *et al.*, 2006; Vié *et al.*, 2009; Hambler *et al.*, 2011; Ashraf *et al.*, 2011; Yousaf *et al.*, 2011; Ismat *et al.*, 2013). The Shatt Al-Arab River is an estuary ecosystem, exposed to freshwater reduction from upstream and increasing salinity from the Arabian Gulf. In the last decade, many native species decreased in the Shatt Al-Arab River as a result of tide front elevation from the Arabian Gulf and reduction of freshwater inflow from upstream (Yaseen *et al.*, 2017). Many native fish species decreased in number or were absent in their regular environment. The *Luciobarbus barbulus*, locally named Nabash, is one of the Iraqi native fish species that was recorded on the red list (IUCN, 2011). Also, The *L. barbulus* is one of the important ecological and commercial fish species. Repeatedly, it was often absent from fishing products. In management of aquatic ecosystem and biodiversity conservation, detection of living species in the water bodies was one of the important steps particularly in the susceptible environment (Ficetola *et al.*, 2008). Natural waters always contain the genetic DNA of living organisms that exist in. The separated cells from these organisms called environmental DNA (eDNA) (Ruppert *et al.*, 2019). The eDNA can be used to confirm the existence of species in water bodies (Rees *et al.*, 2014). Detection of existence of fish species is one of the important aspects in the aquatic environment management (Reilly *et al.*, 2020). Any genetic materials of the epithelial cells from fish fauna could be used as a beneficial source for species detection. Therefore, eDNA method would confirm the presence of certain species. This method including water sampling from the aquatic environment and analyzing for existence of genetic materials that referred to the target species (Jerde *et al.*, 2011). Utilizing genetic materials for biodiversity studies instead of overfishing or drying the water bodies would ease the fieldwork. Also, invasive species can be monitored by eDNA (Wang *et al.*, 2023). In the beginning, this technique was used to investigate the extinct plants, birds, and mammals in the sediment (Willerslev *et al.*, 2003). After that, the method was used on various environmental samples, including terrestrial, water, and ice cores (Hofreiter *et al.*, 2003; Haile *et al.*, 2007, 2009; Willerslev *et al.*, 2007; Ficetola *et al.*, 2008; Matisoo-Smith *et al.*, 2008; Jerde *et al.*, 2011). Epithelial cells that Separate from aquatic organisms to the environment are the source of the eDNA (Hinlo *et al.*, 2017). Therefore, the DNA extracted from the water samples represents the fish species in the water bodies. Even small volumes of pond water contain enough genetic materials (Ficetola *et al.*, 2008). This method depends on collecting of water samples from the water body and investigating of DNA for the target species (Jerde *et al.*, 2011). The eDNA would degrade in a short time, so its presence would be evidence for existence of its belonging species (Pascher *et al.*, 2022). The eDNA breaks down in water within a few days (Turner *et al.*, 2015). Thus, the presence of DNA that belong to an organism in the aquatic environment confirms its recent existence. To identify species, the sequence of a particular gene is compared to that in the NCBI database. Therefore, this study aims to investigate of existence of *L. barbulus* in the Shatt Al-Arab River using an environmental DNA method and depending on mitochondrial *COI* gene sequence.

Materials and Methods

Four stations were chosen in the Shatt Al-Arab River as in the map (Figure 1). Four liters of water were collected from each station. Water samples were frozen at -20˚C till filtration time. Samples were filtered by a vacuum pump with 0.45 µm filter papers and a Buchner conical flask. Thereafter, the precipitates on the filter papers were repeatedly washed with distilled water and preserved in 70% ethanol alcohol till the genomic DNA isolated and purified by using Geneaid™ DNA Isolation Kit -Tissue (GET150) Taiwan, following the manufacturer's instructions. Electrophoresis was run on 0.08% agarose gel with ethidium bromide dye. UV light plate used to analyze DNA integrity and Nanodrop for the exact concentration. The *COI* gene amplified using the polymerase chain reaction (PCR) technique. The PCR cycler was run as follows; one cycle for initial denaturation at 95 ˚C for 5 minutes, 30 cycles for the second stage which included denaturation at 95 ˚C for 1 minute, annealing at 59 ˚C for 45 seconds and extension at 72 ˚C for 1 minute. Moreover, one cycle for final extension at 72 ˚C for 6 minutes. After that, the PCR product electrophoresed on 2% agarose gel with ethidium bromide dye using

