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Original Research Paper

First Report of Molecular Identification Based on Melanophores Pattern to Determine Species Composition of Nike Fish Schooling in Gorontalo Bay, Indonesia

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Abstract: This study aims to identify species that make up the schooling of nike fish in the waters of Gorontalo Bay using an approach to differences in melanophore patterns and molecular analysis with the CO1 gene. Initial identification was morphologically carried out based on differences in melanophore patterns found in individuals composing schooling of nike fish. Sampling was done randomly by picking up nike fish of approximately 100-150 grams from the catch of fishermen during the period of its appearance in Gorontalo Bay on October 5-11 2018. Samples **were** grouped into 5 groups, each of which **was** coded as N1, N2, N3 , N4, and N5. From each group of samples, each individual fish was taken and preserved in 70% alcohol for molecular analysis. The results showed that the nike school in Gorontalo Bay consisted of several species successfully identified, *Sicyopterus pugnans*, *S. cynocephalus*, *Belobranchus segura*, and *Bunaka gyrinoides*..

Keywords: *goby, melanophore, molecular, morphology, nike fish*

Introduction

Nike is the local name of small fish found in the sea and the estuary of Bone River, Gorontalo, Indonesia (Olii *et al*., 2017) and is one of the local mainstay fisheries resources that are relatively small in size compared to other types of fish. This type of fish is known as an unique sort of fish in the Gorontalo Bay. Presently, the fish is one of the economically valuable commodities that prompts high fishing intensity. Ellien & Keith (2016) also reported that in Reunion Island (Mascarene Archipelago, Indian Ocean), some amphidromous fish are targeted by traditional intensive fisheries, at their postlarva stage, when they migrate back to the river.

Besides having a relatively small size of around 2-4 cm, this sort of fish also only appears at certain times. Tantu (2001) mentioned that nike fish appear every latter period of lunar phase at the end of the night and its presence in the form of schooling which consists of two species, namely juvenile of *Awaous melanocephalus* (99%) and the rest are juvenile of *Eleotris fusca* which is a group of whitebite fish from the gobiidae family.

Larson (2001) suggests that gobiids are widespread throughout the world and are mostly found in marine estuary waters. Maie *et al*. (2009) added that fish from the gobi group in Hawaiian Waters lived and developed in marine which initially hatched their larvae in the next river. The larvae were brought into the sea by currents and then lived and developed over a period of time until they reached the juvenile phase, then it will return to its habitat in

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freshwater. Olii *et al*. (2017) stated that the distribution of nike fish in the waters of Gorontalo Bay tends to approach the estuary since its appearance until it disappears from the waters. Pasisingi & Abdullah (2017) states that the period of emergence of nike fish every month in Gorontalo Bay starts from the bay area moving towards the estuary.

Nike is a small, slightly whitish and transparent and scaly fish that forms schooling in the waters. Apparently, the schooling of fish is a group of fish consisting of the same species. McDowall (2009) suggests that schooling of gobi fish consists of different species. Gobiidae fish typically do not only consist of one type of juvenile or adult fish. After Tantu (2001) research on the species that make up the schooling of nike fish in Gorontalo waters has never been conducted again. Yamasaki *et al*. (2011) stated that small undeveloped gobi larvae could be distinguished from the melanophores arrangement.

A large number of species of gobiidae (Tracker & Roje, 2011) allows the existence of similarity or genetic proximity and this can enable the occurrence of natural fertilization. The life cycle passes through several environments that have the potential to change its morphology. In addition, fish that are morphologically dissimilar might still from the same species. In addition to morphological characters in fish, there are also genetic characters that provide genetic information (Purnama *et al*., 2019).

Along with the development of technology, the process of identifying species using morphological characters has limitations including being strongly influenced by environmental conditions, making them vulnerable to change. Identification of species based on genetic molecular character is considered more accurate because the gene is attached since a species is spawned from its parent. Information about genetic diversity can be obtained by analyzing genes that encode proteins from mitochondrial DNA (Purnama *et al*., 2019).

The use of mitochondrial DNA in the identification of genetically species has been carried out with various methods of identification and target genetic markers. The use of mitochondrial DNA has many advantages compared to the use of core DNA in the process of species identification. The advantages are seen from the number of copies obtained from one cell; small in size and haploid (only inherited by the female parent or maternal inheritance), so it does not allow recombination; evolve faster than core DNA, so it can differentiate to species level (Teletchea, 2009; Buklin *et al*., 2011).

