Research Article

Genetic divergence in *Mugil cephalus* & *Liza ramada* based on PCR–RFLP analysis of mtDNA segments

Heba A.M. Abd El-kader, Aboelhassan M. D., Karima, F. Mahrous*
Department of Cell Biology, National Research Center, El Buhouth St., 12311 Dokki, Egypt.
***Corresponding author's E-mail:** l_fathy@yahoo.com

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ABSTRACT

Restriction fragment length polymorphism (PCR-RFLP) and sequence analysis of mitochondrial DNA (mtDNA) genes (12S rRNA & 16S rRNA) were used to study genotypic differences between two species of Egyptian Grey mullets *(M. cephalus* and *L. ramada).* The amplified fragments of 12S rRNA (430 bp) and 16S rRNA (636 bp) were digested with three restriction enzymes (*Hinf*I, *Xba*I and *Taq*I). Obtained results of 12S rRNA gene revealed that no recognition sites for *Hinf*I and *Xba*I enzymes while *Taq*I enzyme showed monomorphic genotype (CC) in two mullet species. For 16S rRNA gene *Hinf*I and *Xb*I enzymes showed the same specific pattern among the two examined mullet species whilst *Taq*I enzyme showed the appearance of three genotypes (CC, CT and TT) in *M. cephalus* and *L. ramada* with highest allele frequency for allele C (0.85) in *L. ramada* and highest allele frequency for allele T (0.30) in *M. cephalus.* The blast and nucleotide sequence alignment of 16S rRNA showed complete identity between *M. cephalus* and *L. ramada* and in comparison with accession numbers KP018403 and JQ060860 showed five mutations at different positions recorded four transition mutations and only one transversion mutation for KP018403 and four transition mutations and a deletion mutation for JQ060860.We concluded that 16S rRNA mtDNA region has been shown to be a good marker to differentiate between two mullet species examined *Mugil cephalus* and *Liza ramada* farmed in Egypt and between *Mugil cephalus* and other Mugilid species.

Keywords: Grey mullets, 12S rRNA, 16S rRNA, PCR-RFLP, sequencing.

INTRODUCTION

The family Mugilidae including Grey mullets, a family of teleostean fishes, are distributed worldwide populate marine and freshwater environments in all modest and equatorial regions[1] and they have great importance in aquaculture and fishery trade of many countries as well as protein source. The ultimate contemporary classification showed that the Mugilidae containing 72 species belonging to seventeen genera[2], the most commonly distributing species of mullets are *Liza ramada* 24 (*L. ramada*) and *Mugil cephalus*[18] (*M. cephalus*).[3] Earlier studies reported that Egyptian water includes five species of Grey mullets.[4-6]

In previous studies, the phylogenetic relationships between Grey mullets have been inspected according to morphological characters[7-9], unfortunately these studies are controversial due to its conservative external morphology, therefore, it is difficult to get a specific key character help in determining the phylogenetic relationships among species.[10,11] Recently, a great deal of interest fastens on using molecular techniques to study genetic variety and evolutionary relationships among mullet species.[3]

In terms of genetic studies, several molecular markers have been used for identification of mullet species including *in situ* hybridization techniques (karyotype analysis)[12,13], allozyme electrophoresis[14-16], *mtDNA* analysis [17, 18], nucleic acid data[19, 15], and 16Sr RNA *mtDNA* analysis.[20,15] Nowadays one of the most widely used techniques is *mtDNA* analysis[21], where *mtDNA* has become one of the most effective genetic marker for the study of evolution and genetic differentiation among species because of absence of recombination, its maternal inheritance, characterization by minimal replacement and exchange rate in *mtDNA* nucleotides compared to nuclear DNA.[22] *MtDNA* coded for the most important ribosomal RNAs (12S rRNA and 16S rRNA) which occupying 1/16 and 1/10 of mitochondrial genome, respectively and important for the translation of messenger RNAs into mitochondrial proteins.[23] In addition, *mtDNA* high mutation rates resulting in intraspecific variance in short evolutionary times. The most widely used technique to identify this variation is Polymerase Chain Reaction-based Restriction Fragment Length Polymorphism (PCR-RFLP).[24]

Therefore, in the present study, we use PCR-RFLP of *mtDNA* genes (12S rRNA and 16S rRNA) to investigate the genotypic differences between two species of Egyptian Grey mullets (*M. cephalus* and *L. ramada*).

