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Molecular Characterization of *Scomberomorus maculates* (Ikan Tenggiri), *Atule mate* (Ikan Selar), and *Decapterus russelli* (Ikan Selayang) Species of Fish From Malaysia Marine Ecosystem Using DNA Sequence of Mitochondrial DNA (mtDNA) Cytochrome Oxidase Subunit I (COI) (DNA Barcoding)

Dissertation submitted in partial fulfillment for
Degree of Bachelor of Science in Forensic Science

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ABSTRACT

This study was undertaken to design a set of common primer to amplify the cytochrome oxidase I (COI) barcoding region of marine fish species. The primer set namely ST F1 5'CACAAAGACATCGGCACCC3' (forward) and ST F3 5'AAGAATCAA/GAATAG/AGTGTTG3' (reverse) were able to amplify the three fish species namely *Scomberomorus maculates* (ikan Tenggiri), *Atule mate* (ikan Selar), and *Decapterus russelli* (ikan Selayang) chosen for this study. The sequence data generated were compared and analyzed using the sequence of COI gene of Gobio gobio (AB 239596) as a reference. On analysis between the three species *Scomberomorus maculates* and *Atule mate* have the highest percentage of similarities (76.91%), followed by *Atule mate* and *Decapterus russelli* (69.29%), and *Scomberomorus maculates* and *Decapterus russelli* (64.45%) (Table 5). The sequence data generated were used to study their phylogenetic relationship by constructing a rooted neighbor joining (NJ) dendrogram (Fig. 18).

INTRODUCTION

Taxonomists so far described approximately 1.9 million species out of 11 million estimated (Chapman 1991). Traditionally, morphology was a key factor in describing and naming species within the field of taxonomy. This long-standing approach, starting with Aristotle and becoming organized due to Linnaeus is very tedious and a matter of subjectivity since it is up to the taxonomist to choose those morphological characters believed to delineate species (Coyne & Orr 2004). As a result, it took 250 years for traditional taxonomy to provide descriptions for less than quarter of the world species (Packer *et al.* 2009).

It is suggested that a final taxonomic system for the animal kingdom will probably include at least 10 million species partitioned among more than a million genera. Given such high diversity, there is a growing realization that it is critical to seek technological assistance for its initial description and its subsequent recognition (Godfray 2002; Blaxter 2003). A ground-breaking approach to species identification was brought by Hebert *et al.* (2003) who proposed the use of a small segment of mitochondrial genome for species identification across phyla from the entire animal kingdom and termed the procedure as DNA barcoding.

The reasons for selection of mitochondrial (mtDNA) over nuclear DNA include uniparental inheritance (in majority of animal phyla), high evolutionary rate, lack of introns, large copy numbers in every cell, and limited recombination. The proposal of cytochrome oxidase I (COI) as the target gene for DNA barcoding was not an arbitrary choice since decades of research showed a useful phylogenetic signal for both above and below species level.

Furthermore, the universal primers designed were capable to recover the 5'end of COI in most animal phyla. According to the barcoding approach, species could be identified based on a barcoding gap between intra and interspecific genetic distances by using a threshold value of 2–3% (Hebert *et al.* 2003) or a 10-fold value (Hebert *et al.* 2004) for species delimitation.

Although numerous studies used molecular methods for species identification prior to the DNA barcoding, it is still a unique concept with manifold attributes. Initially proposed only for animal taxa, a DNA-based identification system was soon found to be successful in land plants, algae (Hollingsworth *et al.* 2009), fungi (Saunders 2005), whether using only COI or in combination with other mitochondrial regions or nuclear DNA. DNA barcoding brings a few major assets. It implies standardization, for example the same DNA fragments used within a taxon, which allows comparisons between datasets of various researchers, revealing cases of synonymy, potential cryptic species or genetically distinct populations.

Vouchers are permanently stored, ideally in a DNA-friendly manner, in museum collections, publicly accessible for future reference. This is what differentiates between the previous techniques which lack the possibility to retrieve the sequences for sequences deposited in public databases (GenBank), therefore resulting in impossible taxonomic verifications and growing concerns about the documentation of scientific data (Pleijel *et al.* 2008).

