Unveiling the DNA Barcoding of Threadfin Breams (Nemipteridae) at Oeba Fish Landing Site and Oesapa Fish Market in Kupang, East Nusa Tenggara

Umbu Domu Wora[1], Diah Permata Wijayanti[1]*, Ita Widowati[1], Fanny Iriany Ginzel[2], Nining Nursalim[3], Nenik Kholilah[3], Muggi Bachtiar[4]

[1]Department of Marine Science, Faculty of Fisheries and Marine Sciences, Universitas Diponegoro
Jl. Prof. Jacub Rais, Tembalang Semarang, Jawa Tengah 50265 Indonesia
[2]Aquatic Resource Management, Christian University of Artha Wacana
Kupang, East Nusa Tenggara, Indonesia
[3]Diponegoro Marine Biodiversity Project Laboratory, Universitas Diponegoro
Jl. Prof. Soedharto SH, Tembalang Semarang, Jawa Tengah 50265 Indonesia
[4]Graduate School of Engineering and Science, University of The Ryukyus
Nishihara, Okinawa 903-0123, Japan
Email: diahpermata@lecturer.undip.ac.id

Abstract

Threadfin breams (Nemipteridae) are demersal fish species that constitute a significant catch for East Nusa Tenggara fishermen at the Oeba Fish Landing Site and Oesapa Fish Market, where they are landed year-round. Over the years, there has been a noticeable increase in the capture of threadfin breams, raising concerns about the potential impact on their genetic diversity. The ongoing trend could affect the region's overall population structure of threadfin breams. This study addresses the need to identify threadfin breams in the landing above sites of threadfin breams through molecular analysis of mtDNA COI. The research involved the examination of 24 samples obtained from the Oeba Fish Landing Site and Oesapa Fish Market. The analysis revealed the presence of five distinct threadfin bream species: Nemipterus hexodon, N. japonicus, N. zysron, N. aurora, and Pristipomoides typus. The genetic distance between individual threadfin breams ranged from 0-0.8%, indicating a relatively close genetic relationship within the population. Also, phylogenetic tree reconstruction further delineated five distinct clades based on the species obtained from the samples. Given these findings, the study emphasizes the importance of sustainable threadfin bream capture to preserve genetic diversity. The results underscore the need for ongoing monitoring and management strategies to ensure the threadfin bream population's long-term health and stability. Additionally, the study suggests that a more in-depth analysis of genetic diversity and the environmental factors influencing this species is warranted for a comprehensive understanding and effective conservation measures.

Keywords*: Threadfin bream, genetic diversity, mtDNA COI, Oesapa and Oeba, Kupang*

Introduction

Threadfin bream fish, a species from the Nemipteridae family, is widely distributed across the Indo-Pacific Region (Russel and Nemipteriade, 2001). These fish inhabit the seafloor near coastal waters, especially around coral reefs, sandy substrates, and muddy bottoms, typically at depths ranging from 10 to 50 meters (Roul *et al*., 2022). The Nemipteridae family comprises 67 species, with many of them commonly known as Nemipterus. In Indonesia, they are often referred to as threadfin bream fish, locally known as "*kurisi fish*" (Junaedi *et al*., 2020). Threadfin breams fish are omnivores, feeding various foods, including plankton, crustaceans, mollusks, and algae. They tend to congregate in large groups and engage in reproduction to increase their offspring (Persada, 2016). Threadfin bream fish are found in various marine regions in Indonesia, including Sulawesi, Maluku, Java, Sumatra, Irian Jaya, and Nusa Tenggara (Priyanie, 2006).

Threadfin bream fish are economically significant and widely utilized by the local population for consumption and various processed products. The fish are characterized by their white-flesh fish, high protein, and low-fat flesh, making them a valuable resource (Malau *et al*., 2022). Threadfin bream fishes constitute a significant portion of fisheries products, as highlighted by Ninef et al. (2019). Local fisheries actively target several species using various methods such as bottom trawls, handline, longline, gill nets, lift nets, surrounding nets, drive-in nets, fish stakes, and traps (Russel, 2001). It stands out as one of the extensively captured species, catering to local consumption and export production, such as surimi (Pangsorn et al., 2007). Nemipterids are popular food

*) Corresponding author

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choices and are available in the market in fresh, dry-salted, dry-smoked, fermented, and steamed forms (Russel, 1990).

