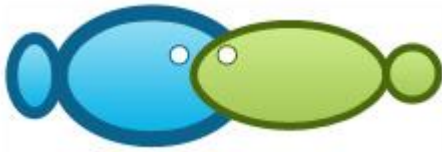Gambar 1. Submit Awal ke Jurnal AACL Bioflux

Determination of Morphological Alteration Based on Molecular Analysis and Melanophore Pattern of Nike Fish from Sea to Estuary Area of Gorontalo Bay, Indonesia

Femy M. Sahami, Rene C. Kepel[2], Abdul H. Olii[1]; Silvester B. Pratasik[2]

[2]Department of Aqutic Resources Management, Faculty of Fisheris and Marine Science, Gorontalo State University, Jl. Jendral Sudirman. No. 6, Gorontalo City, Gorontalo Province, Indonesia; [2]Faculty of Fisheries and Marine Science, Sam Ratulangi University, Jl. Kampus Unsrat Bahu, Manado City, 95115, North Sulawesi Province, Indonesia. Corresponding author: F. M. Sahami, femysahami@yahoo.co.id

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 of melanophore pattern. The small size of this fish (larval stage/post-larva) causes it be tough to analyze morphometric and meristic aspects. Therefore, the melanophore pattern approach and molecular analysis using the CO1 gene were used to identify these fish species. Sampling was carried out on 5 to 11 October 2018 in the Gorontalo Bay area to the mouth of Bone Bolango river when this type of fish appeared. Samples of nike fish were grouped based on differences in melanophore patterns approach in the body and we found there were five different groups and coded N1, N2, N3, N4, and N5. The molecular analysis using the CO1 gene were used to identify these fish species. Genetic investigation showed that from five groups of samples, there were found two groups of samples identified (N3 and N5) that had g different melanophore patterns were the same species, *Belobranchus segura*. Morphological changes have occurred due to an increase in the melanofor pattern of nike species when they migrate from the sea to the river mouth.
Key Words: melanophore, molecular, amphidromus gobie, nike fish

Introduction. Nike is the name of a small type of fish that is around 2-4 cm at length which appears in the waters in a large number carried out by Gorontalo people and its surroundings. These fish do not appear every day in the sea throughout the year but only once a month. Its emergence is unpredicted usually occurs at the end of each month in the calendar of the Hijri year or in the new moon time. The duration of the appearance of this fish at the location of its arrest is also not fixed, which is around three until seven days per the appearance period.

Scientific information about this nike is still not widely publicized. Scientific studies of this type of fish in Gorontalo waters are still rare. Thus far, information about the existence of these fish species has only been obtained from the community. Important aspects of the existence of these fish species can be a source of biodiversity whose existence needs to be preserved.

Nike in Gorontalo waters consists of two species, namely *Awaous melanocephalus* and *Eleotris frusca*. Among the schooling, *A. melanocephalus* is the main constituent species that is 99%, while *E. frusca* is only a supporter species. Furthermore, the inhabited area is from the coast to the upstream (Tantu 2001). *A. melanocephalus* is an amphidromous fish. When these adults spawn in freshwater, the eggs are placed on the substrate at the bottom of the water, after the eggs hatch the larvae drift into the sea, then after a while in the waters, the juvenile returns to the river where the parent originates (Yamasaki et al 2011). Fish from the gobii group in Hawaiian waters lived and developed in the waters of the sea, initially the fish hatched larvae in the river, and by river currents, the larvae were carried into the sea, living and developing into juveniles, then back to their habitat in freshwater (Maie et al 2009). The

distribution of nike fish larvae that move from sea waters to river mouths is influenced by internal factors and external factors.

Gobiidae is one of the largest acanthomorph fish groups, which number approximately 1120 species from 30 genera that have been described and many more that have not been described (Thacker & Roje 2011). A large amount of gobi fish causes genetic similarity and can allow the occurrence of fertilization naturally. This fish in its life cycle passes through several environments that can allow morphological changes, but might be morphologically dissimilar but still the same species.

The high demand for these fish causes high fishing activity and exploitation. And the improper management will accelerate extinction as well. All waters cannot be managed directly (Sahami et al 2017). The response of organisms to the environment becomes significant information in an effort to manage waters. The differences in the topography of Nike fishing grounds can allow for differences in adaptation and ecology and behavior that may be expressed through changes in color and size. Due to their small size and often cryptic ecologies, the full extent of gobiid diversity often goes unnoticed (Thacker & Roje 2011). An effort to hypothesize relationships among the gobioid groups have been hampered by the prevalence of reductive evolution among goby species, such reduction can make identification of informative morphological characters particularly difficult (Thacker 2003).

Molecular and morphological studies can help determination of characteristics of nike fish in the Gorontalo Bay, making it easier for management. 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 moleculer analysis and melanophore patterns along the body of nike fish that migrate from the sea to estuaries in Gorontalo waters using molecular analysis.

Material and Method

Sampling. The study started from October 2018 to January 2019. The sampling of these fish from fishmen was carried out randomly during the period of capture in Leato waters from 5 to 11 October 2018. A map of the sampling location is shown in Fig.1. Sampling was carried out at the Integrated Laboratory of the Faculty of Fisheries and Marine Sciences, Gorontalo State University by sorting and grouping it according to the appearance of the melanophore pattern on the fish's body. Samples of nike fish were grouped based on differences in melanophore patterns in the body and we found there were five different groups and coded N1, N2, N3, N4, and N5. From each group of fish, 5 individuals were taken and filled in the sample bottles which were preserved in 70% alcohol. Furthermore, samples were analyzed for genetic identification in the Papua State University genetic laboratory, Manokwari. The small size of this fish (larval stage/post-larva) causes it be tough to analyze morphometric and meristic aspects. Therefore, the melanophore pattern approach and molecular analysis using the CO1 gene were used to identify these fish species.
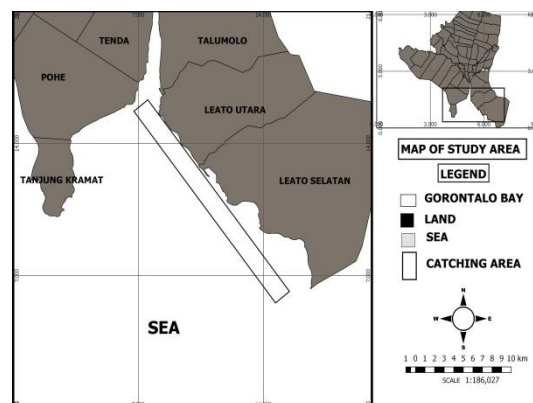

Figure 1. Map of Sampling Site in Gorontalo Bay

Molecular Analysis. DNA genome isolation of samples was conducted using an Isolation kit, which was produced 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 2008), namely (Forward) BCL Fish: 5 '-TCAACYAATCAYAAAGATATYGGCAC-3' and (Reverse) Fish BCH: 5 '-ACTTCYGGGTGRCCRAARAATCA-3'. PCR products were then electrophoresed and photographed above 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 through PT Genetics Science Indonesia by sending samples consisting of 30 µl of PCR DNA products, 10 µl of forward primer and 10 µl reverse primer. 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 analyzed to see samples that had genetic similarity. To find out the relationship level between samples, further analysis was carried out using phylogenetic trees using Maximum Likelihood Method with 1000 bootstraps using MEGA 6.0 software.

Results and Discussion.

The results indicate that nike fish schooling consists of 2 groups which morphologically are dissimilar and have different melanophore pattern yet genetically are the same species. The proofreading results from the forward and reserve sequences which were 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	CCTTTATCTTGTCTTCGGTGCCTGAGCCGGGATAGTGGGCACCGCTTTAAGC CTACTTATCCGCGCTGAACTAAGTCAACCTGGCGCACTCCTAGGAGATGACC AAATCTATAATGTTATCGTTACCGCCCACGCGTTCGTAATAATTTTCTTTATAG TAATACCAATTATGATTGGCGGATTTGGTAACTGACTAATCCCCTTAATGATT GGCGCCCCAGACATGGCCTTCCCACGAATAAACAACATAAGTTTCTGACTTC TCCCGCCATCTTTCCTCCTCCTTTTAGCATCCTCTGGAGTAGAAGCAGGGGC CGGAACAGGGTGAACCGTCTACCCGCCCCTAGCGGGCAACCTCGCCCACGC AGGCGCCTCTGTGGACCTAACAATCTTTTCACTACACCTAGCAGGGGTGTCC TCAATTCTTGGAGCAATTAATTTCATTACCACAATTATTAACATAAAACCTCCG GCAATTTCCCAATACCAAACGCCCTTGTTCGTCTGAGCCGTTCTAATTACAGC CGTCTTATTACTATTATCCCTTCCCGTACTTGCTGCTGGCATCACAATGCTACT TACAGATCGAAATTTAAATACGACGTTCTTTGACCCGGCCGGGGGTGGGGAC CCAATCTTATACCAACACCTTTTC
N5	CCTTTATCTTGTCTTCGGTGCCTGAGCCGGGATAGTGGGCACAGCTTTAAGC CTACTTATCCGCGCTGAACTAAGTCAACCTGGCGCACTCCTAGGAGATGACC AAATCTATAATGTTATCGTTACCGCCCACGCGTTCGTAATAATTTTCTTTATAG TAATACCAATTATGATTGGCGGATTTGGTAACTGACTAATCCCCTTAATGATT GGCGCCCCAGACATGGCCTTCCCACGAATAAACAACATAAGTTTCTGACTTC TCCCGCCATCTTTCCTCCTCCTTTTAGCATCCTCTGGAGTAGAAGCAGGGGC CGGAACAGGGTGAACCGTCTACCCGCCCCTAGCGGGCAACCTCGCCCACGC AGGCGCCTCTGTGGACCTAACAATCTTTTCACTACACCTAGCAGGGGTGTCC TCAATTCTTGGAGCAATTAATTTCATTACCACAATTATTAACATAAAACCTCCG GCAATTTCCCAATACCAAACGCCCTTGTTCGTCTGAGCCGTTCTAATTACAGC CGTCTTATTACTATTATCCCTTCCCGTACTTGCTGCTGGCATCACAATGCTACT TACAGATCGAAATTTAAATACGACGTTCTTTGACCCGGCCGGGGGTGGGGAC CCAATCTTATACCAACACCTTTTC

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	Gen	Accession Number	Max Score	Query cover	Identity
1	Sequence N3: *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%
1	Sequence N5: *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%

Table 2 shows that the two samples based on genetic testing with mitochondrial CO1 are the same species as *Belobranchus segura*. Further analysis was carried out with the phylogenetic tree to show the kinship relationships between samples (N3 and N5) and several species available in the NCBI database as presented in Fig.2.
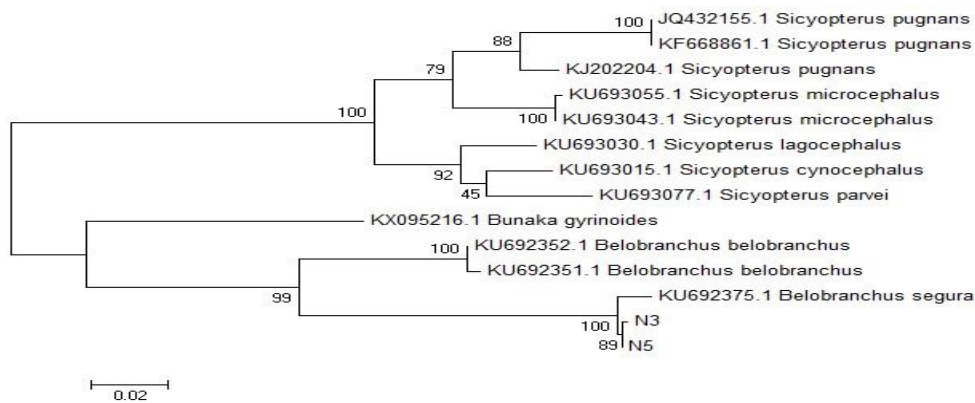

Figure 2. Phylogenetic trees of N3 and N5 samples were compared with several species in the NCBI database

The genetic analysis illustrates that the two samples are similar species although they had different morphology particularly the melanophore patterns. This modification might be an adaptation of species when migrating from sea water to fresh water which is part of its life development. When entering a river mouth, post-larva undergoes changes in morphology, physiology, and behavior (Keith et al 2008). The difference in melanophore patterns from samples N3 and N5 is schematically presented in Figure 3.

Sample Code	Picture (Personal documentation)	Sketch
N3		
N5		

Fig.3. Schematic morphological alterations in the samples N3 and N5 based on the shape of the melanofor 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 the river (Fig. 4). Sample N5 is N3 that has developed and has undergone alterations in the melanophore structure. This can be possible because the N3 sample was taken from the catch on 8 October 2018, while the N5 sample was found in the sample conducted on 11 October 2018. This supports Valade et al (2009) statement that there has been a change in the appearance of chromatophore in the body *Sicyopterus langocephalus* larvae, which starts from the head area then spreads throughout the body along with the larvae age. The results of this study illustrate that there has been a variation in the pattern of melanophore from the species of nike *Belobranchus segura* when migrating from sea water to

the mouth of the river. When still in the sea the melanophore arrangement is still minor, but when it will enter the river mouth the melanophore arrangement has spread along its body.
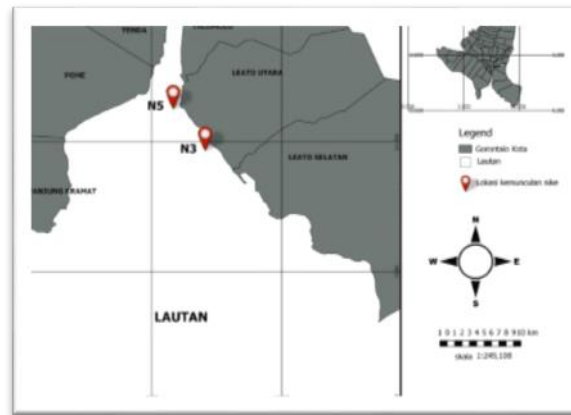

Figure 4. Area sampling of N3 and N5

Hawaiian gobioid fish are amphidromous and have one phase of life in the sea (Hobson & Smith 2007) They are in the pelagic phase of the sea for several months before juveniles in the river (Teichert et al 2016). Nike fishing in the Gorontalo Bay from the first, second and third days has a tendency to shift closer to the river mouth which indicates the migration of nike from sea water to fresh water (Olii et al 2017, Pasisingi & Abdullah 2018).

Changes in color morphology or melanophore patterns of nike species *Belobranchus segura* from the sea with melanophore content which becomes slightly more when approaching the estuary is a form of adaptation in order to enter a new aquatic environment or is part of the stages of its development. Distribution of species along the river is determined by postlarva color aggregation (Nishimoto & Fitzsimons 1986)

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 & Baras 2001). Although the lives of amphidromous fish are strongly related to the conditions of the oceans and rivers, 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 is related to their phase of development (Fitzsimon & McRae 2007). Post-larvae return to rivers where they are recruited and grown to reproductive stages (Ellien et al 2016).

