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SPECIES CHECKLIST AND DNA BARCODING OF MARINE FISHES FROM PULAU TINGGI, JOHOR, MALAYSIA

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Abstract: Since there was a lack of study on the diversity and genetics of marine fishes in Pulau Tinggi, Mersing, Johor could be found to date, this study was conducted to provide a baseline data of species checklist of marine fishes in Pulau Tinggi and to obtain DNA barcodes of the fish specimens using cytochrome c oxidase subunit I (COI) gene. Sample collection in this study was conducted starting from July to August 2019. Spinning rod (angling), hand-net (manual sampling) and gillnet (mid-water column) were used as sampling methods in this study. All specimens obtained in this study were first identified based on morphological features, followed by a molecular approach for unidentified individuals. The morphological and molecular classification proved that all 183 individuals of marine fishes obtained in this study belonged to 83 species, 58 genera, 36 families, 9 orders and 2 classes. Among 83 species obtained in this study, 74 (89%) possess important values for human use. According to IUCN Red List online platform, 22 species are classified as Not Evaluated (NE), one species is classified as Data Deficient (DD), 56 species are classified as Least Concern (LC), three species are classified as Near Threatened (NT) and the remaining one species is classified as vulnerable (VU). A total of seven bidirectional CO1 barcode sequences were obtained from seven species, six genera and six families. All the sequences obtained have significant E values, which were zero, with percent identity values being more than 98% and query coverage values being more than 89%. The blast results for all the sequences provided correct identification until the species level. For future research, this study has updated the latest checklist of marine fishes from Pulau Tinggi, Mersing, Johor, Malaysia.

Keywords: Pulau Tinggi, marine fishes, species checklist, DNA barcoding.

Introduction

Malaysia, which falls under the Sundaland region is also part of the South China Sea. Malaysia has been regarded as one of the biodiversity hotspots and listed as one of 17 megadiverse countries worldwide (Myers *et al*., 2000). The marine and coastal areas of Malaysia are thought to have a great diversity of marine life within its coral reefs, mangrove forests, mud flats, sea grass areas and sandy beaches (Mazlan *et al*., 2005). The first reference material listed 710 marine fish species from Malaysian waters and adjacent seas (Mohsin *et al*., 1996). The second edition book estimated 2,243 fish species inhabiting Malaysian waters (Ambak *et al*., 2010).

On the east coast of Peninsular Malaysia, which faces the South China Sea, particularly in Terengganu, about 441 species have been catalogued (Matsunuma *et al*., 2011). Another known marine and coastal area along the east coast of the Malay Peninsula is the East Johor Island Archipelago (EJIA) which is regarded as Taman Laut Sultan Iskandar (TLSI). One of the biggest islands in the TLSI is Pulau Tinggi (2°18'N; 104°07'E), which consists of well-developed coral reef ecosystems that may attract a variety of coral and rocky reef-associated fishes (Reef Check Malaysia, 2019). Even though some studies have been done to observe fish groups occupying coral reef areas in Pulau Tinggi, no documented study has been found on the richness and genetics of marine fishes on this island. This island has also been subjected to a growing tourism industry. Various development and activities on this island may strongly induce pressure directly or indirectly toward its local marine fish, thus, increasing the challenges for conservation efforts.

Many effective conservation actions depend on accurate species identification (Bakar *et al*., 2018). Over the past decade, DNA barcoding has played a facilitatory role in establishing a rapid, accurate and cost-effective system for identifying species including marine fishes (Zhang *et al*., 2011; Bingpeng *et al*., 2011). Arising integration of molecular and traditional taxonomic methods provides a powerful and standardized approach for marine species identification that helps fisheries and conservation programs (Hebert *et al*., 2003; Costa & Carvalho, 2007; Sheraliev & Peng, 2021; Ahmed *et al*., 2021). Accurate identification is important in determining the exact species richness present in an area, thus, helping in the future study of species diversity in that area. For example, a previous study bore witness to the utility of DNA barcoding in revealing the overlooked diversity of Malaysian commercial snapper (*Lutjanus*) (Bakar *et al*., 2018). Accordingly, this study aimed to investigate the species checklist of marine fishes in Pulau Tinggi, Johor and to obtain the DNA barcodes of the fish specimens using the cytochrome c oxidase I (COI) mitochondrial DNA (mtDNA) gene.

