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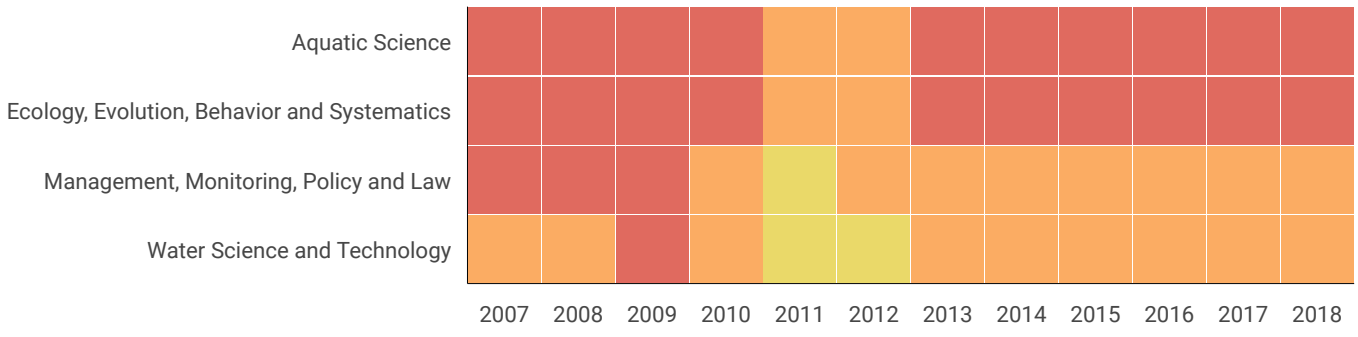

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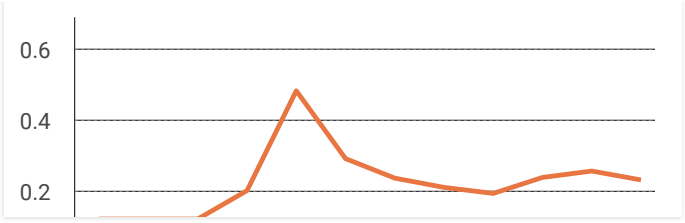

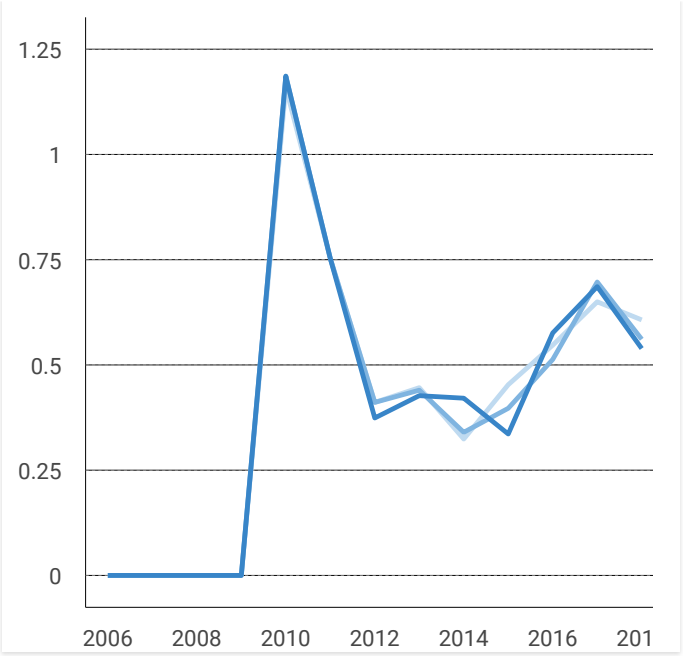

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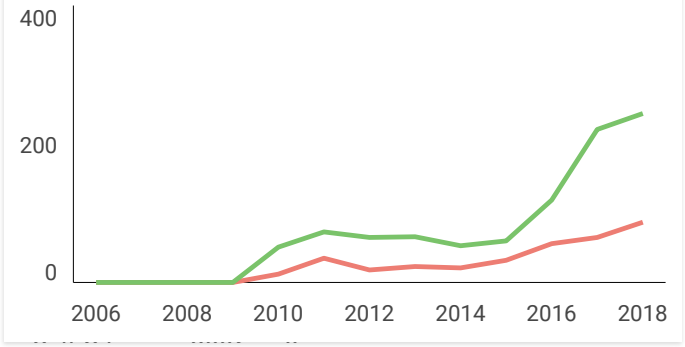