The distribution of nike fish tends to approach the estuary since its appearance time until it disappears (Olii et al 2017). Based on the results of this study it can be concluded that in fact, nike fish do not disappear from the waters as what nike fishing community consider. There had been a morphological alteration in color due to an increase in the number of melanophores.
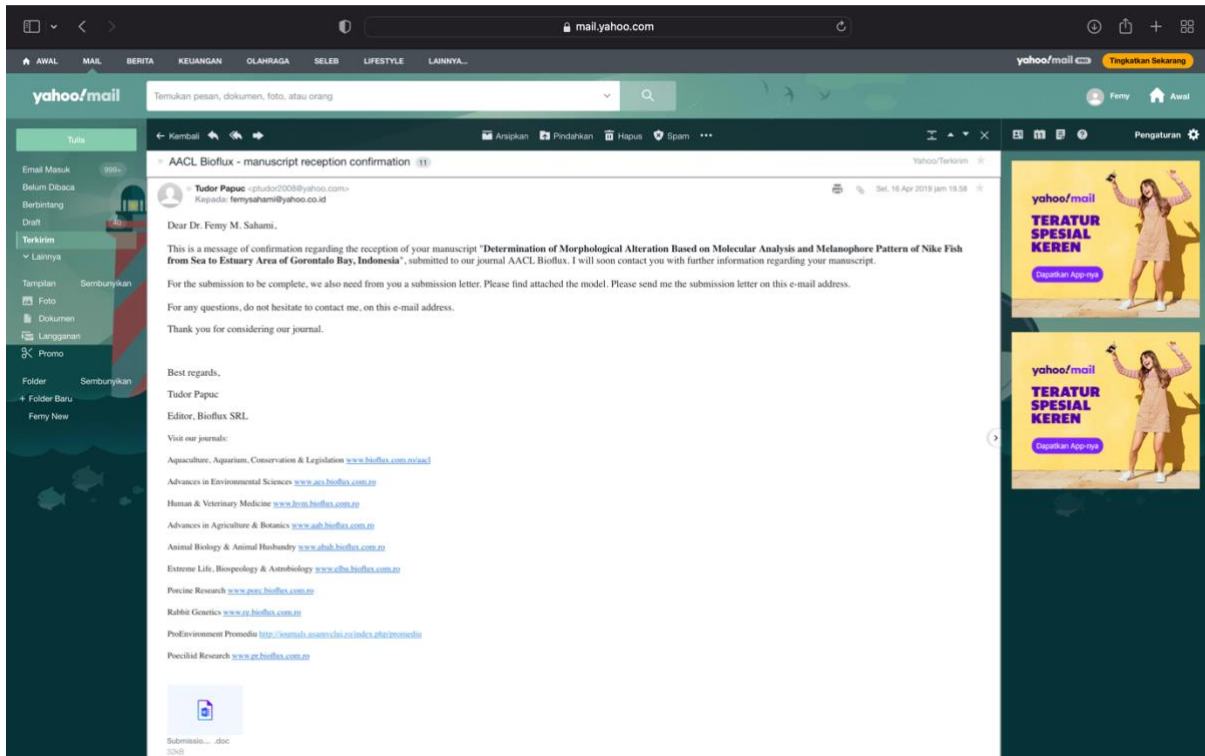
Conclusions. In conclusion, samples N3 and N5 that have different melanophore patterns are genetically the same species, *Belobrancianus segura*. The morphological changes of nike fish of this species are indicated by differences in melanophore patterns, that was an increase in the number and spread of melanophore on the surface of the body when this species has entered the river mouth.
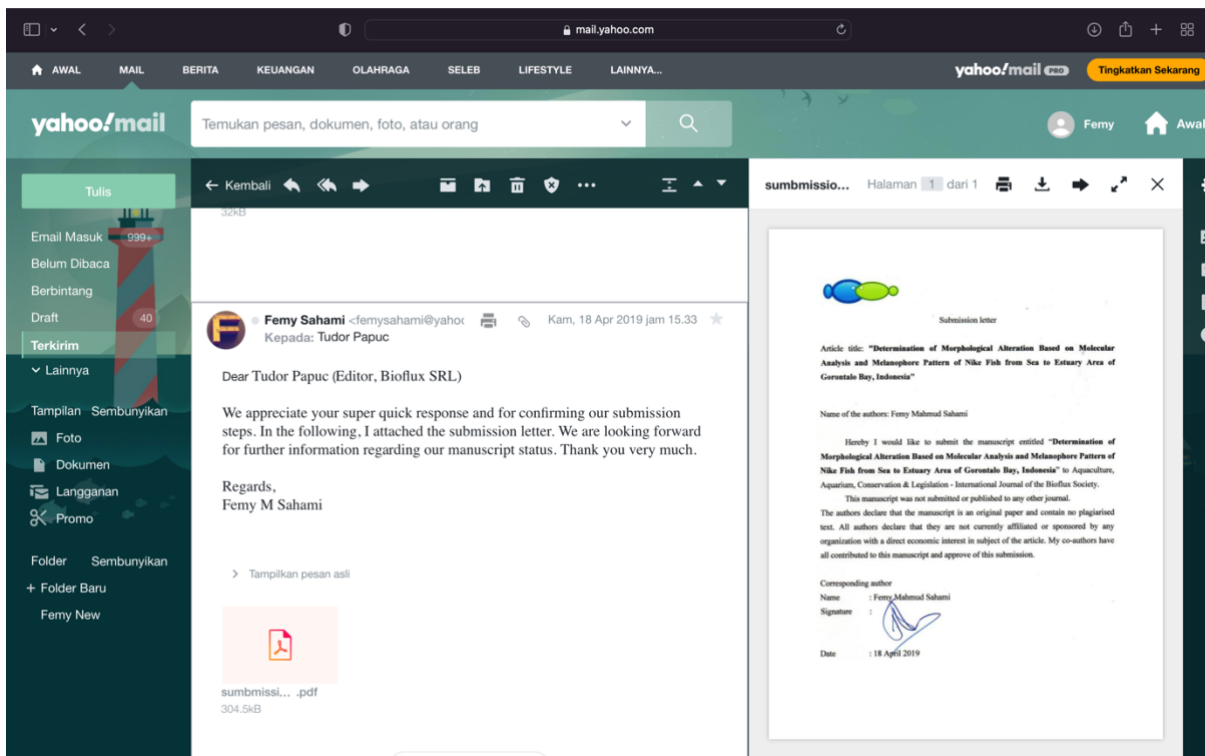
References
Baldwin C.C., Mounts J.H., Smith D.G., Weigt L.A., 2008 Genetic identification and color descriptions of early life-history stages of Belizean Phaeoptyx and Astrapogon (Teleostei: Apogonidae) with comments on identification of adult Phaeoptyx. Zootaxa: 1–22.
Bucklin A., Steinke D., Blanco-Bercial L., 2011 DNA barcoding of marine metazoa. Annual Rev. Marine Sci 3: 471-508.
Ellien C., Werner U., Keith P., 2016 Morfological Change During the Transition from Freshwater to Sea Water in an Amphidromous Goby, *Sicyopterus lagocephalus* (Pallas 1770) (Teleostei). Ecology of Freshwater Fish: 48-59.
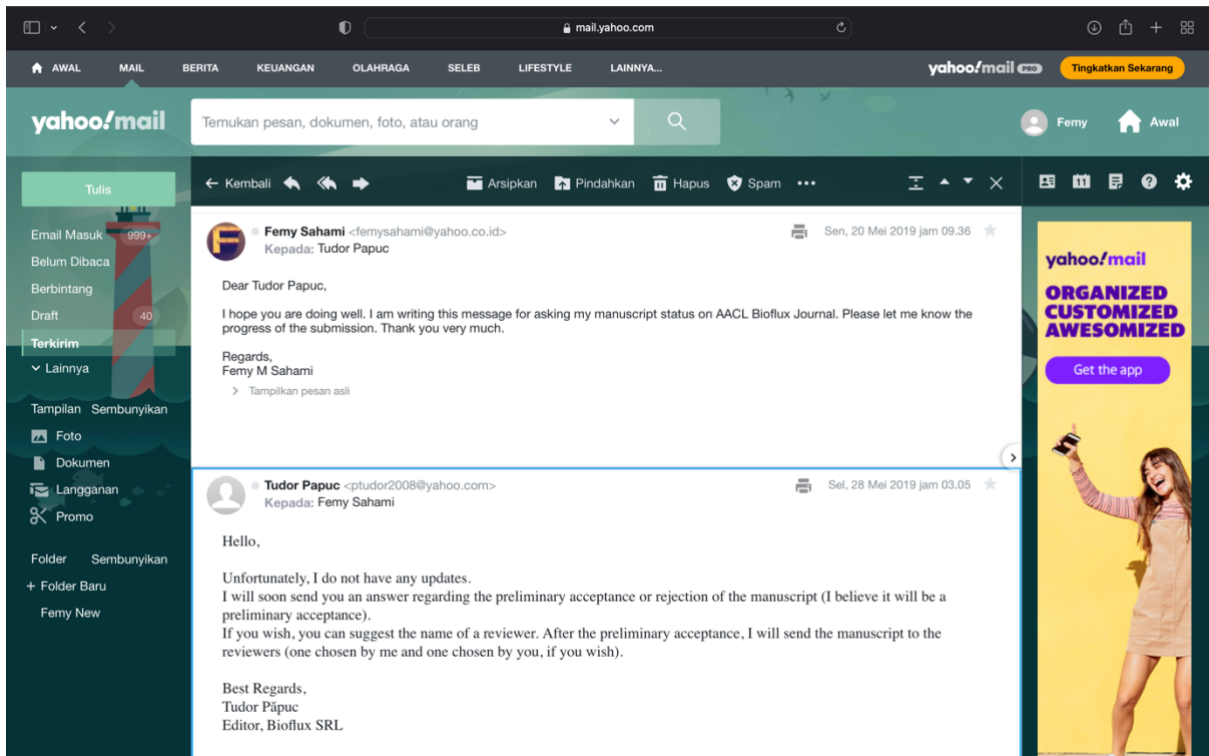
Folmer O., Black M., Hoeh W., Lutz R., Vrijenhoek R., 1994 DNA Primers for Amplification of Mitochondrial Cytochrome C Oxidase Subunit I from Diverse Metazoan Invertebrates. Molecular Marine Biology and Biotechnology 3(5): 294-299

Hebert P.D.N., Ratnasingham S., de Waard J.R., 2003 Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceeding of the Royal Society B 270 (1): S96-S99.

Larson H. K. 2001 *A Revision of the Gobiid Fish Genus Mugilogobius (Teleostei: Gobioidei) and Its Systematic Placement.* Australia: The Western Australian Museum. ISBN 07307 12923.

Maie T, Wilson M., Schoenfuss H., Blob R., 2009 Feeding kinematics and performance of Hawaiian stream Gobies, *Awaous guamensis* and *Lentipes concolor*: linkage of functional morphology. J of Morpho: 344-356.

McDowall R.M. 2009 Early Hatch: A Strategy for Safe Downstream Larval Transport in Ampidromi Fishes. Rev Fish Biol Fish: 1-9

Morgulis A., Coulouris G., Raytselis Y., Madden T.L., Agarwala R., Schäffer A.A., 2008 Database Indexing for Production MegaBLAST Searches. Bioinformatics 24: 1757-1764.

Olii A.H., Sahami F. M., Hamzah S.N., Pasisingi N., 2017 Preliminary findings on distribution pattern of larvae of nike fish (Awaous sp.) in the estuary of Bone River, Gorontalo Province, Indonesia. AACL Bioflux 10: 1110-1118.

Olii A.H., Sahami F. M., Hamzah S.N., Pasisingi N., 2019 Molecular Approach to Identify Gobioid Fishes, "Nike" and "Hundala" (Local Name), from Gorontalo Waters, Indonesia. Journal of Biological Science DOI:10.3844/0jbsci.

Pasisingi N., Abdullah S., 2018 (in Indonesian) Pattern of nike fish (Gobiidae) occurrence in the Gorontalo Bay, Indonesia. Depik 7:111-118.

Purnama A. A., Mubarak J., Daruwati I., Roslim D. I., Elvyra R., 2019 First Report of Morphological and Molecular Identification of Greater Scissotail Rasbora caudimaculata from Rokan Hulu District, Riau Province, Indonesia. AACL Bioflux 34-41.

Tantu F. 2001 (in Indonesia) Nike (Order of Gobioidea) in the Bone Gorontalo Estuary. Tesis. Manado: Program Pasca Sarjana, Universitas Sam Ratulangi Manado.

Tamura K. D., Peterson N., Stecher G., Nei M., Kumar S., 2011 MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28: 2731-2739

Teletchea F., 2009 Molecular identification methods of fish species: Reassessment and possible applications. Rev. Fish Biol. Fisheries 265-293.

Thacker C.E., 2003. Moleculer Phylogeny of The Gobioid Fishes (Teleostei: Perciformes: Gobioidei). 26:354-368.

Thacker C. E., Roje D. M., 2011 Phylogeny of Gobiidae and identification of gobiid lineages. Systematics and Biodiversity 9(4):329–347.

Yamasaki N., Kondo M., Maeda K., Tachihara K., 2011 Reproductive biology of three amphidromous gobies, *Sicyopterus japonicus*, *Awaous melanocephalus*, and *Stenogobius* sp., on Okinawa Island. Cybium 35(4): 345-359.

Valade P., Lord C., Grondin H., Bosc P., Takillebois L., M, I., 2009 Early Life History and Description of Larval Stages of An Amphidromous Goby, *Sicyopterus lagocephalus* (Pallas, 1767) (Teleostei: Gobiidae: Sicydiinae). Cybium 33: 309-319.

Zhang Z., Schwartz S., Wagner L., Miller W., 2000 A greedy algorithm for aligning DNA sequences". J Comput Biol 7(1-2): 203-14.
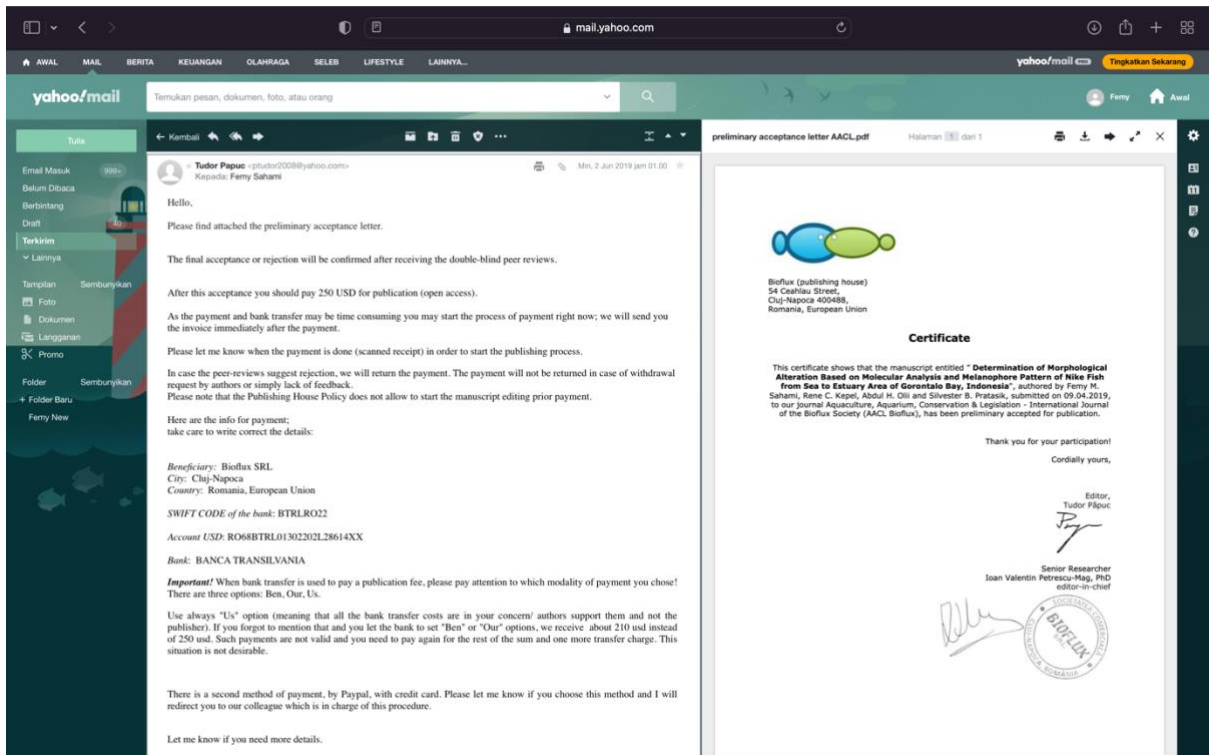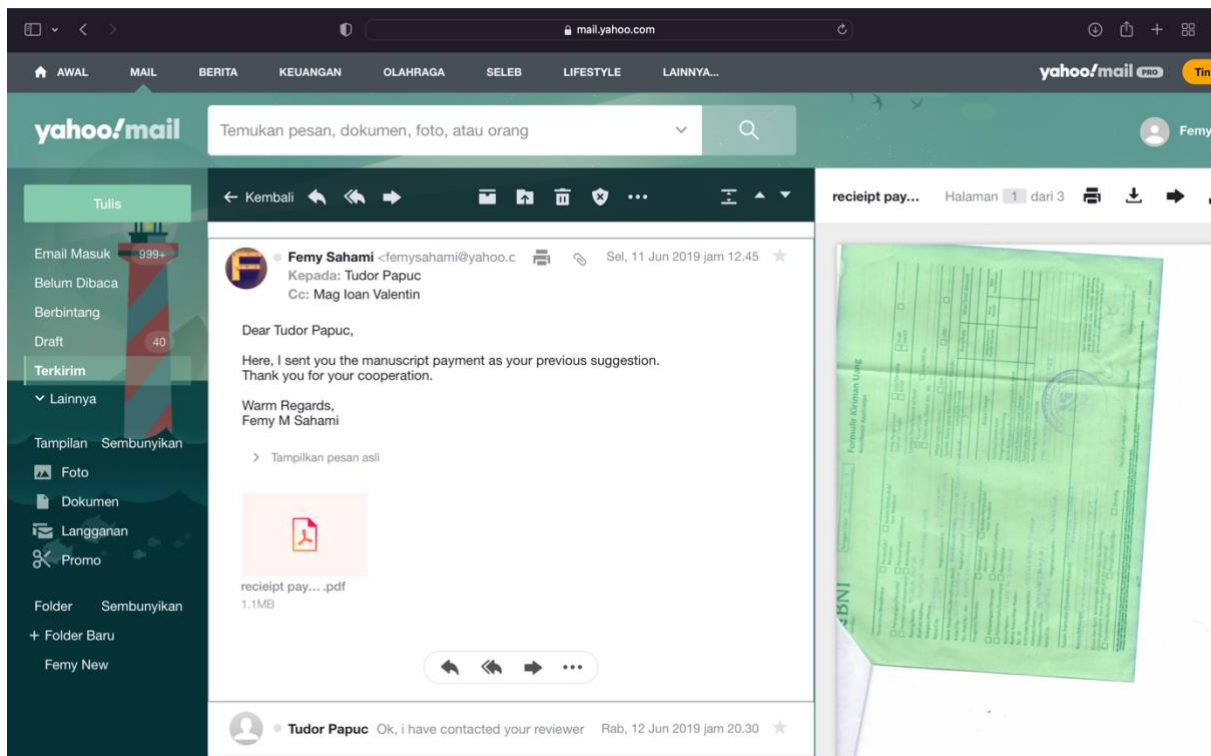
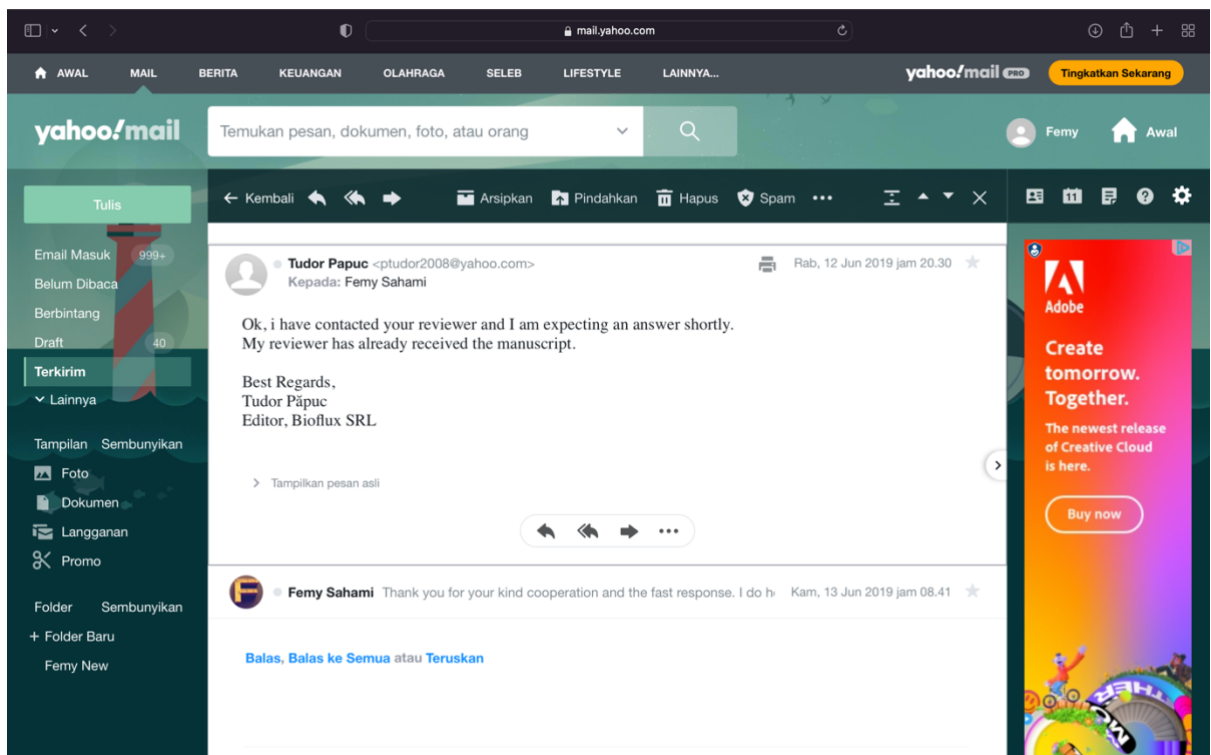Gambar 2. Respon Awal dari Editor

Gambar 3. Korespondensi

Gambar 4

Dear Tudor Papuc,

I hope you are doing well. I am writing this message for asking my manuscript status on AACL Bioflux Journal. Please let me know the progress of the submission. Thank you very much.