MATERIALS AND METHODS

Fish samples and DNA extraction

Total of 80 samples of two species of Egyptian Grey mullets (*M. cephalus* and *L. ramada*) were used randomly from El-Manzla lack in order to study Genetic polymorphism.

Blood sampling and DNA extraction

Blood samples were collected in tubes containing EDTA as anticoagulant and transported to the laboratory under cooled conditions. DNA was extracted and purified from

blood samples using the whole blood salting out technique described by Miller *et al*. [25]. DNA concentration and purity was determined using UV spectrophotometer at optical density of 260 and 280 nm.

Amplification of mitochondrial genes

Polymerase chain reaction was carried out using primers for *mtDNA* genes 12S rRNA and 16S rRNA. The designed primer sequences for 12S rRNA gene were: 5' AAACTAGGATTAGATACCCTATTAT 3' (forward) and 5' AAGAGCGACGGGCGATGTGT 3' (reverse). Where as the designed primer sequences for 16S rRNA gene were: 5'CGCCTGTTTAACAAAAACAT3' (forward) and 5'CCGGTCTGAACTCAGATCATGT3' (reverse) (Fermentas) as described by Bouchon *et al*.[26]. Amplification was carried out in 15 µl reaction mixture containing 1.5 µl of template (50 ng) DNA; 1.5 µl of 10X buffer (100 mM Tris-HCl, pH 8·3, 15 mM $MgCl_2$, 500 mM KCl); 1.5 µl of each primer (100 pmol); 1.5 µl of a 0.2 mM solution of (dNTPs); 1.25 units of *Taq* DNA polymerase. PCR parameters consisted of denaturation at 93°C for 1 minute followed by 30 cycles of 93°C for 30sec, 48°C for 30sec and 72°C for 30sec terminated with elongation at 72°C for 10 min.The PCR product were electrophoresed on 2% agarose gel and stained with ethidium bromide to check amplification product.

Restriction fragment length polymorphism (RFLP)

Amplified PCR products of approximately 430 bp and 636 bp size of the 12S rRNA and 16S rRNA, respectively were digested using three restriction enzymes (*Hinf*I, *Taq*I, *Xba*I) (Fermentas). 5µl of PCR product was digested with 0.5 µl of FastDigest restriction enzymes for 5 min at 37°C. The digested products were electrophoresed on a 2% agarose gel, the bands were visualized under UV light and the gels were photographed using gel documentation system (Bio-Rad, USA). DNA fragments sizes were estimated by their comparison with 100 bp standard molecular size marker

DNA sequencing

The PCR product obtained for 12S rRNA and 16S rRNA genes were purified and sequenced using both reverse and forward primers of the respective mitochondrial genes. Sequence analysis and alignment were compared to Genbank data in NCBI/BlAST/blastn of Mugilids for 12S rRNA and 16S rRNA mtDNA sequences to detect different genotypes.

RESULTS

Genotypic differences between two Mugilid species *M. cephalaus and L. ramada* collected from Egypt were studied using PCR amplification of 12S rRNA and 16S rRNA mitochondrial genes due to their importance in species identification.

PCR product of 430 bp size amplification was observed using 12S rRNA primer (Fig. 1), whereas 636 bp size amplification was observed using 16S rRNA primers (Fig. 2) in two Grey mullet species *M. cephalus, L. ramada.* These PCR amplified fragments (430 bp and 636 bp) were digested with three different restriction enzymes; *Hinf1, Taq*I and *Xba1*to assay the presence of recognition sites.

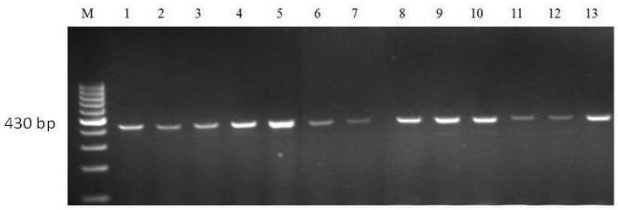

Figure 1: Amplified PCR product of 12S rRNA mitochondrial gene in Grey Mullet species. Lane M: 100 bp marker. Lanes from 1-13 PCR products of 430 bp in Grey Mullet species *M. cephalus* and *L. ramada*.

