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RESEARCH PAPER

Impact of Enzymes and Primers on the PCR Amplification of Some Goatfishes

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Abstract

The polymerase chain reaction (PCR) is one of the most powerful techniques in molecular biology. In DNA isolation, substances co-extracted from biological samples, such as lipids, polysaccharides, and humic acids, influence amplification of the target gene and act as PCR inhibitors. Here, two instruments were used to measure concentrations and purities of DNA extracted from three species of the family Mullidae (*Mullus barbatus*, Linnaeus, 1758; *Mullus surmuletus*, Linnaeus, 1758; *Upeneus moluccensis*, Bleeker, 1855). In order to investigate PCR amplification differences between these species of goatfishes and to optimize protocols for PCR amplification, two PCR enzymes with different annealing temperatures were tested by amplifying the mitochondrial COI gene. The high lipid level of *M. surmuletus* species acted as a polymerase chain reaction inhibitor. The findings presented herein will enable other researchers to choose enzymes and primers appropriate for their studies instead of merely adjusting PCR annealing temperatures.

Keywords: Mediterranean Sea, Egypt, family Mullidae, PCR.

Introduction

Polymerase Chain Reaction (PCR) has become a fundamental technique in many genetic applications. Chemical inhibitors reduce the effectiveness of PCR amplification of target genes from genomic DNA (gDNA) of many environmental specimens (Schneider, Enkerli, & Widmer, 2009). Inhibitors can affect any step of PCR, but normally inhibit the polymerase from amplifying the target DNA, preventing cell lysis necessary for gDNA extraction, sequestering nucleic acids by binding them and blocking their capture (Schrader, Schielke, Ellerbroek, & Johne, 2012; Wilson, 1997). These mechanisms of interference are as diverse as the chemical structures of the inhibitors, many of which are prevalent in nature, including carbohydrates like glycogen, fats, various proteins, phenolic compounds, and humic acids (Maurer, 2011; Schrader *et al*., 2012; Wilson, 1997). Due to the existence of inhibitors and the necessity of modifying steps for PCR processing, the vast majority of PCR inhibitors have been considered incidental contaminants. Since many of these inhibitors have other primary biological functions, the interference they cause during PCR is basically incidental. This has resulted in a paucity of studies aimed at understanding the potential role of such inhibitors in controlling DNA replication (El-Maklizi, Ouf, Ferreira, Hedar, & Cruz-Rivera, 2014).

During a population genetics structure study of the three dominant species of the family Mullidae (*Mullus barbatus*, *Mullus surmuletus* and *Upeneus moluccensis*) in Egyptian waters of the Mediterranean Sea, variable PCR amplification results were obtained for the COI gene. In this study, two instruments were used to measure concentrations and purities of extracted DNA. In addition, two enzymes for PCR amplification were employed in order to compare DNA concentrations and purities, to investigate PCR amplification differences among the three goatfish species, and to develop an optimal PCR protocol for these fishes.

Materials and Methods

Three species of the family Mullidae (goatfishes) (*Mullus barbatus*, Linnaeus, 1758; *Mullus surmuletus*, Linnaeus, 1758; and *Upeneus moluccensis*, Bleeker, 1855) were examined in the present study. Total genomic DNA was extracted from small pieces (~0.25 mg) of muscle using a DNeasy Blood & Tissue Kit (QIAGEN) following the manufacturer's protocol. Genomic DNA concentrations were measured using a NanoDrop 2000 spectrophotometer, and a Qubit® 3.0 Fluorometer with the high sensitivity assay kit.

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The cytochrome oxidase subunit I (COI) region was amplified using primer sets Ward, Zemlak, Innes, Last, and Hebert (2005) (Table 1) and PCR was carried out using a HotStarTaq Master Mix Kit (QIAGEN) for twenty four specimens (8 specimens from each species). PCR was carried out in 20 µL total volume, containing 10 µL of HotstarTaq master mix, 1 µL of each primer (10 pm/µL), an appropriate amount of DNA (~20 ng/µL), and sterilized distilled water up to 20 µL. PCR thermal cycler conditions were set to one cycle for an initial denaturation at 95°C for 15 min, followed by 35 cycles at 94°C for 45 s, annealing at 52°C for 45 s, 72°C for 1 min and final extension at 72°C for 10 min. For *M. surmuletus*, we applied gradient PCR (gradient annealing temperature shown on Figure 3B using HotStarTaq with the same condition as mentioned above.