Regards,
Femy M Sahami

Hello,

Unfortunately, I do not have any updates.
I will soon send you an answer regarding the preliminary acceptance or rejection of the manuscript (I believe it will be a preliminary acceptance).
If you wish, you can suggest the name of a reviewer. After the preliminary acceptance, I will send the manuscript to the reviewers (one chosen by me and one chosen by you, if you wish).

Best Regards,
Tudor Păpuc
Editor, Bioflux SRL

Gambar 4. Korespondensi

Gambar 5

Hello,

Please find attached the preliminary acceptance letter.

The final acceptance or rejection will be confirmed after receiving the double-blind peer reviews.

After this acceptance you should pay 250 USD for publication (open access).

As the payment and bank transfer may be time consuming you may start the process of payment right now; we will send you the invoice immediately after the payment.

Please let me know when the payment is done (scanned receipt) in order to start the publishing process.

In case the peer-reviews suggest rejection, we will return the payment. The payment will not be returned in case of withdrawal request by authors or simply lack of feedback.
Please note that the Publishing House Policy does not allow to start the manuscript editing prior payment.

Here are the info for payment;
take care to write correct the details:

Beneficiary: Bioflux SRL
City: Cluj-Napoca
Country: Romania, European Union

SWIFT CODE of the bank: BTRLRO22

Account USD: RO68BTRL01302202L28614XX

Bank: BANCA TRANSILVANIA

Important! When bank transfer is used to pay a publication fee, please pay attention to which modality of payment you chose! There are three options: Ben, Our, Us.

Use always "Us" option (meaning that all the bank transfer costs are in your concern/ authors support them and not the publisher). If you forgot to mention that and you let the bank to set "Ben" or "Our" options, we receive about 210 usd instead of 250 usd. Such payments are not valid and you need to pay again for the rest of the sum and one more transfer charge. This situation is not desirable.

There is a second method of payment, by Paypal, with credit card. Please let me know if you choose this method and I will redirect you to our colleague which is in charge of this procedure.

Let me know if you need more details.

preliminary acceptance letter AACL.pdf

Bioflux (publishing house)
54 Ceahlau Street,
Cluj-Napoca 400488,
Romania, European Union

Certificate

This certificate shows that the manuscript entitled " **Determination of Morphological Alteration Based on Molecular Analysis and Melanophore Pattern of Nike Fish from Sea to Estuary Area of Gorontalo Bay, Indonesia**", authored by Femy M. Sahami, Rene C. Kepel, Abdul H. Olii and Silvester B. Pratasik, submitted on 09.04.2019, to our Journal Aquaculture, Aquarium, Conservation & Legislation - International Journal of the Bioflux Society (AACL Bioflux), has been preliminary accepted for publication.

Thank you for your participation!

Cordially yours,

Editor,
Tudor Păpuc

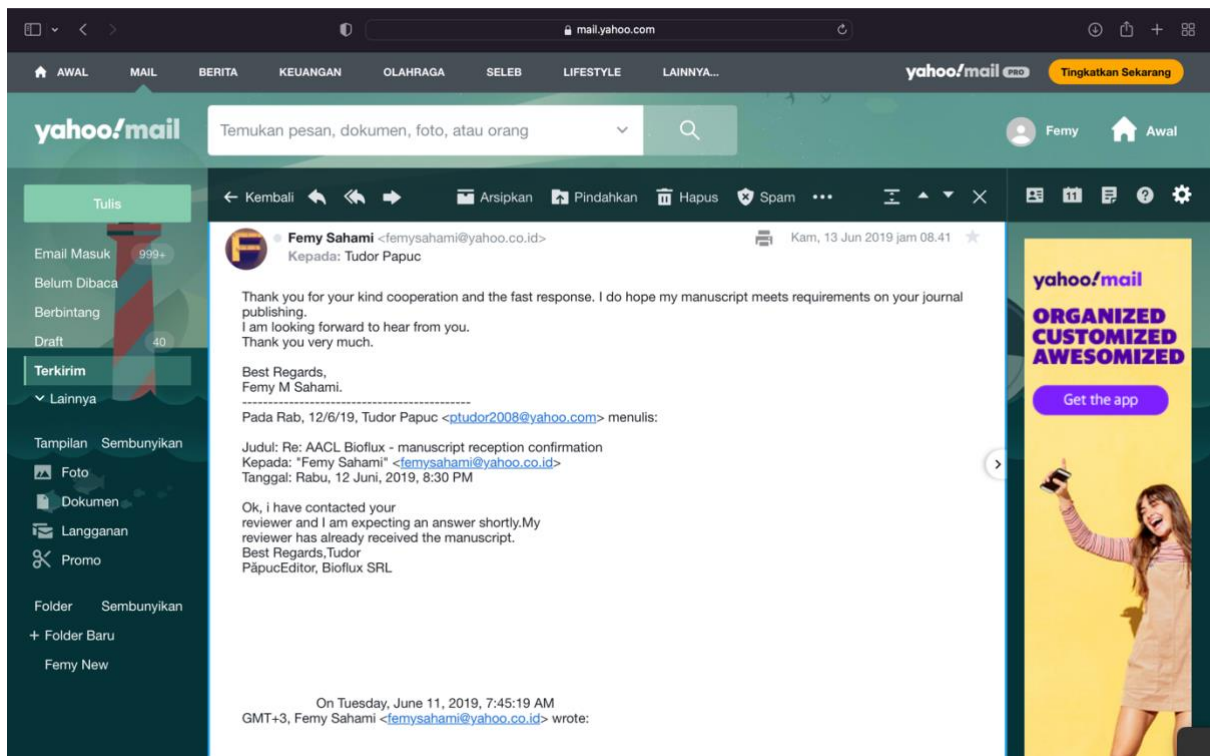Senior Researcher
Ioan Valentin Petrescu-Mag, PhD
editor-in-chief

Gambar 5. Korespondensi
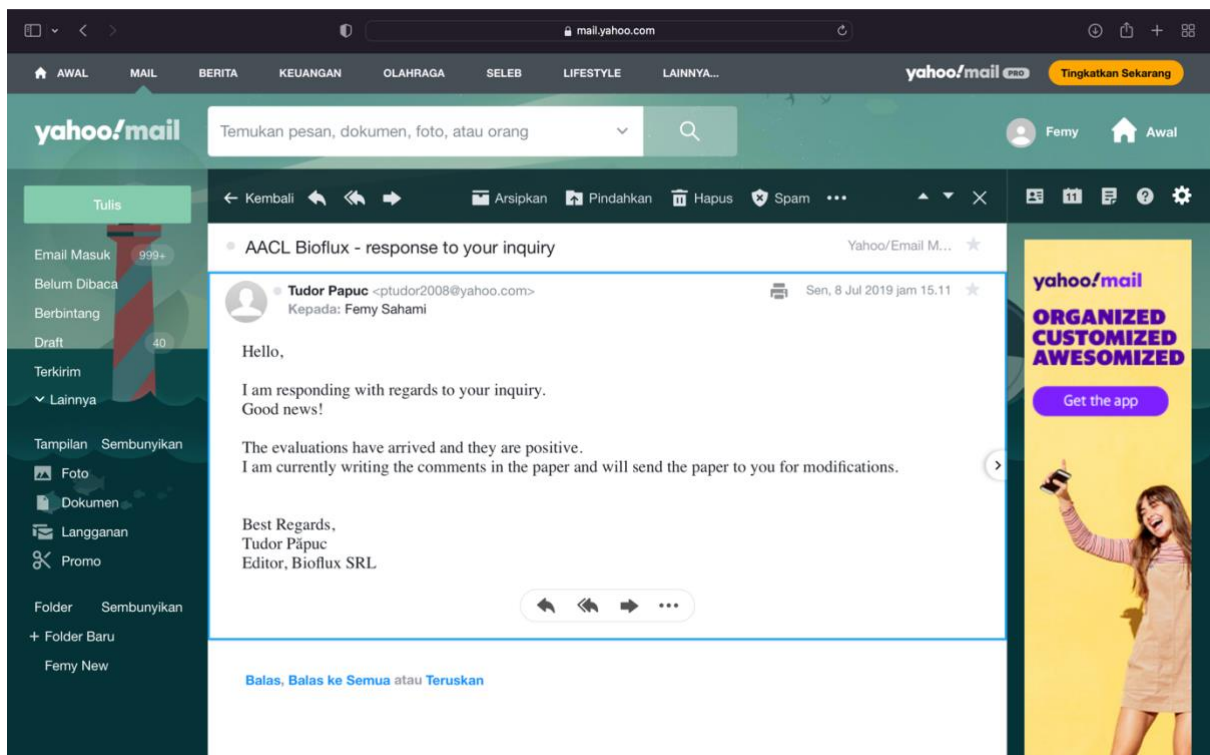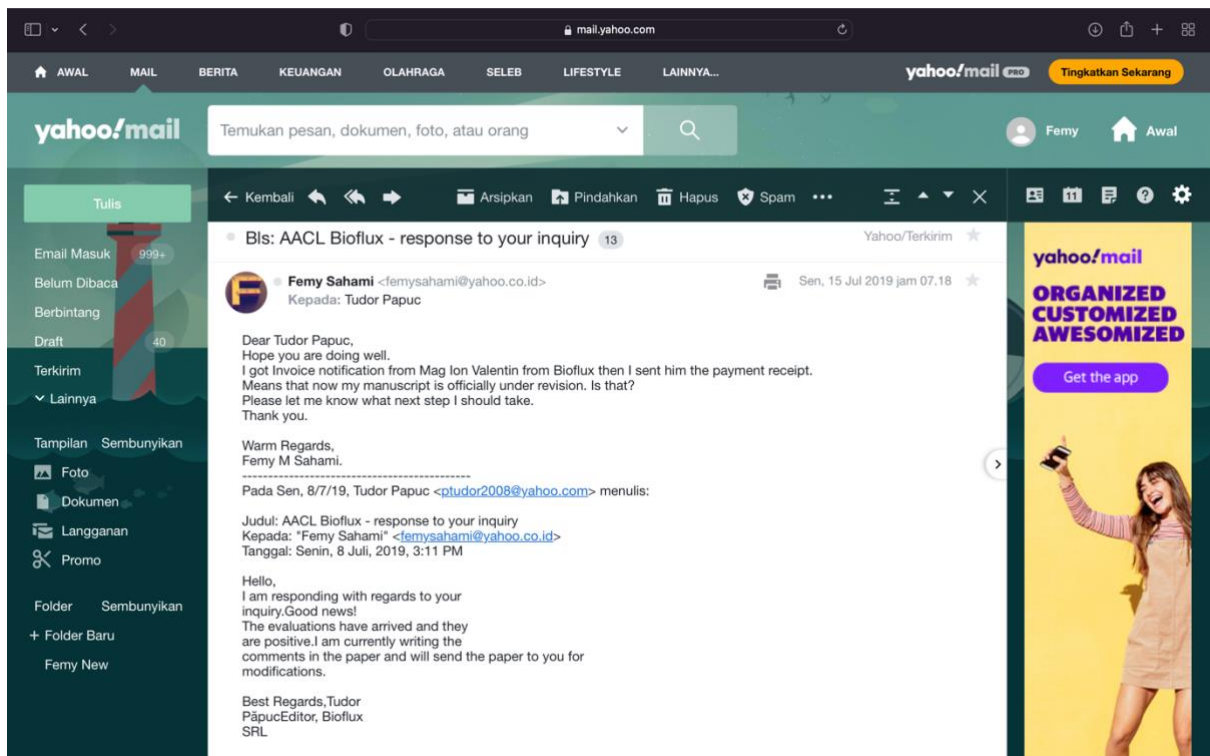
Gambar 6. Korespondensi

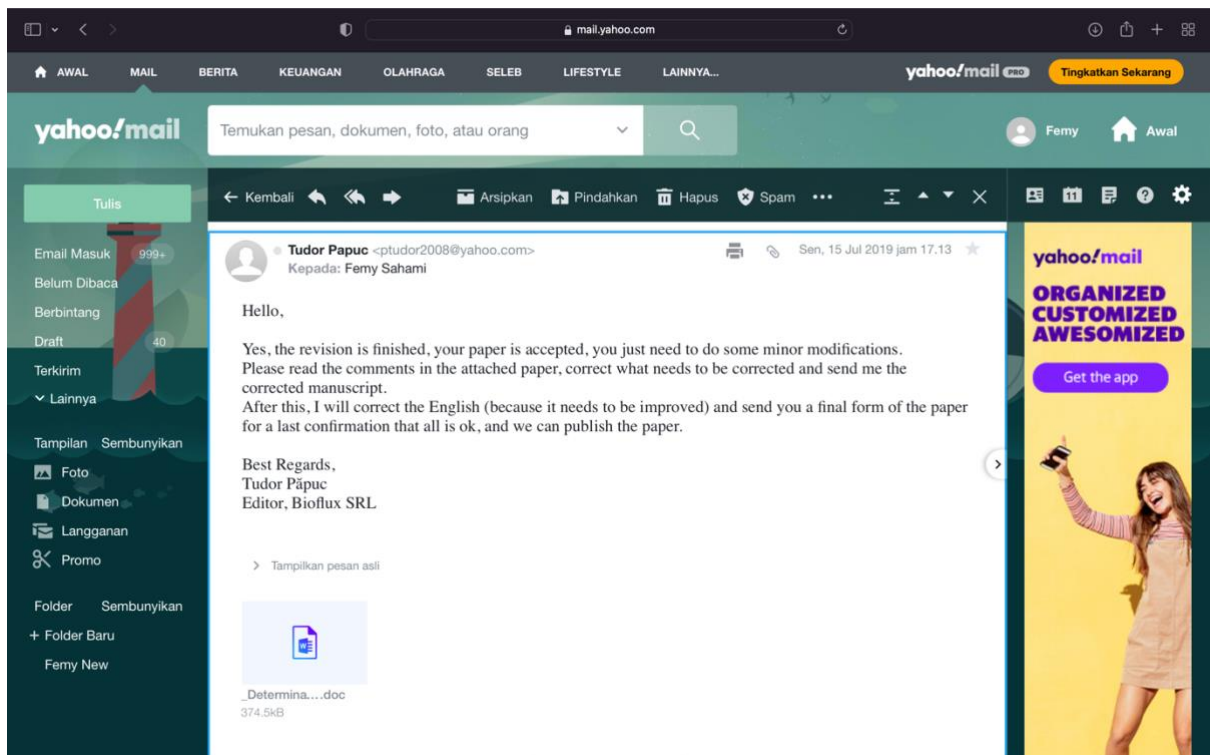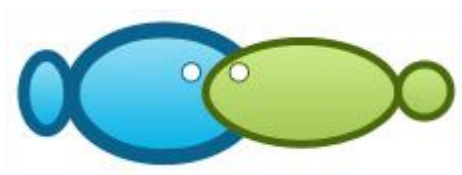Gambar 7. Korespondensi

Gambar 8. Korespondensi

Gambar 9. Korespondensi

Gambar 10. Korespondensi

Gambar 11. Korespondensi

Determination of Morphological Alteration Based on Molecular Analysis and Melanophore Pattern of Nike Fish from Sea to Estuary Area of Gorontalo Bay, Indonesia

Femy M. Sahami, Rene C. Kepel[2], Abdul H. Olii[1], Silvester B. Pratasik[2]

[2] Department of Aqutic Resources Management, Faculty of Fisheris and Marine Science, Gorontalo State University, Jl. Jendral Sudirman. No. 6, Gorontalo City, Gorontalo Province, Indonesia; [2] Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia. Corresponding author: F. M. Sahami, femysahami@yahoo.co.id

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 of melanophore pattern. The small size of this fish (larval stage/post-larva) causes it be tough to analyze morphometric and meristic aspects. Therefore, the melanophore pattern approach and molecular analysis using the CO1 gene were used to identify these fish species. Sampling was carried out on 5 to 11 October 2018 in the Gorontalo Bay area to the mouth of Bone Bolango river when this type of fish appeared. Samples of nike fish were grouped based on differences in melanophore patterns approach in the body and we found there were five different groups and coded N1, N2, N3, N4, and N5. The molecular analysis using the CO1 gene were used to identify these fish species. Genetic investigation showed that from five groups of samples, there were found two groups of samples identified (N3 and N5) that had g different melanophore patterns were the same species, *Belobranchus segura*. Morphological changes have occurred due to an increase in the melanofor pattern of nike species when they migrate from the sea to the river mouth.
Key Words: melanophore, molecular, amphidromus gobie, nike fish

Introduction. Nike is the name of a small type of fish that is around 2-4 cm at length which appears in the waters in a large number carried out by Gorontalo people and its surroundings. These fish do not appear every day in the sea throughout the year but only once a month. Its emergence is unpredicted usually occurs at the end of each month in the calendar of the Hijri year or in the new moon time. The duration of the appearance of this fish at the location of its arrest is also not fixed, which is around three until seven days per the appearance period.

Scientific information about this nike is still not widely publicized. Scientific studies of this type of fish in Gorontalo waters are still rare. Thus far, information about the existence of these fish species has only been obtained from the community. Important aspects of the existence of these fish species can be a source of biodiversity whose existence needs to be preserved.