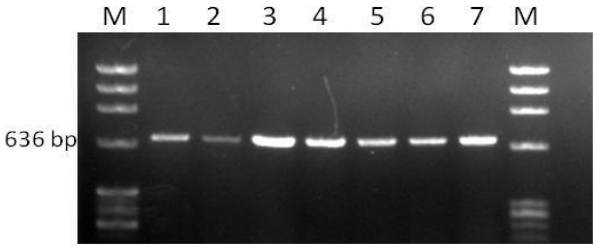

Figure 2: Amplified PCR product of 16S rRNA mitochondrial gene in Grey Mullet species. Lane M: Phi-X174 Hae III marker. Lanes 1-7 PCR product 636 bp in Grey Mullet species *M. cephalus* and *Liza ramada*.

Digestion results with *Hinf*I *and Xba*I enzymes showed no recognition sites in 12S rRNA gene in two mullet species. While digestion of 12S rRNA mitochondrial gene with *Taq*I restriction enzyme exhibited appearance of one recognition site (TˆCGA) recording one genotype that was homozygous CC with two digested segments at 302 bp and 128 bp in two tested species (Fig. 3). For 16S rRNA gene *Hinf*I and *Xba*I restriction enzymes showed the same specific pattern among the two examined mullet species *M. Cephalus* and *L. ramada.* while *Taq*I restriction enzyme showed three different genotypes in the two examined mullet, homozygous genotype TT at 636 bp that did not recognize the *Taq*I restriction site and detected only in *M. cephalus* species and this genotype was absent in *L. ramada*. Whilst the other two genotypes were cut with *Taq*I restriction enzyme according to the presence of restriction site (TˆCGA) (Fig. 4).These two genotypes were homozygous genotype CC with two digested bands at 522 and 114 bp and heterozygous genotype CT with three digested bands (636, 522 and 114 bp). Examined Mugilid species revealed the two genotypes CC and CT.

The blast and alignment of nucleotide sequence of 16S rRNA mitochondrial gene for two species were performed with accession numbers KP018403 *M. cephalus* mitochondrial complete genome and JQ060860 *M. liza* isolate 295 16S rRNA gene partial mitochondrial sequence that covered 636 bp (Fig. 5 a, b). The analysis of nucleotide sequences announced complete identity

between *M. cephalus*, *L. ramada* (Fig. 6). The comparison of nucleotide sequences of *M. cephalus* and *L. ramada* with KP018403 *M. cephalus* mitochondrial complete genome showed differences at five positions 120 (C/T), 288 (G/C), 290 (A/G), 461 (T/C) and 617 (G/A) as described by graphical figure 7a., recording four transition mutations at 120 (C/T), 290 (A/G), 461 (T/C) and 617 (G/A) and only one transversion mutation at 288 (G/C). Contrary the comparison with JQ060860 *M. liza* isolate 295 16S rRNA gene partial mitochondrial sequence, five mutations were detected at positions 51 (C/T), 120 (C/T), 373 (C/T), 377(C/-) and 378 (C/T) that proved by graphical figure 7 b. All observed mutations were transition mutations at 51 (C/T), 120 (C/T), 373 (C/T) and 378 (C/T) except the mutation at position 377 which represented a deletion in our sequences

Table 1: Genotype and allele frequencies of 16S rRNA

Mullet species	Genotype frequencies			Allele frequencies	
	CC	CT	TT	C	T
Liza Ramada	0.70	0.30	-	0.85	0.15
Mugil Cephalus	0.47	0.47	0.06	0.70	0.30
Total	0.56	0.40	0.04	0.76	0.24

Table 1 represents the genotype and allele frequencies of16S rRNA gene in *M. cephalus* and *L. ramada*, from which we observed that the highest genotype frequency was for CT genotype in *M. cephalus* (0.47) and for CC genotype in *L. ramada* (0.70), in contrast the lowest genotype frequency for TT genotype was in *M. cephalus* (0.06) that lacked in *L. ramada*. The highest allele frequency was for allele C in *L. ramada* (0.85) and the lowest allele frequency was for allele T (0.15) in the same species.

