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DNA BARCODING TO RESOLVE MORPHOLOGICAL AMBIGUITY OF YELLOWFIN SNAPPER, *Lutjanus xanthopinnis* FROM TERENGGANU

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Abstract: *Lutjanus* species belong to the family Lutjanidae, where some of the species, such as *L. lutjanus*, *L. vitta*, and *L. johnii*, are common species in Malaysia. The fish, also known as snapper, can be discovered in abundance in most fish landings, including at Terengganu. *Lutjanus xanthopinnis*, commonly known as yellowfin snapper, is another fish commonly found in Malaysian water, especially Terengganu. However, their morphological characteristics were doubted, as it is hard to distinguish by morphometric and meristic identification alone. This study was conducted by collecting 100 Lutjanid samples with mixed freshness grades from 11 locations consisting of four districts: Besut, Kuala Nerus, Kuala Terengganu, and Marang, Terengganu. These 11 locations consisted of fish landing ports, fish stalls, and fish markets in the identification using the Cytochrome Oxidase 1 (CO1) gene marker for the five morphological ambiguous samples. The initial external examination of the samples based on the colour of the stripes led to the identification of the 30 Lutjanid samples as *L. xanthopinnis*. Further identification of 30 Lutjanid samples via 40 morphometrics and nine meristic characters led to only 25 individuals from the 30 samples fully confirming their identity as *L. xanthopinnis*. However, five of the samples have several gill rakers, soft dorsal, and anal fin rays that are not similar yet overlap with the previous reports. Hence, these five samples were taken to undergo molecular identification via DNA barcoding using the CO1 gene. The Neighbour Joining Method phylogenetic tree with the Kimura-2 Parameter (K2P) model revealed that the five samples were differentiated into three clades, which were *L. xanthopinnis*, *L. vitta*, and *L. lutjanus*. Molecular identification for the five samples also revealed that three samples were identified as *L. xanthopinnis*. In contrast, the remaining two were identified as *L. vitta* and *L. lutjanus*, respectively, with 99% to 100% similarity. The study has demonstrated that molecular identification via DNA barcoding is a valuable complementary tool to morphological identification, enabling more accurate species identification. It is recommended that fresh samples and more samples are collected from other states. Furthermore, the use of DNA barcoding should be continued in species validation to achieve the highest possible level of accuracy.

Keywords: Molecular identifications, morphological characteristics, *Lutjanus xanthopinnis*, cytochrome c oxidase 1, Terengganu.

Introduction

There are 17 recognised genera and 112 species of snapper (Family: Lutajnidae) associated mainly with coral reefs in the tropical and subtropical Atlantic and Indo-Pacific regions (Preveenraj, 2018). *Lutjanus* sp. is one of the genera in the family Lutjanidae. This genus has been widely circulated throughout coastal reefs, sandy bays, and estuaries. According to a study by Chong *et al*. (2010), 10 species of *Lutjanus* are discovered in Malaysian waters, and they are considered one of the most commercially important fish species, including Terengganu. There were seven lutjanid fish found in Terengganu, which are *L. argentimaculatus*, *L. fulviflamma*, *L. lutjanus*, *L. malabaricus*, *L. quinquelineatus*, *L. russellii*, and *L. vitta* (Matsunuma *et al*., 2011). According to data provided by the Department of Fisheries

Malaysia (DOF, 2021), 2,265 tonnes of snappers landed in Malaysia, and 497 tonnes (21.94%) of snappers landed in Terengganu. These snappers were recorded under Jenahak and Remong/ Kunyit-kunyit. Many species in this genus have very similar morphologies, such as the red snapper and the yellow-striped snapper complex reviewed in previous studies by Iwatsuki *et al*. (2015). Note that close similarities between species make it difficult for fisheries personnel and even experienced taxonomists to reliably identify species based on external characteristics (Bakar *et al*., 2018). A previous study named a new snapper species, *Lutjanus xanthopinnis*, based on specimens from Japan, Taiwan, Indonesia, and Sri Lanka that were thought to be *L. madras* (Valenciennes, 1831). The study also updated the description of the real *L. madras* and made genetic comparisons with other yellow-striped fish species. The *Lutjanus madras* species, believed to have migrated significantly from its original habitat in the Indian and Western Pacific Oceans, was diagnosed and described in a survey of the genus by Allen and Talbot (1985). On the other hand, Iwatsuki *et al*. (2015) reported some morphological characters between *L. xanthopinnis* and *L. madras*, as presented in Table 1 below.