Nike in Gorontalo waters consists of two species, namely *Awaous melanocephalus* and *Eleotris frusca*. Among the schooling, *A. melanocephalus* is the main constituent species that is 99%, while *E. frusca* is only a supporter species. Furthermore, the inhabited area is from the coast to the upstream (Tantu 2001). *A. melanocephalus* is an amphidromous fish. When these adults spawn in freshwater, the eggs are placed on the substrate at the bottom of the water, after the eggs hatch the larvae drift into the sea, then after a while in the waters, the juvenile returns to the river where the parent originates (Yamasaki et al 2011). Fish from the gobii group in Hawaiian waters lived and developed in the waters of the sea, initially the fish hatched larvae in the river, and by river currents, the larvae were carried into the sea, living and developing into juveniles, then back to their habitat in freshwater (Maie et al 2009). The distribution of nike fish larvae that move from sea waters to river mouths is influenced by internal factors and external factors.

Gobiidae is one of the largest acanthomorph fish groups, which number approximately 1120 species from 30 genera that have been described and many more that have not been described (Thacker & Roje 2011). A large amount of gobi fish causes genetic similarity and can allow the occurrence of fertilization naturally. This fish in its life cycle passes through several environments that can allow morphological changes, but might be morphologically dissimilar but still the same species.

The high demand for these fish causes high fishing activity and exploitation. And the improper management will accelerate extinction as well. All waters cannot be managed directly (Sahami et al 2017). The response of organisms to the environment becomes significant information in an effort to manage waters. The differences in the topography of Nike fishing grounds can allow for differences in adaptation and ecology and behavior that may be expressed through changes in color and size. Due to their small size and often cryptic ecologies, the full extent of gobiid diversity often goes unnoticed (Thacker & Roje 2011). An effort to hypothesize relationships among the gobioid groups have been hampered by the prevalence of reductive evolution among goby species, such reduction can make identification of informative morphological characters particularly difficult (Thacker 2003).

Molecular and morphological studies can help determination of characteristics of nike fish in the Gorontalo Bay, making it easier for management. 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 estuaries in Gorontalo waters using molecular analysis.

Material and Method

Sampling. The study started from October 2018 to January 2019. The sampling of these fish from fishmen was carried out randomly during the period of capture in Leato waters from 5 to 11 October 2018. A map of the sampling location is shown in Fig.1. Sampling was carried out at the Integrated Laboratory of the Faculty of Fisheries and Marine Sciences, Gorontalo State University by sorting and grouping it according to the appearance of the melanophore pattern on the fish's body. Samples of nike fish were grouped based on differences in melanophore patterns in the body and we found there were five different groups and coded N1, N2, N3, N4, and N5. From each group of fish, 5 individuals were taken and filled in the sample bottles which were preserved in 70% alcohol. Furthermore, samples were analyzed for genetic identification in the Papua State University genetic laboratory, Manokwari. The small size of this fish (larval stage/post-larva) causes it be tough to analyze morphometric and meristic aspects. Therefore, the melanophore pattern approach and molecular analysis using the CO1 gene were used to identify these fish species.
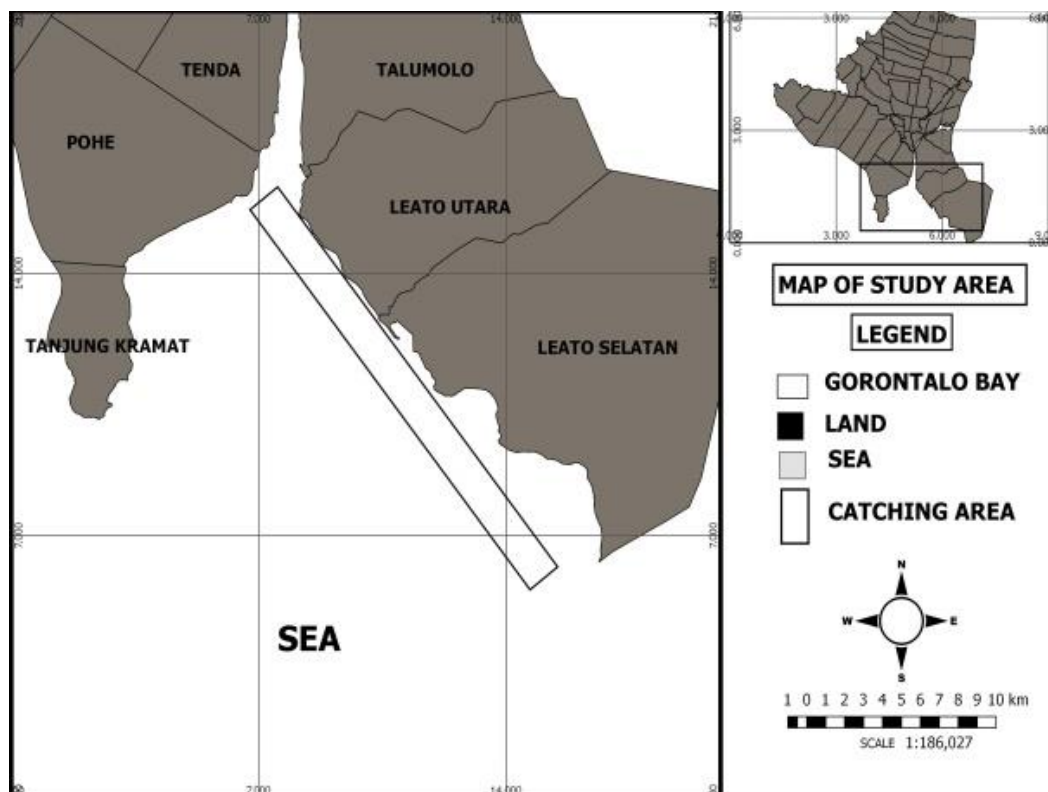

Figure 1. Map of Sampling Site in Gorontalo Bay

Molecular Analysis. DNA genome isolation of samples was conducted using an Isolation kit, which was produced 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 2008), namely (Forward) BCL Fish: 5 '-TCAACYAATCAYAAAGATATYGGCAC-3' and (Reverse) Fish BCH: 5 '-ACTTCYGGGTGRCCRAARAATCA-3'. PCR products were then electrophoresed and photographed above 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 through PT Genetics Science Indonesia by sending samples consisting of 30 µl of PCR DNA products, 10 µl of forward primer and 10 µl reverse primer. 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 analyzed to see samples that had genetic similarity. To find out the relationship level between samples, further analysis was carried out using phylogenetic trees using Maximum Likelihood Method with 1000 bootstraps using MEGA 6.0 software.

Results and Discussion. The results indicate that nike fish schooling consists of 2 groups which morphologically are dissimilar and have different melanophore pattern yet genetically are the same species. The proofreading results from the forward and reserve sequences which were 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	CCTTTATCTTGTCTTCGGTGCCTGAGCCGGGATAGTGGGCACCGCTTTAAGC CTACTTATCCGCGCTGAACTAAGTCAACCTGGCGCACTCCTAGGAGATGACC AAATCTATAATGTTATCGTTACCGCCCACGCGTTCGTAATAATTTTCTTTATAG TAATACCAATTATGATTGGCGGATTTGGTAACTGACTAATCCCCTTAATGATT GGCGCCCCAGACATGGCCTTCCCACGAATAAACAACATAAGTTTCTGACTTC TCCCGCCATCTTTCCTCCTCCTTTTAGCATCCTCTGGAGTAGAAGCAGGGGC CGGAACAGGGTGAACCGTCTACCCGCCCCTAGCGGGCAACCTCGCCCACGC AGGCGCCTCTGTGGACCTAACAATCTTTTCACTACACCTAGCAGGGGTGTCC TCAATTCTTGGAGCAATTAATTTCATTACCACAATTATTAACATAAAACCTCCG GCAATTTCCCAATACCAAACGCCCTTGTTCGTCTGAGCCGTTCTAATTACAGC CGTCTTATTACTATTATCCCTTCCCGTACTTGCTGCTGGCATCACAATGCTACT TACAGATCGAAATTTAAATACGACGTTCTTTGACCCGGCCGGGGGTGGGGAC CCAATCTTATACCAACACCTTTTC
N5	CCTTTATCTTGTCTTCGGTGCCTGAGCCGGGATAGTGGGCACAGCTTTAAGC CTACTTATCCGCGCTGAACTAAGTCAACCTGGCGCACTCCTAGGAGATGACC AAATCTATAATGTTATCGTTACCGCCCACGCGTTCGTAATAATTTTCTTTATAG TAATACCAATTATGATTGGCGGATTTGGTAACTGACTAATCCCCTTAATGATT GGCGCCCCAGACATGGCCTTCCCACGAATAAACAACATAAGTTTCTGACTTC TCCCGCCATCTTTCCTCCTCCTTTTAGCATCCTCTGGAGTAGAAGCAGGGGC CGGAACAGGGTGAACCGTCTACCCGCCCCTAGCGGGCAACCTCGCCCACGC AGGCGCCTCTGTGGACCTAACAATCTTTTCACTACACCTAGCAGGGGTGTCC TCAATTCTTGGAGCAATTAATTTCATTACCACAATTATTAACATAAAACCTCCG GCAATTTCCCAATACCAAACGCCCTTGTTCGTCTGAGCCGTTCTAATTACAGC CGTCTTATTACTATTATCCCTTCCCGTACTTGCTGCTGGCATCACAATGCTACT TACAGATCGAAATTTAAATACGACGTTCTTTGACCCGGCCGGGGGTGGGGAC CCAATCTTATACCAACACCTTTTC

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	Gen	Accession Number	Max Score	Query cover	Identity
1	Sequence N3: *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%
1	Sequence N5: *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%

Table 2 shows that the two samples based on genetic testing with mitochondrial CO1 are the same species as *Belobranchus segura*. Further analysis was carried out with the phylogenetic tree to show the kinship relationships between samples (N3 and N5) and several species available in the NCBI database as presented in Fig.2.

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

The genetic analysis illustrates that the two samples are similar species although they had different morphology particularly the melanophore patterns. This modification might be an adaptation of species when migrating from sea water to fresh water which is part of its life development. When entering a river mouth, post-larva undergoes changes in morphology, physiology, and behavior (Keith et al 2008). The difference in melanophore patterns from samples N3 and N5 is schematically presented in Figure 3.

Sample Code	Picture (Personal documentation)	Sketch
N3		
N5		

Fig.3. Schematic morphological alterations in the samples N3 and N5 based on the shape of 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 the river (Fig. 4). Sample N5 is N3 that has developed and has undergone alterations in the melanophore structure. This can be possible because the N3 sample was taken from the catch on 8 October 2018, while the N5 sample was found in the sample conducted on 11 October 2018. This supports Valade et al (2009) statement that there has been a change in the appearance of chromatophore in the body *Sicyopterus langocephalus* larvae, which starts from the head area then spreads throughout the body along with the larvae age. The results of this study illustrate that there has been a variation in the pattern of melanophore from the species of nike *Belobranchus segura* when migrating from sea water to the mouth of the river. When still in the sea the melanophore arrangement is still minor, but when it will enter the river mouth the melanophore arrangement has spread along its body.

Figure 4. Area sampling of N3 and N5

Hawaiian gobioid fish are amphidromous and have one phase of life in the sea (Hobson & Smith 2007) They are in the pelagic phase of the sea for several months before juveniles in the river (Teichert et al 2016). Nike fishing in the Gorontalo Bay from the first, second and third days has a tendency to shift closer to the river mouth which indicates the migration of nike from sea water to fresh water (Olii et al 2017, Pasisingi & Abdullah 2018).

Changes in color morphology or melanophore patterns of nike species *Belobranchus segura* from the sea with melanophore content which becomes slightly more when approaching the estuary is a form of adaptation in order to enter a new aquatic environment or is part of the stages of its development. Distribution of species along the river is determined by postlarva color aggregation (Nishimoto & Fitzsimons 1986)

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 & Baras 2001). Although the lives of amphidromous fish are strongly related to the conditions of the oceans and rivers, 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 is related to their phase of

development (Fitzsimon & McRae 2007). Post-larvae return to rivers where they are recruited and grown to reproductive stages (Ellien et al 2016).

The distribution of nike fish tends to approach the estuary since its appearance time until it disappears (Olii et al 2017). Based on the results of this study it can be concluded that in fact, nike fish do not disappear from the waters as what nike fishing community consider. There had been a morphological alteration in color due to an increase in the number of melanophores.

Conclusions. In conclusion, samples N3 and N5 that have different melanophore patterns are genetically the same species, *Belobrancianus segura*. The morphological changes of nike fish of this species are indicated by differences in melanophore patterns, that was an increase in the number and spread of melanophore on the surface of the body when this species has entered the river mouth.

References

Baldwin C.C., Mounts J.H., Smith D.G., Weigt L.A., 2008 Genetic identification and color descriptions of early life-history stages of Belizean Phaeoptyx and Astrapogon (Teleostei: Apogonidae) with comments on identification of adult Phaeoptyx. Zootaxa: 1–22.

Bucklin A., Steinke D., Blanco-Bercial L., 2011 DNA barcoding of marine metazoa. Annual Rev. Marine Sci 3: 471-508.

Ellien C., Werner U., Keith P., 2016 Morfological Change During the Transition from Freshwater to Sea Water in an Amphidromous Goby, *Sicyopterus lagocephalus* (Pallas 1770) (Teleostei). Ecology of Freshwater Fish: 48-59.

Folmer O., Black M., Hoeh W., Lutz R., Vrijenhoek R., 1994 DNA Primers for Amplification of Mitochondrial Cytochrome C Oxidase Subunit I from Diverse Metazoan Invertebrates. Molecular Marine Biology and Biotechnology 3(5): 294-299

Hebert P.D.N., Ratnasingham S., de Waard J.R., 2003 Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceeding of the Royal Society B 270 (1): S96-S99.

Larson H. K. 2001 *A Revision of the Gobiid Fish Genus Mugilogobius (Teleostei: Gobioidei) and Its Systematic Placement.* Australia: The Western Australian Museum. ISBN 07307 12923.

Maie T, Wilson M., Schoenfuss H., Blob R., 2009 Feeding kinematics and performance of Hawaiian stream Gobies, *Awaous guamensis* and *Lentipes concolor*: linkage of functional morphology. J of Morpho: 344-356.

McDowall R.M. 2009 Early Hatch: A Strategy for Safe Downstream Larval Transport in Ampidromi Fishes. Rev Fish Biol Fish: 1-9

Morgulis A., Coulouris G., Raytselis Y., Madden T.L., Agarwala R., Schäffer A.A., 2008 Database Indexing for Production MegaBLAST Searches. Bioinformatics 24: 1757-1764.

Olii A.H., Sahami F. M., Hamzah S.N., Pasisingi N., 2017 Preliminary findings on distribution pattern of larvae of nike fish (Awaous sp.) in the estuary of Bone River, Gorontalo Province, Indonesia. AACL Bioflux 10: 1110-1118.

Olii A.H., Sahami F. M., Hamzah S.N., Pasisingi N., 2019 Molecular Approach to Identify Gobioid Fishes, "Nike" and "Hundala" (Local Name), from Gorontalo Waters, Indonesia. Journal of Biological Science DOI:10.3844/0jbsci.

Pasisingi N., Abdullah S., 2018 (in Indonesian) Pattern of nike fish (Gobiidae) occurrence in the Gorontalo Bay, Indonesia. Depik 7:111-118.

Purnama A. A., Mubarak J., Daruwati I., Roslim D. I., Elvyra R., 2019 First Report of Morphological and Molecular Identification of Greater Scissotail Rasbora caudimaculata from Rokan Hulu District, Riau Province, Indonesia. AACL Bioflux 34-41.

Tantu F. 2001 (in Indonesia) Nike (Order of Gobioidea) in the Bone Gorontalo Estuary. Tesis. Manado: Program Pasca Sarjana, Universitas Sam Ratulangi Manado.

Tamura K. D., Peterson N., Stecher G., Nei M., Kumar S., 2011 MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28: 2731-2739

Teletchea F., 2009 Molecular identification methods of fish species: Reassessment and possible applications. Rev. Fish Biol. Fisheries 265-293.

Thacker C.E., 2003. Moleculer Phylogeny of The Gobioid Fishes (Teleostei: Perciformes: Gobioidei). 26:354-368.

Thacker C. E., Roje D. M., 2011 Phylogeny of Gobiidae and identification of gobiid lineages. Systematics and Biodiversity 9(4):329–347.

Yamasaki N., Kondo M., Maeda K., Tachihara K., 2011 Reproductive biology of three amphidromous gobies, *Sicyopterus japonicus*, *Awaous melanocephalus*, and *Stenogobius* sp., on Okinawa Island. Cybium 35(4): 345-359.

Valade P., Lord C., Grondin H., Bosc P., Takillebois L., M, I., 2009 Early Life History and Description of Larval Stages of An Amphidromous Goby, *Sicyopterus lagocephalus* (Pallas, 1767) (Teleostei: Gobiidae: Sicydiinae). Cybium 33: 309-319.

Zhang Z., Schwartz S., Wagner L., Miller W., 2000 A greedy algorithm for aligning DNA sequences". J Comput Biol 7(1-2): 203-14.

Gambar 12. Korespondensi

Determination of Morphological Alteration Based on Molecular Analysis and Melanophore Pattern of The Migrating Nike Fish in Gorontalo Bay, Indonesia

Femy M. Sahami[1], Rene C. Kepel[2], Abdul H. Olii[1], Silvester B. Pratasik[2]

[1] Department of Aquatic Resources Management, Faculty of Fisheries and Marine Science, Gorontalo State University, Jl. Jendral Sudirman. No. 6, Gorontalo City, Gorontalo Province, Indonesia; [2] Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia. Corresponding author: F. M. Sahami, femysahami@yahoo.co.id

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 of melanophore pattern. The small size of this fish (larval stage/post-larva) causes it be tough to analyze morphometric and meristic aspects. Therefore, the melanophore pattern approach and molecular analysis using the CO1 gene were used to identify these fish species. Sampling was carried out on 5 to 11 October 2018 in the Gorontalo Bay area to the mouth of Bone Bolango river when this type of fish appeared. Samples of nike fish were grouped based on differences in melanophore patterns approach in the body and we found there were five different groups and coded N1, N2, N3, N4, and N5. The molecular analysis using the CO1 gene were used to identify these fish species. Genetic investigation showed that from five groups of samples, there were found two groups of samples identified (N3 and N5) that had g different melanophore patterns were the same species, *Belobranchus segura*. Morphological changes have occurred due to an increase in the melanophore pattern of nike species when they migrate from the sea to the river mouth.
Key Words: melanophore, molecular, amphidromus gobiidae, nike fish

Introduction. Nike is the name of a small type of fish that is around 2-4 cm at length which appears in the waters in a large number carried out by local fishermen. These fish do not appear every day in the sea throughout the year but only once a month. Its emergence is unpredicted usually occurs at the end of each month in the calendar of the Hijri year or in the new moon time. The duration of the appearance of this fish at the location of its arrest is also not fixed, which is around three until seven days per the appearance period.