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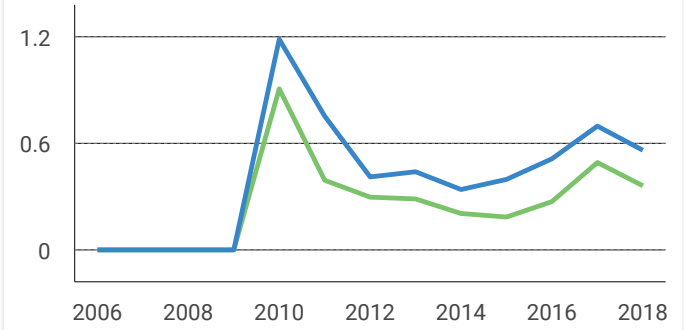

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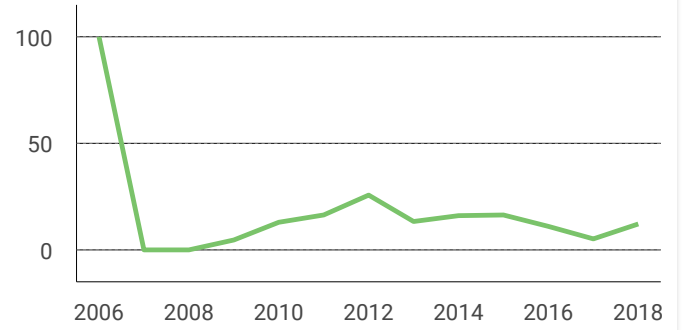

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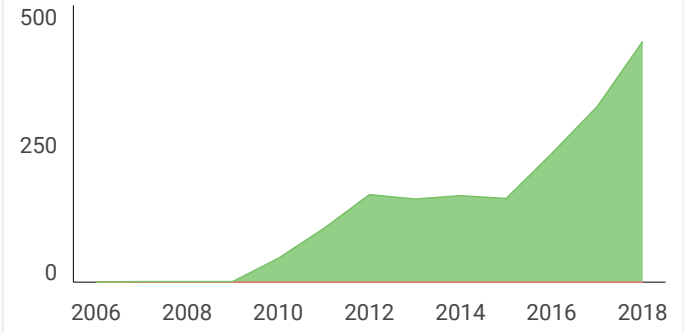

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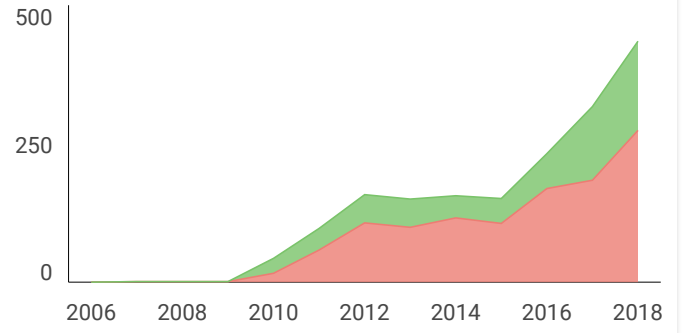

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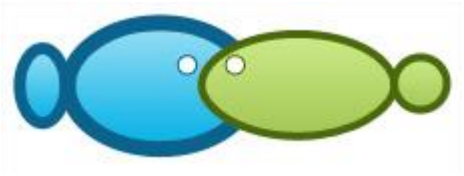

Determination of morphological alteration based on molecular analysis and melanophore pattern of the migrating Nike fish in Gorontalo Bay, Indonesia

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Abstract. Nike is a term for minuscule fish that frequently appear in Gorontalo waters. This type of fish belongs to the amphidromous goby group. This study aims to describe the morphological alterations of Nike fish from the sea to the estuary area of Gorontalo waters based on molecular analysis and different melanophore pattern. The small size of this fish (larval stage and post-larva) cause difficulties in analysing morphometric and meristic aspects. Therefore, the melanophore pattern approach and molecular analysis using the COI gene were used to identify and study the fish species. Sampling was carried out from 5 to 11 October 2018 in Gorontalo Bay area and the mouth of Bone Bolango river. The study was carried out when this type of fish appeared in the research area. Samples of Nike fish were grouped based on differences in melanophore patterns in the body. It was found there were five different groups. The groups were coded N1, N2, N3, N4 and N5. The molecular analysis using the COI gene was used to identify the fish species. Genetic investigations showed that from five groups, there were two groups (N3 and N5) that had different melanophore patterns, but belong to the same species, *Belobranchus segura*. Morphological changes have occurred due to an increase in the melanophore patterns of Nike species when they migrate from the sea to the river.
Key Words: amphidromus gobiidae, melanophore, molecular, Nike fish.

