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DNA Barcoding of Commercial Fish Sold in Muar Fish Market, Johor

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Abstract: Due to the high demand in the fisheries market, species identification is vital for commercial fish to avoid mislabeling and fraud in marketplaces. The increasingly threatening human activities like overfishing, blast fishing, poison fishing, and trawling have become the major threats to fish and caused an urgent need for documentation of both marine and freshwater fishes. Commercial fish products sometimes are sold by their parts, such as fins which further create confusion for identification purposes. Morphological identification also requires highly skilled ichthyologists to avoid misidentification. Thus, identification through DNA barcoding can be utilized as it holds the potential for accurate and rapid identification. Here we identified the commercial marine fish species sold in Muar Fish Market, Johor, using the Cytochrome Oxidase subunit I (COI) gene of mitochondrial DNA (mtDNA). A 700 base-pair sequence of the COI region was targeted by amplifying extracted DNA from 28 fin samples collected from the fish market. From 28 samples, we successfully sequenced 16 samples, and by using phylogenetic analysis, (Neighbor-Joining (NJ), Maximum Parsimony (MP) and Bayesian Inference (BI)) we successfully identified the samples belonging to nine families (Scombridae, Platycephalidae, Carangidae, Ariidae, Polynemidae, Coryphaenidae, Sphyraenidae, Muraenesocidae, Engraiulidae), 12 genera and 16 species. This study reports the first documentation of commercial fish sold in Muar through the DNA barcoding technique. This technique should be expanded further to identify any species with important conservation implications, such as endangered species, for improved management of fisheries in Malaysia.

Keywords: Commercial fish, fish market, DNA barcoding, fisheries, Muar

1. Introduction

Malaysia is home to a total of 1951 freshwater and marine fish species living in both marine and coral habitats, which are classified into 704 genera and 186 families, where half of the species (48%) are endangered, and over 27% of them require urgent scientific investigations to determine their conservation status [1]. Malaysia's fisheries sector remains important as fish product outputs have increased in the last few years [2]. However, issues such as overfishing,

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blast fishing, poison fishing, and trawling are considered significant threats and are badly affecting Malaysia's marine species. To conserve marine and freshwater fish, strict enforcement of fishery regulations and management and more national marine parks gazettement are required [3]. The most critical stage in conserving and managing species is the accuracy in the identification and delimitation of fish species [4]. Therefore, additional research, observation, and data collection are necessary to identify the fish species caught and commercialized in the fish market to comprehend and ascertain the exact situation of marine fishery stocks [5].

Identification of species is essential in biodiversity studies because the recognition is fundamental for studies involving the extinction of some genetic variants and subsequent loss of intra-specific diversity, with unpredictable effects on species biodiversity [6]. Knowing the correct species of fish can prevent commercial fraud, especially when it involves fish of higher economic value. Previously, morphological identification was used because it can be easily measured and determined using photographs or scientific drawings accessible in the literature [7]. Several fish morphological features can be quantified using lateral views, such as the body's shape, size of fins, mouth, and eye size [8]. Unfortunately, some limitations appear when using this approach, especially for the species with similar morphological characters, and are difficult to identify based on morphological characteristics alone [9].

The changes and variations in fish species' morphological characteristics, especially during the stages of ontogenetic development, served as a challenge to identify them based on morphology, which may lead to controversial classification [10]. Thus, molecular analysis through DNA barcoding was significantly developed as the assay has high sensitivity in detecting DNA sequences and diversity of DNA sequences can support fish identification [11]. The technique can increase the accuracy and effectiveness of identifying fish by using a reliable and comprehensive reference database [11,12]. [13] advocated DNA barcoding using the mitochondrial Cytochrome Oxidase Subunit I gene (COI), which sparked the international drive to barcode all the fish species. The intraspecific diversity of the COI gene in animals is lower compared to interspecific diversity, making it a good marker for classifying and identifying vertebrates and invertebrates [11,14]. The effectiveness of DNA barcoding also depends on the extensive bank of reference sequences deposited in the reference library, GenBank, and Barcode of Life Database (BOLD).