The PCR protocol for the Ex-Taq was carried out in 50 µL total volume, containing 0.25 µL of TaKaRa Ex Taq (5 units/µL), 5 µL of 10X Ex Taq buffer, 5 µL dNTP Mixture (2.5 mM each), 1.5 µL of each primer (10 pm/µL), an appropriate amount of DNA (~50 ng/µL), and sterilized distilled water up to 50 µL. PCR thermal cycler conditions were set to one cycle for an initial denaturation at 94°C for 1 min, followed by 30 cycles at 94°C for 30 s, annealing at 52°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min. These PCR conditions for the HotStarTaq and the Ex-Taq were applied using primer sets FishF2 and FishR2 for *M. surmuletus* (Table 1). DNA bands (PCR products) were examined using 1.5% agarose gel electrophoresis at 100 v for 20 min.

One-way analysis of variance (ANOVA) was used to assess data among groups (goatfishes) and treatments (instruments).

Results

Genomic DNA concentrations were measured with two instruments, the Qubit and NanoDrop (Table 2 and Figure 1). For *M. barbatus*, DNA concentrations ranged from 9.32-19.10 ng/µL with the

Table 1. List of DNA primer sets for the COI gene used in the present study

Primer name	Sequences	%GC	Length
FishF1	5'-TCAACCAACCACAAAGACATTGGCAC-3'	46%	26bp
FishR1	5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'	46%	26bp
FishF2	5'-TCGACTAATCATAAAGATATCGGCAC-3'	38%	26bp
FishR2	5'-CTTCAGGGTGACCGAAGAATCAGAA-3'	48%	25bp

Table 2. DNA concentrations from goatfish muscle samples, determined using Qubit and NanoDrop instruments

Species	Sample ID	DNA Conc.(ng/µL)		260/280	260/230
		Qubit	NanoDrop		
Mullus barbatus	MBAE1	9.32	37.20	1.93	2.26
	MBAE2	13.00	88.50	1.97	2.08
	MBAE3	19.10	180.10	1.55	1.18
	MBAE4	14.40	123.70	1.99	2.10
	MBAE5	14.60	84.30	1.96	2.04
	MBAE6	15.20	162.70	2.02	2.13
	MBAE7	14.00	55.70	1.85	1.42
	MBAE8	12.10	82.90	1.98	2.17
	Mean	13.97	101.89	1.91	1.92
	Standard Deviation	±2.79	±49.94	±0.15	±0.39
Mullus surmuletus	MSUE1	14.00	270.40	2.07	2.18
	MSUE2	11.70	35.70	1.92	2.24
	MSUE3	14.40	130.90	2.00	2.21
	MSUE4	11.30	55.70	1.95	2.13
	MSUE5	14.10	227.50	2.05	2.16
	MSUE6	10.30	54.40	1.98	2.09
	MSUE7	10.60	61.10	1.96	2.29
	MSUE8	15.60	76.70	1.94	2.06
	Mean	12.75	114.05	1.98	2.17
	Standard Deviation	±2.00	±88.56	±0.05	±0.08
Upeneus moluccensis	UMOE1	6.08	268.00	0.85	1.11
	UMOE2	8.44	76.70	1.23	1.03
	UMOE3	7.00	27.70	1.90	1.80
	UMOE4	1.88	11.10	1.70	0.90
	UMOE5	2.24	12.50	1.77	1.12
	UMOE6	4.28	15.10	1.76	1.07
	UMOE7	9.00	57.20	1.57	1.39
	UMOE8	3.86	141.40	1.32	0.76
	Mean	5.35	76.21	1.51	1.15
	Standard Deviation	±2.70	±89.27	±0.35	±0.32