Materials and Methods

Procedures

Study Sites

Sample collection in this study was conducted in Pulau Tinggi, Johor (Figure 1) from July to August 2019. Pulau Tinggi (2°18'N; 104°07'E) is located about 30 kilometres southeast of Mersing, Johor on the east coast of Peninsular Malaysia that faces the South China Sea (Azman *et al*., 2008; Amanda, 2018). Pulau Tinggi covered a total area of about 16 square kilometres and was gazetted as Marine Park in 1994 under the Fisheries Act 1985 (Amended 1993) (Azman *et al*., 2008). The white sandy beaches along the coastline of Pulau Tinggi are dotted with caves (Escobar *et al*., 2003). It is surrounded by clear turquoise sea waters supporting rocky, coral reefs, sea grasses and mangroves (Azman *et al*., 2008; Reef Check Malaysia, 2019). The interior part of the terrestrial ecosystem in Pulau Tinggi is mostly covered with secondary lowland dipterocarp rainforest but there is still part of the primary forest that remains virgin (Escobar *et al*., 2003). Petrographically, this island is dominated by a pyroclastic rock type that varies from millimetres to 5 centimetres in size (Ghani, 2006). Unpublished data for Ar-Ar whole rock dating indicated that the age of the Pulau Tinggi volcanic was approximately 85 million years ago (Roselee, 2014).

Marine Fish Sampling

In this study, spinning rods (angling), small hand-net (manual sampling) and gillnet (mid-water column) were used to catch fish. Application of "catch and release" practice was incorporated in this study since there was the same morphospecies of fish caught during the sampling period. A spinning rod for bottom angling or line angling was used at the available jetty nearby Shaz Resort Pulau Tinggi while a

Figure 1: Pulau Tinggi, Mersing, Johor
Source: Google Earth Pro version 7.3.4.8248

floater was used during the angling along the beach. This method was performed when the tide was changing from low tide to high tide, both during the daytime and nighttime. The flesh of shrimp, squid and fish was used as the bait for this fishing method. Besides that, the fish that were trapped in the tide pools along the intertidal zone in front of Kampung Tanjung Balang were manually collected using a small hand net. This method was performed during low tide, where fish were usually trapped in temporary ponds created when sea water turns to low tide in the daytime and nighttime (White *et al*., 2015). Furthermore, a gill net of 15 m long and 1.2 m width with a mesh size of 1.5 inches was used to catch fish and was set up approximately 25 m from the shore in front of Kampung Tanjung Balang for about 6 hours during the daytime and nighttime sampling, once a week.

The fish collected from all gear types during the field study were sorted and identified based on the morphological features by referring to Matsunuma *et al*. (2011), Moore and Colas (2016), FishBase online platform and other available references prior to the molecular approach for unknown fish individuals.

DNA Barcoding

DNA barcoding was done as a complementary method to morphological identification to confirm the species status of unknown individuals, especially for specimens with a deformed body. A portion of muscle tissue or a fin clip fragment from each fish specimen was dissected or removed with a sterile blade and preserved in 95% ethanol (Bingpeng *et al*., 2018). Total genomic DNA was extracted from each muscle tissue or fin clip sample using the Tissue Genomic DNA Extraction Mini Kit by Favorgen. The quality and quantity of the extracted total genomic DNA were determined through electrophoresis (on 1% agarose gel, with FloroSafe DNA Stain). The exTEN 2x PCR Master Mix (200 reactions) was used for polymerase chain reaction (PCR). Approximately 655 base pairs fragment of the 5′ end region of the protein-coding mitochondrial cytochrome c oxidase I (COI) gene was amplified using the primer set described by Ward *et al*. (2005) and Wang *et al*. (2012). PCR amplification was carried out in a thermal cycler, where the general protocol of the PCR kit (i.e., exTEN 2x PCR Master Mix) was followed, with annealing thermal condition at 54°C (Wang *et al*., 2012).