Scientific information about this nike is still not widely publicized. Scientific studies of this type of fish in Gorontalo waters are still rare. Thus far, information about the existence of these fish species has only been obtained from the community. Important aspects of the existence of these fish species can be a source of biodiversity whose existence needs to be preserved.

Nike in Gorontalo waters consists of two species, namely *Awaous melanocephalus* and *Eleotris frusca*. Among the schooling, *A. melanocephalus* is the main constituent species that is 99%, while *E. frusca* is only a supporter species. Furthermore, the inhabited area is from the coast to the upstream (Tantu 2001). *A. melanocephalus* is an amphidromous fish. When these adults spawn in freshwater, the eggs are placed on the substrate at the bottom of the water, after the eggs hatch the larvae drift into the sea, then after a while in the waters, the juvenile returns to the river where the parent originates (Yamasaki et al 2011). Fish from the gobiid group in Hawaiian waters lived and developed in the waters of the sea, initially the fish hatched larvae in the river, and by river currents, the larvae were carried into the sea, living and developing into juveniles, then back to their habitat in freshwater (Maie et al 2009). The distribution of nike fish larvae that move from sea waters to river mouths is influenced by internal factors and external factors.

Gobiidae is one of the largest acanthomorph fish groups, which number approximately

1120 species from 30 genera that have been described and many more that have not been described (Thacker & Roje 2011). A large amount of gobiid fish causes genetic similarity and can allow the occurrence of fertilization naturally. This fish in its life cycle passes through several environments that can allow morphological changes, but might be morphologically dissimilar but still the same species.

The high demand for these fish causes high fishing activity and exploitation. And the improper management will accelerate extinction as well. All waters cannot be managed directly (Sahami et al 2017). The response of organisms to the environment becomes significant information in an effort to manage waters. The differences in the topography of Nike fishing grounds can allow for differences in adaptation and ecology and behavior that may be expressed through changes in color and size. Due to their small size and often cryptic ecologies, the full extent of gobiid diversity often goes unnoticed (Thacker & Roje 2011). An effort to hypothesize relationships among the gobioid groups have been hampered by the prevalence of reductive evolution among goby species, such reduction can make identification of informative morphological characters particularly difficult (Thacker 2003).

Molecular and morphological studies can help determination of characteristics of nike fish in the Gorontalo Bay, making it easier for management. 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 estuaries in Gorontalo waters using molecular analysis.

Material and Method

Sampling. The study started from October 2018 to January 2019. The sampling of these fish from fishmen was carried out randomly during the period of capture in Leato waters from 5 to 11 October 2018. A map of the sampling location is shown in Fig.1. The collected samples were put in a plastic bottle and palced into ice box. The sample arrangement was carried out at the Integrated Laboratory of the Faculty of Fisheries and Marine Sciences, Gorontalo State University by sorting and grouping it according to the appearance of the melanophore pattern on the fish's body. Samples of nike fish were grouped based on differences in melanophore patterns in the body and we found there were five different groups and coded N1, N2, N3, N4, and N5. From each group of fish, 5 individuals were taken and filled in the sample bottles which were preserved in 70% alcohol. Furthermore, samples were analyzed for genetic identification in the Papua State University genetic laboratory, Manokwari. The small size of this fish (larval stage/post-larva) causes it be tough to analyze morphometric and meristic aspects. Therefore, the melanophore pattern approach and molecular analysis using the CO1 gene were used to identify these fish species.
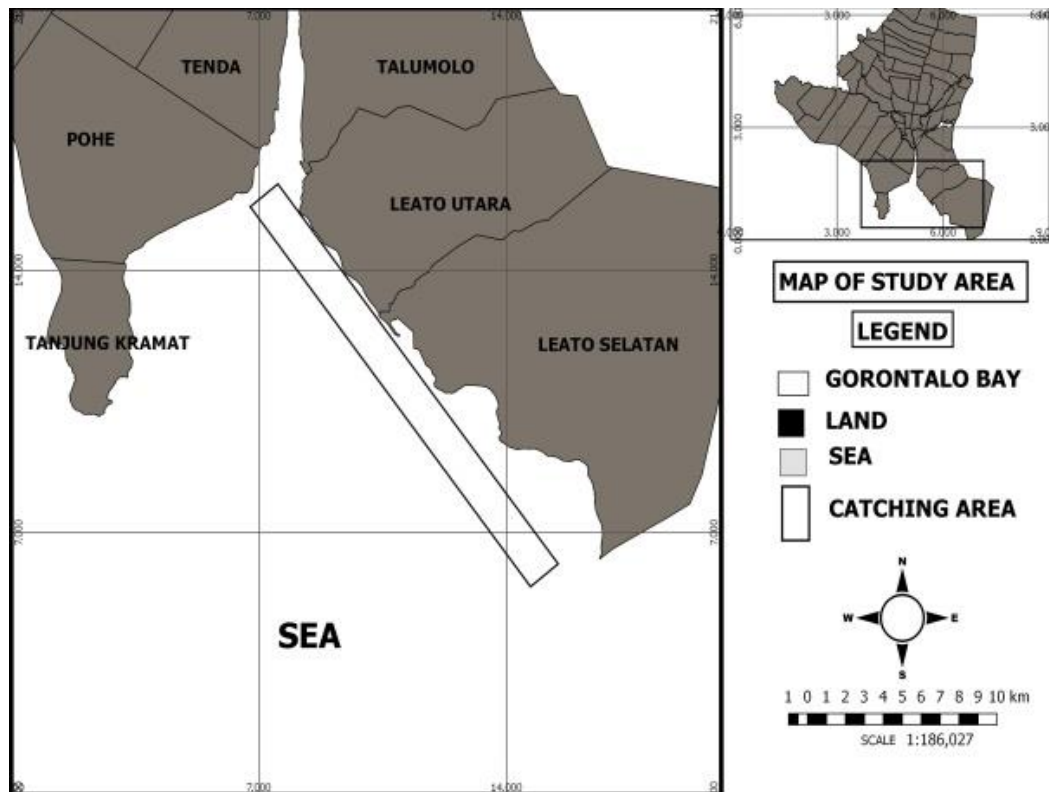
Figure 1. Map of Sampling Site in Gorontalo Bay

Molecular Analysis*.* DNA genome isolation of samples was conducted using an Isolation kit, which was produced 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 2008), namely (Forward) BCL Fish: 5 '-TCAACYAATCAYAAAGATATYGGCAC-3' and (Reverse) Fish BCH: 5 '-ACTTCYGGGTGRCCRAARAATCA-3'. PCR products were then electrophoresed and photographed above 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 through PT Genetics Science Indonesia by sending samples consisting of 30 µl of PCR DNA products, 10 µl of forward primer and 10 µl reverse primer. 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 analyzed to see samples that had genetic similarity. To find out the relationship level between samples, further analysis was carried out using phylogenetic trees using Maximum Likelihood Method with 1000 bootstraps using MEGA 6.0 software.

Results and Discussion. The results indicate that nike fish schooling consists of 2 groups which morphologically are dissimilar and have different melanophore pattern yet genetically are the same species. The proofreading results from the forward and reserve sequences which were 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	CCTTTATCTTGTCTTCGGTGCCTGAGCCGGGATAGTGGGCACCGCTTTAAGC
	CTACTTATCCGCGCTGAACTAAGTCAACCTGGCGCACTCCTAGGAGATGACC
	AAATCTATAATGTTATCGTTACCGCCCACGCGTTCGTAATAATTTTCTTTATAG
	TAATACCAATTATGATTGGCGGATTTGGTAACTGACTAATCCCCTTAATGATT
	GGCGCCCCAGACATGGCCTTCCCACGAATAAACAACATAAGTTTCTGACTTC
	TCCCGCCATCTTTCCTCCTCCTTTTAGCATCCTCTGGAGTAGAAGCAGGGGC
	CGGAACAGGGTGAACCGTCTACCCGCCCCTAGCGGGCAACCTCGCCCACGC
	AGGCGCCTCTGTGGACCTAACAATCTTTTCACTACACCTAGCAGGGGTGTCC
	TCAATTCTTGGAGCAATTAATTTCATTACCACAATTATTAACATAAAACCTCCG
	GCAATTTCCCAATACCAAACGCCCTTGTTCGTCTGAGCCGTTCTAATTACAGC
	CGTCTTATTACTATTATCCCTTCCCGTACTTGCTGCTGGCATCACAATGCTACT
	TACAGATCGAAATTTAAATACGACGTTCTTTGACCCGGCCGGGGGTGGGGAC
	CCAATCTTATACCAACACCTTTTC
N5	CCTTTATCTTGTCTTCGGTGCCTGAGCCGGGATAGTGGGCACAGCTTTAAGC
	CTACTTATCCGCGCTGAACTAAGTCAACCTGGCGCACTCCTAGGAGATGACC
	AAATCTATAATGTTATCGTTACCGCCCACGCGTTCGTAATAATTTTCTTTATAG
	TAATACCAATTATGATTGGCGGATTTGGTAACTGACTAATCCCCTTAATGATT
	GGCGCCCCAGACATGGCCTTCCCACGAATAAACAACATAAGTTTCTGACTTC
	TCCCGCCATCTTTCCTCCTCCTTTTAGCATCCTCTGGAGTAGAAGCAGGGGC
	CGGAACAGGGTGAACCGTCTACCCGCCCCTAGCGGGCAACCTCGCCCACGC
	AGGCGCCTCTGTGGACCTAACAATCTTTTCACTACACCTAGCAGGGGTGTCC
	TCAATTCTTGGAGCAATTAATTTCATTACCACAATTATTAACATAAAACCTCCG
	GCAATTTCCCAATACCAAACGCCCTTGTTCGTCTGAGCCGTTCTAATTACAGC
	CGTCTTATTACTATTATCCCTTCCCGTACTTGCTGCTGGCATCACAATGCTACT
	TACAGATCGAAATTTAAATACGACGTTCTTTGACCCGGCCGGGGGTGGGGAC
	CCAATCTTATACCAACACCTTTTC

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	Gen	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%

Table 2 shows that the two samples based on genetic testing with mitochondrial CO1 are the same species as *Belobranchus segura*. This is the new dicovery related to nike fish data. The previous studies never reported that *B. Segura* was the species member of nike schooling in Gorontalo waters. Further analysis was carried out with the phylogenetic tree to show the kinship relationships between samples (N3 and N5) and several species available in the NCBI database as presented in Fig.2.
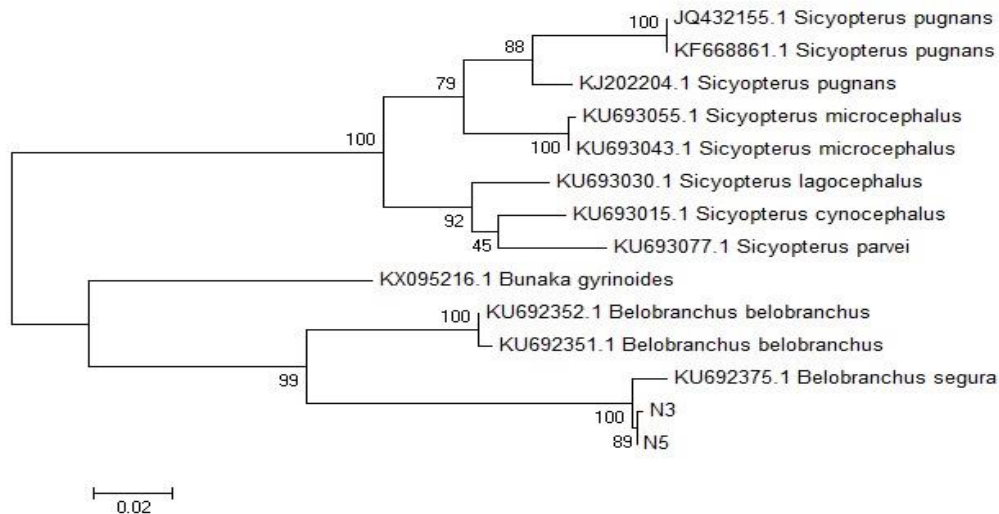
Figure 2. Phylogenetic trees of N3 and N5 samples were compared with several species in the NCBI database.

The genetic analysis illustrates that the two samples are similar species although they had different morphology particularly the melanophore patterns. This modification might be an adaptation of species when migrating from sea water to fresh water which is part of its life development. When entering a river mouth, post-larva undergoes changes in morphology, physiology, and behavior (Keith et al 2008). The difference in melanophore patterns from samples N3 and N5 is schematically presented in Figure 3.

Fig.3. Schematic morphological alterations in the samples N3 and N5 based on the shape of 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 the river (Fig. 4). Sample N5 is N3 that has developed and has undergone alterations in the melanophore structure. This can be possible because the N3 sample was taken from the catch on 8 October 2018, while the N5 sample was found in the sample conducted on 11 October 2018. This supports Valade et al (2009) statement that there has been a change in the appearance of chromatophore in the body *Sicyopterus langocephalus* larvae, which starts from the head area then spreads throughout the body along with the larvae age. The results of this study illustrate that there has been a variation in the pattern of melanophore from the species of nike *Belobranchus segura* when migrating from sea water to the mouth of the river. When still in the sea the melanophore arrangement is still minor, but when it will enter the river mouth the melanophore arrangement has spread along its body.
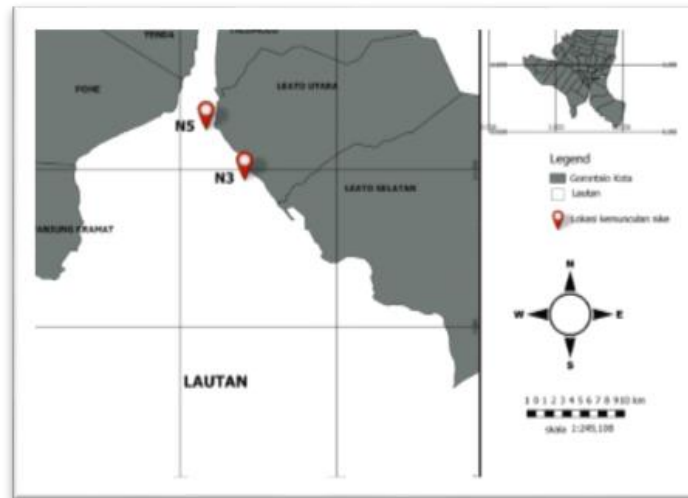
Figure 4. Area sampling of N3 and N5

Hawaiian gobioid fish are amphidromous and have one phase of life in the sea (Hobson & Smith 2007) They are in the pelagic phase of the sea for several months before juveniles in the river (Teichert et al 2016). Nike fishing in the Gorontalo Bay from the first, second and third days has a tendency to shift closer to the river mouth which indicates the migration of nike from sea water to fresh water (Olii et al 2017, Pasisingi & Abdullah 2018).

Changes in color morphology or melanophore patterns of nike species *Belobranchus segura* from the sea with melanophore content which becomes slightly more when approaching the estuary is a form of adaptation in order to enter a new aquatic environment or is part of the stages of its development. Distribution of species along the river is determined by postlarva color aggregation (Nishimoto & Fitzsimons 1986)

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 & Baras 2001). Although the lives of amphidromous fish are strongly related to the conditions of the oceans and rivers, 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 is related to their phase of development (Fitzsimon & McRae 2007). Post-larvae return to rivers where they are recruited and grown to reproductive stages (Ellien et al 2016).

The distribution of nike fish tends to approach the estuary since its appearance time until it disappears (Olii et al 2017). Based on the results of this study it can be concluded that in fact, nike fish do not disappear from the waters as what nike fishing community consider. There had been a morphological alteration in color due to an increase in the number of melanophores.

Conclusions. In conclusion, samples N3 and N5 that have different melanophore patterns are genetically the same species, *Belobrancianus segura*. The morphological changes of nike fish of this species are indicated by differences in melanophore patterns, that was an increase in the number and spread of melanophore on the surface of the body when this species has entered the river mouth.

References

Baldwin C.C., Mounts J.H., Smith D.G., Weigt L.A., 2008 Genetic identification and color descriptions of early life-history stages of Belizean Phaeoptyx and Astrapogon (Teleostei: Apogonidae) with comments on identification of adult Phaeoptyx. Zootaxa: 1–22.

Bucklin A., Steinke D., Blanco-Bercial L., 2011 DNA barcoding of marine metazoa. Annual Review of Marine Science 3: 471-508.

Ellien C., Werner U., Keith P., 2016 Morfological Change During the Transition from Freshwater to Sea Water in an Amphidromous Goby, *Sicyopterus lagocephalus* (Pallas 1770) (Teleostei). Ecology of Freshwater Fish: 48-59.

Folmer O., Black M., Hoeh W., Lutz R., Vrijenhoek R., 1994 DNA Primers for Amplification of Mitochondrial Cytochrome C Oxidase Subunit I from Diverse Metazoan Invertebrates. Molecular Marine Biology and Biotechnology 3(5): 294-299

Hebert P.D.N., Ratnasingham S., de Waard J.R., 2003 Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceeding of the Royal Society B 270 (1): S96-S99.

Larson H. K. 2001 *A Revision of the Gobiid Fish Genus Mugilogobius (Teleostei: Gobioidei) and Its Systematic Placement.* Australia: The Western Australian Museum. ISBN 07307 12923.

Maie T, Wilson M., Schoenfuss H., Blob R., 2009 Feeding kinematics and performance of Hawaiian stream Gobies, *Awaous guamensis* and *Lentipes concolor*: linkage of functional morphology. Journal of Morphology: 344-356.

McDowall R.M., 2009 Early Hatch: A Strategy for Safe Downstream Larval Transport in Ampidromi Fishes. Reviews in Fish Biology and Fisheries: 1-9

Morgulis A., Coulouris G., Raytselis Y., Madden T.L., Agarwala R., Schäffer A.A., 2008 Database Indexing for Production MegaBLAST Searches. Bioinformatics 24: 1757-1764.