Table 1: Morphological characters between *L. xanthopinnis* and *L. madras*

Characteristics	***Lutjanus xanthopinnis***	***Lutjanus madras***
	Iwatsuki *et al*. (2015)	**Iwatsuki *et al*. (2015)**
Dorsal-fin rays	X, 13	X,13
Anal-fin rays	III, 8	III,9
Pored lateral-lines scales	48-50	49-51
Gill rakers, upper and lower limb totals (rudiments) = total including rudiments	22-23	17-22
Scale rows on cheek	4-5	5-7
Scales row above lateral line	6-7	8-10
Preopercle flange	Scaled	No scales
Posterior extension of vomerine tooth patch	Present	Present
Stripes on sides	Thin stripes plus thin mid-lateral yellow stripe	Thin stripes plus broad mid-lateral yellow stripes

Conventionally, species identification should not rely on just morphological and meristic features. Identification via morphometric and meristic characteristics in fish is important, but molecular identification via DNA barcoding needs to be performed for a more decisive confirmation of the fish species. Nevertheless, it has been proven that genetic methods will be beneficial in identifying and classifying specific-level organisms that are not clearly classified. Liu *et al*. (2022) advocated the use of the Cytochrome Oxidase 1 (CO1) gene, which is discovered in mitochondrial DNA, as a global animal biorecognition system, including in fish identification (Arjunaidi *et al*., 2016; Azahar *et al*., 2022; Alzahaby & Biju Kumar, 2023; Shalu *et al*., 2023). Several authors, such as Bakar *et al*. (2018), Velamala *et al*. (2019), Andriyono and Suciyono (2020), and Limmon *et al*. (2020), have conducted molecular studies on fishes from the family Lutjanidae and the results obtained were accurate and as desired. To ensure the fish is identified correctly, it is crucial to compare the results of the two methodologies, molecular and morphological evaluation (Aaron *et al*., 2018). The goal of this paper is to figure out what kind of yellowfin snapper (*L. xanthopinnis*) fish were observed

in Terengganu by looking at their morphology (morphometric and meristic counts) and their CO1 gene (Afriyie *et al*., 2020).

Materials and Methods

Sample Collection

A total of 100 individuals of Lutjanids were sampled from 11 locations in four districts: Besut (n = 1), Kuala Nerus (n = 4), Kuala Terengganu (n = 4), and Marang (n = 2) (Figure 1 and Table 2). This sampling was done from August to October 2022 by visiting fish landing sites and fish markets in Terengganu. The specimens were priorly identified based on the external marking, which has a series of narrow horizontal stripes that are yellow and located below the lateral line (Iwatsuki *et al*., 2015). Within those 100 samples, some samples were not fresh, and discolouration was observed on their bodies. However, the important body marking for identification was still visible. The initial identification led to only 30 samples being identified as *L. xanthopinnis*.