According to data from the Maritime and Fisheries Department of East Nusa Tenggara province, the catch of threadfin bream fish has been increasing annually, with 161.5 tons in 2017, 170.7 tons in 2018, and 336.6 tons in 2019 (BPS NTT, 2019). Uncontrolled harvesting practices can decrease the availability of marine organisms (Ambariyanto, 2017) including threadfin bream fish populations, which, if left unaddressed, could threaten genetic diversity (Saleky *et al*., 2021). One practical approach to this issue is molecular identification (Mopay, 2017).

Morphological identification has several limitations, especially for cryptic organisms (Hebert *et al*., 2003). Cryptic organisms have very similar morphological features, making it challenging to distinguish between different species (Poulin and de Leon, 2017; Moritz *et al*., 2018). Morphological identification in some studies often requires experienced experts due to the difficulty of the organisms under investigation. This has led to molecular identification becoming an alternative, particularly in Indonesia. Molecular identification is easier to learn and can be performed by anyone for research on cryptic organisms like fish (Nursalim *et al*., 2022), Decapoda (Cheng *et al*., 2020; Ambariyanto *et al.,* 2023), Coral (Insafitri *et al.,* 2023), Rajungan (Marita *et al.,* 2023) and Cephalopoda (Kholilah *et al*., 2021a).

Numerous studies on the molecular identification of threadfin bream fish have been conducted in several countries, such as Malaysia (Imtiaz *et al.,* 2016), the Philippines, and China (Guo *et al.,* 2020; Yi *et al.,* 2021). Molecular identification is beneficial in distinguishing morphologically similar organisms or those that are challenging to differentiate visually. Meanwhile, research in East Nusa Tenggara related to both morphology and molecular aspects has been conducted on skipjack tuna (*Katsuwonus pelamis*) (Jatmiko *et al.,* 2019) and boarfish (Nomleni *et al.,* 2022). However, there have been relatively few studies on threadfin bream fish, morphologically and molecularly. Numerous investigations have been undertaken on threadfin bream fish in Indonesia, exemplified by the study in Brondong, East Java, which delves into the sustainable potential of these fish (Widagdo *et al*., 2019), the reproductive capacity of four-tailed threadfin bream fish in Bangka Belitung (Utami et al., 2018) and the breeding characteristics of threadfin bream fish in the waters of Southern Java (Lisamy *et al*., 2023). Consequently, concerns have been raised about the overexploitation of threadfin bream resources in Banten Bay waters (Irnawati and Surilayani, 2021). Notably, there is no reported research on threadfin bream fish in East Nusa Tenggara, specifically in the waters of Kupang Bay and Rote Ndao. Threadfin bream fish are cryptic species, exhibiting a high degree of morphological similarity within the species, making it quite challenging to perform morphological identifications down to the species level (Tang *et al.,* 2006). This underlined the importance of molecular identification to determine the species of threadfin bream fish captured in the waters of East Nusa Tenggara. This research uses the mtDNA COI locus, which is effective and frequently used for fish species identification, as demonstrated in Ning *et al*. (2015) study on threadfin bream fish species identification in the South China Sea. This study aims to identify and examine the genetic diversity of captured threadfin bream fish that was collected from two landing sites, the Oeba Fish Landing Site and Oesapa Fish Market, Kupang, East Nusa Tenggara using the mtDNA COI locus.