Introduction. Among the large range of the life history pattern of tropical fishes, about 200 species undertake diadromus migrations and the most common form of diadromy in the tropics is amphidromy (Milton 2009). Nike fish in this study is an amphidromous species alleged to be a typical species in the Gorontalo region. It is a small fish with a low frequency of occurrence in waters. A temporary assumption is that Nike is a type of fish that migrates for nursery and spawning purposes from seawater to freshwater (Olii et al 2017). Nike are usually 2 to 4 cm in length and appear in some waters in large numbers, being caught by local fishermen. Olii et al (2019) observed the morphological characteristics of some Nike fish, like a small body size, reaching only 3 cm, a transparent body color, no scales, incomplete fins and undeveloped mouth. These fish are not a daily occurrence in the sea throughout the year, but once a month they can be seen by fishermen. Their emergence usually occurs at the end of each month in the calendar of the Hijri year, corresponding to the appearance of the new moon. The duration of the appearance of this fish is not constant, ranging from three to seven days (Olii et al 2017; Pasisingi & Abdullah 2018).

Scientific information about the Nike fish is still scarce, and even less information is found for this fish in Gorontalo Bay, Indonesia. Thus far, information about the existence of the species has only been obtained informally, from the local community. Important aspects of the existence of the fish can be a source of information for preserving the biodiversity in the area. Kumar (2017) states that biodiversity provides ecological services to support human life and well-being.

Nike fish in Gorontalo waters consists of more than one species. Out of these, *Awaous melanocephalus* is the main constituent species, 99% of Nike fish, and *Eleotris frusca* is only a supporter species. Furthermore, the inhabited area is from the coast to the upstream of Bone River (Tantu 2001). *A. melanocephalus* is an amphidromous fish. When the adults spawn in freshwater, the eggs are placed on the riverbed substrate. After the eggs hatch, the larvae drift into the sea. After some time, the juveniles return to the river in which they hatched (Yamasaki et al 2011). Gobiids in Hawaiian waters live and develop in seawater. Initially, the larvae hatch in the river and afterwards they are carried into the sea by river currents, living and developing into juveniles. Then they migrate back to their habitat in freshwater (Maie et al 2009). The distribution of Nike fish larvae moving from seawater to river mouths is influenced by internal and external factors.

Gobiidae is one of the largest acanthomorph fish families, which numbers approximately 1120 species from 30 genera that have been described and many more that have not been yet described (Thacker & Roje 2011). A large number of gobiids present genetic similarities and can allow the occurrence of natural hybridization. The fish passes through several environments throughout its life history, allowing it morphological changes. This sometimes leads to morphological dissimilarities of the same species.

The high demand for these fish causes high fishing activities and exploitation. Wolok et al (2019) stated that within one year of fishing, the average fisherman captures 40 forty buckets of fish with an average selling price of 750000 IDR (50-55 USD) per bucket if sold on the market. So in one year of fishing, fishermen get 30000000 IDR (2100 USD). There has not been a proper management for this resource yet, partially due to lack of scientific data. Therefore, an improper management will potentially threaten the sustainability of Nike fish. The responses of organisms to their environments represent significant information in the effort to manage waters. The differences in the topography of Nike fishing areas might explain differences in adaptation, ecology and behaviour, and differences in colour and size. Due to their small size and often cryptic ecologies, the full extent of gobiid diversity goes unnoticed (Thacker & Roje 2011). Efforts to hypothesize relationships among gobioids have been hampered by the prevalence of reductive evolution among goby species. Such reduction can make the identification of informative morphological characters particularly difficult (Thacker 2003).

Molecular and morphological studies can help the determination of some characteristics of Nike fish in the Gorontalo Bay, making it easier to be managed. Genetic characters can provide fish genetic information and support morphological characters data (Purnama et al 2019). This study aims to determine morphological changes based on molecular analysis and melanophore patterns along the body of Nike fish that migrate from the sea to the estuaries in Gorontalo waters, using molecular analysis.