Figure 1: Map of sampling sites

Table 2: Locations of the sample collection

No.	Location	Latitude	Longitude	Specimen Collected
1	Kompleks Perikanan LKIM Besut	5°49’50.7”N	102°33’44.7”E	3
2	Batu Rakit	5°27’00.3”N	103°02’52.3”E	5
3	Mengabang Telipot	5°25’33.7”N	103°04’26.1”E	6
4	Pantai Tok Jembal	5°24’16.0”N	103°05’56.4”E	5
5	Pasar Batu 6	5°20’47.9”N	103°05’30.1”E	7
6	Pasar Payang	5°20’15.4”N	103°08’09.5”E	4
7	Pasar Chabang Tiga	5.3174° N	103.1252° E	9
8	Kompleks Perikanan LKIM Pulau Kambing	5°19’19.7”N	103°07’42.8”E	48
9	Kompleks Perikanan LKIM Chendering	5°16’16.1”N	103°09’54.4”E	8
10	Kompleks Perikanan LKIM Marang	5°12’08.4”N	103°12’23.3”E	2
11	Pasar Bandar Marang	5°12’10.7”N	103°12’21.0”E	3
	Total			**100**

Morphological Identification

Morphometric Measurements and Meristic Counts

The morphometric measurements and meristic counts were performed according to the key and description by Iwatsuki *et al*. (2015). A total of 40 morphometric characters were measured according to Allen and Talbot (1985), Iwatsuki *et al*. (1993), Iwatsuki *et al*. (2015), and Aaron *et al*. (2018) using manual vernier callipers up to 0.01 mm. Some of the morphometric measurements conducted on the fish are shown in Figure 2, while Table 3 lists all the 40 morphometric characteristics measured in this study. Consequently, nine meristic characters were counted and observed based on Iwatsuki *et al*. (2015) (Table 1).

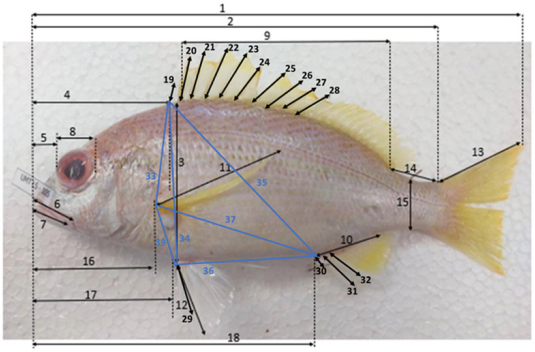

Figure 2: Several morphometric characters that were measured on *Lutjanus* sp.

Table 3: Morphometric characters that were measured in this study

No.	Description	Characters
1	Total length	TL
2	Standard length	SL
3	Body depth	BD
4	Head length	HL
5	Snout length	SL
6	Upper jaw length	JLu
7	Lower jaw length	JLl
8	Eye diameter	ED
9	Dorsal fin length	DFL
10	Anal fin length	AFL
11	Pectoral fin length	PEC L
12	Pelvic fin length	PEL L
13	Caudal fin length	SFL
14	Caudal peduncle length	CPL
15	Caudal peduncle height	CPH
16	Pre-pectoral depth	PrPcD

17	Pre-pelvic length	PrpeD
18	Pre-anal length	PraD
19	First dorsal spine length	DSL1
20	Second dorsal spine length	DSL2
21	Third dorsal spine length	DSL3
22	Fourth dorsal spine length	DSL4
23	Fifth dorsal spine	DSL 5
24	Sixth dorsal spine	DSL6
25	Seventh dorsal spine	DSL7
26	Eighth dorsal spine	DSL 8
27	Ninth dorsal spine	DSL 9
28	Tenth dorsal spine	DSL 10
29	Pelvic spine length	PeSL
30	First anal spine length	ASL1
31	Second anal spine length	ASL2
32	Third anal spine length	ASL3
33	Pectoral fin insertion-dorsal fin origin	PCDD1
34	Pelvic fin insertion-dorsal fin origin	PEDD1
35	Anal fin origin-dorsal fin origin	ADD1
36	Pelvic fin insertion-anal fin origin	PeAD
37	Pectoral fin insertion-anal fin origin	PcAD
38	Pectoral fin insertion-pelvic fin insertion	PcPeD