Materials and Methods

The research was conducted in April 2023, with samples from the Oeba Fish Landing Site and Oesapa Fish Market in Kupang City (Figure 1.). The threadfin bream fish used as research samples were purchased from a fish vendor in the market. Information on fishing locations was also obtained from short interviews with fish sellers. The samples that have been purchased are then identified morphologically using a Reef Fish Identification Tropical Pacific book (Allen *et al*., 2003). For molecular analysis, the fish samples were taken from the muscles near the dorsal fin and preserved in 96% ethanol. Subsequently, the samples were transported to the Integrated Laboratory at Universitas Diponegoro Semarang for molecular identification.

Molecular analysis

Threadfin breams fish flesh samples were extracted using a 10% chelex solution (Whals *et al*., 1991). PCR amplification was performed at the mtDNA COI gene locus using the Fish F1 and R1 primers. The sequence of the forward primer Fish F1 is 5'-TCAACCAACCACAAAGACATTGGGCAC-3', and the reverse primer Fish R1 is 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3. The PCR process involved an initial denaturation at 95ºC for 3 minutes, 35 cycles including denaturation at 94ºC for 30 sec., primer annealing at 55ºC for 30 sec., DNA segment extension at 72ºC for 1 min., and a final extension at 72ºC for 10 min. This PCR amplification process resulted in base pairs approximately 600-700 bp in length, which could be visualized through electrophoresis (Ning *et al*., 2015). Positive (fluorescent) samples were then sent to the

Sequencing Facility (PT. Genetika Science Indonesia) for further processing using the Sanger method.

The data obtained consisted of a pair of ABI format files (forward and reverse) that could be opened using MEGA XI software. Both sequences were aligned using the Clustal W menu in MEGA XI. The alignment results were combined into a single sequence and saved in FASTA file format (Dailami *et al*., 2021). The sequence was compared with the GenBank database using the Basic Local Alignment Search Tool (BLAST) on NCBI (Boratyn *et al*., 2013). The sequence was entered into the BLAST form available at https://blast.ncbi.nlm.nih.gov/Blast.cgi, and the sequence with the highest similarity was downloaded in FASTA file format for use in constructing a phylogenetic tree (Dailami *et al*., 2021). A phylogenetic tree was created using the Maximum Likelihood tree method with the Kimura-2 parameter model and 1000 bootstrap replicates, the model test analysis performed in MEGA. Subsequently, haplotype analysis was performed using DNAsp V6.11.01 software (Rozas, 2009).

Results and Discussion

Based on molecular analysis, five species of (Nemipteridae family) were identified in the Oeba Fish Auction (Oeba Fish Landing Site) and the Oesapa Fish Market, which consists of *Nemipterus japonicus, N. hexodon, N. aurora, N. zysron, and Pristipomoides typus* (Allen et al., 2003) (Figure 2.). The catch of threadfin breams fish landed at Fish Landing Site Oeba are sourced from the waters around Rote Ndao and Kupang Bay, while those at the Oesapa Fish Market come from the Kupang Bay area. The fishing gear used for these operations consists of drifting and bottom gillnets. *N. japonicus* is a benthic fish abundant in coastal waters. This species typically resides on muddy substrates ranging from 5 to 80 meters and feeds on various food sources, including algae and crustaceans. *N. japonicus* has a reddish body, a yellow color from behind the head to the tail fin, and a dorsal fin from the tip of the pectoral fin to the base (Elhaweet, 2013). *N. hexodon* is another benthic species found in shallow waters, primarily around coral reefs at depths of 10 to 80 m. It primarily feeds on plankton, mollusks, and benthic organisms, including *N. hexodon*, with a pinkish body color and yellow lines on the upper side, and red spots (Gustomi, 2019). *N. aurora* is a species found in shallow waters and around coral reefs. This fish usually inhabits depths of 5 to 50 meters and consumes plankton, mollusks, and crustaceans. *N. aurora* has an elongated, round, pinkish body and a red tail (Gustomi, 2019). *N. zysron* is a benthic species that thrives on muddy and sandy bottoms at depths of 10 to 60 m. Its diet mainly consists of algae and plankton. *N. zysron* has an elongated, pinkish, round body, yellow below the eyes, and a reddish tail (Jawad, 2014). *P. typus* is a benthic fish found in shallow waters at 20 to 80 m. It is commonly