Amplified COI fragments were sent to the sequencing service provider, i.e., Apical Scientific Sdn. Bhd.) in suspension form to perform the bidirectional (forward and reverse) sequencing further. CO1 sequences were aligned using the Molecular Evolutionary Genetics Analysis software version 7.0 (MEGA7) (Kumar *et al*., 2016). Confirmation of species identity was done using nucleotide Basic Local Alignment Search Tool (BLAST) search that is available at National Center for Biotechnology Information (NCBI) online platform (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The COI sequences were then translated into amino acids and examined for the presence of stop codons prior to the GenBank submission. BankIt, a NCBI program was used to prepare the sequence data for GenBank submission. Each sequence was aligned and compared with corresponding sequences from the GenBank database to determine the reliability of the sequences.

Results and Discussion

The morphological and molecular classification proved that all 183 individuals of coastal marine fishes recorded in this study represented the native fishes of the South China Sea with 83 species, 58 genera, 36 families and nine orders (Figure 2, Tables 1-2) (Mohsin *et al*., 1996; Randall *et al*., 2000). Among these, three species with nine individuals were classified as class Chondrichthyes while another 174 individuals represented the class Osteichthyes (i.e., the bony fishes), specifically the Teleostei (perfect bone) group.

A number of 24 families (66.7%) with 69 species (83.1%) were classified in order Perciformes, considered the highest taxonomic order in this study. Perciformes possess more than 9,200 species worldwide and it is not just the largest order in fish classification but also the largest order of all vertebrates (Moyle *et al*., 2004). In addition, the published reference

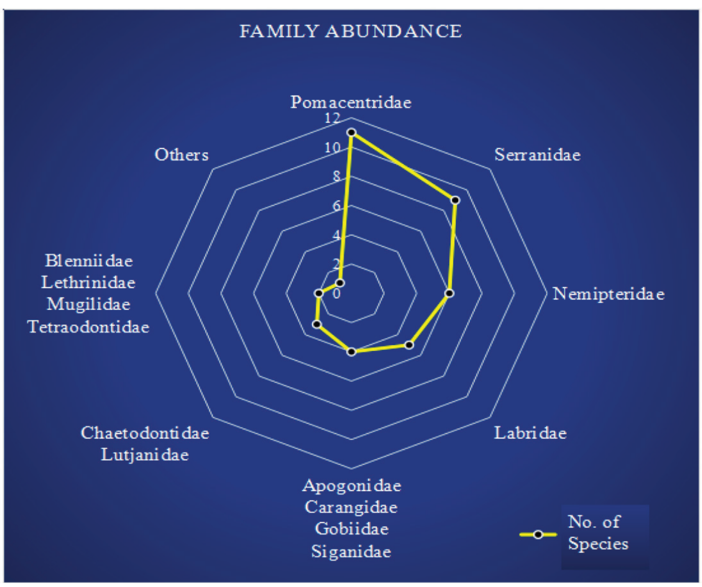

Figure 2: The family abundance of recorded marine fishes in Pulau Tinggi, Mersing, Johor