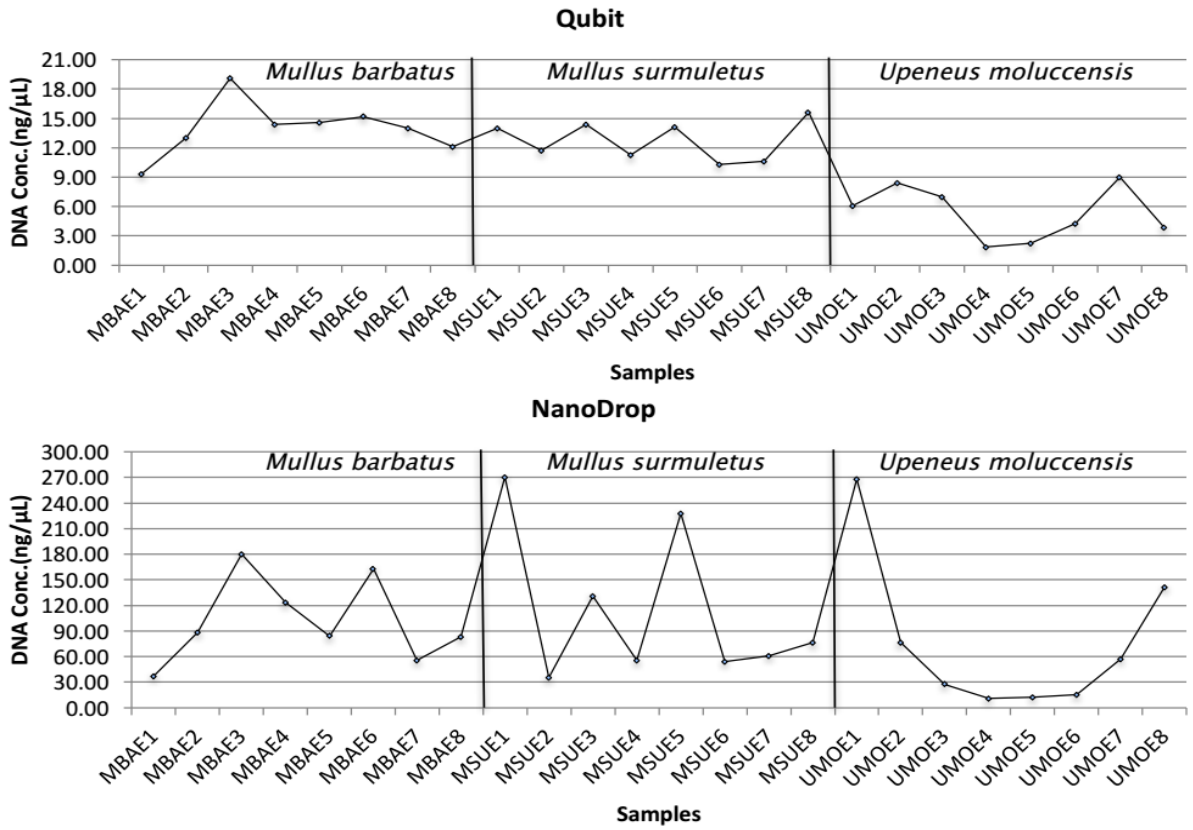

Figure 1. DNAconcentrations among three species of for three species of family Mullidae measured by Qubit and NanoDrop.

Qubit and from 37.20-180.10 ng/µL with the NanoDrop. The lowest DNA purity for this species was 1.55 and the highest was 2.02 at 260/280, whereas at 260/230 lowest and highest values were 1.18 and 2.26, respectively. Genomic DNA extracted from *M. surmuletus* ranged from 10.30-15.60 ng/µL with the Qubit. Measurements with the NanoDrop ranged from 35.70-270.40 ng/µL. The purity evaluation for this species indicated 1.94 as the lowest and 2.07 as the highest value at 260/280, and 2.06 and 2.29 as the lowest and the highest values at 260/230, respectively. Regarding the extracted DNA from *U. moluccensis*, the concentrations measured by Qubit instrument ranged from a minimum of 1.88 ng/µl to a maximum of 9.00 ng/µl. Concurrently, with the NanoDrop instrument, they were ranged from a minimum of 11.10 ng/µl to the maximum of 268.00 ng/µl. The average DNA purity for this species ranged between 0.85 as the lowest value to 1.90 as the highest value 260/280, and it recorded 0.76 and 1.80 as the lowest and the highest values respectively at 260/230. The lowest amounts of DNA were found from *Upeneus moluccensis* (Table 2).

One-way ANOVA showed a significant difference of DNA concentrations between the Qubit and NanoDrop groups (p-value < 0.00; f-ratio = 30.83). However, the p-value was insignificant (p-value < 0.62; f-ratio = 0.49) among goatfishes using Nanodrop, while the Qubit results showed a significant difference (p-value < 0.00; f-ratio = 27.33) between *U. moluccensis* species and the other two groups.

While the amount of DNA isolated from *M. surmuletus* was almost as high as from *M. barbatus* and higher than from *U. moluccensis* (Table 2), the mitochondrial cytochrome oxidase subunit I (COI) gene was successfully amplified only for the latter two species, producing discrete bands with fine resolution. In contrast, no samples of *M. surmuletus* were amplified at an annealing temperature of 52°C used for all three species with the HotStarTaq Master Mix Kit, QIAGEN (Figure 2). *M. surmuletus* species was not amplified with primer set Fish-F1 and Fish-R1. However, it was successfully amplified with primer set Fish-F2 and Fish-R2. HotStarTaq Master Mix Kit-QIAGEN showed no amplification of the target gene at the expected size (~700 bp) (Figure 3a). However, when using TaKaRa Ex-Taq™, many bands were obtained with a downward temperature gradient (55-51.8°C). One faint band of the expected size was seen. In addition, two strong pseudo bands ranged from 400-600 bp and two more very faint pseudo bands ranged between 250-350 bp. Moreover, with an upward temperature gradient (57.1-70°C), the expected band gradually disappeared (Figure 3b).