As proposed by Herbert *et al.* (2003), a single gene sequence should be sufficient to differentiate all or at least the vast majority of animal species and the cytochrome oxidase subunit I (COI) is proposed as the barcoding region for the bioidentification system of animals. The sequence was similar to a barcode with species being delineated by a particular sequence or by a tight cluster of very similar sequences. Empirical support for the barcoding concept ranges from studies of invertebrates (Hebert *et al.* 2004a,b; Hogg & Hebert 2004).

According to FishBase (www.fishbase.org) fishes comprise nearly half of all vertebrate species which includes approximately 15,700 marine and 13,700 freshwater species. Ward *et al.* (2005) carried out a pilot study that compiled barcodes for 200 species commercially important Australian marine fishes. Since then an additional 5000 barcodes have been generated from over 2000 species. However this study does not design efficient effort to hone analytical protocols which is a progress for barcoding (Ivanova *et al.* 2007). Ivanova *et al* (2007) specifically seek to identify protocols that enable both efficient polymerase chain reaction (PCR) amplification of the barcode region to deliver high quality sequence data.

In recent years, DNA barcodes have proved to be a valuable asset in identifying marine organisms, especially in obvious cases where morphological identification is not possible, namely processed seafood. The famous example of fish sold as red-snapper in the US and actually consisting of other species in 77% of cases was soon followed by other studies, which proved that seafood substitutions are common. The extent of this phenomenon on the global market of fresh, smoked or dried fish products varies across continents and the possible

explanations include genuine mislabeling due to morphological similarities between closely related species or fraudulent substitution of expensive species with cheaper variants.

An extreme case of fish substitution had drastic consequences for public health, leading to food poisoning due to puffer fish toxin and the consequent recall of products (Cohen *et al.* 2009). With its power to reveal mislabeled products, DNA barcoding will have multiple implications from food safety and public health, to fisheries management with depletion of fish stocks and conservation in which protected species caught illegally.

LITERATURE REVIEW

More than 40 years ago, starch gel electrophoresis of proteins was first used to identify species (Manwell & Baker, 1963). Nearly 30 years ago, single gene sequence analysis of ribosomal DNA was being used to investigate the evolutionary relationships at a high extraordinary level (Woese & Fox 1977) and mitochondrial DNA approaches dominated molecular systematic in the late 1970s and 1980s (Avise 1994). Tautz *et al.* (2002, 2003) made the case for a DNA-based taxonomic system. DNA sequence analysis has been used for 30 years to assist species identifications, but different sequences have been used for different taxonomic groups.

Hebert *et al.* (2003) proposed that a single gene sequence would be sufficient to differentiate all, or at least the vast majority of animal species. DNA barcoding is the derivation of short DNA sequence(s) that enables species identification, recognition, and discovery in a particular domain of life (e.g., Hebert *et al.* 2003a). The primary purpose of DNA barcoding is to identify an unknown specimen in terms of known classification (Miller 2007, Stoeckle & Hebert 2008). Persis *et al.* (2009) stated that molecular taxonomy using COI sequence which is termed as a DNA is a rigorously standardized sequence of a minimum length and quality from an agreed-upon gene. It is deposited in a major sequence database and attached to a voucher specimen whose origins and current status is recorded.

Herbert *et al.* (2003) proposed the use of the mitochondrial DNA gene cytochrome oxidase subunit I (CO1) as a global bioidentification system for animals. Mitochondrial genes have a high copy number allowing a greater yield of mtDNA to be recovered from trace samples compared to nuclear DNA (Carracedo *et al.* 2000) In addition, mtDNA genes typically lack recombination promoting the loss or fixation of mtDNA haplotypes, reducing within species diversity and thus enabling species identification (Avise *et al.* 1984 & Cronin *et al.* 1991). Herbert *et al* (2003) stated that reasons for choosing mitochondrial (mtDNA) over nuclear DNA include uniparental inheritance (in a majority of animal phyla), high evolutionary rate, lack of introns, large copy numbers in every cell, and limited recombination. This will result in significance variance in mtDNA sequences between species and comparatively small variance within species. They also proposed the use of the mitochondrial DNA gene cytochrome oxidase subunit I (COI) as a global bioidentification system for the animals.