Table 1: Summary of ray-finned fishes in Pulau Tinggi, Mersing, Johor

No.	Families	Scientific Name	Common Name	IUCN Status	Total Species
1	Pomacentridae	*Abudefduf bengalensis*	Bengal sergeant	LC	11
		Abudefduf sexfasciatus	Scissortail sergeant	LC	
		Abudefduf sordidus	Blackspot sergeant	LC	
		Abudefduf vaigiensis	Indo-Pacific sergeant	LC	
		Amphiprion ocellaris	Clown anemonefish	NE	
		Dischistodus fasciatus	Banded damsel	NE	
		Dischistodus perspicillatus	White damsel	NE	
		Dischistodus prosopotaenia	Honey-head damsel	NE	
		Dischistodus pseudochrysopoecilus	Monarch damsel	NE	
		Pomacentrus chrysurus	Whitetail damsel	NE	
		Pomacentrus tripunctatus	Threespot damsel	NE	
2	Serranidae	*Cephalopholis boenak*	Chocolate hind	LC	9
		Cephalopholis formosa	Bluelined hind	LC	
		Epinephelus areolatus	Areolate grouper	LC	
		Epinephelus coioides	Orange-spotted grouper	LC	
		Epinephelus corallicola	Coral grouper	LC	
		Epinephelus erythrurus	Cloudy grouper	LC	
		Epinephelus fuscoguttatus	Brown-marbled grouper	VU	
		Epinephelus quoyanus	Longfin grouper	LC	
		Plectropomus maculatus	Spotted coralgrouper	LC	
3	Nemipteridae	*Nemipterus furcosus*	Fork-tailed threadfin bream	LC	6
		Pentapodus bifasciatus	White-shouldered whiptail	NE	
		Pentapodus trivittatus	Three-striped whiptail	LC	
		Scolopsis ciliata	Saw-jawed monocle bream	LC	
		Scolopsis margaritifera	Pearly monocle bream	LC	
		Scolopsis monogramma	Monogrammed monocle bream	LC	
4	Labridae	*Cheilinus chlorourus*	Floral wrasse	LC	5
		Choerodon anchorago	Orange-dotted tuskfish	LC	
		Halichoeres chloropterus	Pastel-green wrasse	LC	
		Hemigymnus melapterus	Blackeye thicklip	LC	
		Thalassoma lunare	Moon wrasse	LC	

5	Apogonidae	*Apogonichthyoides melas*	Black cardinalfish	NE	4
		Ostorhinchus chrysopomus	Spotted-gill cardinalfish	NE	
		Ostorhinchus endekataenia	Candystripe cardinalfish	NE	
		Pristicon trimaculatus	Three-spot cardinalfish	NE	
6	Carangidae	*Carangoides bajad*	Orange-spotted trevally	LC	4
		Carangoides gymnostethus	Bludger	LC	
		Caranx sexfasciatus	Bigeye trevally	LC	
		Selar boops	Oxeye scad	LC	
7	Gobiidae	*Amblygobius stethophthalmus*	Freckled goby	NE	4
		Gobiodon histrio	Broad-barred goby	LC	
		Istigobius ornatus	Ornate goby	LC	
		Valenciennea muralis	Mural goby	NE	
8	Siganidae	*Siganus fuscescens*	Mottled spinefoot	LC	4
		Sigaus guttatus	Orange-spotted spinefoot	LC	
		Siganus punctatus	Goldspotted spinefoot	LC	
		Siganus virgatus	Barhead spinefoot	LC	
9	Chaetodontidae	*Chaetodon octofasciatus*	Eightband butterflyfish	LC	3
		Chelmon rostratus	Copperband butterflyfish	LC	
		Coradion chrysozonus	Goldengirdled coralfish	LC	
10	Lutjanidae	*Lutjanus carponotatus*	Spanish flag snapper	NE	3
		Lutjanus fulviflamma	Dory snapper	LC	
		Lutjanus russellii	Russell's snapper	LC	
11	Blenniidae	*Blenniella bilitonensis*	Biliton blenniella	LC	2
		Salarias fasciaus	Jewelled blenny	LC	
12	Lethrinidae	*Lethrinus lentjan*	Pink ear emperor	LC	2
		Lethrinus ornatus	Ornate emperor	LC	
13	Mugilidae	*Ellochelon vaigiensis*	Squaretail mullet	LC	2
		Moolgarda seheli	Bluespot mullet	NE	
14	Tetraodontidae	*Arothtron immaculatus*	Immaculate puffer	LC	2
		Arothtron mappa	Map puffer	LC	
15	Caesionidae	*Caesio cuning*	Redbelly yellowtail fusilier	LC	1
16	Centrogenyidae	*Centrogenys vaigiensis*	False scorpionfish	NE	1
17	Diodontidae	*Diodon liturosus*	Black-blotched porcupinefish	NE	1
18	Echeneidae	*Echeneis naucrates*	Live sharksucker	LC	1
19	Gerreidae	*Gerres oyene*	Common silver-biddy	LC	1
20	Holocentridae	*Sargocentron rubrum*	Redcoat	LC	1
21	Kyphosidae	*Kyphosus cinerascens*	Blue sea chub	LC	1
22	Monacanthiae	*Acreichthys tomentosus*	Bristle-tail file-fish	LC	1