Material and Method

Sampling. The study was conducted from October 2018 to January 2019. The sampling of fish from fishmen was carried out randomly from 5 to 11 October 2018, in Leato waters. The sampling location is presented in Figure 1. The collected samples were placed in plastic sterile containers and into an icebox. The samples were sorted and grouped according to the melanophore pattern on the body of the fish at the Integrated Laboratory of the Faculty of Fisheries and Marine Sciences, Gorontalo State University. Samples of Nike fish were sorted in five different groups, coded N1, N2, N3, N4 and N5. From each group of fish, 5 individuals were selected and preserved in sample containers with 70% alcohol. Furthermore, samples were analysed for genetic identification in the Papua State University genetic laboratory, Manokwari. The small size of this fish (larval stage/post-larva) causes difficulties in determining morphometric and meristic aspects. Therefore, the melanophore pattern approach and molecular analysis using the COI gene were used to identify the fish species.

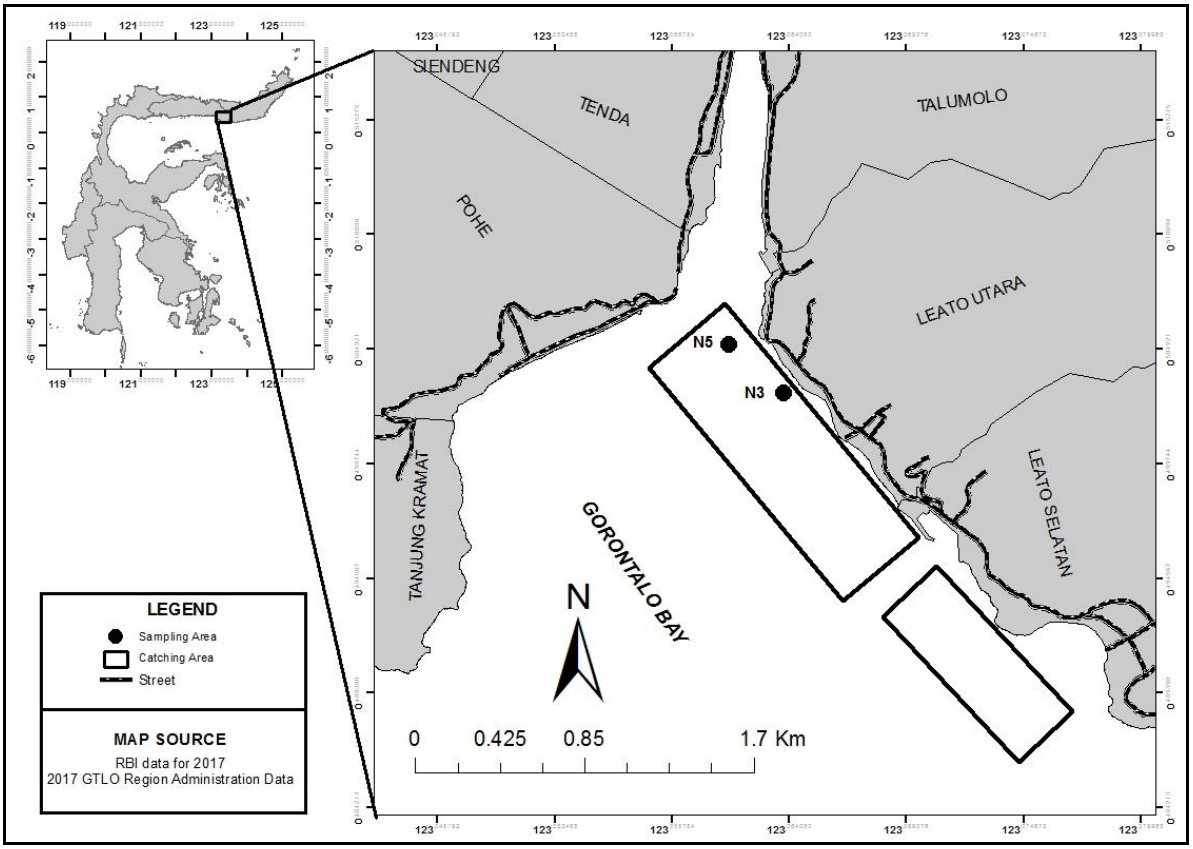

Figure 1. Map of the sampling sites in Gorontalo Bay.