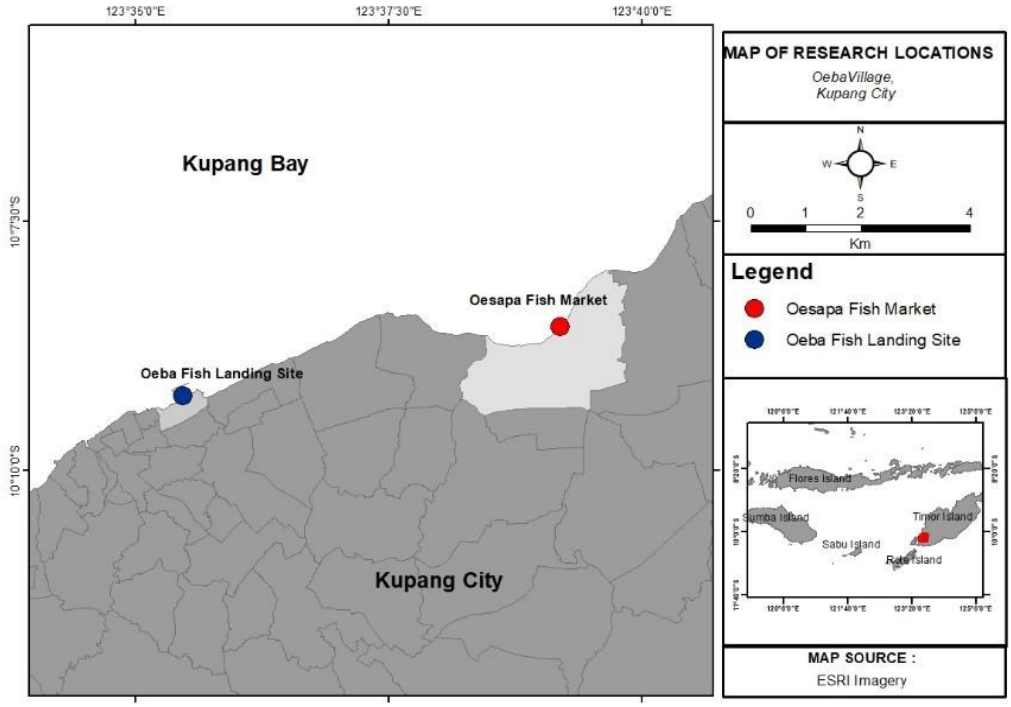

Figure 1. Sampling location, Oeba Fish Landing Site and Oesapa Fish Market in Kupang City, East Nusa Tenggara

encountered near coral reefs and primarily preys on tiny benthic organisms. *P. typus* has a small, round, compact body, red in color, and a longer upper tail (Baranes, 2017).

Molecular analysis using the mtDNA COI locus with lengths ranging from 600-700 base pairs resulted in five species were identified with similarities ranging from 96.83% to 100.00% based on genetic databases in NCBI (Table 1.). Leray *et al*. (2013) proposed a minimum identity percentage of 98% to indicate species similarity. Almost all the samples examined in this study exhibited an identity percentage exceeding 98%. However, sample Oesapa 03 showed a lower value (96.83%). According to Kholilah *et al*. (2021b), the low percentage value may be attributed to limitations in comparative data available in GenBank. Additionally, this could also be due to low quality of sequencing results which depending on contributing factors such as sample quality (purity and DNA concentration) (Chakraborty *et al*., 2006), PCR processes (primer design and PCR cycles), PCR product purification, and sequencing methods, could contribute to this. However, despite BLAST results showing figures below 98%, morphological observations support the identification of the fish species as *N. hexodon*.