The efficacy of COI of fish species have been validated by the studies by Ward *et al.* (2005). They sequenced specimens of three species of Chimaerids, 61 species of sharks and rays, and 143 species of teleosts for the barcode region of COI. The research found that all 207 sequenced species were discriminated unequivocally. COI barcoding of species for identification is far more powerful than protein fingerprinting. Reliable discrimination of *Thunnus* species using conventional protein electrophoresis is difficult (Yearsley *et al.* 1999) but as the same samples were readily identified by COI sequencing (Ward *et al.* 2005).

The primary reason for the selection of COI as the standard barcode gene is the typical pattern of variation observed for numerous species, with marked divergence and lack of overlap between intraspecific (i.e., between individuals of the same species) and interspecific (i.e., between individuals of different species) genetic distances (Hebert *et al.* 2003). Although barcode analysis seeks only to delineate species boundaries, there is clearly some phylogenetic signal in COI sequence data. (Ward *et al.* 2005).

The barcoding data on fish species in Malaysia is scanty. Hence, in this research project it was contemplated to design primers which could amplify the partial segment of COI barcoding region across fish species.

OBJECTIVES OF THE STUDY

The objectives of the study are:

1. To design primers for amplification of partial segment of mithochondrial DNA (mtDNA) cytochrome oxidase subunit I (COI) gene of 3 species of fish namely *Scomberomorus maculates*, *Atule mate*, and *Decapterus russelli*.

2. To study the sequence differences (genetic polymorphism) between 3 species of fish namely *Scomberomorus maculates*, *Atule mate*, and *Decapterus russelli*.

MATERIALS AND METHODS

Sample source

Three species of marine fish which are of commercially available were selected as samples for this research. The scientific names for those species are *Scomberomorus maculates*, *Atule mate*, and *Decapterus russelli*. Their common names are ikan Teggiri, ikan Selar and ikan Selayang respectively.

Reagents

i. Proteinase K (20 mg/µL)
ii. 2 M Sodium acetate
iii. 70% ethanol
iv. Tris EDTA buffer
v. 10% Ammonium persulphate
vi. 10X TBE buffer
vii. 0.5X TBE buffer
viii. Distilled water
ix. 95% alcohol
x. FFV allelic ladder
xi. QIAGEN Kit (ATL buffer, AL buffer, spin column and collection tube, AW1 buffer, AW2 buffer, and AE buffer)

Chemicals

i. Tris base
ii. Sodium acetate (Merck, Germany)
iii. Absolute Acetic acid, CH3COOH
iv. EDTA (Promega, USA)
v. Ethidium bromide (Syne, USA)
vi. Ammomnium persulphate (Promega USA)
vii. Agarose (Promega, USA)
viii. Orange and big dye (Syne, USA)

Apparatus

i. Vortex mixer, EVM-600 ERLA
ii. Microcentrifuge, 24D Spectrafuge
iii. Pipette 1000 µL, 500 µL, 200µL, 100 µL, 20 µL and 10 µL
iv. Parafilm
v. Thermal analyzer
vi. Freezer
vii. Refrigerator
viii. Water bath
ix. Electrophoresis apparatus
x. Power pack
xi. Filter tip

xii. Microwave, ELBA

xiii. 3130*xl* Genetic analyzer\

xiv. Photoprint

xv. UV Transluminator

xvi. Hot plate magnetic stirrer

xvii. Disposable latex glove

Reagent Preparation

Proteinase K (20 µg/ µL)

A total amount of 2 mg of Proteinase K was mixed with 1 mL of deionized water

Triss borate EDTA (TBE, 10X) buffer

10X TBE buffer were prepared by mixing 107.8 g Triss base and 7.44 g of EDTA into 500 mL distilled water. The solution was dissolved using magnetic stirrer. The pH of the solution was measured until stable and 27.5 g of boric acid were added bit by bit until the pH 8.3 was achieved. The volume of the solution was make up to 1000 mL by adding distilled water and autoclaved.

TBE Buffer (0.5X)

TBE Buffer (0.5X) was prepared by mixing 50 mL of 10X TBE stock buffers with 950 mL of distilled water.

3 M sodium acetate

A total amount of 102.025 g of sodium acetate was added to 200 mL dionised water. The pH was adjusted to 5.2 with glacial acetic acid. Solution was made up to 250 mL and autoclaved.

2 M Sodium acetate

A total amount of 16 mL of 3 M Sodium acetate was added to 8 mL of deionised water.