One of the encoding genes in the mtDNA genome includes the cytochrome oxidase subunit 1 (CO1) gene. CO1 is the most conservative protein-coding gene in the animal mitochondrial genome (Brown, 1985 in Folmer *et al*.,1994). Hebert *et al*. (2003) stated that the CO1 gene can be used as DNA barcoding as it has many advantages including the possibility of deletion and insertion in small sequences and the number of conserve parts. Folmer *et al*. (1994) stated that primary CO1 produced an informative sequence for phylogenetic analysis in species with a higher taxonomic level.

Research on the study of the morphological and genetic characteristics of nike in Gorontalo Bay is deficient. Olii *et al*. (2019) in their recent study stated that nike and hundala fish supposedly as *Sicyopterus longifilis* which are considered as Bone River species and the estuary of Gorontalo Bay. Furthermore, Olii *et al*. (2019) stated that a more comprehensive analysis of other divergent gene sequences is needed to convince this species characterization.

Based on the previous description, this study aims to identify species that make up the schooling of nike fish in the sea waters of Gorontalo Bay using a molecular analysis approach with the CO1 gene. This study is accommodating in determining the characteristics of nike in the sea of Gorontalo Bay therefore management and sustainability can be easily carried out in nature. The results of this study can also contribute to the aquatic fauna biodiversity.

Materials and Methods

Sampling

This research was conducted from October 2018 to January 2019 as from the sampling of nike fish to molecular analysis. Sampling was carried out randomly by picking up nike fish of 100-150 grams from the catch of the fishermen at the fishing location in Leato waters during the appearance period on October 5 to 11 year 2018. Map of the sampling is shown in Fig.1. The samples were then examined at the Integrated Laboratory of the Faculty of Fisheries and Marine Sciences of Gorontalo State University by sorting and grouping based on differences in the composition of the melanophore patterns on the body. The grouping results consist of 5 sample groups which

then each group is coded as N1, N2, N3, N4, and N5. For molecular analysis purposes, each group of samples is taken from each group of 5 individuals and filled in a sample bottle and preserved in 70% alcohol. Subsequently, the samples were analyzed for genetic identification in Genetic Laboratory of Papua State University, Manokwari, Indonesia.

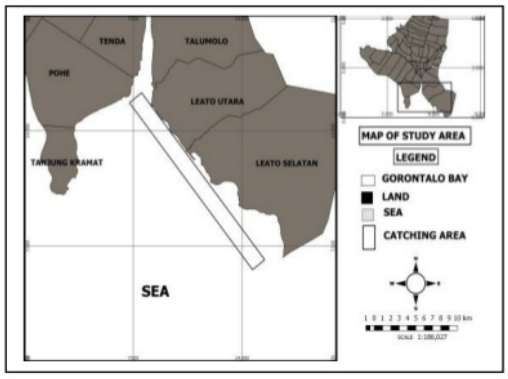

Fig.1: Map of the sampling site

Molecular Analysis

Isolation of the Genome DNA from the sample was carried out using an Isolation Kit, which was produced by Geneaid (Genomic DNA Mini Kit (Tissue)). Isolation method is supported in accordance with the Standard Protocol of the product. The PCR process is conducted using primary pairs referring to Baldwin *et al*. (2008) namely BCL Fish: 5'-TCAACYAATCAYAAAGATATYGGCAC - 3' (Forward) and BCH Fish: 5'-ACTTCYGGGTGRCCRAARAATCA - 3 ' (Reverse). PCR products are electrophoresed and the results are photographed above UV transilluminator (Pacific image, Electronic). The nucleotide sequencing cycle is a method for determining the sequence of nucleotides contained in DNA. DNA samples that have been amplified and electrophoresed are then sequenced. The sequencing process was carried out at the 1st Base Laboratory in Malaysia through PT Genetics Science Indonesia by sending samples consisting of 30 samples of PCR DNA products, forward 10 μl primers and 10 μl reverse primers. Editing and proofreading sequences are performed using MEGA 6.0 software.

Results

Melanophore Patterns of Nike Fish

Nike is a sort of fish that lives in groups. This fish is tiny and appears to consist of only one species in its cluster arrangement (Fig. 2).

Fig. 2: Nike fish group after fishing

However, in detail, the composition of the crowds consists of species that have almost the same shape but have different melanophore patterns. This study initiated the grouping of nike fish based on variances in melanophore patterns and genetically confirmed. Schematic results of melanophore patterns from five nike fish groups are shown in Table 2.

Genetic Identification of Nike

The genetic identification of five types of nike with divergent melanophore patterns formed four dissimilar species, namely N1 for *Sicyopterus pugnans*, N2 for *Sicyopterus cynocephalus*, N3 and N5 for *Belobranchus segura*, and N4 for *Bunaka gyri*noides. DNA Electrophoresis of mitochondrial DNA COI shown in Fig. 3, with amplicon lengths of ~ 600-700 bp.