These overall molecular findings are concordant with the morphological features as it was described above. These results further showed that two species of threadfin breams, *N. hexodon* and *N. japonicus* was landed in Oesapa Fish Market, while five species, *N. hexodon*, *N. zysron*, *N. aurora*, *P. typus*, and *N. japonicus* was landed in Oeba Fish Landing Site. Threadfin bream fish has also been identified using samples from Pondicherry coastal waters, with the same results for *N. hexodon*, *N. zysron*, and *N. japonicus* (Ravitchandirane *et al*., 2012).

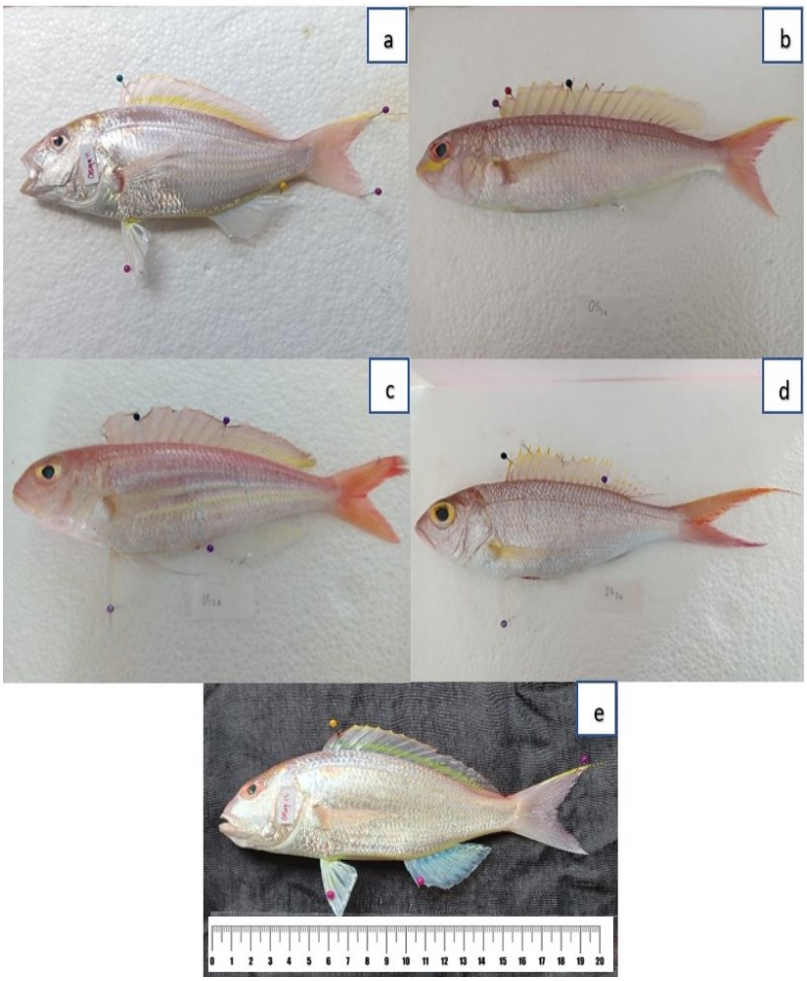

Figure 2. Morphological identification of threadfin breams, a. *Nemipterus hexodon,* b. *N. zysron,* c. *N. aurora,* d. *Pristipomoides typus,* and e. *N. japonicus*

Table 1. Species identification of threadfin breams fish using BLAST method in GeneBank database