70% Ethanol

A total amount of 350 mL of absolute ethanol was mixed with 150 mL dionised water.

Ethidium bromide stock solution

An amount of 1 g of ethidium bromide was dissolved in 100 mL of dionised water and stored in an amber bottle.

Orange G loading dye

An amount of 0.125 g of orange G and 20 g of sucrose was dissolved in 30 mL of distilled water. Distilled water was added to make up the solution to 50 mL. It was then aliquoted into 1.5 mL microcentrifuge tube and kept at 4°C for further use.

100 base pair DNA ladder

The 100 bp ladder (0.1 μg/μL, Fermentas) consists of 10 chromatography purified individual DNA fragments between 100 and 1000 bp.

Methodology

DNA extraction method

Tissue or gills of each of the three species were used for extracting genomic DNA. DNA extraction from fish tissue and gill was done using the QIAGEN kit following the recommended procedures of the manufacturer of the kit. Tissue or gill weighing 25 µg were minced with sterile surgical blade on glass plate and transferred into 1.5 mL microfuge tube. 180 µL of ATL buffer was added to the tissue and gill sample. Then 20 µL of AL buffer was added into the sample and 200 µL of 90% ethanol was added and mixed well and then incubated at 70°C for 10 minutes. After that, each of the samples was transferred into the spin column and centrifuged for a minute at 8000 rpm. The spin column was then transferred into new collection tube. Then, 500 µL of AW1 buffer was added to the column and the column was centrifuged at 8000 rpm for a minute. Next, the spin column was transferred into new collection tube and 500 µL AW2 buffer was added to the column and it was then centrifuged at 14000 rpm for 3 minutes. After 3 minutes of centrifugation, the spin column was again transferred into new collection tube and centrifuged for another minute at 14000 rpm. After that, the spin column was taken out and placed into a new 1.5 mL microfuge tube. 80 µL of AE buffer was added to column and the column was left at room temperature for 3 minutes. After that, the column was centrifuged at 8000 rpm for a minute. The spin column was removed from the collection tube and discarded. The collection tube containing genomic DNA was stored in the freezer at -20°C.

Agarose gel electrophoresis of extracted DNA

The presence of extracted DNA was checked using agarose gel electrophoresis. A 1% agarose gel was prepared by adding 0.6 g of agarose powder into 60 mL of 0.5X TBE buffer (50 mL of 10X TBE buffer and 950 mL of water) in a bottle. The mixture was heated in microwave oven for 3 minutes and then was allowed to cool under the running tap water. After that 2 µL of ethidium bromide was added into the solution. The solution was then poured onto a gel plate with the comb inserted into its place and the gel was left for 30-45 minutes to solidify. When the gel was ready, comb was removed and the gel was transferred into the electrophoresis tank. Then, 0.5X TBE buffer was poured into the tank until the gel was submerged. 3 µL of samples were mixed with 2 µL of orange G dye and the mixture of each of the samples was loaded into the well. Then, 1 µL (0.1 µg/µL) of 100 bp DNA ladder was also loaded into a well. Electrophoresis was performed for 30 minutes at 80 V. The presences of DNA were observed under UV light by using Image Analyzer (UVP Bioimagine System).

DNA Quantification

The extracted genomic DNA samples from 3 different species of fish were quantified using the spectrophotometer. A total amount of 5 µL of DNA sample was mixed with 45 µL of TBE buffer (pH 8.0) and the optical density (OD) value was observed under 260 nm wavelengths. The quantified DNA was diluted with TE buffer (pH 8.0) so that the diluted DNA was approximately 15-20 ng/ µL)

Designing primers

Custom oligo primers were designed using published database on mtDNA Cytochrome Oxidase I (COI) gene of reference sequences from gene bank. These sequences were aligned in BioEdit sequence alignment software and sequence which showed identities and similarities with most of the species were chosen for primer pairs. During the primer design, the routine precaution namely non-complementary between forward and reverse primer sequences, percentage of G+C content and the melting temperature (T_m) were taken into consideration. The primer sequences used in this study are shown in Table 1 including the information of G+C content and T_m value.