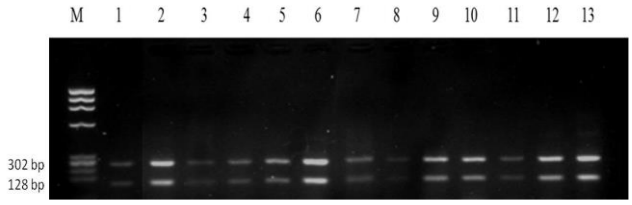

Figure 3: *Taq1* restriction pattern of Gry Mullet species 12S rRNA gene. Lane M: Phi-X174 HaeIII marker. Lanes 1–13: Homozygous genotype showed two restricted fragments at 128 and 302-bp for both studied species.

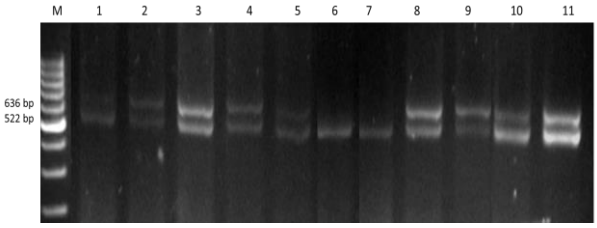

Figure 4: *Taq1* restriction pattern of Gry Mullet species 16S rRNA gene. Lane M: 100 bp. Lanes 7, 8& 9 homozygous genotype (CC) showed two restricted fragments at 522 and 114 bp; Lanes 2, 3, 4, 5& 6 heterozygous genotype (CT) showed three fragments at 522, 114 & 636 bp; Lane1 homozygous genotype (TT) at 636 bp.

The overall frequencies of C and T alleles in tested Mugilid species were 0.76 and 0.24, respectively.

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Query  1     CGCCTGTTTACCAAAAACATCGCCTCTTGTAAACCTCACATAAGAGGTCCTGCCTGCCCA  60
Sbjct  2031  CGCCTGTTTACCAAAAACATCGCCTCTTGTAAACCTCACATAAGAGGTCCTGCCTGCCCA  2090

Query  61    GTGACCCCTGTTCAACGGCCGCGGTATTTTAACCGCGCAAAGGTAGCGCAATCACTTGTT  120
Sbjct  2091  GTGACCCCTGTTCAACGGCCGCGGTATTTTAACCGCGCAAAGGTAGCGCAATCACTTGTC  2150

Query  121   CCTTAAATGAGAACCAGTATGAATGGCTAGACGAGGGCTTAACTGTCTCCTTTTCCCAAC  180
Sbjct  2151  CCTTAAATGAGAACCAGTATGAATGGCTAGACGAGGGCTTAACTGTCTCCTTTTCCCAAC  2210

Query  181   CAATGAAATTGATCTTCCCGTGCAGAAGCGGGAATACTAACATAAGACGAGAAGACCCTG  240
Sbjct  2211  CAATGAAATTGATCTTCCCGTGCAGAAGCGGGAATACTAACATAAGACGAGAAGACCCTG  2270

Query  241   CGGAGCTTTAGACGCCAGAACAGATCACGTCAAATACCTCTCTCAAACAGGTAACAACAA  300
Sbjct  2271  CGGAGCTTTAGACGCCAGAACAGATCACGTCAAATACCTCTCTCAAAGAAGTAACAACAA  2330

Query  301   ATGAACCCTGTTCCACGTCTTAGGTTGGGGCGACCACGGTGAACAGAAAAACCCCCGCGT  360
Sbjct  2331  ATGAACCCTGTTCCACGTCTTAGGTTGGGGCGACCACGGTGAACAGAAAAACCCCCGCGT  2390

Query  361   GGACTGAGAGCATATATTCACACTTATTAATACTGCTTCTCACAACCATGAGCTACAGCT  420
Sbjct  2391  GGACTGAGAGCATATATTCACACTTATTAATACTGCTTCTCACAACCATGAGCTACAGCT  2450

Query  421   CTAAATAACAGAACTTCTGACCaaaaaaaTGATCCGGCAACGCCGATTAACGGACCAAGT  480
Sbjct  2451  CTAAATAACAGAACTTCTGACCAAAAAAATGATCCGGCAATGCCGATTAACGGACCAAGT  2510