No	Sample code	Blast result	Accession number	Ident (%)	Accession number ref.
1	Oesapa 01	*N. hexodon*	OR539540	99.69%	OQ386781.1
2	Oesapa 02	*N. hexodon*	OR539541	99.85%	OQ386781.1
3	Oesapa 03	*N. hexodon*	OR539542	96.83%	KY362813.1
4	Oesapa 04	*N. japonicus*	OR539543	99.09%	EU871686.1
5	Oesapa 05	*N. hexodon*	OR539544	99.84%	OQ386768.1
6	Oesapa 06	*N. japonicus*	OR539545	99.52%	MK843715.1
7	Oesapa 07	*N. japonicus*	OR539546	100.00%	GU674068.1
8	Oesapa 08	*N. japonicus*	OR539547	100.00%	GU674068.1
9	Oesapa 09	*N. japonicus*	OR539548	99.37%	KY371816.1
10	Oesapa 10	*N. hexodon*	OR539549	99.84%	OQ385999.1
11	Oeba 1a	*N. zysron*	OR539550	100.00%	KY362889.1
12	Oeba 1b	*N. zysron*	OR539551	100.00%	GU674246.1
13	Oeba 1c	*N. zysron*	OR539552	100.00%	GU674449.1
14	Oeba 2a	*N. aurora*	OR539553	98.12%	OQ385711.1
15	Oeba 2b	*N. aurora*	OR539554	98.12%	OQ386196.1
16	Oeba 2c	*N. aurora*	OR539555	98.12%	OQ385815.1
17	Oeba 3a	*P. typus*	OR539556	100.00%	OQ387716.1
18	Oeba 3b	*P. typus*	OR539557	100.00%	OQ386445.1
19	Oeba 3c	*P. typus*	OR539558	100.00%	JN311852.1
20	Oeba 4d	*P. typus*	OR539559	100.00%	OQ387716.1
21	Oeba I	*N. hexodon*	OR539560	99.84%	OQ385999.1
22	Oeba II	*N. hexodon*	OR539561	99.84%	OQ386768.1
23	Oeba III	*N. japonicus*	OR539562	99.23%	EU871686.1
24	Oeba IV	*N. hexodon*	OR539563	99.84%	OQ386781.1

Genetic distances within the same species ranged from 0-0,8%. The lowest genetic distance was observed among samples of *N. zysron* and *P. typus*, while the highest was among *N. hexodon* samples (Table 2.). Lower genetic distances indicate closer genetic relationships and potential common ancestry, while higher genetic distances indicate more distant relationships (Dwifajri, 2022). As the genetic distance increases within the 0-0.8% range, it indicates a gradual accumulation of genetic differences among individuals or populations within the species. The higher the percentage, the greater the genetic divergence. A genetic distance of 0.8% does not necessarily mean that the individuals are different species. This result is in line with the journal on Caesio cuning fish where a genetic distance value of 1-3% is the same sepsis (Nursalim *et al*., 2020), where the sepsis limit is considered the same as a genetic difference value of 3% (Leray *et al*., 2013). It signifies a level of genetic diversity within the boundaries of a single species. Diverse populations or groups within a species can naturally accumulate genetic differences over time due to factors like geographic isolation, adaptation to local environments, or other evolutionary processes (Kawecki and Ebert, 2004; Rundle and Nosil, 2005; Charlesworth and Charlesworth, 2007; Hartl and Clark, 2007; Futuyma, 2009).

The observed genetic distances between species in the study revealed significant divergence, ranging from 11.6% to 23.5% (Table 2.); of particular note was the substantial genetic separation between *N. aurora* and *P. typus*, registering the highest distance at 23.5%. This finding highlighted a notable genetic differentiation between these two species, suggesting a considerable evolutionary divergence (Kimura, 1983; Nei, 1987). Such pronounced genetic distances indicate distinct lineages and imply prolonged periods of independent evolutionary trajectories. The elevated genetic divergence between *N. aurora* and *P. typus* could be attributed to various factors, including historical isolation, differing ecological niches, and potential reproductive barriers (Coyne and Orr, 2004). These results contribute valuable insights into the genetic landscape of these species, highlighting the intricate patterns of divergence that have evolved. Further exploration of

Table 2. Genetic distance between-within species

Species	Genetic distance within species	Genetic distance between species			
		N. hexodon	*N. japonicus*	*N. zysron*	*N. aurora*
N. hexodon	0.008				
N. japonicus	0.004	0.165			
N. zysron	0.000	0.201	0.116		
N. aurora	0.001	0.124	0.167	0.196	
P. typus	0.000	0.225	0.226	0.209	0.235