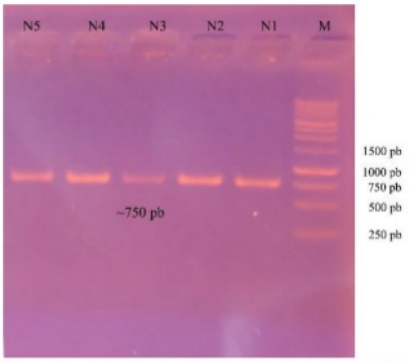

Fig. 3: Electrophoresis results of nike fish

The results of the comparison of genetic identification of each sample with the sequence of NCBI nucleotide databases are shown in Table 1, while the results of the identification of five varieties of nike with dissimilar melanophore patterns.

Table 1: Comparison of each sample sequence with NCBI nucleotide database sequences

No	Species	Gen	Accession Number	Max Score	Query cover	Identity
	N1 sequence					
1	*Sicyopterus pugnans*	COI	KJ202204.1	1199	100%	99%
2	*Sicyopterus pugnans*	COI	KF668861.1	1044	100%	95%
3	*Sicyopterus pugnans*	COI	HQ639045.1	1038	100%	95%
4	*Sicyopterus pugnans*	COI	JQ432155.1	1037	99%	95%
5	*Sicyopterus pugnans*	COI	HQ639044.1	1033	100%	95%
	N2 sequence					
1	*Sicyopterus cynocephalus*	COI	KU693015.1	1194	99%	99%
2	*Sicyopterus cynocephalus*	COI	KU693016.1	1177	99%	99%
3	*Sicyopterus lagocephalus*	COI	KU693030.1	1055	100%	96%
4	*Sicyopterus lagocephalus*	COI	KU693023.1	1055	100%	96%
5	*Sicyopterus lagocephalus*	COI	KF482068.1	1055	100%	96%
	N3 sequence					
1	*Belobranchus segura*	COI	KU692375.1	1166	99%	99%
2	*Belobranchus segura*	COI	KU692374.1	1166	99%	99%
3	*Belobranchus segura*	COI	KU692367.1	1166	99%	99%
4	*Belobranchus segura*	COI	KU692362.1	1166	99%	99%
5	*Belobranchus segura*	COI	KU692372.1	1160	99%	99%
	N4 sequence					
1	*Bunaka gyrinoides*	COI	KX095216.1	1164	96%	99%
2	*Belobranchus belobranchus*	COI	KU692351.1	726	99%	87%
3	*Hypseleotris galii*	COI	KJ669480.1	723	100%	87%
4	*Hypseleotris galii*	COI	KJ669479.1	723	99%	87%
5	*Belobranchus belobranchus*	COI	KU692352.1	721	99%	87%
	N5 sequence					
1	*Belobranchus segura*	COI	KU692375.1	1171	99%	99%
2	*Belobranchus segura*	COI	KU692374.1	1171	99%	99%
3	*Belobranchus segura*	COI	KU692367.1	1171	99%	99%
4	*Belobranchus segura*	COI	KU692362.1	1171	99%	99%
5	*Belobranchus segura*	COI	KU692372.1	1166	99%	99%

Table 1 shows that sequences of N3 and N5 have the same species as *Belobranchus segura*, while in Table 2 it is shown that samples N3 and N5 have diverse patterns. The connection between five types of nike with different melanophore patterns compared to some of the nike species available in the NCBI database is depicted in the phylogenetic tree on Figure 4.

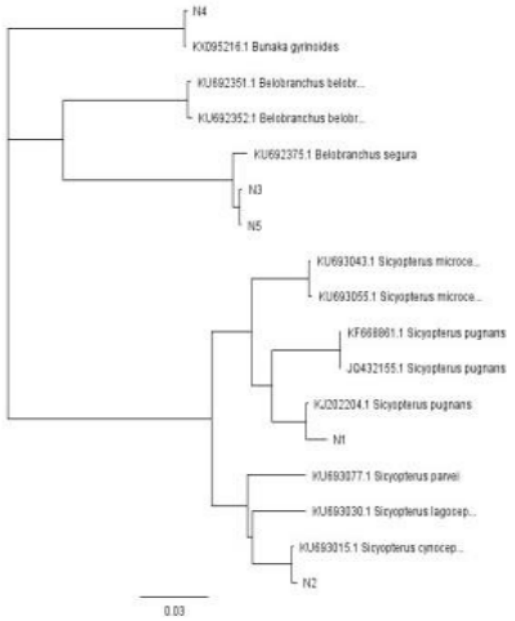

Fig. 4: Phylogenetic tree of Nike compared to some species in NCBI

The phylogenetic tree shows that the five types of nike are divided into three monophyletic clades specifically the first clade consists of *Bunaka gyrinoides* (N4), the second clade contains the genus *Belobranchus* namely *Belobranchus segura* (N3) and *Belobranchus belobranchusi* (N5), and the third clade comprises of the genus *Sicyopterus* namely *Sicyopterus pugnans* (N1), *Sicyopterus cynocephalus* (N2), and *Sicyopterus microcephalus*.