Query  481   TACCCCAGGGATAACAGCGCAATCCTCTTTAAGAGTCCATATCGACAAGAGGGTTTACGA  540
Sbjct  2511  TACCCCAGGGATAACAGCGCAATCCTCTTTAAGAGTCCATATCGACAAGAGGGTTTACGA  2570

Query  541   CCTCGATGTTGGATCAAGACATCCTAATGGTGCAACCGCTATTAAGGGTTCGTTTGTTCA  600
Sbjct  2571  CCTCGATGTTGGATCAAGACATCCTAATGGTGCAACCGCTATTAAGGGTTCGTTTGTTCA  2630

Query  601   ACGATTAAAGTCTTACATGATCTGAGTTCAGACCGG  636
Sbjct  2631  ACGATTAAAGTCTTACGTGATCTGAGTTCAGACCGG  2666
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Figure 5a: The blast and alignment of nucleotide sequence of 16S rRNA mitochondrial gene for two species *M. cephalus* and *L. ramada* with accession number KP018403 *M. cephalus* mitochondrial complete genome.

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Query  1       CGCCTGTTTACCAAAAACATCGCCTCTTGTAAACCTCACATAAGAGGTCCTGCCTGCCCA 60
                                                                 |||||||||
Sbjct  743     CGCCTGTTTACCAAAAACATCGCCTCTTGTAAACCTCACATAAGAGGTCCGGCCTGCCCA 803

Query  61      GTGACCCCTGTTCAACGGCCGCGGTATTTTAACCGCGCAAAGGTAGCGCAATCACTTGTT 120
                                                                 |||||||||
Sbjct  804     GTGACCCCTGTTCAACGGCCGCGGTATTTTAACCGCGCAAAGGTAGCGCAATCACTTGTC 863

Query  121     CCTTAAATGAGAACCAGTATGAATGGCTAGACGAGGGCTTAACTGTCTCCTTTTCCCAAC 180
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  864     CCTTAAATGAGAACCAGTATGAATGGCTAGACGAGGGCTTAACTGTCTCCTTTTCCCAAC 923

Query 181      CAATGAAATTGATCTTCCCGTGCAGAAGCGGGAATACTAACATAAGACGAGAAGACCCTG 240
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  924      CAATGAAATTGATCTTCCCGTGCAGAAGCGGGAATACTAACATAAGACGAGAAGACCCTG 992

Query  241     CGGAGCTTTAGACGCCAGAACAGATCACGTCAAATACCTCTCTCAAACAGGTAACAACAA 300
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  993      CGGAGCTTTAGACGCCAGAACAGATCACGTCAAATACCTCTCTCAAACAGGTAACAACAA 1052

Query  301     ATGAACCCTGTTCCACGTCTTAGGTTGGGGCGACCACGGTGAACAGAAAAACCCCCGCGT 360
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1053     ATGAACCCTGTTCCACGTCTTAGGTTGGGGCGACCACGGTGAACAGAAAAACCCCCGCGT 1113

Query  361     GGACTGAGAGCATATA-TTCACACTTATTAATACTGCTTCTCACAACCATGAGCTACAGC 420
                |||||||||||  ||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  1114     GGACTGAGAGCACATACCTCACACTTATTAATACTGCTTCTCACAACCATGAGCTACAGC 1174

Query  421     TCTAAATAACAGAACTTCTGACCaaaaaaaTGATCCGGCAACGCCGATTAACGGACCAAG 480
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1175     TCTAAATAACAGAACTTCTGACCAAAAAAATGATCCGGCAACGCCGATTAACGGACCAAG 1235

Query  481     TTACCCCAGGGATAACAGCGCAATCCTCTTTAAGAGTCCATATCGACAAGAGGGTTTACG 540
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1236     TTACCCCAGGGATAACAGCGCAATCCTCTTTAAGAGTCCATATCGACAAGAGGGTTTACG 1295

Query  541     ACCTCGATGTTGGATCAAGACATCCTAATGGTGCAACCGCTATTAAGGGTTCGTTTGTTC 600
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1296     ACCTCGATGTTGGATCAAGACATCCTAATGGTGCAACCGCTATTAAGGGTTCGTTTGTTC  1355

Query  601     AACGATTAAAGTCTTACATGATCTGAGTTCAGACCGG  637
                |||||||||||||||||||||||||||||||||||||
Sbjct  1356     AACGATTAAAGTCTTACATGATCTGAGTTCAGACCGG  1392
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Figure 5b: the blast and alignment of *M. cephalus* and *L. ramada* with accession number JQ060860 *M. liza* isolate 295 16S rRNA gene partial mitochondrial sequence describing the detected five SNP differences between studied species and KP018403 and JQ060860.