Molecular Analysis. DNA genome isolation of samples was conducted using an Isolation Kit by Geneaid - Genomic DNA Mini Kit (Tissue). The isolation method carried out refers to the product standard protocol. The PCR process was carried out using the primary pair (Baldwin et al 2009), namely (Forward) BCL Fish: 5'-TCAACYAATCAYAAAGATATYGGCAC-3' and (Reverse) Fish BCH: 5'-ACTTCYGGGTGRCCRAARAATCA-3'. PCR products were then electrophoresed and photographed above an UV Transilluminator (Pacific image, Electronic). The nucleotide sequencing cycle is a method for determining the sequence of nucleotides contained in DNA. The DNA samples that had been amplified and electrophoresed were subsequently sequenced. The sequencing process was carried out at the First Base Laboratory in Malaysia by PT Genetics Science Indonesia. Samples consisting of 30 µL of PCR DNA products, 10 µL of forward primer and 10 µL of reverse primer were sent to the laboratory. Editing and proofreading sequences were performed using MEGA 6.0 software. The proofreading results from the forward and reverse sequences were combined into a sequence. Then the sequence results were analysed to find genetic similarities. To find out the relationship level among samples, further analyses were carried out based on phylogenetic trees with the Maximum Likelihood Method with 1000 bootstraps using MEGA 6.0 software.

Results and Discussion. The results indicate that Nike fish sampled consist of 2 morphologically dissimilar groups, with different melanophore patterns, but both groups are genetically the same species. The proofreading results from the forward and reserve sequences combined with the sequence of the two samples (N3 and N5) are presented in Table 1.

Table 1

Proofreading results of forward and reserve sequence of samples N3 and N5

Sample code	*Proof reading results*
N3	CCTTTATCTTGTCTTCGGTGCCTGAGCCGGGATAGTGGGCACCGCTTTAAGCCT ACTTATCCGCGCTGAACTAAGTCAACCTGGCGCACTCCTAGGAGATGACCAAAT CTATAATGTTATCGTTACCGCCCACGCGTTCGTAATAATTTTCTTTATAGTAATAC CAATTATGATTGGCGGATTTGGTAACTGACTAATCCCCTTAATGATTGGCGCCCC AGACATGGCCTTCCCACGAATAAACAACATAAGTTTCTGACTTCTCCCGCCATCT TTCCTCCTCCTTTTAGCATCCTCTGGAGTAGAAGCAGGGGCCGGAACAGGGTGA ACCGTCTACCCGCCCCTAGCGGGCAACCTCGCCCACGCAGGCGCCTCTGTGGA CCTAACAATCTTTTCACTACACCTAGCAGGGGTGTCCTCAATTCTTGGAGCAATT AATTTCATTACCACAATTATTAACATAAAACCTCCGGCAATTTCCCAATACCAAAC GCCCTTGTTCGTCTGAGCCGTTCTAATTACAGCCGTCTTATTACTATTATCCCTTC CCGTACTTGCTGCTGGCATCACAATGCTACTTACAGATCGAAATTTAAATACGAC GTTCTTTGACCCGGCCGGGGGGTGGGGACCCAATCTTATACCAACACCTTTTC
N5	CCTTTATCTTGTCTTCGGTGCCTGAGCCGGGATAGTGGGCACAGCTTTAAGCCT ACTTATCCGCGCTGAACTAAGTCAACCTGGCGCACTCCTAGGAGATGACCAAAT CTATAATGTTATCGTTACCGCCCACGCGTTCGTAATAATTTTCTTTATAGTAATAC CAATTATGATTGGCGGATTTGGTAACTGACTAATCCCCTTAATGATTGGCGCCCC AGACATGGCCTTCCCACGAATAAACAACATAAGTTTCTGACTTCTCCCGCCATCT TTCCTCCTCCTTTTAGCATCCTCTGGAGTAGAAGCAGGGGCCGGAACAGGGTGA ACCGTCTACCCGCCCCTAGCGGGCAACCTCGCCCACGCAGGCGCCTCTGTGGA CCTAACAATCTTTTCACTACACCTAGCAGGGGTGTCCTCAATTCTTGGAGCAATT AATTTCATTACCACAATTATTAACATAAAACCTCCGGCAATTTCCCAATACCAAAC GCCCTTGTTCGTCTGAGCCGTTCTAATTACAGCCGTCTTATTACTATTATCCCTTC CCGTACTTGCTGCTGGCATCACAATGCTACTTACAGATCGAAATTTAAATACGAC GTTCTTTGACCCGGCCGGGGGTGGGGACCCAATCTTATACCAACACCTTTTC

The sequences produced are then compared with sequences contained in bank gene deposits (NCBI nucleotide databases). The results are presented in Table 2.