Therefore, this study focused on the Muar fish market in Johor due to the trade of fish species mainly from the Straits of Malacca. Until now, there has been a lack of documentation regarding the fish sold in the Muar fish market, leading to a lack of record of fish diversity in Muar. Thus, this study aims to document the commercial marine fish species sold by the fishermen in Muar using DNA barcoding of the COI gene. This data is vital for fisheries conservation and management in Johor, especially for species listed as threatened, endangered, or critically endangered by the IUCN Red List.

2. Methodology

2.1 Sample Collection

A total of 28 samples of fish were collected from the Muar fish market (Figure 1). The purpose of the study was explained, and permission was obtained from the fisherman and fishmonger before sample collections were conducted. All fin samples were collected and stored in a labeled vial with 95% ethanol and deposited in Molecular Genetic Laboratory, Universiti Tun Hussein Onn Malaysia. The samples were stored at -20°C to prevent DNA degradation.

Fig 1 - The location of Muar fresh market

2.2 DNA Extraction and Amplification

The DNA from fish samples was successfully extracted using Innuprep DNA Mini Kit (Analytik Jena, Germany), according to the protocol provided by the manufacturer. Extracted DNA was then quantified using Implen NanoPhotometer N50 for DNA concentration and purity. Approximately ~710 bp were amplified using forward primer (LCO1490 5′-GGTCAACAAATCATAAAGATATTGG-3′) and reverse primer (HCO2198 5′-TAAACTTCAGGGTGACCAA AAAATCA-3′) as described by [15].

A total of 25 µL reaction mixture was used in total for the PCR reaction, which contained 12.5 µL Mastermix My Taq Red Mix, 6.5 µL ddH_2O, 1.5 µL of each primer, and 3 µL DNA template. The thermal cycling conditions consisted of an initial step of 2 min at 95°C followed by 35 cycles of denaturation at 94°C (0.5 min), annealing at 45°C (1 min), extension at 72°C (1 min) and final extension at 72°C for 5 min and held at 4°C. PCR products were visualized on 1.5% agarose gel and sequenced. The unpurified DNA products were sent to Apical Scientific Sdn. Bhd. in Shah Alam, Selangor, Malaysia, for Sanger sequencing.

2.3 Sequence Data Analysis

The raw sequences obtained were analyzed and edited using Bioedit Sequence Alignment Editor (BioEdit 7.2) software. For species identification, amplified sequences were compared with reference sequences in the GenBank database (https://blast.ncbi.nlm.nih. gov/Blast.cgi) using the basic local alignment search tool (BLAST). The conservation status of each identified species was further compared using the IUCN Red List of Threatened Species.

2.4 Phylogenetic Assessment

The phylogenetic analysis was conducted based on three approaches, including Neighbor-Joining (NJ), Maximum Parsimony (MP), and Bayesian Inference (BI), to analyze the taxonomic positions of the fish species. Neighbor-Joining (NJ) and Maximum Parsimony (MP) analyses were conducted using MEGA X software, in which Kimura-2-Parameter model was used for the NJ tree, and the tree bisection and reconnection (TBR) algorithm was used for the MP tree with 1000 bootstrap replications. On the other hand, Bayesian inference was conducted using MrBayes V 3.2.2, in which Metropolis-coupled Markov chain Monte Carlo (MCMC) was run with 1 million generations to approximate the posterior probabilities of trees.

3. Result

From 28 samples extracted, only 25 samples were successfully amplified, while three samples (S14, S20, S28) showed poor quality after PCR amplification. Another nine samples (S4, S11, S12, S13, S19, S21, S23, S24, and S27) were excluded from the analysis because of poor sequence quality based on the chromatograms obtained. Hence, 16 COI sequences were successfully identified up to the species level, consisting of nine families, 12 genera, and 16 species (Table 1). Of 16 species, only four were listed as Least Concern (LC) by IUCN Redlist, belonging to Coryphaenidae, Carangidae, and Ariidae families. At the same time, three species from the Scombridae family were classified as data deficient (DD). Nine samples were not assessed and categorized as not evaluated (NE) under IUCN Redlist of Threatened Species. GenBank database revealed the identical percentage with the maximum identity in the range 75%-100%, in which 13 species except for S5 (75.36%), S9 (92.85%), and S16 (91.85%) are supported by more than 95% of query cover percentage. One sample, which is S25, showed only 44% of query cover with more than 95% of identical scores and matched up to the species level of *Congresox talabonoides.* All the samples obtained showed consistent results, as confirmed by the high similarity of the sequences obtained here with reference sequences from GenBank. The NJ, MP, and BI phylogenetic trees which exhibited identical topological formations and were summarized in a single phylogenetic tree (Figure 2). All the phylogenetic trees showed division between each species based on the formation of sub-clade supported by more than 80% of bootstrap values. The phylogenetic analysis has successfully confirmed the species identification, as indicated by the BLAST results from the GenBank database.