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cgcctgtttaccaaaaacatcgcctcttgtaaacctcacataagaggtcctgcctgccca
gtgacccctgttcaacggccgcggtattttaaccgcgcaaaggtagcgcaatcacttgtt
ccttaaatgagaaccagtatgaatggctagacgagggcttaactgtctccttttcccaac
caatgaaattgatcttcccgtgcagaagcgggaatactaacataagacgagaagaccctg
cggagctttagacgccagaacagatcacgtcaaatacctctctcaaacaggtaacaacaa
atgaaccctgttccacgtcttaggttggggcgaccacggtgaacagaaaaaccccccgcgt
ggactgagagcatatattcacacttattaatactgcttctcacaaccatgagctacagct
ctaaataacagaacttctgaccaaaaaaatgatccggcaacgccgattaacggaccaagt
taccccagggataacagcgcaatcctctttaagagtccatat^cgacaagagggtttacg
acctcgatgttggatcaagacatcctaatggtgcaaccgctattaagggttcgtttgttc
aacgattaaagtcttacatgatctgagttcagaccgg
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Figure 6: Complete nucleotide sequence of 16S rRNA mitochondrial gene of *M. cephalus* and *Liza ramada* recorded 636 bp nucleotides showing the forward primer sequence of 20 bp and reverse primer sequence of 22 bp.

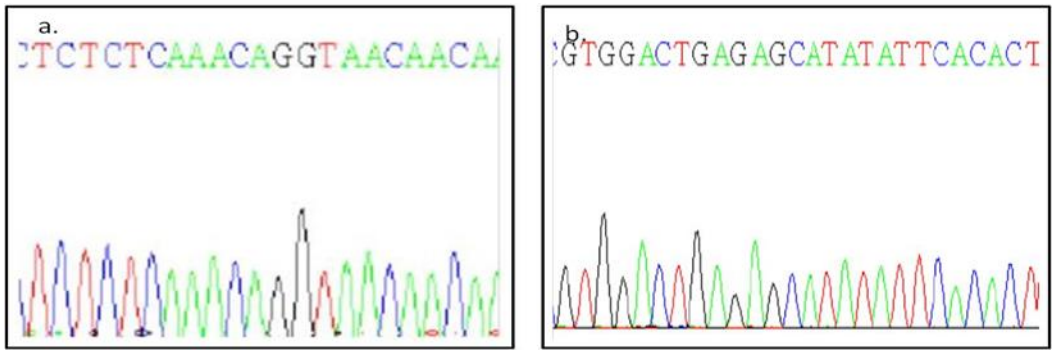

Figure 7a: Graphical description for nucleotide sequence of 16S rRNA which approved the mutation sites at positions 288 (G/C) and 290 (A/G) in comparison of our studied species with accession number KP018403, whereas **b.** approved the mutation sites at positions 373 (C/T), 377(C/-) and 378 (C/T) in comparison with accession number JQ060860.

DISCUSSION

The study of population genetic is of major value for fishery managers and ecologists. It is impossible to determine suitable administration measures in the lack of a clear estimation of the stock structure. In the last decade, many insights have been provided into the systematic of fishes at all taxonomic levels through molecular and phylogenetic studies.

In the current study, PCR amplification of 430 bp was observed using 12S rRNA primers and 636 bp was observed using 16S rRNA primers in two examined Grey mullet species which are in the same size range as conveyed in earlier studies on brackish water finifishes[27] and results reported by Papasotiropoulos *et al.*[18] where the PCR product size was 630 bp in *M. cephalus* for 16S rRNA gene while in other mullet species (*L. salines, L. aurata*, *L. ramada, M. cephalus & C. labrosus*) PCR product size was 450 bp for 12S rRNA gene and 600 bp for 16S rRNA gene. Klossa-Kilia *et al.*[28] found that the sizes of the PCR-amplified mtDNA segments for all *Atherina boyeri* populations studied, were about 450 bp for 12S rRNA, 600 bp for 16S rRNA.