100 bp ladder to check the exact band size. The primers that used to amplify and sequence of the *Cytochrome C Oxidase (COI)* gene, which are designed by NCBI blast- Primer using the *L. barbulus COI* sequence as a template, were as follows; ZLb-F-5'-CGGAATAGTGGGAACTGCCT-3' and ZLb-R-5'-GAATGCTATGTCTGGGGCT-'3. Subsequently, the amplified gene segment was sequenced, Wuhan Benagen Technology Company Ltd, Wuhan Laboratory. Chromas software was used to manage the sequence. The processed sequence then checked with the NCBI Blast database. The Neighbour-joining tree created by NCBI-Blast (NCBI, 2024). While the sequence alignment revealed by Clustal Omega (2024).

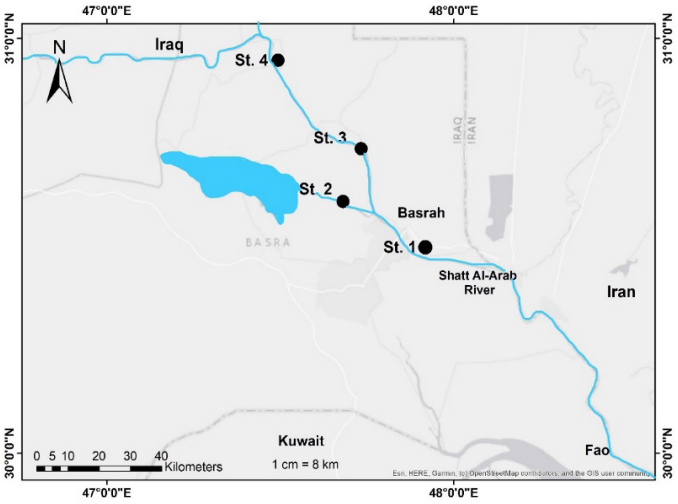

Figure 1: *The sampling stations of the Shatt Al-Arab River. The black spots represent the sites of samples.*

Results and Discussion

The results of three samples sequencing confirmed that the COI gene sequence belongs to *L. barbulus* with a length, after processing, 206 base pair. The sequence was checked with NCBI blast and the results matched the sequence of the *L. barbulus* query that covered 100% identity as in Table 1. On the other hand, the sequence alignment revealed that the *L. barbulus COI* sequence obtained by eDNA was matched with *L. barbulus COI* record in the GenBank. Neighbour joining tree showed that the sample sequence clustered with the sequences of the same species of Iraqi and Iranian inland waters OM669701.1, KY249567.1, KM590436.1 (Figure 3). While there is only 99% identity with *COI* sequences of the morphologically similar species *Luciobarbus xanthopterus* OM669699.1, KM590446.1 (Figure 2 and Table 1). Then the *COI* gene sequence of *L. barbulus* obtained by

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PQ608516.1  1    CGGAATAGTGGGAACTGCCTTAAGCCTTCTCATTCGAGCCGAATTAAGCCAACCCGGATC  60
KM590436.1  71   ............................................................  130
KY249567.1  6    ............................................................  65
OM669701.1  13   ............................................................  72
KM590446.1  71   ............................................................  130
OM669699.1  12   ............................................................  71

PQ608516.1  61   ACTTCTAGGTGATGATCAAATTTATAATGTTATTGTTACTGCTCACGCCTTCGTGATAAT  120
KM590436.1  131  ............................................................  190
KY249567.1  66   ............................................................  125
OM669701.1  73   ............................................................  132
KM590446.1  131  ............................................................  190
OM669699.1  72   ............................................................  131