4. Discussion

The approach using DNA barcoding in fish identification can be exploited further due to its ability to identify up to species level, as proven by [16], which focused on the commercial fish products collected from the Malaysian fish market. Due to the difficulties in identifying fish species using morphological identification, DNA barcoding can serve as a solution by using DNA extracted from a small quantity of samples and an established universal PCR primer, as proven by [14] and [17] in identifying the species of shark from fins. DNA barcoding can also overcome the challenge of identifying processed food, especially fish products[18]. Species identification using the COI gene or other acceptable primer also depends on the availability and comprehensiveness of public reference databases such as GenBank or BOLD [17,19,20]. More studies have also been conducted on a larger scale in India [21], Taiwan [11] and Japan [22].

Table 1 - The IUCN status and GenBank results from the species identified using COI sequences

Sample No	Family	Species	Common name	IUCN Status	Query cover	Identical percentage	Accession number
S1	Scombridae	*Rastrelliger brachysoma*	Short mackerel	DD	96 %	99.57%	EU555283.1
S3		*Scomberomorus plurilineatus*	Queen mackerel	DD	90%	99.70%	MH230978.1
S5		*Scomberomorus plurilineatus*	Queen mackerel	DD	93%	75.36%	MH235714.1
S2	Platycephalidae	*Platycephalus cultellatus*	Flathead fish	NE	98%	99.14%	MW423368.1
S6	Carangidae	*Megalaspis cordyla*	Torpedo scad	LC	94%	99.56%	MT410983.1
S10		*Atule mate*	Yellowtail scad	LC	97%	98.86%	KM522838.1
S7	Ariidae	*Nemapteryx nenga*	Sea catfish	NE	91%	99.85%	MH235676.1
S8		*Nemapteryx caelata*	Engraved sea catfish	NE	95%	97.95%	KY026490.1
S9		*Nemapteryx caelata*	Engraved sea catfish	NE	92%	92.85%	KY026490.1
S17		*Plicofollis dussumieri*	Blacktip sea catfish	LC	92%	99.70%	JN312820.1
S15	Polynemidae	*Eleutheronema rhadinum*	East Asian fourfinger threadfin	NE	99%	98.87%	MW630081.1
S16		*Eleutheronema rhadinum*	East Asian fourfinger threadfin	NE	99%	91.85%	MW630081.1
S18	Coryphaenidae	*Coryphaena hippurus*	Common dolphinfish	LC	95%	99.28%	KF719178.1
S22	Sphyraenidae	*Sphyraena putnamae*	Sawtooth barracuda	NE	96%	99.13%	MZ068239.1
S25	Muraenesocidae	*Congresox talabonoides*	Indian pike conger	NE	44%	95.26%	MK777079.1
S26	Engraiulidae	*Stolephorus tri*	Spined anchovy	NE	90%	99.08%	MW498817.1