Molecular Identification

DNA Extraction and DNA Quantification, CO1 Amplification, and DNA Sequencing

Muscle tissue was used for DNA extraction using the salt extraction method according to the protocol by Muhammad *et al*. (2016). The eluted DNA was then quantified using a 7,415 nanoscanning micro-volume spectrophotometer (Jenway, United Kingdom) and stored in a -20°C freezer. The samples were amplified using primers: FishF1 (5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3') and FishR1 (5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3') (Ward *et al*., 2005) with an amplicon of approximately 650 bp in the SuperCycler® (Kyratec, USA). Consequently, the PCR was performed in a 25.0 volume containing 6.0 µL ddH2O, 13.0 µL PCR premix (PCR Buffer, dNTP, Taq Polymerase), 0.5 each primer (10 µM), and 5.0 µL DNA template. The PCR cycling conditions were as follows: A 2-minute initial denaturation at 95°C, followed by 40 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 54°C, extension for 1 minute at 72°C, and final extension for 10 minutes at 72°C (Aaron *et al*., 2018). The five PCR products were visualised using a 2% agarose gel. PCR products that achieved the desired band size were sent for unidirectional sequencing using the forward primer, FishF1, at Apical Scientific Sdn. Bhd.

Data Analysis

The received sequences were aligned, cleaned from noisy ends and stop codons, and trimmed into equal sizes using Molecular Evolutionary Genetics Analysis version 11 (MEGA v11.0) software (Tamura *et al*., 2021). The trimmed sequences were identified using the nucleotide Basic Local Alignment Search Tool (BLAST) from NCBI GenBank. Note that top matches with at least 97% sequence similarity are generally used as criteria for probable species identification (Aaron *et al*., 2018; Mwita & Chuhila, 2023). A phylogenetic tree through the neighbour-joining method using the Kimura-2 Parameter (K2P) model was constructed with 1,000 replications. An outgroup and sister group, along with several identical species with the same sequences, were included in the phylogenetic tree construction.

Results and Discussion

Morphometric Measurements and Meristic Counts

The weight of all 30 samples ranged from 47.00 g to 280.30 g, with a mean of 100.95 ± 52.12 g. Based on the 38 morphometric characters measured in Table 1, 25 of the 30 samples fully confirmed their identity as *L. xanthopinnis*. Nevertheless, five Lutjanid samples did not fall within the range of meristic counts stated by Iwatsuki *et al*. (2015) for *L. xanthopinnis*. These five samples were UMT LS-16, UMT LS-17, and UMT LS-29 from LKIM Pulau Kambing, UMT LS-36 from Pasar Payang, and UMT LS-67 from Pasar Chabang Tiga. The meristic counts of these five samples were also compared with the meristic counts of *L. lutjanus*, *L. vitta*, and *L. madras*. However, none of the five samples fell within the range of meristic counts for the remaining three *Lutjanus* species. Hence, it does not lead to identifying any of the three species. The morphometric measurements that were taken for the 25 Lutjanid samples were recorded in Table 4 below.

Table 4: Morphometric measurement of a total of 30 *Lutjanus xanthopinnis* samples

No.	Descriptions	Characters	Mean ± SD	Range (cm)
1	Total length	TL	18.52 ± 2.78	15.00-27.60
2	Standard length	SL	15.37 ± 2.46	12.00-23.00
3	Body depth	BD	5.35 ± 1.17	2.50-8.80
4	Head length	HL	5.40 ± 0.92	4.00-8.50
5	Snout length	SL	1.67 ± 0.35	1.10-2.50
6	Upper jaw length	JLu	2.17 ± 0.41	1.40-3.40
7	Lower jaw length	JLl	1.66 ± 0.60	1.00-4.00
8	Eye diameter	ED	1.46 ± 0.20	1.00-2.00
9	Dorsal fin length	DFL	7.93 ± 1.94	6.00-16.50
10	Anal fin length	AFL	2.43 ± 0.44	2.00-3.50
11	Pectoral fin length	PEC L	4.31 ± 0.79	3.00-6.40
12	Pelvic fin length	PEL L	2.86 ± 0.59	2.00-4.40
13	Caudal fin length	SFL	3.75 ± 0.89	2.50-6.50
14	Caudal peduncle length	CPL	2.60 ± 0.60	1.50-4.40
15	Caudal peduncle height	CPH	1.97 ± 0.54	1.50-3.60
16	Pre-pectoral depth	PrPcD	4.47 ± 0.12	4.00-4.50