PQ608516.1  121  CTTCTTTATAGTAATGCCTATTCTAATTGGAGGATTTGGGAACTGACTTGTACCATTAAT  180
KM590436.1  191  ............................................................  250
KY249567.1  126  ............................................................  185
OM669701.1  133  ............................................................  192
KM590446.1  191  ............................................................  250
OM669699.1  132  ............................................................  191

PQ608516.1  181  AATTGGAGCCCCAGACATAGCATTCC  206
KM590436.1  251  .........................  276
KY249567.1  186  .........................  211
OM669701.1  193  .........................  218
KM590446.1  251  .........................T.  274
OM669699.1  192  .........................T.  215
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Figure 2: *Multiple sequence alignment of Luciobarbus barbulus COI obtained by eDNA analysis from the north part of Shatt AL Arab River with five various COI sequences of genus Luciobarbus from Iraqi and Iranian inland waters.*

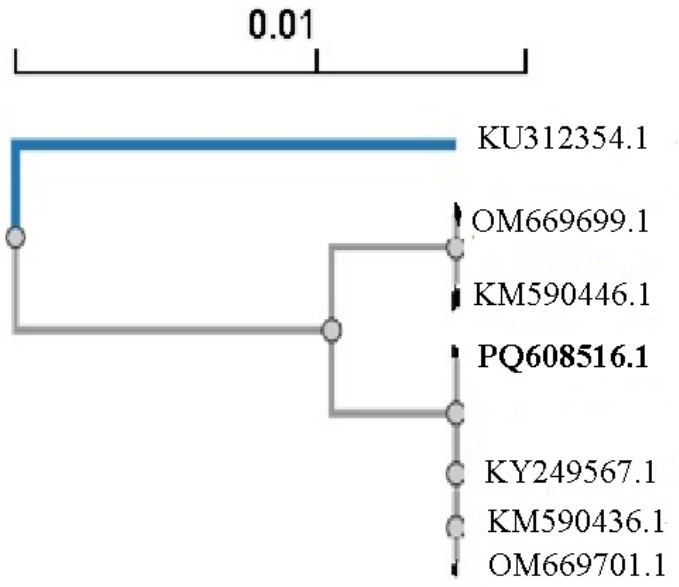

Figure 3: *The phylogenetic tree. The tree showed genetic distant among and L. barbulus fish species of Iraq created by the eDNA method (PQ608516.1), in the current study, and three COI sequences records of the same species in the Blast (OM669701.1, KY249567.1, KM590436.1) of Iraq and neighbor waterbodies of Iran in addition to two COI sequences of L. xanthopterus (OM669699.1, KM590446.1) of the same genus from the surrounding areas. Capoeta damascina fish species COI gene sequence (KU312354.1) used as outgroup. built by Clustal Omega.*

eDNA deposited in the GenBank with an Accession PQ608516.1. Depending on the results of this study, *L. barbulus* was exist in the upper part of the river in stations three and four. Simultaneously, the lower station (station 1) in Basra centre gave negative results in PCR analysis. The absence of *L. barbulus COI* gene sequence in the lower station maybe attributed to increase of salinity that made the environment unsuitable for this species. As for the station 2 in the

Table 1: *Comparison of L. barbulus COI sequence values obtained by eDNA analysis PQ608516.1 and three COI sequences of the same species in addition to two species of L. xanthopterus of Iraqi and neighboring Iranian inland waters.*

Acc. Ver.	Scientific name	% identity	Alignment length	Mis-matches	Gap opens	S. start	S. end	country	Bit score
PQ608516.1	*L.barbulus*	100	206	0	0	1	206	Current Study-eDNA	NA
KY249567.1	*L. barbulus*	100	206	0	0	6	211	Islamic Republic of Iran	381
OM669701.1	*L. barbulus*	100	206	0	0	13	218	Republic of Iraq	381
KM590436.1	*L. barbulus*	100	206	0	0	71	276	Islamic Republic of Iran	381
OM669699.1	*L. xanthopterus*	99	206	1	0	71	274	Republic of Iraq	377
KM590446.1	*L. xanthopterus*	99	206	1	0	12	215	Islamic Republic of Iran	377