The results related to restriction enzyme (*Hinf*I) for the two tested genes (12S rRNA, 16S rRNA) showed no recognition sites for 12Sr RNA gene in*M. cephalus and L. ramada* which parallel to the results recorded by Papasotiropoulos *et al.*[18] where no recognition sites were detected for 12S rRNA using *Hinf*I enzyme in five Mugilid species examined. Hisar *et al.*[29] revealed that *Hinf*I enzyme unable to distinguish between five examined mullet fish species. For 16S rRNA gene, *Hinf*I showed the same restriction pattern in two mullet species, as well as Papasotiropoulos *et al.*[18] notified that *Hinf*I showed the same restriction pattern (A: at 500 bp & 130 bp; B: 220, 200, 130, 50 bp & C: 270, 200, 130 bp) in five mullet species examined *(L. salines, L. aurata, L. ramada, M. cephalus & C. labrosus*). Semina *et al.*[21] revealed that *Hinf*I enzyme produced genetic differences between 12S rRNA and 16S rRNA genes in *M. cephalus* samples examined. Whilst, Trape *et al.*[30] stated that no restriction sites detected for 16S rRNA gene using *Hinf1* enzyme in Grey mullet species.

Whereas the analysis results using *Xba*I enzyme showed that no recognition sites detected for 12S rRNA gene while for 16S rRNA gene monomorphic specific pattern was observed among two mullet species examined which concordant with earlier studies by Klossa-Kilia *et al.*[28], *Xba1* enzyme showed uniqueness for 16S rRNA gene haplotype A (two restriction bands at 300 bp & 150 bp, haplotype B (no restriction site 450 bp) and for 12S rRNA gene no recognition sites were recorded in the five Grey mullet species examined and Ahmed *et al.*[31] declared that *Xba*I yielded a monomorphic pattern "N" and a polymorphic pattern "A" among mtDNA D-loop region in Hilsa shad (*Tenualosa ilisha*) population. while, Mazumder and Alam[32] notified that using *Xba*I enzyme to differentiate among *T. ilisha* populations yielded high levels of haplotype and gene diversity. Also, Juskeviciute and Paulauskas[33] revealed that *Xba*I enzyme gave restriction patterns that differed among five species of small rodent tested.

For *Taq*I enzyme, we observed that *Taq*I yielded a monomorphic genotype (CC) for 12S rRNA in the two examined mullet species, however for 16S rRNA gene it yielded a polymorphic patterns (CC, CT, TT) among the tested Mugilid species which in agreement with results of Papasotiropoulos *et al.*[18] for 12S rRNA it showed one haplotype A (275,175) among five mullet species, for 16S rRNA it showed three haplotypes (A*, A, B). Klossa-Kilia *et al.*[28] revealed that *Taq*I enzyme yielded polymorphic patterns for 12S rRNA and 16S rRNA to distinguish between *Atherina boyeri* populations, In contrast, Hisar *et al.*[29] revealed that *Taq*I enzyme cannot differentiate between five mullet species examined.

Concerning the results of 16S rRNA genotype frequency in two mullet species, the frequency of heterozygous genotype (CT) was 0.28, 0.12 in *M. cephalus* and *L. ramada,* respectively, while homozygous genotype frequency (CC) was the same in two tested Mugilid (0.28) and lower (TT) genotype frequency was recorded in *M. cephalus* (0.04) that lacked in *L. ramada*. Which compatible with results of Semina *et al.*[21] where the genotype frequency founded on RFLP analysis of 12S/16S rRNA varied from 0.27 between *M. cephalus* and *L. haematocheila* to 0.10 between *L. haematocheila* and *L.*

aurata and inconsistent to Mamuris *et al.*[34] who inspected that RFLP analysis of 12S/16S rRNA region created 6 and 4 different haplotypes in Red stripped mullets where different haplotypes were patent in *Mullus surmuletus* (ranging from 1.07 to 1.46) and in *M. barbatus* (ranging from 2.10 to 4.00) which is higher than our results and the other results recorded by Caldara *et al.*[10], Papasotiropoulos *et al.*[18] and Erguden *et al.*[35] where the haplotype frequency using 16S rRNA gene among Mugilid species examined was (0.88). These findings confirmed by the fact that our study is established on short fragments (400-636 bp) and highly conservative 12S/16S rRNA region.