17	Pre-pelvic length	PrpeD	4.73 ± 0.09	4.70-5.00
18	Pre-anal length	PraD	8.90 ± 0.27	8.00-9.00
19	First dorsal spine length	DSL1	0.50 ± 0.00	0.50-0.50
20	Second dorsal spine length	DSL2	1.44 ± 0.17	1.00-1.50
21	Third dorsal spine length	DSL3	1.48 ± 0.07	1.20-1.50
22	Fourth dorsal spine length	DSL4	1.95 ± 0.15	1.50-2.00
23	Fifth dorsal spine	DSL 5	1.50 ± 0.00	1.50-1.50
24	Sixth dorsal spine	DSL6	1.50 ± 0.00	1.50-1.50
25	Seventh dorsal spine	DSL7	1.00 ± 0.00	1.00-1.00
26	Eighth dorsal spine	DSL 8	1.00 ± 0.00	1.00-1.00
27	Ninth dorsal spine	DSL 9	1.00 ± 0.00	1.00-1.00
28	Tenth dorsal spine	DSL 10	1.00 ± 0.00	1.00-1.00
29	Pelvic spine length	DSL 11	N/A ± N/A	N/A-N/A
30	First anal spine length	PeSL	1.53 ± 0.12	1.50-2.00
31	Second anal spine length	ASL1	0.48 ± 0.07	0.20-0.50
32	Third anal spine length	ASL2	1.01 ± 0.04	1.00-1.20
33	Pectoral fin insertion-dorsal fin origin	ASL3	1.00 ± 0.00	1.00-1.00
34	Pelvic fin insertion-dorsal fin origin	PCDD1	3.39 ± 0.21	3.00-3.50
35	Anal fin origin-dorsal fin origin	PEDD1	4.98 ± 0.09	4.50-5.00
36	Pelvic fin insertion-anal fin origin	ADD1	6.10 ± 0.37	6.00-7.50
37	Pectoral fin insertion-anal fin origin	PeAD	4.02 ± 0.09	4.00-4.50
38	Pectoral fin insertion-pelvic fin insertion	PcAD	5.03 ± 0.12	5.00-5.50

Table 5 below presents the meristic count conducted on five morphologically ambiguous characters from the sample of this study, compared to the other three *Lutjanus* species from previous studies that did not fall into the range of the four *Lutjanus* species.

The analysis revealed that the most significant difference was observed in the number of gill rakers. Samples UMT LS-16, UMT LS-17, and UMT LS-29 exhibited fewer gill rakers compared to the results reported by Iwatsuki *et al*. (2015). However, the number of gill rakers for UMT LS-36 matched the value reported for *L. vitta* by Matsunuma *et al*. (2011). Additionally, the number of gill rakers in sample UMT LS-67 matched the values reported for *L. lutjanus* by Matsunuma *et al*. (2011) and Iwatsuki *et al*. (2015). Generally, the meristic count for other meristic characters was not significant, and some characters, such as the number of hard dorsal and anal rays, overlapped. These results indicate that the meristic count of gill rakers can be a more accurate method for the morphological identification of Lutjanids (Simon *et al*., 2010; Meshram *et al*., 2021). However, relying solely on morphological characters for Lutjanids is insufficient, and molecular identification must be performed to ensure high accuracy of species identification. Hence, these five samples initially identified as *L. xanthopinnis* were further identified molecularly via the DNA barcoding method using the CO1 gene.