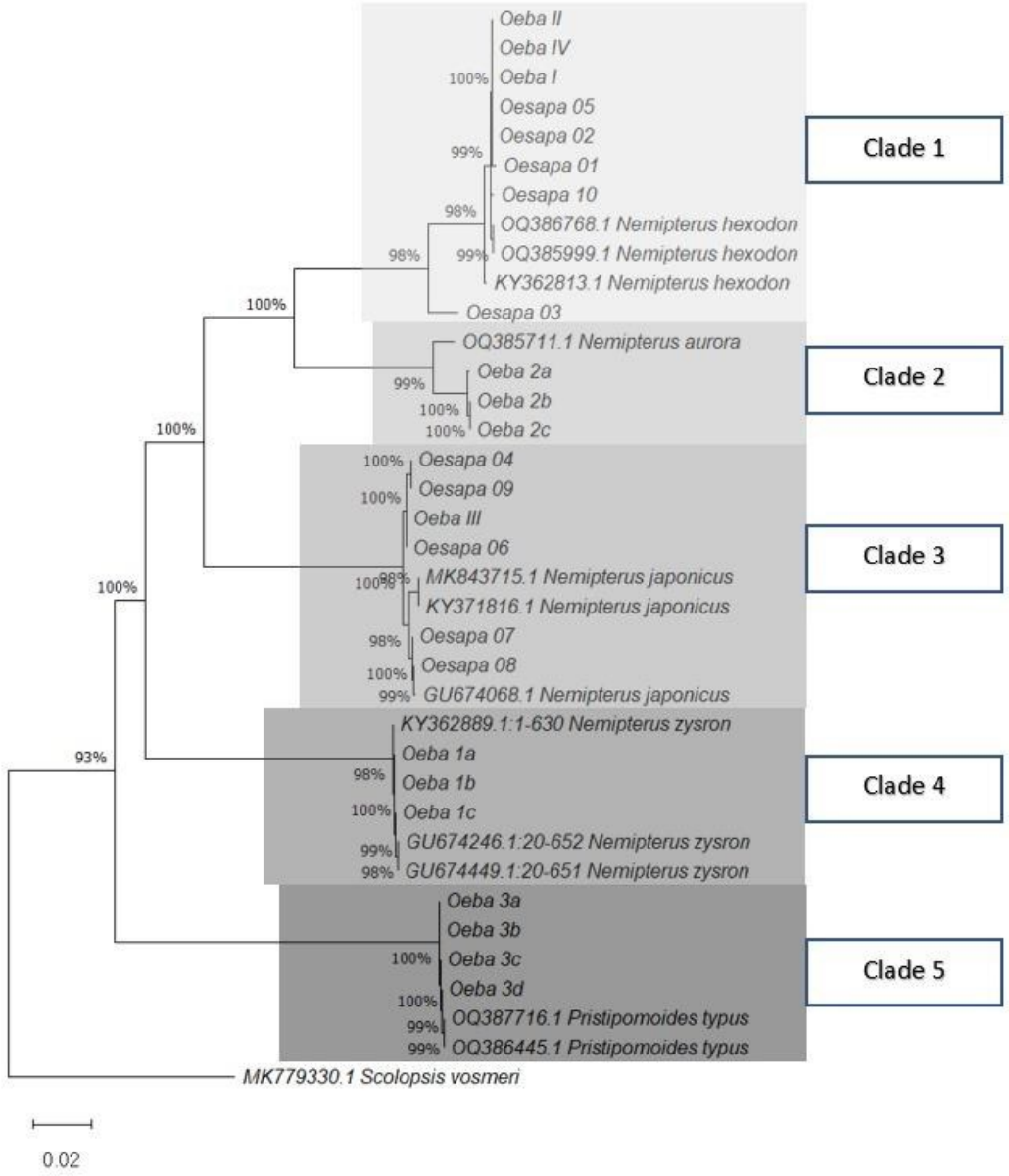

Figure 3. The phylogenetic tree was constructed using the Maximum Likelihood tree method with the Kimura-2 parameter model and subjected to 1000 bootstrap replicates.

the specific genetic markers and mechanisms driving these differences would offer a more comprehensive understanding of the evolutionary dynamics between *N. aurora* and *P. typus*.