23	Mullidae	*Upeneus tragula*	Freckled goatfish	LC	1
24	Paralichthyidae	*Pseudorhombus arsius*	Largetooth flounder	NE	1
25	Pegasidae	*Pegasus volitans*	Longtail seamoth	DD	1
26	Pempheridae	*Pempheris oualensis*	Blackspot sweeper	NE	1
27	Pomacanthidae	*Pomacanthus annularis*	Bluering angelfish	LC	1
28	Pseudochromidae	*Congrogadus subducens*	Carpet eel-blenny	NE	1
29	Scaridae	*Scarus ghobban*	Blue-barred parrotfish	LC	1
30	Sillaginidae	*Sillago aeolus*	Oriental sillago	NE	1
31	Soleidae	*Pardachirus pavoninus*	Peacock sole	LC	1
32	Synanceiidae	*Synanceia horrida*	Estuarine stonefish	LC	1
33	Terapontidae	*Terapon jarbua*	Jarbua terapon	LC	1

Note: NE=Not Evaluated, DD=Data Deficient, LC=Least Concern, VU=Vulnerable

Table 2: Summary of cartilaginous fishes in Pulau Tinggi, Mersing, Johor

No.	**Families**	**Scientific Name**	**Common Name**	**IUCN Status**	**Total**
1	Dasyatidae	*Taeniura lymma*	Ribbontail stingray	NT	1
2	Carcharhinidae	*Carcharhinus melanopterus*	Blacktip reef shark	NT	1
3	Scyliorhinidae	*Atelomycterus marmoratus*	Coral catshark	NT	1

Note: NT=Near Threatened

material by Mohsin *et al*. (1996) also showed that the Perciformes were recorded as the highest order in their study, with 449 species classified into 60 families found in Malaysia and its neighbouring countries.

Regarding family abundance, the highest number of species in this study belonged to the family Pomacentridae (Damselfishes and Anemonefishes), with 11 species recorded throughout the sampling session. This was followed by the family Serranidae (Groupers) with nine species. According to Moyle *et al*. (2004), nearly 57% of all Perciformes species are dominated by just eight families, including Pomacentridae and Serranidae. In addition, Pomacentridae and Serranidae species are considered prolific fishes that can be found associated with tropical and temperate reefs as well as inshore environments (Moyle *et al*., 2004; Matsunuma *et al*., 2011). Besides that, Nemipteridae (Threadfin Breams and Monocle Bream) was considered the third largest family caught in this study with six species total. This was followed by five species from the family Labridae (Wrasses). Families that shared the same number of species were the Apogonidae (Cardinalfishes), Carangidae (Jacks), Gobiidae (Gobies) and Siganidae (Rabbitfishes) with four species each while Chaetodontidae (Butterflyfishes) and Lutjanidae (Snappers) shared three species in total for each family. Furthermore, four families possessed two species each, which were Blenniidae (Blennies), Lethrinidae (Emperors), Mugilidae (Mullets) and Tetraodontidae (Puffers). Another 22 families including the cartilaginous fishes had the least species recorded with each family showing only one species found throughout the sampling period.

Accordingly, FishBase online platform was used to assess the importance of marine fishes recorded in this study to humans. It shows that 74 species (89%) recorded in this study possess values important for human uses, either as wild fisheries products, commercial aquaculture, commercial ornamental fish, game fish or traditional medicine purpose or overlap between these categories. Among these, 63 species were recognized as wild fisheries products and classified into three ascending groups based on their importance, which were subsistence fishes (nine species), minor commercial fishes (25 species) and major commercial fishes (29 species). Besides that, nine fish in this study are also being recognized as aquaculture species. Furthermore, 37 fish recorded in this study are used as ornamental fish that act as decoration for public or private aquariums. Moreover, ten species were classified as game fish based on the FishBase online platform classification. Among all the fish species obtained in this study, only one species, *Pegasus volitans* (Longtail seamoth) was recognized as being used in Traditional Chinese Medicine (TCM) in dried form (Osterhage *et al*., 2016).