Table 5: Meristic count taken on five morphological ambiguous characters from the samples of this study and compared with the meristic counts of other four *Lutjanus* species from the previous study

Characteristics	Sample ID Iwatsuki *et al.* (2015) Iwatsuki *et al.* (2015)			*Lutjanus xanthopinnis*	*Lutjanus vitta*		*Lutjanus lutjanus*		*Lutjanus madras*
				Matsunuma *et al.* (2011)	Iwatsuki *et al.* (2015)	Matsunuma *et al.* (2011)	Iwatsuki *et al.* (2015)		
	UMT- LS 16,17, 29	UMT LS-36	UMT LS-67						
Dorsal-fin rays	X,13	X,14	X, 14	X, 13	X,12-13	X,12-13	X-XII,12	X-XII,12	X,13
Anal-fin rays	III,9	III,9	III, 8	III, 8	III,8	III,8-9	III,8	III,8	III,8
Pored lateral-lines scales	50,50,50	50	50	48-50	49-52	47-50	48-50	48-50	49-51
Gill rakers, upper and lower limb totals (rudiments) = total including rudiments	6 + 9 = 15	7 + 9 = 16	7 + 17 = 24	22-23	17-22	15-19	24-26	23-27	22-23
Scale rows on cheek	7	5	6	5	5-7	N/A	5-8	N/A	4-5
Scales row above lateral line	6	6	5	6-7	8-10	N/A	4 or 5	N/A	6-7
Preopercle flange	Scaled	Scaled	No Scales	Scaled	Scaled	N/A	No scales	N/A	No scales
Posterior extension of vomerine tooth patch	Presence	Presence	Presence	Presence	Presence	N/A	Presence	Presence	Presence
Stripes on sides	Thin stripes plus thin mid-lateral yellow stripe.	Thin stripes plus broad mid-lateral dark stripes.	Thin stripes plus broad mid-lateral yellow stripes.	Thin stripes plus thin mid-lateral yellow stripe.	Thin stripes plus broad mid-lateral dark stripes.	Dark brown to blackish stripe on side from eye to upper half of caudal peduncle.	Thin stripes plus broad mid-lateral yellow stripes.	Broad yellow to brownish stripe from eye to caudal fin base.	Thin stripes plus broad mid-lateral yellow stripes.

Molecular Identification

All five samples were successfully amplified at a size of 634 bp. The constructed neighbour-joining tree with the K2P model and a bootstrap value of 1,000 revealed there was the presence of three clusters between the five samples (Figure 3). Samples UMT LS-16, UMT LS-17, and UMT LS-29 were clustered into a single clade (Clade 2) with other GenBank *L. xanthopinnis* sequences that came from Bangladesh, Indonesia, and Japan, which shows the three samples are *L. xanthopinnis*. However, UMT LS-36 formed a cluster with *L. vitta* sequences from NCBI GenBank originating from Australia, China, and Indonesia (Clade 1). Meanwhile, UMT LS-67 formed another cluster with *L. lutjanus* sequences from GenBank that originated from China, Indonesia, and Japan (Clade 3). This indicates that those samples are

L. vitta and *L. lutjanus*, respectively. *Epinephelus lanceolatus* was chosen as the outgroup since the fish came from a different family compared to the study species, while *L. Johnii* was chosen as the sister group because it comes from the same family as the fish species studied in the study. Furthermore, NCBI BLAST results also prove that UMT LS-16, UMT LS-17, and UMT LS-29 were identified as *L. xanthopinnis*, while UMT LS-36 and UMT LS-67 were identified as *L. vitta* and *L. Lutjanus*, respectively. All the samples were identified at a percentage of identical ≥ 99%. The five samples were submitted to GenBank, and their accession numbers were as follows: UMT LS-16, UMTLS-17, UMT LS-29 (OQ439420-OQ439422), UMT LS-36 (OQ443123), and UMT LS-67 (OQ443124).