Discussion

Many biological samples contain substances co-extracted with DNA that have inhibit PCR. Although

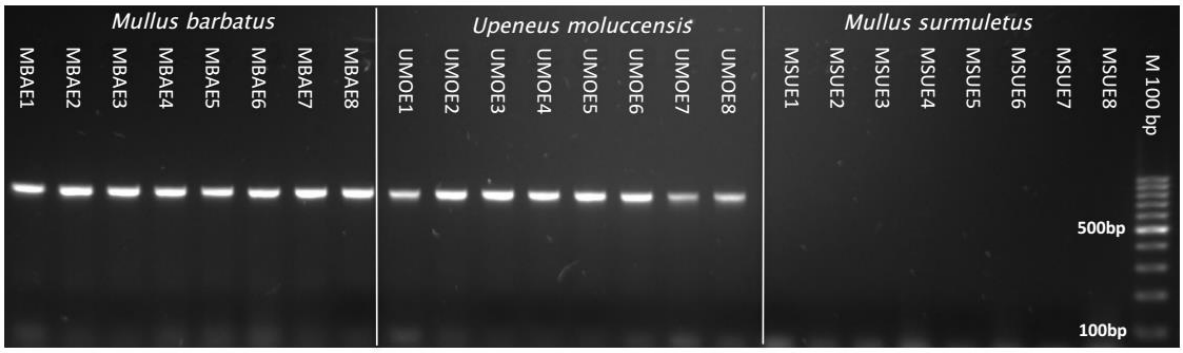

Figure 2. Agarose gel electrophoresis image for three species of family Mullidae using cytochrome oxidase subunit I (COI). M100bp:100 bp DNA ladder. Amplified at annealing 52℃[HotStarTaq Master Mix Kit (QIAGEN)].