Table 2

Comparison sequences of sample and NCBI nucleotide database

No.	*Species*	*Gene*	*Accession Number*	*Max Score*	*Query cover*	*Identity*
			Sequence N3:			
1	*Belobranchus segura*	COI	KU692375.1	1166	99%	99%
2	*B. segura*	COI	KU692374.1	1166	99%	99%
3	*B. segura*	COI	KU692367.1	1166	99%	99%
4	*B. segura*	COI	KU692362.1	1166	99%	99%
5	*B. segura*	COI	KU692372.1	1160	99%	99%
			Sequence N5:			
1	*Belobranchus segura*	COI	KU692375.1	1171	99%	99%
2	*B. segura*	COI	KU692374.1	1171	99%	99%
3	*B. segura*	COI	KU692367.1	1171	99%	99%
4	*B. segura*	COI	KU692362.1	1171	99%	99%
5	*B. segura*	COI	KU692372.1	1166	99%	99%

The two samples are both *Belobranchus segura* based on genetic testing with mitochondrial COI. This is a new discovery related to Nike fish data. The previous studies never reported that *B. Segura* is a member species of the Nike group schooling in Gorontalo waters. Further analysis was carried out with the phylogenetic tree to show the kinship relations between samples (N3 and N5) and several species available in the NCBI database (Figure 2).

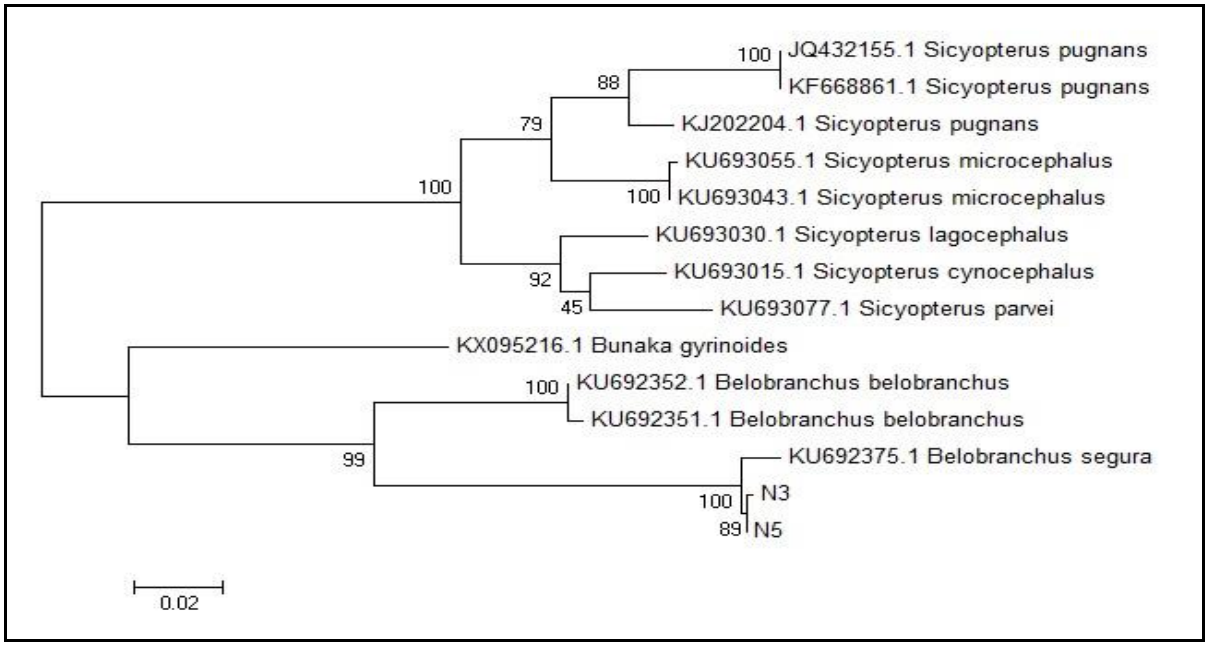

Figure 2. Phylogenetic trees of N3 and N5 samples compared with several species in the NCBI database.