The molecular identification method was proven more reliable in species identification, especially for fish. This was because molecular identification could distinguish fish species with overlapping or missing identification morphological features (Basith *et al*., 2021; Hassan *et al*., 2024). Furthermore, molecular methods via DNA barcoding rely on the abundantly available mitochondrial DNA (mtDNA) CO1 gene in the cell instead of delicate, crucial morphological features (Eissa *et al*., 2021; Mwita & Chuhila, 2023). For instance, the absence or damage to these morphological features, such as what has occurred to the five Lutjanid samples in this study, can make the identification process harder and might also lead to species misidentification (Wang *et al*., 2018; Fadli *et al*., 2020; Sawalman & Maduppa, 2020). Hence, molecular identification via DNA barcoding that utilises the CO1 gene is more reliable for identifying a fish species. This was due to the fact that molecular identification was not affected by the absence of important identification morphological features but by the presence of CO1 in the cell. Despite DNA barcoding being a more accurate identification tool, it only serves as a complementary tool to validate the identified species with higher accuracy (Wang *et al*., 2018; Ahmed *et al*., 2021; Tadmor-Levi *et al*., 2022).

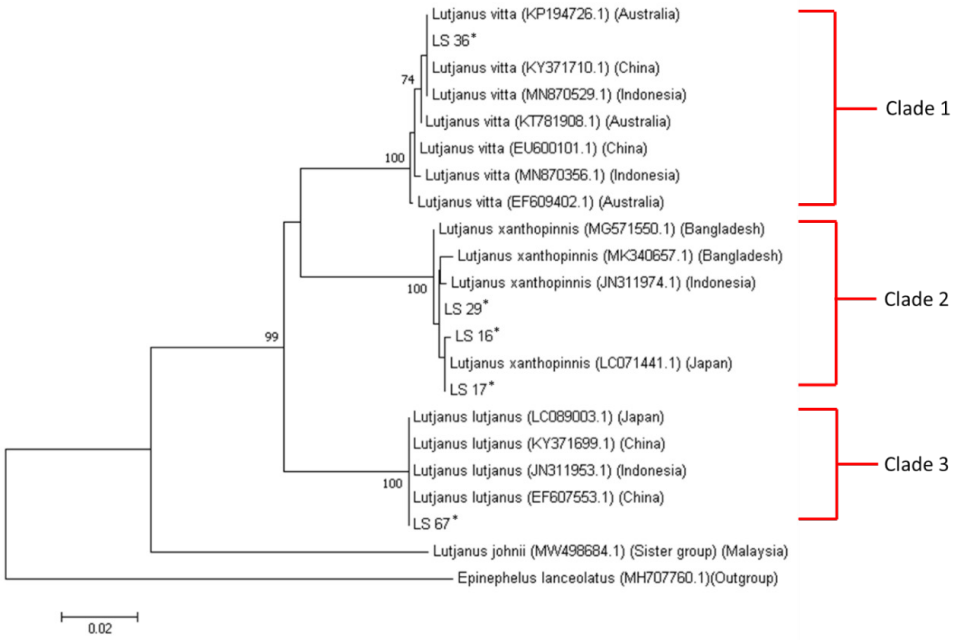

Figure 3: Neighbour-joining phylogenetic tree between the *Lutjanus* spp. samples from this study in Terengganu (samples labelled as LS--*) and the *Lutjanus* spp. Taken from GenBank

Conclusions

In conclusion, 28 samples were successfully identified as *L. xanthopinnis*, while the remaining samples were identified as *L. vitta* (n = 1) and *L. lutjanus* (n = 1). DNA barcoding, which serves as a complementary tool in molecular identification, has been proven to have higher accuracy in species identification compared to morphological identification. This was due to the ambiguity of the morphology in all five samples, which prevented effective morphological identification. To increase the accuracy of the results, it is recommended that samples should be taken from other states for morphological analysis. Additionally, fresh samples should be used for future morphological analysis, as their morphological features are less likely to be affected by the degradation process. Finally, verifying species identification through the molecular method using CO1 should be continued to ensure high accuracy in confirming the fish's identity.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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