Garmat-Ali branch, it showed damaged DNA at the DNA extraction stage. The pollution resulted from domestic effluents near the station 2 could be resulted in genomic degradation. In addition, the genome should be well-preserve at -20°C shortly after the sample collection. Otherwise, it will be destroyed by enzymes activity (Chatterjee and Walker, 2017). The results of the current study referred to the existence of *L. barbulus* in two stations of the selected four. The effect of salt tide in the lower part of the Shatt Al-Arab River and the domestic pollution in the Garmat Ali Branch Station caused a reduction in water quality, which did not fit the demands of *L. barbulus* living habitat. The results confirmed that the eDNA method is a suitable and friendly tool to monitoring and verify of certain species of aquatic environment. It is no need to catch many of fishes by using nets or any other method that would often deadly for fish. Furthermore, using eDNA requires less effort, time, and money at sampling. This is the first time in Iraq that used the eDNA to detect fish species, and the results revealed that the environmental DNA is a successful method in investigating of fish species that existence in the Shatt Al-Arab River. In addition, the results of present study proved that this method is reliable in revealing underwater living organisms although the sampling stations, the filtering, and preservation of the filtered matters have a significant role in the DNA extraction and sequencing (Hinlo *et al.*, 2017). Many of studies have used the eDNA method that was a useful method in detecting fish species and that agreed with our study (Wang *et al.*, 2023). Nakamichi *et al.* (2023), they used eDNA to detect rare species of exotic eels and an endangered fish in a River from Fukushima, Japan. On the other hand, LeBlanc *et al.* (2020) used eDNA for detection of marine invasive invertebrate species in eastern Canada. Furthermore, a study by Xin *et al.* (2024) showed the effectiveness of eDNA in exploring of diversity and distribution of fish communities in the Liaohe National Parkark, China. The eDNA offers a spatial vision by detecting more cryptic fish species and would resolve the early life stage of fish species in the exact habitat (Chapman and Sullivan, 2022). In the other side, traditional monitoring methods, such as fishing or pond harvesting, are spending cost, efforts and difficulties in differentiation of cryptic species or immature life stages (Rishan *et al.*, 2023). The eDNA is an upgraded capability to monitor the aquatic habitat and great biomarker for species and population diversity (Bohmann *et al.*, 2014). The technique is useful in confirming of presence of fish species while there are limitations that obstruct obtain on confirmed results such as water quality, the flow rate and pollution.

Conclusions and Recommendation

From this study, it can be concluded that the native species left that part of the Shatt Al-Arab River where increasing water salinity resulted in unsuitable habitat. On the other hand, domestic pollution could also be reducing the opportunity of success of eDNA method. This study showed that the eDNA is a valuable tool for exploring fish species in the Shatt Al-Arab River. Therefore, we recommended the use of eDNA method to investigating the Shatt Al-Arab ecosystem for exotic fish species. The protocol needs less effort, cost, workdays and has more accurate results. Moreover, the eDNA method is suitable to use in relatively standard environments while it is not suitable in polluted or unstable environments.

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Novelty Statement

This study is the first one in Iraq that used Environmental DNA method to searching for fish species in Iraqi ecosystem. Previous studies in Iraq never mentioned to use this method.

Author's Contribution

Mustafa S.F. Ziyadi: Designed the experimentation.
Rafid M. Karim and Mustafa S.F. Ziyadi: Performed animal experiments and editing a preliminary draft of manuscript.
Rafid M. Karim and Amar Yasser Jassim: Analyzed the data and wrote the manuscript.

Conflict of interest
The authors have declared no conflict of interest.

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