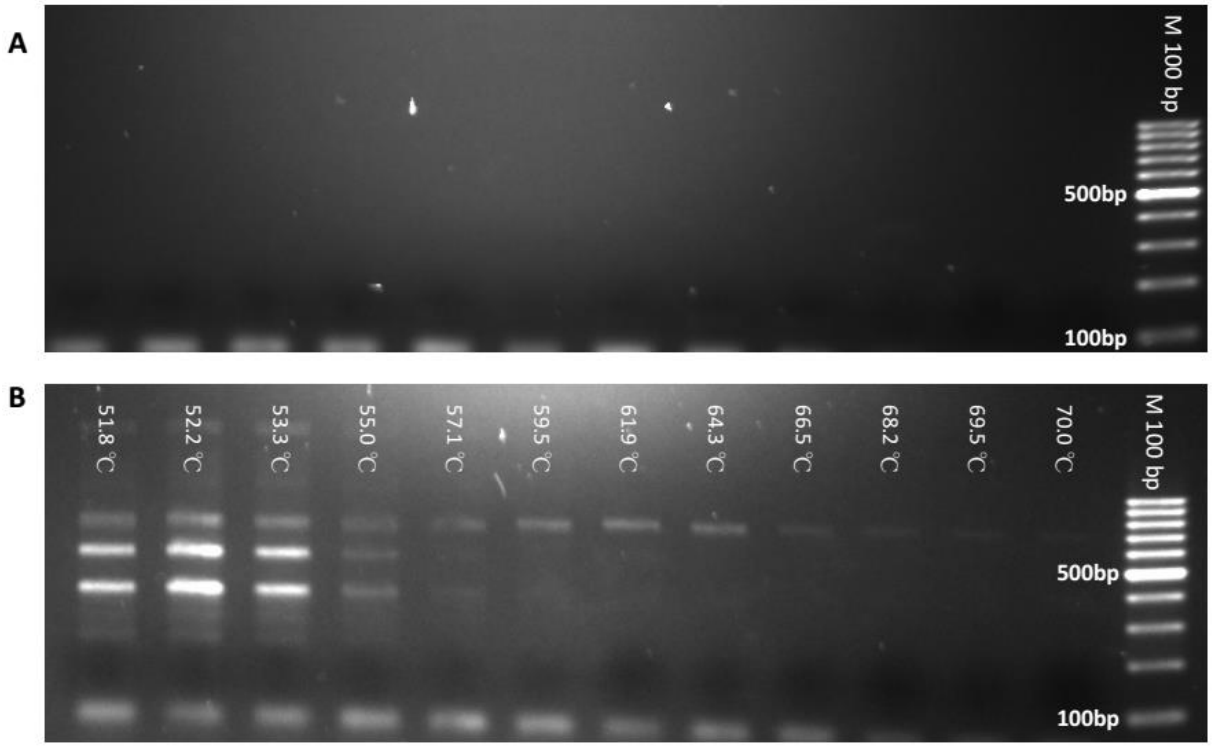

Figure 3. Agarose gel electrophoresis image for the gradient PCR of *Mullus surmuletus*. A- HotStarTaq Master Mix Kit (QIAGEN); B- TaKaRa Ex Taq.

the presence of such inhibitors is well documented, their mechanisms of action are still unclear (Opel, Chung, & McCord, 2010). DNA concentrations and purities were measured using two different instruments for the three mullid species. Amounts of DNA measured in all the samples using the Qubit were less than amounts measured using the NanoDrop. However, the accuracy of the Qubit measurements was greater, based upon the means and the standard deviations, a finding also reported by Neill, McPartlin, Arthure, Riedel, and Nd (2011). Sensitivity differences between the Qubit and NanoDrop instruments may result in apparent gDNA concentration differences among samples. However, variations in gDNA concentrations and purities had no effect on PCR amplification. Therefore, initial DNA concentration differences are not the main reason for non-amplified gDNA.

The gene COI was successfully amplified with from *M. barbatus* and *U. moluccensis*, but was not amplified from *M. surmuletus*. Ozan (2016) reported that using FishF1 and FishR1, the COI gene from *M. surmuletus* cannot be amplified We wondered why this primer set (FishF1 and FishR1) didn't show similar amplification for two congeneric species? Possibly, unsuccessful amplification of the COI gene in *Mullus surmuletus* was due to high GC content or primer length (Table 1). GC bonds are stronger than AT bonds (Mamedov *et al*., 2008). Therefore, the gene amplification cannot by achieved using normal

PCR techniques (Kumar & Kaur, 2014).

The Taq enzymes used for the PCR reaction also has an important role in polymerase efficiency. When HotStarTaq Master Mix was used, no amplification of COI from *Mullus surmuletus* occurred, while it was successfully amplified from the other two species. In contrast, when TaKaRa Ex-Taq^{TM} was used for PCR, amplification occurred and several different bands of the gene were seen. Thus, the Taq polymerase used for PCR may also affect DNA polymerization (Abu Al-Soud & Radstrom, 1998; Opel *et al*., 2010; Purzycka, Olewiecki, Soltyszewski, Pepinski, & Janica, 2006).

Differences of chemical composition between the three goatfishes might influence the PCR amplifications. Feeding habits of the dominant three species in the Egyptian Mediterranean water show that *Mullus barbatus* mainly feeds on crustaceans and polychaetes (Chérif *et al*., 2011), while the gut contents of *Upeneus moluccensis* mainly consisted of crustaceans, molluscs, teleosts, polychaetes, and bivalves (Golani & Galil, 1991). Unlike the latter two species, *M. surmuletus* has a narrow range of prey items, such as Mysidacea and amphipods crustaceans (Dulcic, 2002). Lipid content and fatty acid profiles of the three species are quite different, considering that the body chemical composition of fish, particularly lipid content, depends on sex, age, seasonal changes and feeding habits (Ackman, 1989; Yeannes & Almandos, 2003). Öksüz, Özyılmaz, and Küver (2011) found that lipids of *M. surmuletus* include higher levels of DHA (Docosahexaenoic Acid, C22: 6n3) and EPA (Eicasapentaenoic Acid, C20: 5n3) fatty acids than does *U. moluccensis*. In addition, lipid levels in *M. surmuletus* are higher than those in *M. barbatus* (Polat, Kuzu, Özyurt, & Tokur, 2009). This high level of lipids has a role in blocking PCR as one of polymerase chain reaction inhibitors.

The present study concluded that the differences of the body chemical composition between three species of family Mullidae have an effect on blocking PCR amplification of cytochrome oxidase subunit one gene (COI). Higher levels of lipid in *M. surmuletus* than in *M. barbatus* may play an important role as one of PCR inhibitors.

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Conflict of Interest

All authors declare they have no conflicts of interest.

Ethical Approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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