In addition, the conservation status of the fish species recorded in this study was assessed using the International Union for Conservation of Nature (IUCN) Red List online platform (https://www.iucnredlist.org/). A number of 22 species are classified as Not Evaluated (NE), one species is classified as Data Deficient (DD), 56 species are classified as Least Concern (LC), three species are classified as Near Threatened (NT) and the remaining one species is classified as vulnerable (VU). The single species categorized as DD is *P. volitans*, a rare species found on coastal marine and the species inhabits bays and estuaries with sandy, muddy, grassy bottoms. Three species classified as NT in this study are all from the cartilaginous fish group known as *Taeniura lymma* (Ribbontail stingray), *Carcharhinus melanopterus* (Blacktip reef shark) and *Atelomycterus marmoratus* (Coral catshark). *Epinephelus fuscoguttatus* (Brown-marbled grouper) is the only species recorded in this study with VU status. This species is relatively uncommon and typically forms transient spawning aggregations during a few months of the year.

Among the 83 species of marine fishes recorded in this study, 76 species (92.6%) were successfully identified based on the morphological features by referring to Matsunuma *et al*. (2011), Moore and Colas (2016), FishBase online platform, and others related references during the field study. However, another seven species (8.4%) were hard to be identified morphologically due to the limited knowledge of fish morphological taxonomy. Therefore, the remaining seven species were identified through DNA barcoding of COI.

A total of seven bidirectional COI barcode sequences were obtained from seven species, six genera and six families. The length of the sequences ranges from 669 to 714 base pairs. Table 3 shows the BLAST results of the COI barcode sequences obtained in this study containing the local extreme metrics (E value, Percent Identity and Maximum Score), total metrics (Total Score and Query Coverage), length of base pair, accession number of the corresponding sequence and also the species identity. All the sequences showed significant E values, which were zero. In terms of percent identity, the mean of similarity of the query sequences to the target sequences can be considered high with 99.59% (ranging from 98.47% to 100%) while the mean of query sequences covered by the target sequences was 94.57% by average (ranging from 89% to 100%). The nucleotide BLAST results provided correct identification until the species level. A morphological confirmation followed a nucleotide BLAST search that provided the species names.

Conclusion

This study concluded that both objectives were successfully achieved based on the updated checklist of marine fish species inhabiting the inshore area of Pulau Tinggi, Mersing, Johor and the generation of DNA barcodes of the unknown fish specimens obtained using the cytochrome c oxidase I mitochondrial DNA gene. This study also suggested that DNA barcoding can close the gaps in morphologically-based identification systems. Along with the information from DNA

Table 3: Nucleotide Basic Local Alignment Search Tool (BLAST) results

Code	Base Pairs	Max Score	Total Score	Query Coverage (%)	E Value	Percent Identity (%)	Accession No. of Corresponding Sequence	Species Identity
006A	708	1166	1166	92%	0.0	98.93%	HQ564373.1	*Pomacentrus tripunctatus*
018A	694	1277	1277	100%	0.0	99.86%	KJ202140.1	*Caranx sexfasciatus*
037A	714	1199	1199	91%	0.0	99.85%	JN313083.1	*Centrogenys vaigiensis*
090A	702	1166	1166	89%	0.0	100%	KY849534.1	*Moolgarda seheli*
092A	681	1149	1149	95%	0.0	98.47%	KP194814.1	*Pomacentrus chrysurus*
096A	681	1205	1205	95%	0.0	100%	KJ937293.1	*Pempheris oualensis*
104A	669	1236	1236	100%	0.0	100%	AB890096.1	*Pristicon trimaculatus*

barcoding as the complement, an integrative taxonomic approach can be raised to identify marine fishes. Thus, the integration is beneficial in fisheries management and conservation of marine fish in the coastal region of Pulau Tinggi. This study also shows the importance of marine fish species that inhabit an inshore area of Pulau Tinggi to humans. The area can be vulnerable to the increasing trend of mass tourism and related activities such as establishing resorts, facilities and various water sports could cause acute and chronic effects on the coastal area in Pulau Tinggi if proper management and conservation are not in place. Therefore, the area needs a good management plan so that the marine fish resources will not be negatively affected and will be continuously available to support the livelihood of Pulau Tinggi local people.

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