Olii A.H., Sahami F. M., Hamzah S.N., Pasisingi N., 2017 Preliminary findings on distribution pattern of larvae of nike fish (Awaous sp.) in the estuary of Bone River, Gorontalo Province, Indonesia. AACL Bioflux 10: 1110-1118.

Olii A.H., Sahami F. M., Hamzah S.N., Pasisingi N., 2019 Molecular Approach to Identify Gobioid Fishes, "Nike" and "Hundala" (Local Name), from Gorontalo Waters, Indonesia. Journal of Biological Science DOI:10.3844/0jbsci.

Pasisingi N., Abdullah S., 2018 Pattern of nike fish (Gobiidae) occurrence in the Gorontalo Bay, Indonesia. Depik 7:111-118. [in Indonesian]

Purnama A. A., Mubarak J., Daruwati I., Roslim D. I., Elvyra R., 2019 First Report of Morphological and Molecular Identification of Greater Scissotail Rasbora caudimaculata from Rokan Hulu District, Riau Province, Indonesia. AACL Bioflux 34-41.

Tantu F., 2001 Nike (Order of Gobioidea) in the Bone Gorontalo Estuary. Tesis. Manado: Program Pasca Sarjana, Universitas Sam Ratulangi Manado. [in Indonesian]

Tamura K. D., Peterson N., Stecher G., Nei M., Kumar S., 2011 MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28: 2731-2739

Teletchea F., 2009 Molecular identification methods of fish species: Reassessment and possible applications. Reviews in Fish Biology and Fisheries: 265-293.

Thacker C.E., 2003. Moleculer Phylogeny of The Gobioid Fishes (Teleostei: Perciformes: Gobioidei) 26:354-368.

Thacker C. E., Roje D. M., 2011 Phylogeny of Gobiidae and identification of gobiid lineages. Systematics and Biodiversity 9(4):329–347.

Yamasaki N., Kondo M., Maeda K., Tachihara K., 2011 Reproductive biology of three amphidromous gobies, *Sicyopterus japonicus*, *Awaous melanocephalus*, and *Stenogobius* sp., on Okinawa Island. Cybium 35(4): 345-359.

Valade P., Lord C., Grondin H., Bosc P., Takillebois L., M, I., 2009 Early Life History and Description of Larval Stages of An Amphidromous Goby, *Sicyopterus lagocephalus* (Pallas, 1767) (Teleostei: Gobiidae: Sicydiinae). Cybium 33: 309-319.

Zhang Z., Schwartz S., Wagner L., Miller W., 2000 A greedy algorithm for aligning DNA sequences". Journal of Computational Biology 7(1-2): 203-14.

Authors:

Femy M. Sahami, Department of Aquatic Resources Management, Gorontalo State University, Jl. Jendral Sudirman, No.6, 96128, Gorontalo City, Indonesia, e-mail: femysahami@yahoo.co.id

Rene C. Kepel, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia, e-mail: renecharleskepel65@gmail.com
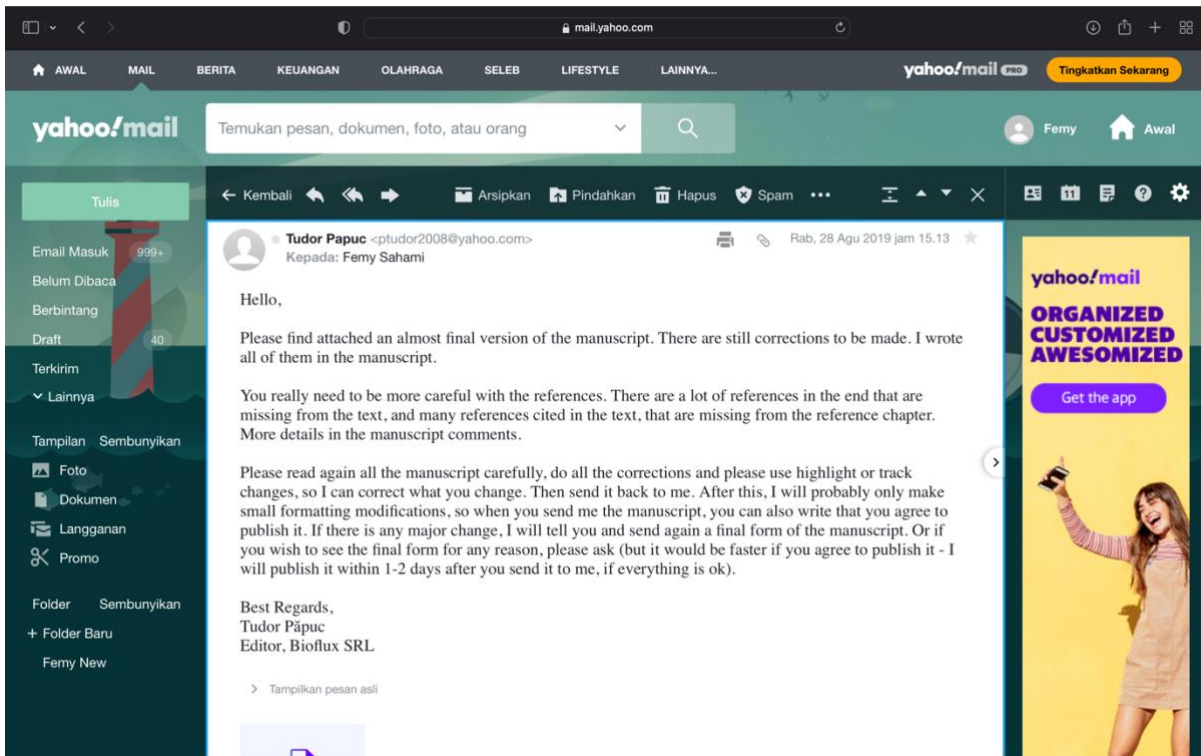
Abdul Hafidz Olii, Department of Aquatic Resources Management, Gorontalo State University, Jl. Jendral Sudirman, No.6, 96128, Gorontalo City, Indonesia, e-mail: oliihafidz@gmail.com

Silvester B. Pratasik, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia, e-mail: spjong07@yahoo.com
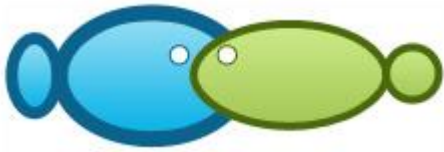
How to cite this article:
Sahami F. M., Kepel R. C., Olii A. H., Pratasik S. B., 2019 Determination of Morphological Alteration Based on Molecular Analysis and Melanophore Pattern of the Migrating Nike Fish in Gorontalo Bay, Indonesia. AACL Bioflux xx(x): xxxxxxx.

Gambar 13. Korespondensi

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

Femy M. Sahami[1], Rene C. Kepel[2], Abdul H. Olii[1], Silvester B. Pratasik[2]

[1] Department of Aquatic Resources Management, Faculty of Fisheries and Marine Science, Gorontalo State University, Gorontalo City, Indonesia; [2] Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia. Corresponding author: F. M. Sahami, femysahami@yahoo.co.id

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. Nike is the name of a small type of fish, usually 2 to 4 cm in length, which appear in some waters in a large numbers and are caught by local fishermen. 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.

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 species can be a source of information for preserving the biodiversity in the area.

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 (Tantu 2001). *A. melanocephalus* is an amphidromous fish. When the adults spawn in freshwater, the eggs are placed on the substrate on the riverbed. 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, after which 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, which can allow morphological changes. This sometimes leads to morphological dissimilarities in the same species.

The high demand for these fish causes high fishing activity and exploitation. Improper management will accelerate the extinction of nike fish. Not all waters can be managed directly (Sahami et al 2017). The responses of organisms to their environment represent significant information in the effort to manage waters. The differences in the topography of nike fishing might explain differences in adaptation, ecology and behaviour, and also differences expressed through changes in colour and size. Due to their small size and often cryptic ecologies, the full extent of gobiid diversity goes unnoticed (Thacker & Roje 2011). An effort 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 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 estuaries in Gorontalo waters, using molecular analysis.

Material and Method

Sampling. The study conducted from October 2018 to January 2019. The sampling of these fish from fishmen was carried out randomly during the period of capture in Leato waters from 5 to 11 October 2018. A map of the sampling location is shown in Figure 1. The collected samples were placed in plastic sterile bottles 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 grouped based on differences in melanophore patterns in the body 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.
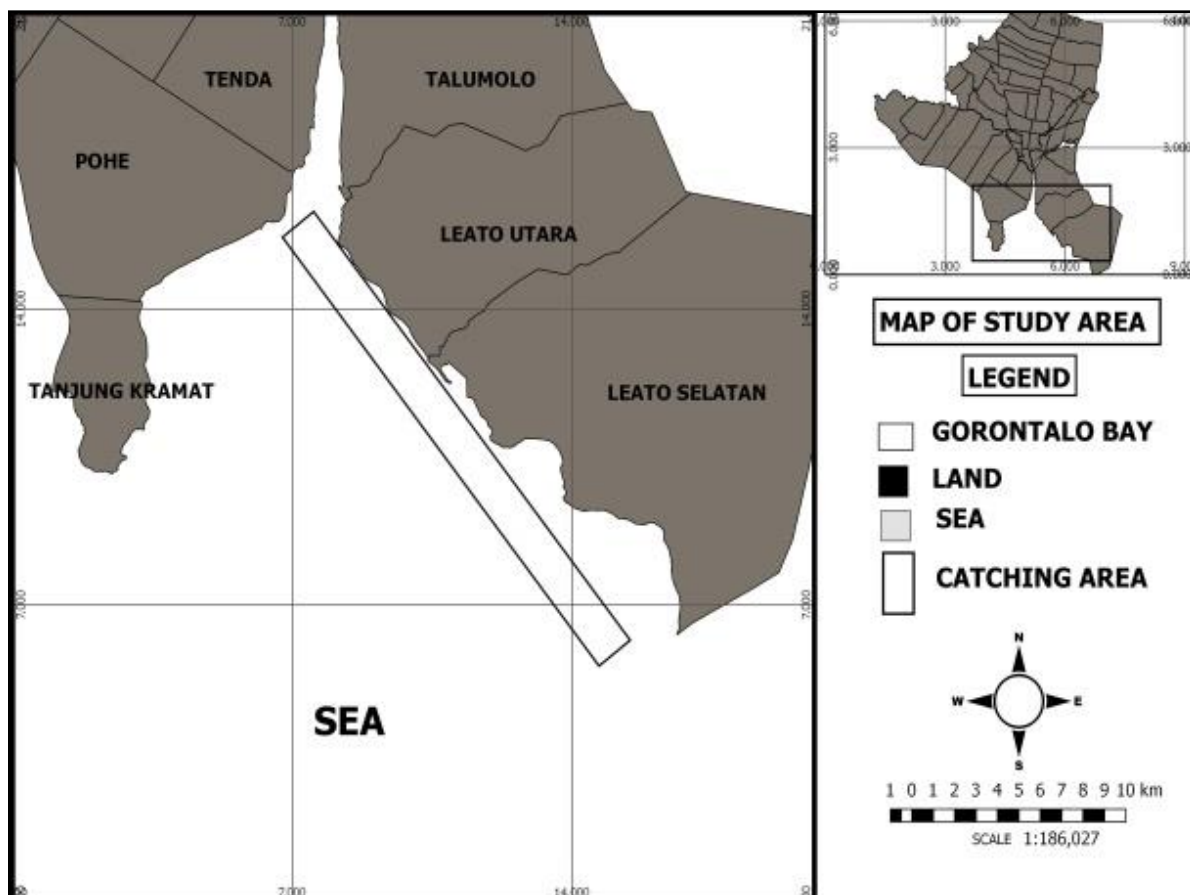
Figure 1. Map of the sampling sites in Gorontalo Bay.

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 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 which 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	CCTTTATCTTGTCTTCGGTGCCTGAGCCGGGATAGTGGGCACCGCTTTAAGCCTAC TTATCCGCGCTGAACTAAGTCAACCTGGCGCACTCCTAGGAGATGACCAAATCTAT AATGTTATCGTTACCGCCCACGCGTTCGTAATAATTTTCTTTATAGTAATACCAATT ATGATTGGCGGATTTGGTAACTGACTAATCCCCTTAATGATTGGCGCCCCAGACAT GGCCTTCCCACGAATAAACAACATAAGTTTCTGACTTCTCCCGCCATCTTTCCTCCT CCTTTTAGCATCCTCTGGAGTAGAAGCAGGGGCCGGAACAGGGTGAACCGTCTAC CCGCCCCTAGCGGGCAACCTCGCCCACGCAGGCGCCTCTGTGGACCTAACAATCT TTTCACTACACCTAGCAGGGGTGTCCTCAATTCTTGGAGCAATTAATTTCATTACCA CAATTATTAACATAAAACCTCCGGCAATTTCCCAATACCAAACGCCCTTGTTCGTCT GAGCCGTTCTAATTACAGCCGTCTTATTACTATTATCCCTTCCCGTACTTGCTGCTG GCATCACAATGCTACTTACAGATCGAAATTTAAATACGACGTTCTTTGACCCGGCC GGGGGTGGGGACCCAATCTTATACCAACACCTTTTC
N5	CCTTTATCTTGTCTTCGGTGCCTGAGCCGGGATAGTGGGCACAGCTTTAAGCCTAC TTATCCGCGCTGAACTAAGTCAACCTGGCGCACTCCTAGGAGATGACCAAATCTAT AATGTTATCGTTACCGCCCACGCGTTCGTAATAATTTTCTTTATAGTAATACCAATT ATGATTGGCGGATTTGGTAACTGACTAATCCCCTTAATGATTGGCGCCCCAGACAT GGCCTTCCCACGAATAAACAACATAAGTTTCTGACTTCTCCCGCCATCTTTCCTCCT CCTTTTAGCATCCTCTGGAGTAGAAGCAGGGGCCGGAACAGGGTGAACCGTCTAC CCGCCCCTAGCGGGCAACCTCGCCCACGCAGGCGCCTCTGTGGACCTAACAATCT TTTCACTACACCTAGCAGGGGTGTCCTCAATTCTTGGAGCAATTAATTTCATTACCA CAATTATTAACATAAAACCTCCGGCAATTTCCCAATACCAAACGCCCTTGTTCGTCT GAGCCGTTCTAATTACAGCCGTCTTATTACTATTATCCCTTCCCGTACTTGCTGCTG GCATCACAATGCTACTTACAGATCGAAATTTAAATACGACGTTCTTTGACCCGGCC GGGGGTGGGGACCCAATCTTATACCAACACCTTTTC

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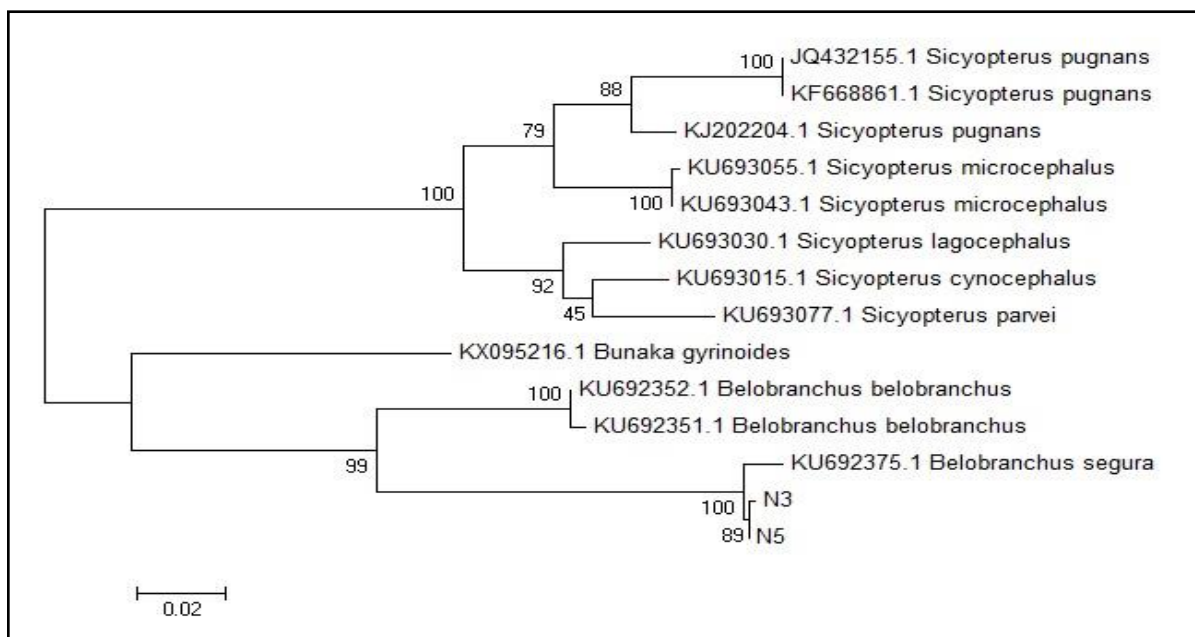
Figure 2. Phylogenetic trees of N3 and N5 samples compared with several species in the NCBI database.

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 difference 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 river mouth (Figure 4). 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.
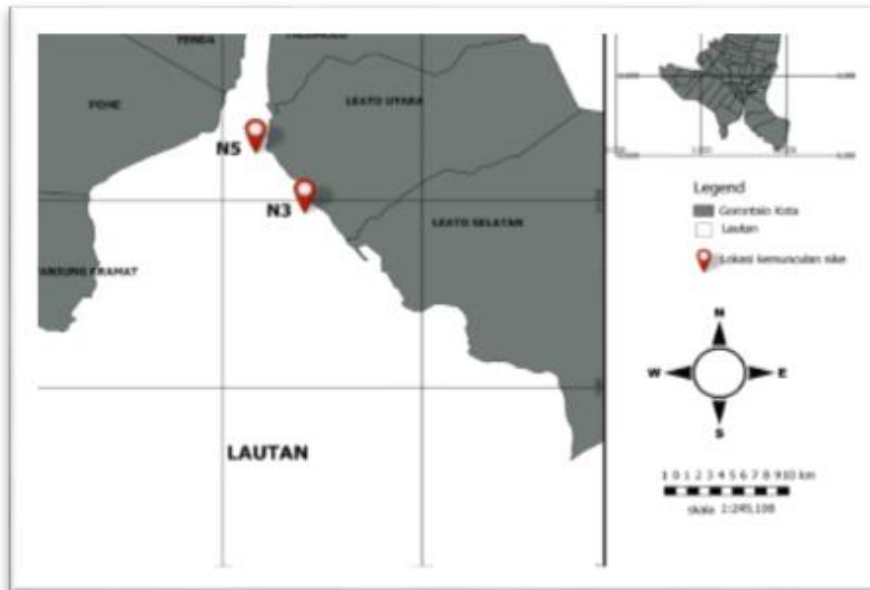
Figure 4. Area sampling of N3 and N5

Hawaiian gobioid fish are amphidromous and have one life stage in the sea (Hobson & Smith 2007). They stay in the pelagic sea zone for several months before migrating in the river (Teichert et al 2016). Nike fishing in the Gorontalo Bay from the first, second and third days has a tendency to shift closer to the river mouth 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 distribution of the species along the river is determined by post larva color aggregation (Nishimoto & Fitzsimons 1986).