Discussion

The results of this study indicate that there are five groups (N1, N2, N3, N4, and N5) identified based on the different melonophore pattern approaches, but the results of molecular analysis merely display four species, namely *Sicyopterus pugnans, Sicyopterus cynocephalus, Belobranchus segura,* and *Bunaka gyrinoides*. Furthermore, *Sicyopterus longifilis* were not identified in this study although Olii *et al*. (2019) reported in previous study that nike fish is a larval stadia of "hundala" fish (*Sicyopterus longifilis*). There

are two things that might cause this results. Firstly, the difference in the time or period of sampling. Secondly, *Sicyopterus longifilis* probably does not appear at the time of sampling in this study. Olii *et al.* (2019) conducted their sampling in April 2018, while this study was carried out in October 2018.

Genetic identification of N3 and N5 using the NCBI nucleotide database sequence shows that they are the similar species as *Belobranchus segura*. Identification using morphological approach and the melanophore pattern displays that samples N3 and N5 have different appearance of melanophore patterns (Table 2). This dissimilarity might be affected by alterations of melanophore during the development of the larvae. N5 sample is the same species as N3 that has developed and has undergone changes in the melanophore structure. This is possible since N3 sample was taken on October 8, 2018, while N5 sample was found on October 11, 2018. Valade *et al.* (2009) described changes in the appearance of chromatophore in the larvae of *Sicyopterus langocephalus* which starts from the head area then spreads along the body alongside with the larvae age. In addition, N3 and N5 sampling locations were different, the N3 samples were taken in the sea, while the N5 samples were found in estuary area. Pasisingi & Abdullah (2017) describe that the period of emergence of nike fish monthly in Gorontalo Bay starts from the sea area and moves towards the estuary. Keith *et al.* (2008) stated that when entering a river mouth, postlarva undergoes alterations in morphology, physiology and behavior. Postlarvae returns to rivers where they recruit adult reproductive stages (Ellien, Werner, & Keith, 2016). This supports the results of the analysis that N5 is a developed larva of N3.

The categorization of nike fish based on the melanophore pattern for molecular identification purposes is problematic as this type of fish is still in the postlarva stage, hence it requires accuracy in visual observation. Genetic identification based on differences in melanophore patterns in nike and other fish larvae in Gorontalo Bay has never been done. Up to now, scientific information about identification morphometrically and molecularly has been widely published only in adult gobi fish, while for small size fish such as nike or other types of small fish have not been broadly published. Larson (2001) describes that the systematics and phylogeny of the gobi are acknowledged to be difficult and have been variously described by "infamous and unwieldy morass" (Springer, 1983), "chaotic" (Gosline, 1971), "insane world" (Birdsong *in* Winterbottom, 1984) and " troublesome" (Birdsong *et al.,* 1998) and part of the problem has been attributed to the tiny size of these fishes. Thacker (2003) reported that effort to hypothesize relationships among the gobioid groups have been hindered by the prevalence of reductive evolution among goby spesies; such reduction can make identification of informative morphological characters mostly problematic.

The results of this study have shown that the schooling of nike in Gorontalo Bay is not only composed of two species, namely *Awaous melanocephalus* and *Eleotris frusca* as has been suggested by Tantu (2001). There might be other numerous species making up nike schooling that have not been described as well as Olii *et al.* (2019) discovered that the nike fish in Gorontalo Bay are larvae of *Sicyopterus longifilis*. In addition, Tracker & Roje (2011) states that the gobiidae is one of the largest acanthomorph fish groups consisting of roughly 1120 species from 30 genera that have been described and many more that have not been described. Therefore, biologically and ecologically, this type of nike are essential to recognize in order to stock management and biodiversity interests.

Conclusion

Based on the results of this study we can conclude that the schooling of nike fish in Gorontalo Bay consists of several species and identified 4 species were *Sicyopterus pugnans, Sicyopterus cynocephalus, Belobranchus segura,* and *Bunaka gyrinoides*. To obtain more comprehensive results, further research is needed by taking into account the time and season of its appearance.

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