The genetic analysis illustrates that the two samples are the same species, even though they have different morphologies, particularly in the melanophore patterns. This modification might be an adaptation of the species when migrating from seawater to fresh water, as part of its development. When entering a river mouth, the juvenile undergoes changes in morphology, physiology and behavior (Keith et al 2008). The differences in melanophore patterns from samples N3 and N5 is schematically presented in Figure 3.

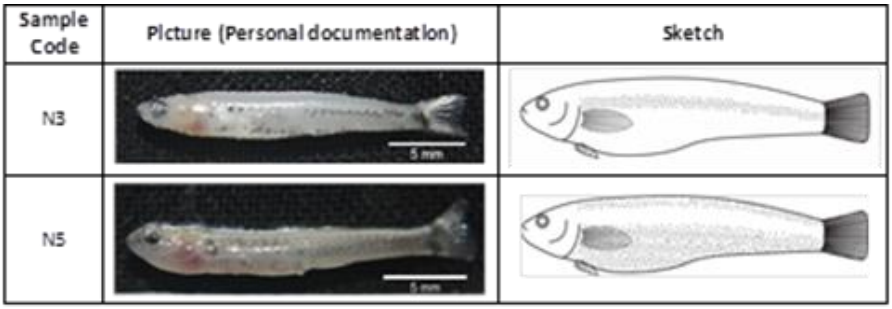

Figure 3. Schematic morphological alterations in N3 and N5 samples, based on the melanophore pattern.

In addition, geographically, N3 and N5 are found in different locations. N3 (N 00°30,122' E 123°03,895'26.2) was found in the sea area, while N5 (N 00°30,305' E123° 03,739'26.2) was found at the mouth of Bone River (Figure 1). Sample N5 is former N3 that has developed and has undergone alterations in the melanophore structure. This is possible because the N3 samples were collected in 8 October 2018, while the N5 samples were collected in 11 October 2018. This supports the findings of Valade et al (2009), which observed a change in the appearance of chromatophores in the body of *Sicyopterus langocephalus* larvae, starting from the head and spreading throughout the body during the larvae stage. The results of this study illustrate that there has been a variation in the melanophore pattern of the species *Belobranchus segura* when migrating from sea to river. When still in the sea, the melanophore arrangement is not yet dispersed, but when it enters the river, the melanophore arrangement spreads along the body.

Hawaiian gobioid fish are amphidromous and have one life stage in the sea (Hobson et al 2007). They stay in the pelagic sea zone for several months before migrating in the river (Teichert et al 2016). Nike fish has a tendency to shift closer to the river mouth in the days after its appearance in the Gorontalo Bay, which indicates the migration of Nike from sea water to fresh water (Olii et al 2017; Pasisingi & Abdullah 2018).

Melanophore patterns of *Belobranchus segura* migrating from sea to rivers are characterized by a slight increase in coloration. This is visible when approaching the estuary, being either a form of adaptation in order to enter a new aquatic environment or a part of its development stages.

The occurrence of migratory behavior has a genetic basis in freshwater fish, although it is clear from various studies that genetic signals for migratory behavior may be strongly influenced by environmental and developmental factors (Lucas et al 2001). Although the life of amphidromous fish is strongly related to environmental conditions, they vary in ecology and behavior and the causative factors that drive juveniles to move upstream into the adult habitat are not fully understood. This migration could be related to the development stage (Fitzsimons et al 2007). Milton (2009) informs that amphidromous fishes spawn in freshwater and the larvae migrate to the sea, then back to freshwater. *Sicyopterus lagocephalus* as an amphidromous goby species, meets drastic changes of habitat to fulfil its life cycle. Adults live and spawn in rivers, where eggs hatch into larvae that reach the sea. Post-larvae return to rivers where they are recruited and grown to reproductive stages (Ellien et al 2014). The distribution of the species along the river is determined by post larva color aggregation (Nishimoto & Fitzsimons 1986).

Conclusions. Samples N3 and N5, with different melanophore patterns, are genetically the same species, *Belobranchus segura*. The morphological changes of this species from the Nike fish group are indicated by the differences in melanophore patterns, with an increase in the number and spread of melanophores on the surface of the body when the species enters the river mouth. Also, Nike fish do not disappear from the waters, as the Nike fishing community considers.

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