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 & Baras 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 (Fitzsimon & McRae 2007). Post-larvae return to rivers where they are recruited and grown to reproductive stages (Ellien et al 2014).

The distribution of nike fish tends to approach the estuaries since its appearance time until it disappears (Olii et al 2017). Based on the results of this study it can be concluded that there are morphological alterations in color due to an increase in the number of melanophores. Also, nike fish do not disappear from the waters, as the nike fishing community considers.

Conclusions. **S**amples N3 and N5, with different melanophore patterns, are genetically the same species, *Belobrancianus 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.

References

Baldwin C. C., Mounts J. H., Smith D. G., Weigt L. A., 2009 Genetic identification and color descriptions of early life-history stages of Belizean *Phaeoptyx* and *Astrapogon* (Teleostei: Apogonidae) with comments on identification of adult *Phaeoptyx*. Zootaxa 2008:1–22.

Bucklin A., Steinke D., Blanco-Bercial L., 2011 DNA barcoding of marine metazoa. Annual Review of Marine Science 3:471-508.

Ellien C., Werner U., Keith P., 2014 Morfological Change During the Transition from Freshwater to Sea Water in an Amphidromous Goby, *Sicyopterus lagocephalus* (Pallas 1770) (Teleostei). Ecology of Freshwater Fish 25(1):48-59.

Folmer O., Black M., Hoeh W., Lutz R., Vrijenhoek R., 1994 DNA Primers for Amplification of Mitochondrial Cytochrome C Oxidase Subunit I from Diverse Metazoan Invertebrates. Molecular Marine Biology and Biotechnology 3(5):294-299.

Hebert P. D. N., Ratnasingham S., de Waard J. R., 2003 Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceeding of the Royal Society B 270(1): S96-S99.

Larson H. K., 2001 A Revision of the Gobiid Fish Genus Mugilogobius (Teleostei: Gobioidei) and Its Systematic Placement. Western Australian Museum, Perth, W. A., 233 p.

Maie T., Wilson M., Schoenfuss H., Blob R., 2009 Feeding kinematics and performance of Hawaiian stream Gobies, *Awaous guamensis* and *Lentipes concolor*: linkage of functional morphology. Journal of Morphology 270(3):344-356.

McDowall R. M., 2009 Early Hatch: A Strategy for Safe Downstream Larval Transport in Ampidromi Fishes. Reviews in Fish Biology and Fisheries 19:1-9.

Morgulis A., Coulouris G., Raytselis Y., Madden T. L., Agarwala R., Schäffer A. A., 2008 Database Indexing for Production MegaBLAST Searches. Bioinformatics 24:1757-1764.

Olii A. H., Sahami F. M., Hamzah S. N., Pasisingi N., 2017 Preliminary findings on distribution pattern of larvae of nike fish (*Awaous* sp.) in the estuary of Bone River, Gorontalo Province, Indonesia. AACL Bioflux 10:1110-1118.

Olii A. H., Sahami F. M., Hamzah S. N., Pasisingi N., 2019 Molecular Approach to Identify Gobioid Fishes, "Nike" and "Hundala" (Local Name), from Gorontalo Waters, Indonesia. Journal of Biological Science 19(1):52-56.

Pasisingi N., Abdullah S., 2018 [Pattern of nike fish (Gobiidae) occurrence in the Gorontalo Bay, Indonesia]. Depik 7:111-118. [in Indonesian]

Purnama A. A., Mubarak J., Daruwati I., Roslim D. I., Elvyra R., 2019 First Report of Morphological and Molecular Identification of Greater Scissotail *Rasbora caudimaculata* from Rokan Hulu District, Riau Province, Indonesia. AACL Bioflux 12(1):34-41.

Tantu F., 2001 [Nike (Order of Gobioidea) in the Bone Gorontalo Estuary]. Tesis. Manado: Program Pasca Sarjana, Universitas Sam Ratulangi Manado. [in Indonesian]

Tamura K. D., Peterson N., Stecher G., Nei M., Kumar S., 2011 MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28:2731-2739.

Teletchea F., 2009 Molecular identification methods of fish species: Reassessment and possible applications. Reviews in Fish Biology and Fisheries 19:265-293.

Thacker C. E., 2003. Moleculer Phylogeny of The Gobioid Fishes (Teleostei: Perciformes: Gobioidei) 26:354-368.

Thacker C. E., Roje D. M., 2011 Phylogeny of Gobiidae and identification of gobiid lineages. Systematics and Biodiversity 9(4):329–347.

Yamasaki N., Kondo M., Maeda K., Tachihara K., 2011 Reproductive biology of three amphidromous gobies, *Sicyopterus japonicus*, *Awaous melanocephalus*, and *Stenogobius* sp., on Okinawa Island. Cybium 35(4):345-359.

Valade P., Lord C., Grondin H., Bosc P., Takillebois L. M. I., 2009 Early Life History and Description of Larval Stages of An Amphidromous Goby, *Sicyopterus lagocephalus* (Pallas, 1767) (Teleostei: Gobiidae: Sicydiinae). Cybium 33:309-319.

Zhang Z., Schwartz S., Wagner L., Miller W., 2000 A greedy algorithm for aligning DNA sequences". Journal of Computational Biology 7(1-2):203-214.

Authors:
Femy M. Sahami, Department of Aquatic Resources Management, Gorontalo State University, Jl. Jendral Sudirman, No.6, 96128, Gorontalo City, Indonesia, e-mail: femysahami@yahoo.co.id
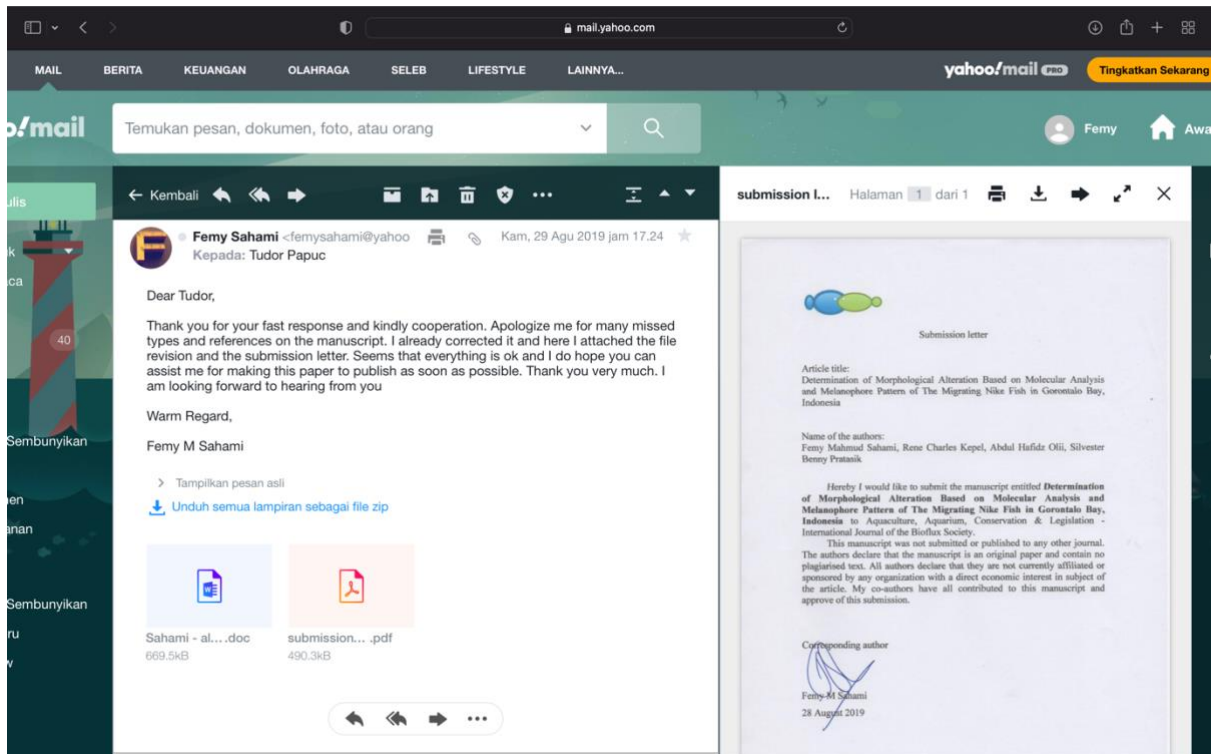
Rene C. Kepel, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia, e-mail: renecharleskepel65@gmail.com
Abdul Hafidz Olii, Department of Aquatic Resources Management, Gorontalo State University, Jl. Jendral Sudirman, No.6, 96128, Gorontalo City, Indonesia, e-mail: oliihafidz@gmail.com
Silvester B. Pratasik, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia, e-mail: spjong07@yahoo.com

How to cite this article:
Sahami F. M., Kepel R. C., Olii A. H., Pratasik S. B., 2019 Determination of Morphological Alteration Based on Molecular Analysis and Melanophore Pattern of the Migrating Nike Fish in Gorontalo Bay, Indonesia. AACL Bioflux xx(x): xxxxxxx.

[1] Department of Aquatic Resources Management, Faculty of Fisheries and Marine Science, Gorontalo State University, Jl. Jendral Sudirman. No. 6, Gorontalo City, Gorontalo Province, Indonesia; [2] Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia. Corresponding author: F. M. Sahami, femysahami@yahoo.co.id

Gambar 14. Korespondensi

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

Femy M. Sahami[1], Rene C. Kepel[2], Abdul H. Olii[1], Silvester B. Pratasik[2]

[1] Department of Aquatic Resources Management, Faculty of Fisheries and Marine Science, Gorontalo State University, Gorontalo City, Indonesia; [2] Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia. Corresponding author: F. M. Sahami, femysahami@yahoo.co.id

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 life history pattern of tropical fishes, about 200 spesies from families undertake diadromus migration and the most common form of diadromy in the tropics is amphidromy (Milton 2009). Nike fish in this study is an amphidormy species which is alleged to be a typical species in Gorontalo region, is a small fish whose frequency of occurrence in waters is very limited. A temporary assumption is that nike is a type of fish that migrates for nursery and spawning purpose from seawater to freshwater (Olii et al 2017). Nike is the name of a small type of fish, usually 2 to 4 cm in length, which appear in some waters in a large numbers and are caught by local fishermen. 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 of the appearance period (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 species can be a source of information for preserving the biodiversity in the area. Kumar (2018) stated that the biodiversity provides ecological service 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 substrate on the riverbed. 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, after which 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, which can allow morphological changes. This sometimes leads to morphological dissimilarities in the same species.

The high price as an impact of The high demand for these fish causes high fishing activity and exploitation. Wolok et al (2019) stated that within 1 (one) year of arrest, the average fisherman gets 40 (forty) buckets with an average selling price of 750,000 IDR per bucket if sold on the market. So in 1 (One) year of arrest, fishermen get 30,000,000 IDR. All this time, there are no proper management for this resource due to lack of scientific data. Therefore, an Improper management potentially will threaten will accelerate the extinction sustainability of nike fish. The responses of organisms to their environment represent significant information in the effort to manage waters. The differences in the topography of nike fishing might explain differences in adaptation, ecology and behaviour, and also differences expressed through changes in colour and size. Due to their small size and often cryptic ecologies, the full extent of gobiid diversity goes unnoticed (Thacker & Roje 2011). An effort 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 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 estuaries in Gorontalo waters, using molecular analysis.

Material and Method

Sampling. The study conducted from October 2018 to January 2019. The sampling of these fish from fishmen was carried out randomly during the period of capture in Leato waters from 5 to 11 October 2018. A map of the sampling location is shown in Figure 1. The collected samples were placed in plastic sterile bottles 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 grouped based on differences in melanophore patterns in the body 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.
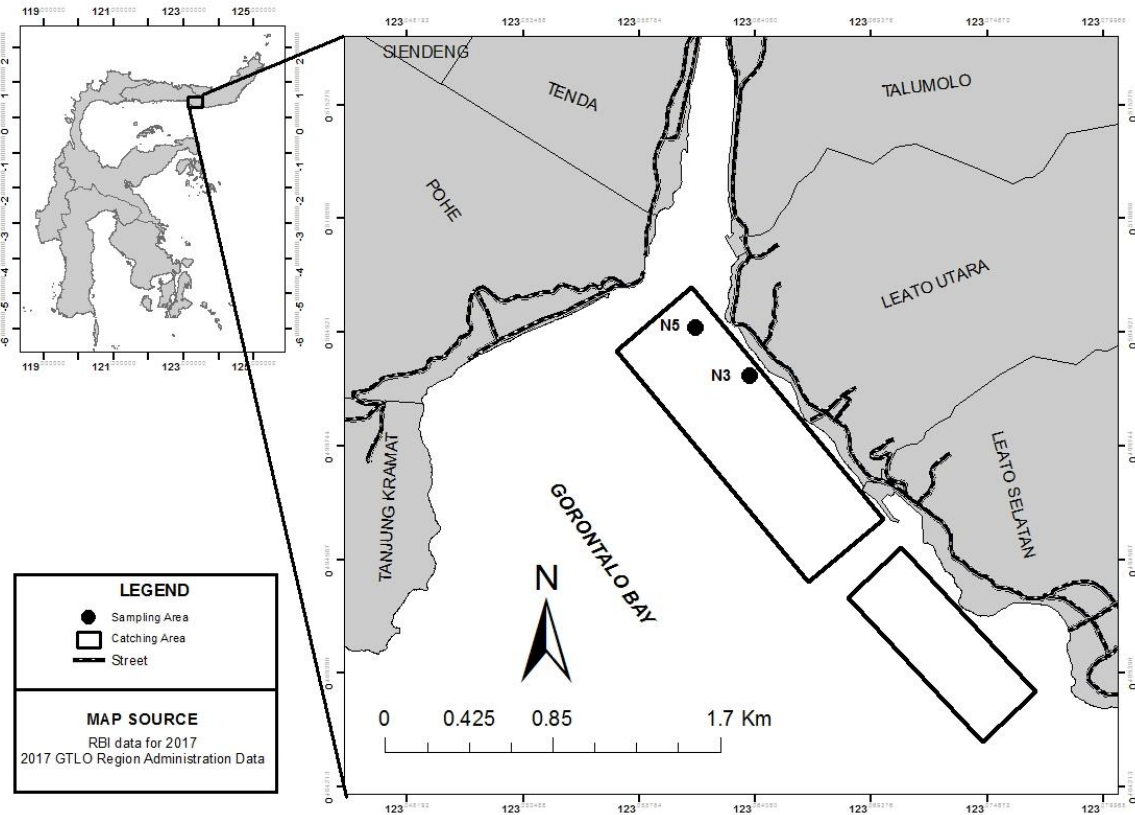
Figure 1. Map of the sampling sites in Gorontalo Bay.

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 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 which 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	CCTTTATCTTGTCTTCGGTGCCTGAGCCGGGATAGTGGGCACCGCTTTAAGCCTAC TTATCCGCGCTGAACTAAGTCAACCTGGCGCACTCCTAGGAGATGACCAAATCTAT

	AATGTTATCGTTACCGCCCACGCGTTCGTAATAATTTTCTTTATAGTAATACCAATT
	ATGATTGGCGGATTTGGTAACTGACTAATCCCCTTAATGATTGGCGCCCCAGACAT
	GGCCTTCCCACGAATAAACAACATAAGTTTCTGACTTCTCCCGCCATCTTTCCTCCT
	CCTTTTAGCATCCTCTGGAGTAGAAGCAGGGGCCGGAACAGGGTGAACCGTCTAC
	CCGCCCCTAGCGGGCAACCTCGCCCACGCAGGCGCCTCTGTGGACCTAACAATCT
	TTTCACTACACCTAGCAGGGGTGTCCTCAATTCTTGGAGCAATTAATTTCATTACCA
	CAATTATTAACATAAAACCTCCGGCAATTTCCCAATACCAAACGCCCTTGTTCGTCT
	GAGCCGTTCTAATTACAGCCGTCTTATTACTATTATCCCTTCCCGTACTTGCTGCTG
	GCATCACAATGCTACTTACAGATCGAAATTTAAATACGACGTTCTTTGACCCGGCC
	GGGGGTGGGGACCCAATCTTATACCAACACCTTTTC
N5	CCTTTATCTTGTCTTCGGTGCCTGAGCCGGGATAGTGGGCACAGCTTTAAGCCTAC
	TTATCCGCGCTGAACTAAGTCAACCTGGCGCACTCCTAGGAGATGACCAAATCTAT
	AATGTTATCGTTACCGCCCACGCGTTCGTAATAATTTTCTTTATAGTAATACCAATT
	ATGATTGGCGGATTTGGTAACTGACTAATCCCCTTAATGATTGGCGCCCCAGACAT
	GGCCTTCCCACGAATAAACAACATAAGTTTCTGACTTCTCCCGCCATCTTTCCTCCT
	CCTTTTAGCATCCTCTGGAGTAGAAGCAGGGGCCGGAACAGGGTGAACCGTCTAC
	CCGCCCCTAGCGGGCAACCTCGCCCACGCAGGCGCCTCTGTGGACCTAACAATCT
	TTTCACTACACCTAGCAGGGGTGTCCTCAATTCTTGGAGCAATTAATTTCATTACCA
	CAATTATTAACATAAAACCTCCGGCAATTTCCCAATACCAAACGCCCTTGTTCGTCT
	GAGCCGTTCTAATTACAGCCGTCTTATTACTATTATCCCTTCCCGTACTTGCTGCTG
	GCATCACAATGCTACTTACAGATCGAAATTTAAATACGACGTTCTTTGACCCGGCC
	GGGGGTGGGGACCCAATCTTATACCAACACCTTTTC

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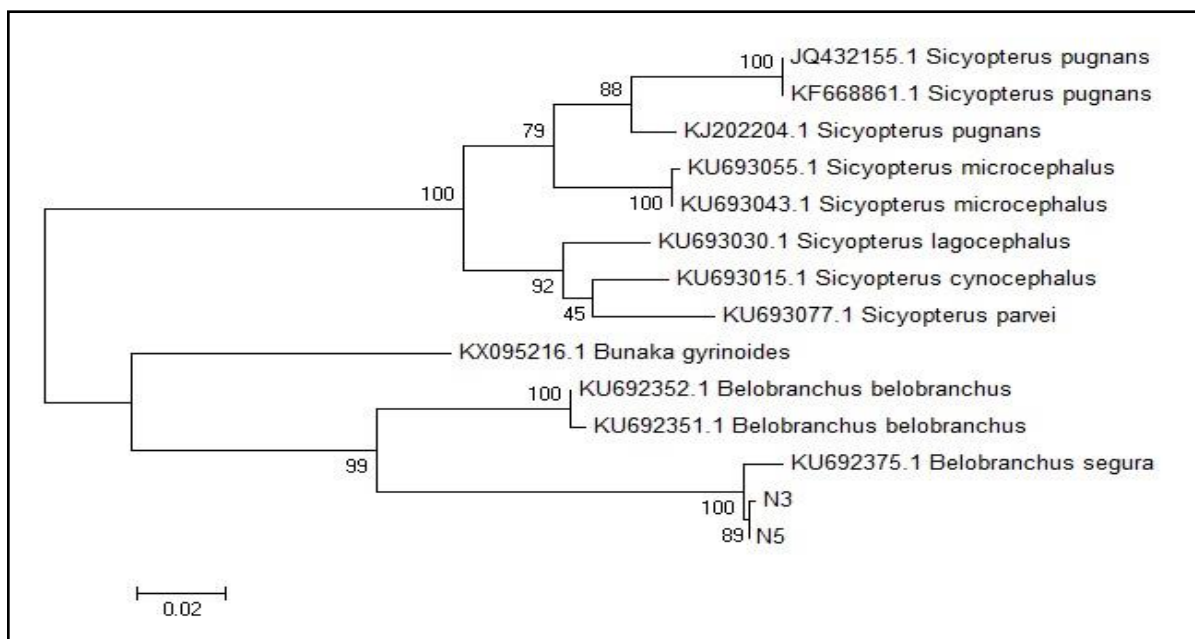
Figure 2. Phylogenetic trees of N3 and N5 samples compared with several species in the NCBI database.