Threadfin bream fish landed at the Oeba Fish Landing Site and Oesapa Fish Market, consisting of 10 haplotypes (Table 3.). There was a sharing of haplotypes between haplotype two and haplotype five

Table 3. Haplotype threadfin breams fish

Haplotype	Number of sample	Sample code
Nemipterus hexodon		
Hap-1	1	Oesapa 01
Hap-2	6	Oesapa 02, 05, 10, Oeba I, II, IV
Hap-3	1	Oesapa 03
Nemipterus japonicus		
Hap-1	2	Oesapa 04, 09
Hap-2	2	Oesapa 06, Oeba III
Hap-3	2	Oesapa 07, 08
Nemipterus zysron		
Hap-1	3	Oeba 1a, 1b, 1c
Nemipterus aurora		
Hap-1	1	Oeba 2a
Hap-2	2	Oeba 2b, 2c
Pristipomoides typus		
Hap-1	4	Oeba 3a, 3b, 3c, 3d

from both locations. Threadfin bream fish *N. hexodon* and *N. japonicus* have shared haplotypes even from these 2 locations. Meanwhile, *N. zysron*, *N. aurora* and *P. typus* share haplotypes in haplotypes 1, 2 and 3, and were only found in samples from Oeba Fish Landing.

Phylogenetic reconstruction revealed that *N. hexodon* from Oeba Fish Landing Site and Oesapa Fish Market were in the same clade as *N. hexodon* accession number OQ386768 from Philippine waters. *N. aurora* from Oesapa and Oeba were in the same clade as *N. aurora*, accession number OQ385711 from the Philippines. *N. japonicus* from Oeba and Oesapa were also in the same clade as *N. japonicus*, accession number MK843715 from waters in China. *N. zysron* from Oeba formed a clade with *N. zysron* accession number KY362889 from Indian waters. *P. typus* from Oeba was in the same clade as P. typus accession number OQ387716 from Philippine waters. This indicates that the clade is formed based on species similarity. The phylogenetic tree (Figure 3) demonstrated that *N. hexodon* and *N. japonicus* were in different clades. The phylogenetic tree is divided into five clades, each representing the same species. Ingroup and outgroup data were obtained from NCBI, and their addition to the phylogenetic tree aimed to polarize threadfin bream fish samples. The branches showed strong support with bootstrap values of 93-100% at each node, indicating a high confidence level in the constructed tree (Saleky and Dailami, 2021).

The research findings show that *N. hexodon* species landed at the Oeba Fish Landing Site share haplotypes with *N. hexodon* species from the Oesapa fish market. Similarly, *N. japonicus* from Oeba threadfin bream fish shares haplotypes with *N. japonicus* species from Oesapa Fish Market. Meanwhile, *N. zysron,* and *P. typus* only have one haplotype from Oeba. Furthermore, *N. aurora* is divided into two haplotypes and originates only from Oeba. The sharing of haplotypes in these fishes result from genetic sharing between the locations (Diaz-Ferguson, 2010). Based on the number of haplotypes formed, more threadfin bream fish are obtained from the Oeba Fish Landing Site than from the Oesapa fish market. This can happen because three species of kurisi fish *N. zysron, N. aurora,* and *P. typus* are only found in this location.

Conclusion

Five threadfin bream fish species were successfully identified in the Oeba Fish Landing Site and Oesapa Fish Market, namely *N. hexodon*, *N. japonicus*, *N. zysron*, *N. aurora*, and *P. typus*. The phylogenetic tree also indicates that these five species belong to separate clades, despite their morphological similarities. Sustainable fishing practices are crucial to protect genetic diversity in these species. Furthermore, in-depth analysis of genetic diversity and the environmental factors influencing these species warrants further research.

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