Table 1. The oligo primers set used to amplify partial segment of mtDNA COI of *Scomberomorus maculates, Atule mate*, and *Decapterus russelli.*

Primers	**Primer sequence**	**Tm value (°C)**	**Percentage of G+C content (%)**
ST F1	5'CACAAAGACATCGGCACCC3'	60	57.9
ST R3	5'AAGAATCAA/GAATAG/AGTGTTG3'	52	30.0

Polymerase Chain Reaction (PCR) amplification

DNA amplification was carried out in a PCR mixture volume of 25 µL in a 0.5 mL PCR tube. The required amount of PCR mixture was calculated as shown in Table 2. The samples were then centrifuged briefly to bring the content to the bottom of the tube. The protocol used for amplification is shown in Table 3.

Table 2. The components of PCR reaction

PCR mixture components	Volume per sample (µL)
PCR master mix	21.0
Forward primer	1.5
Reverse primer	1.5
DNA sample/10ng	1.0
Total volume	25

Table 3: Amplification protocol

Initial incubation	Cycling for 30 cycles	Final extension	Hold step
95°C for 3 minutes	95°C, 30 seconds 50°C, 30 seconds 72°C for 1 minute	72°C, 7 minutes	4°C

Agarose gel electrophoresis of amplified PCR products

The amplified PCR products were checked using agarose gel electrophoresis. A 1.2% agarose gel was prepared by adding 0.6 g of agarose powder into 50 mL of 0.5X TBE buffer. The mixture was boiled in microwave oven for three minutes. The agarose solution was allowed to cool under the running tap water. After the solution was cooled, 2 µL of ethedium bromide was added and the solution was then poured onto the gel plate with the comb inserted. The gel was left to solidify for half an hour. When the gel was ready, comb was removed and 0.5X TBE buffer was poured into the electrophoresis tank until the gel was completely submerged. 3 µL of PCR products were mixed with 2 µL of orange G dye and then the mixture was loaded into a well. Then, 1 µL (0.1 µg/µL) of 100 bp DNA ladder was loaded into a well. Electrophoresis was performed for 30 minutes at 80 V. The presences of amplified DNA products were observed under UV light by using Image analyzer.

Purification of PCR Product

The amplified PCR products were purified using Gene ALL PCR SV protocol kit following the recommendations of the manufacturer. In a 1.5 mL microfuge tube containing 20 µL of amplified DNA sample and 250 µL of PB buffer were mixed before transferring into SV column. The mixture was vortexed and centrifuged at 12000 rpm for 30 seconds. The pass through was discarded, and the SV column was reinserted into the same collection tube. After that, 350 µL of NW buffer was added into the column and then it was centrifuged for 30 seconds at 12000rpm. The pass-through was discarded and SV column was inserted into the same collection tube and centrifuged for another minute at 12000 rpm. Then, the SV column was transferred into a 1.5 mL microfuge tube and 30 µL of elution buffer (EB) was applied to the center membrane of SV column. The tubes were kept at room temperature for 1 minute. After 1 minute, the tubes were centrifuged at 12000 rpm for 1 minute. Then the column was removed from the tube which contained the purified products. The purified product was then examined by electrophoresis using 1.2% agarose gel in 0.5X TBE buffer.

Gel Electrophoresis of purified PCR products

The purified PCR products were checked using the agarose gel electrophoresis. A 1.2% of agarose gel was prepared by adding 0.6 g of agarose powder into 50 mL of 0.5X TBE buffer. The mixture was boiled in microwave oven for 3 minutes. The agarose was allowed to cool under the running tap water. After that 2 µL of ethidium bromide was added into the solution. The solution was poured into a gel plate with the comb already inserted into its place and left for half an hour to solidify. When the gel was ready, the comb was removed and the gel was put into the electrophoresis tank. Then 0.5X TBE buffer was poured into the tank until the gel submerged. A total of 3 µL of PCR product was mixed with 2 µL of orange dye and then the mixture was loaded into a well. Then, 1 µL of 100 bp DNA ladder was loaded into a well. Electrophoresis was performed for 30 minutes at 80 V. The presences of purified amplified DNA products for sequencing were observed under UV light by using Image analyzer (UVP Bioimagine System).

Termination Cycle

Termination cycle of the purified PCR products was carried out in a final volume of 10 µL in a 0.5 mL tube containing 3 µL of purified PCR products, 3.75 µL of distilled water, 1 µL of primer (each tube contained only one type of primer F1 or R4), 1.75 µL of 5X buffer and 0.5 µL of Big Dye. Each of the tube was placed into PCR machine. The cycle sequencing amplification condition was as follows: 96°C for 1 minute, 35 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes and a final hold at 4°C.