*LC: least concern, DD: data deficient, NE: not evaluated

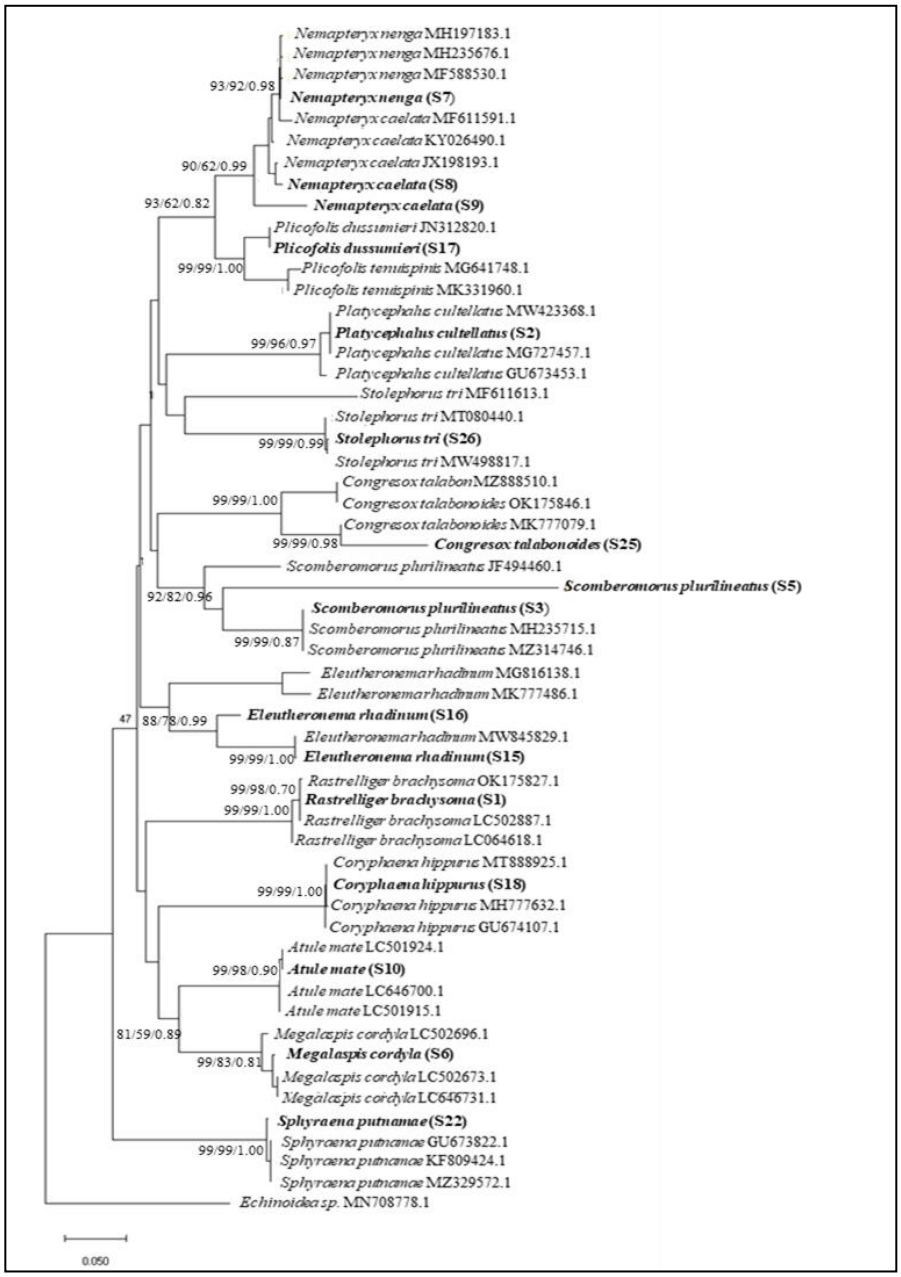

Fig 2 - Summary of Neighbor-Joining, Maximum Parsimony, and Bayesian Inference phylogenetic trees to determine the taxonomic position of fish species from Muar Fish Market. Values above the branch represent bootstrap values for NJ/bootstrap values for MP/posterior probability for BI.

This study's finding also contributed to classifying the status of fish species sold in Muar Fish Market by using the IUCN Red List, which can be used as the main indicator for the conservation status of the fish species. For example, the study by [14] identified six samples of shark fins from local markets in Sabah and Sarawak categorized as endangered. Other than that, [16] reported one critically endangered and three endangered species were found in commercial fish products in Malaysian fish markets. The government and authorities can also use the data generated by this study and use molecular identification to overcome the misidentification of fish and fish products sold in the local fish market. Thus, by conducting more study, observation, and data collection of marine fishes in Malaysia, we can identify the fish species being sold in the market to understand and determine their conservation status, ensure fishery stocks, and maintain the ecosystem balances.

5. Conclusion

We can conclude that DNA barcoding is an accurate, sensitive, and rapid approach to fish identification, especially for trace samples. Molecular identification can identify fish products without their complete morphological characteristics up to the species level, granted the information are available in the public reference database. This

technique should be expanded to further understand the dynamics of Malaysia's fisheries sector, determining their origin, species diversity, and conservation status.

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