According to our results, the analysis of nucleotide sequences of 16S rRNA mitochondrial gene between two mullet species *M. cephalus*, *L. ramada* revealed complete identity which analogous to previous studies by Cataudella *et al.*[36] and Ohno[37] who suggested that the karyotype of *M. cephalus* was recognized as the ancestor of all teleosts and in relation to the karyoevolutive pattern that anticipated by the above authors revealed that the karyotypes of *Liza* and *Chelon* species might have acquired by translocation affair from an ancestral karyotype similar to that found in *M. cephalus* while Papasotiropoulos *et al.*[17] reported that greatest genetic differentiation was observed between *M. cephalus* and the other *liza* species studied (*C. labrosus* and *L. aurata*) using three (12s rRNA, 16s rRNA, and COI) mtDNA segments. As well as, the results obtained by using PCR-RFLP of mtDNA gene segments evidenced that *M. cephalus* was the most discrete one from other *liza* species examined (*C.labrosus, L. aurata, L. ramada and L. saliens*)[18] which similar to the results recorded by Rossi *et al.*[15] by using 16s mt-rRNA and allozyme data. Martin[38] concluded that faster substitution rate which distinguishes *M. cephalus* species resulting in highest genetic differentiation in comparison to other studied mullet species which could be due to a combined effect of saturation of signal and nucleotide bias.

The blast and alignment of nucleotide sequence of 16S rRNA mitochondrial gene of 636 bp with accession number KP018403 *M. cephalus* mitochondrial complete genome manifested differences at five positions (four transition mutations at 120 (C/T), 290 (A/G), 461 (T/C) and 617 (G/A) and only one transversion mutation at 288 (G/C)) and with JQ060860 *M. liza* isolate 295 16S rRNA gene partial mitochondrial sequence recorded five mutations (four transition mutations at 51 (C/T), 120 (C/T), 373 (C/T), 378 (C/T) and one deletion at position 377). Semina *et al.*[21] stated that the level of distinction detected between *Mugil cephalus* and *Liza aurata* has been reported to be in the range of 22% ,similarly Papasotiropoulos *et al.*[18] and Erguden *et al.*[35] recorded the percentage of diversity between *Mugil cephalus and Liza* species as 14.2%, 17.2%, respectively. Caldara *et al.*[10] found that the alignment of partial 16S rRNA between eight Mugilid species that covers 788 bp reveals a modest amount of guanine (G,21.4%) and amplitude of adenine (A,31%) and it contained 121 mutableand thrift informative sites. Additionally, Shekhar *et al.*[27] announced that alignment of 575 bp of 16S rRNA of *Mugil cephalus* and *Liza* species showed 25 gaps at nucleotide position 238, 337-356 and 408-411, insertion/deletion at position (337-356 & 238) were detected in examined *Liza* species which indicating the useful of this region to distinguish *Mugil cephalus* and *Liza* species. Also, sequence alignment of 16s rRNA gene between three *liza* species of 550 bp resulted in 531 monomorphic and 17 polymorphic and two sites were gaps, the analysis revealed that there were 537 identical, 10 transitional and one transversional pairs of nucleotides. 16S rRNA phylogenetic analysis can be used to identify Mugilid species[19] which in accordance with prior studies by Papsotiropoulos *et al.*[18] and Rossi *et al.*[15] which based on analysis of 16S rRNA gene to distinguish between species of Mugilidae family.

CONCLUSION

Several studies have been successfully used variable mitochondrial and nuclear DNA regions for the identification of fish species. To be reliable, the PCR-RFLP and sequencing techniques become one of the most effective tools that must be applied to DNA regions which are highly conserved within species and at the same time variable between species to be diagnostic and facilitate the persistence of genetic differentiation between populations. In our study, the 16S rRNA mtDNA region has been shown to be a good marker to differentiate between two mullet species examined *M. cephalus* and *L. ramada* farmed in Egypt and between *M. cephalus* and other Mugilid species, which means that 16S rRNA is an effective molecular tool to identify *M. cephalus* species.

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