Ethanol Precipitation

To purify the amplification product for sequencing, ethanol precipitation was performed after the termination cycle was completed. A total 10 µL of the cycle sequence product was transferred into a 1.5 mL microfuge tube and then 3 µL of 2 M sodium acetate (pH 5.2) and 50 µL of cold ethanol were added to the sample. The mixture was vortexed and left at room temperature for 30 minutes. The mixture was the centrifuged at 6000 rpm at 4°C for 40 minutes and the supernatant was discarded. Then 100 µL of cold 70% ethanol was added into the tube, and it was vortexed and centrifuged at 3000 rpm at 4°C for 10 minutes and this step was repeated twice. The supernatant was discarded and the tube was left at room temperature for 30 minutes to dry. A total of 10 µL of Hi-Di was added to the sample and the tubes were later placed into the thermal cycler for 3 minutes at 94°C. Then the sample was loaded into a 96 well plates and sequenced using Genetic Analyzer 3130XL.

Analysis of the sequences

Sequencing of forward and reverse strands was carried out to avoid ambiguity in sequencing. The analyses of all three species sequence were done using BioEdit and MEGA software. Using the BioEdit software, the forward sequence was aligned with its respective reverse complement of the reverse sequence to check for fidelity of sequencing.

RESULTS

The genomic DNA was extracted from the three marine fish species namely *Scomberomorous maculates*, *Atule mate*, and *Decapterus russelli* and preparatory agarose gel electrophoresis was run as mentioned under materials and methods. Ethidium bromide stained genomic DNA is shown in the electropherogram in Figure 1.

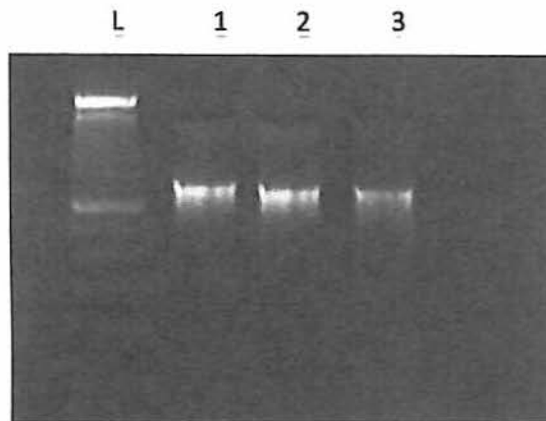

Figure 1: Electropherograph showing extracted genomic DNA. The lanes were labeled as L, 1, 2 and 3. L reference to DNA ladder, Lane 1, 2, and 3 represent the extracted DNA from *Scomberomorus maculates*, *Atule mate*, and *Decapterus russelli* respectively.

The primer set F1 and R3 were able to amplify the partial fragments of mithochondrial DNA (mtDNA) COI gene of each of the three species of marine fish name *Scomberomorous maculates*, *Atule mate* and *Decapterus russelli*. The electrophoresis analyzed was shown in Figure 2.

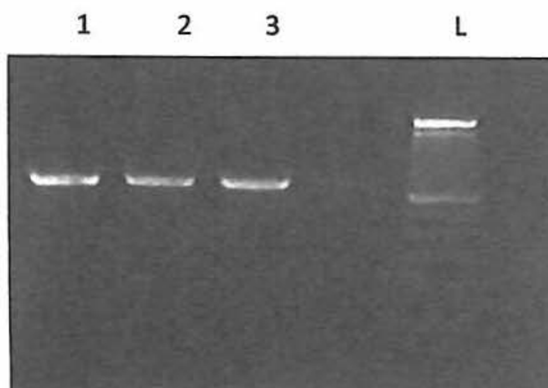

Figure 2: Electropherograph showing PCR products generated after DNA amplification of mtDNA COI gene of the studied species using F1 and R3 primers set. The lanes were labeled as L, 1, 2, and 3. L reference to 100bp DNA ladder and Lane 1, 2, and 3 represent the amplified DNA from *Scomberomorus maculates*, *Atule mate*, and *Decapterus russelli* (respectively).