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 difference 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 river mouth (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 fishing in the Gorontalo Bay from the first, second and third days of

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 & Baras 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) informed that amphidromous fishes spawn in freshwater and the larvae migrate to the sea before migrating back to freshwater. *Sicyopterus lagocephalus* as an amphidromous goby species needs drastic change of habitat to fulfil its life cycle: adults live and spawn in rivers, where eggs hatch into prolarvae that have to reach the sea to acquire characteristics of planktonic larvae. 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).

The distribution of nike fish tends to approach the estuaries since its appearance time until it disappears (Olii et al 2017). Based on the results of this study it can be concluded that there are morphological alterations in color due to an increase in the number of melanophores. Also, nike fish do not disappear from the waters, as the nike fishing community considers.

Conclusions. **S**amples N3 and N5, with different melanophore patterns, are genetically the same species, ~~*Belobrancianus*~~ *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.

References

Baldwin C. C., Mounts J. H., Smith D. G., Weigt L. A., 2009 Genetic identification and color descriptions of early life-history stages of Belizean *Phaeoptyx* and *Astrapogon* (Teleostei: Apogonidae) with comments on identification of adult *Phaeoptyx*. Zootaxa 2008:1–22.

Ellien C., Werner U., Keith P., 2014 Morfological Change During the Transition from Freshwater to Sea Water in an Amphidromous Goby, *Sicyopterus lagocephalus* (Pallas 1770) (Teleostei). Ecology of Freshwater Fish 25(1):48-59.

Fitzsimons, J.M., McRae M.G., Nishimoto R.T 2007 [Behavioral ecology of indigenous stream fishes in Hawai'i]. In: [Biology of Hawaiian Streams and Estuaries]. Evenhuis N.L & Fitzsimons J.M. (eds.), pp. 11–21. Bishop Museum Bulletin in Cultural and Environmental Studies.Hobson, K.A., Smith, R.J.F., Sorensen, P 2007 [Applications of stable isotope analysis to tracing nutrient sources to Hawaiian gobioid fishes and other stream organisms]. In: [Biology of Hawaiian Streams and Estuaries]. Evenhuis N.L & Fitzsimons J.M. (eds.), pp. 99–111. Bishop Museum Bulletin in Cultural and Environmental Studies.

Keith P., Hoareau T., Lord C., Ah-Yane O., Gimmoneau G., Robinet T., Valade P 2008. Characterisation of post-larval to juvenile stages, metamorphosis, and recruitment of an amphidromous goby, *Sicyopterus lagocephalus* [Pallas, 1767] (Teleostei: Gobiidae: Sicydiinae). Marine and Freshwater Research 59 (10): 876-889.

Kumar, A 2018 Biodiversity conservation and management in India, Brazil and South Africa. Biodiversity: Law, Policy and Governance. Tandon, U. (Ed.), Parasaran, M. (Ed.), Luthra, S. (Ed.). Routledge in association with GSE Research, London.

Lucas, M.C., Baras E 2001 Migration of freshwater fishes. MPG Books Ltd., Bodmin, Cornwall, Great Britain.

Maie T., Wilson M., Schoenfuss H., Blob R., 2009 Feeding kinematics and performance of Hawaiian stream Gobies, *Awaous guamensis* and *Lentipes concolor*: linkage of functional morphology. Journal of Morphology 270(3):344-356.

Milton, D. A 2009 Living in two worlds: Diadromous fishes, and factors affecting population connectivity between tropical rivers and coasts. Ecological connectivity among tropical coastal ecosystems, ed. I. Nagelkerken 325-355.

Nishimoto, R.T., Fitzsimons J.M 1986 [Courtship, territoriality, and coloration in the endemic Hawaiian freshwater goby, *Lentipes concolor*]. In: [Indo-Pacific fish biology]. Uyeno, T., Arai R., Taniuchi T., & Matsuura K. (eds.), pp. 811–817. Proceedings of the second International Conference on Indo-Pacific fishes, Ichthyological Society of Japan, Tokyo.

Olii A. H., Sahami F. M., Hamzah S. N., Pasisingi N., 2017 Preliminary findings on distribution pattern of larvae of nike fish (*Awaous* sp.) in the estuary of Bone River, Gorontalo Province, Indonesia. AACL Bioflux 10:1110-1118.

Olii A. H., Sahami F. M., Hamzah S. N., Pasisingi N., 2019 Molecular Approach to Identify Gobioid Fishes, "Nike" and "Hundala" (Local Name), from Gorontalo Waters, Indonesia. Journal of Biological Science 19(1):52-56.

Pasisingi N., Abdullah S., 2018 [Pattern of nike fish (Gobiidae) occurrence in the Gorontalo Bay, Indonesia]. Depik 7:111-118. [in Indonesian]

Purnama A. A., Mubarak J., Daruwati I., Roslim D. I., Elvyra R., 2019 First Report of Morphological and Molecular Identification of Greater Scissotail *Rasbora caudimaculata* from Rokan Hulu District, Riau Province, Indonesia. AACL Bioflux 12(1):34-41.

Tantu F., 2001 [Spatial-Temporal Abbundance of Nike (Order of Gobioidea) in the Bone Gorontalo Estuary]. Master Thesis. Manado: Program Pasca Sarjana, Universitas Sam Ratulangi Manado pp. 45 [in Indonesian]

Thacker C. E., 2003. Moleculer Phylogeny of The Gobioid Fishes (Teleostei: Perciformes: Gobioidei). Molecular Phylogenetics and Evolution 26:354-368.

Teichert, N., Valade P., Grondin H., Trichet E., Sardenne F., Gaudin P 2016 Pelagic larval traits of the amphidromous goby *Sicyopterus lagocephalus* display seasonal variations related to temperature in La Réunion Island. Ecology of Freshwater Fish 25: 234-247.

Thacker C. E., Roje D. M., 2011 Phylogeny of Gobiidae and identification of gobiid lineages. Systematics and Biodiversity 9(4):329–347.

Valade P., Lord C., Grondin H., Bosc P., Takillebois L. M. I., 2009 Early Life History and Description of Larval Stages of An Amphidromous Goby, *Sicyopterus lagocephalus* (Pallas, 1767) (Teleostei: Gobiidae: Sicydiinae). Cybium 33:309-319.

Wolok, T., Fachrussyah Z.C., Yantu I 2019 Technical And Economic Analysis Of Catching Equipment Totaluo In Nike Fishing (*Awaous melanocephalus*) In Gorontalo City. Jambura Science of Management 1 (2): 65-71.

Yamasaki N., Kondo M., Maeda K., Tachihara K., 2011 Reproductive biology of three amphidromous gobies, *Sicyopterus japonicus*, *Awaous melanocephalus*, and *Stenogobius* sp., on Okinawa Island. Cybium 35(4):345-359.

Authors:
Femy Mahmud Sahami, Department of Aquatic Resources Management, Gorontalo State University, Jl. Jendral Sudirman, No.6, 96128, Gorontalo City, Indonesia, e-mail: femysahami@yahoo.co.id
Rene Charles Kepel, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia, e-mail: renecharleskepel65@gmail.com
Abdul Hafidz Olii, Department of Aquatic Resources Management, Gorontalo State University, Jl. Jendral Sudirman, No.6, 96128, Gorontalo City, Indonesia, e-mail: oliihafidz@gmail.com
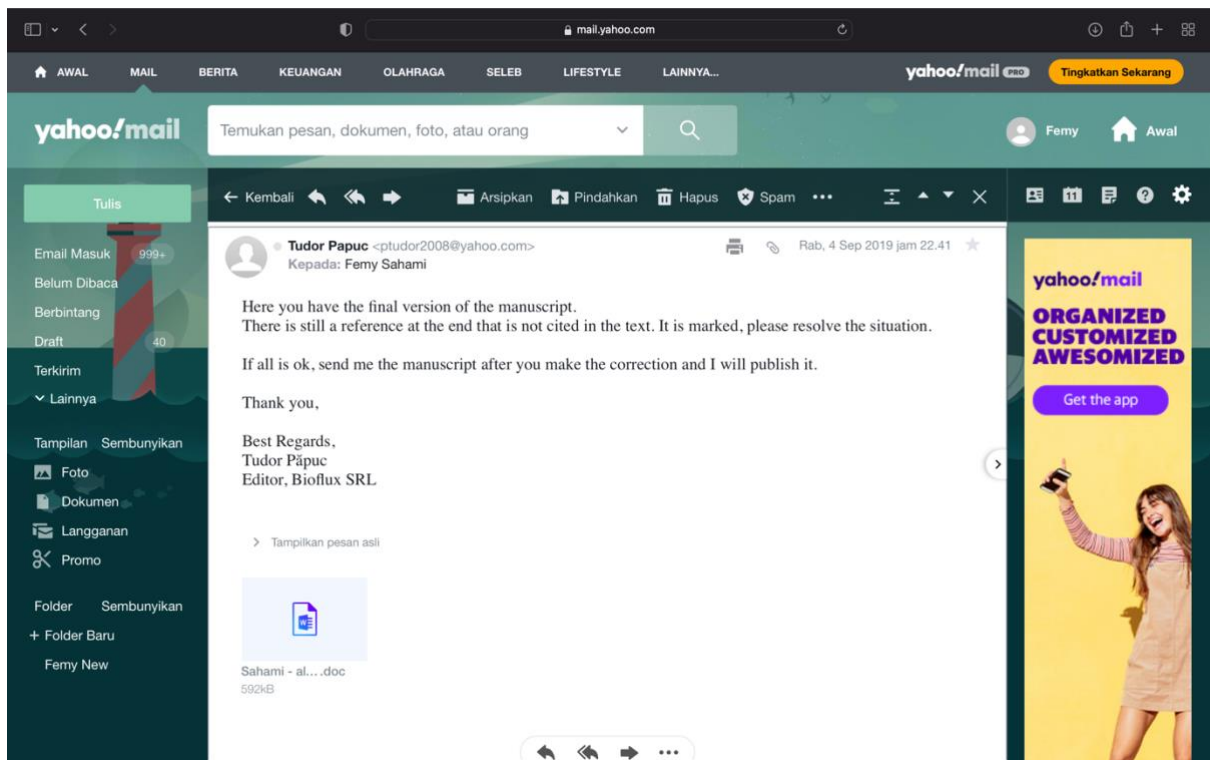Silvester Benny Pratasik, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia, e-mail: spjong07@yahoo.com

[1] Department of Aquatic Resources Management, Faculty of Fisheries and Marine Science, Gorontalo State University, Jl. Jendral Sudirman. No. 6, Gorontalo City, Gorontalo Province, Indonesia; [2] Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia. Corresponding author: F. M. Sahami, femysahami@yahoo.co.id

Gambar 15. Korespondensi

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

Femy M. Sahami[1], Rene C. Kepel[2], Abdul H. Olii[1], Silvester B. Pratasik[2]

[1] Department of Aquatic Resources Management, Faculty of Fisheries and Marine Science, Gorontalo State University, Gorontalo City, Indonesia; [2] Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia. Corresponding author: F. M. Sahami, femysahami@yahoo.co.id

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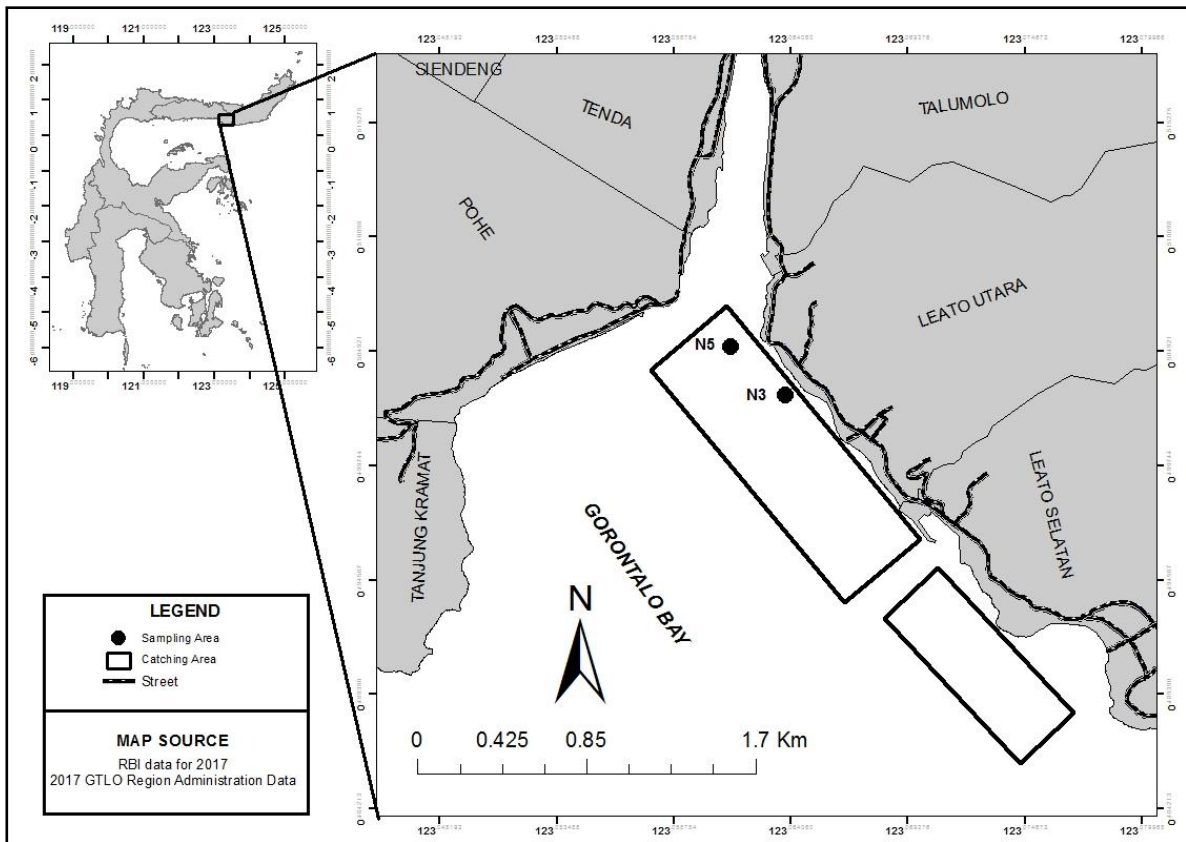
Figure 1. Map of the sampling sites in Gorontalo Bay.

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	CCTTTATCTTGTCTTCGGTGCCTGAGCCGGGATAGTGGGCACCGCTTTAAGCCTAC TTATCCGCGCTGAACTAAGTCAACCTGGCGCACTCCTAGGAGATGACCAAATCTAT AATGTTATCGTTACCGCCCACGCGTTCGTAATAATTTTCTTTATAGTAATACCAATT ATGATTGGCGGATTTGGTAACTGACTAATCCCCTTAATGATTGGCGCCCCAGACAT GGCCTTCCCACGAATAAACAACATAAGTTTCTGACTTCTCCCGCCATCTTTCCTCCT CCTTTTAGCATCCTCTGGAGTAGAAGCAGGGGCCGGAACAGGGTGAACCGTCTAC CCGCCCCTAGCGGGCAACCTCGCCCACGCAGGCGCCTCTGTGGACCTAACAATCT TTTCACTACACCTAGCAGGGGTGTCCTCAATTCTTGGAGCAATTAATTTCATTACCA CAATTATTAACATAAAACCTCCGGCAATTTCCCAATACCAAACGCCCTTGTTCGTCT GAGCCGTTCTAATTACAGCCGTCTTATTACTATTATCCCTTCCCGTACTTGCTGCTG GCATCACAATGCTACTTACAGATCGAAATTTAAATACGACGTTCTTTGACCCGGCC GGGGGTGGGGACCCAATCTTATACCAACACCTTTTC
N5	CCTTTATCTTGTCTTCGGTGCCTGAGCCGGGATAGTGGGCACAGCTTTAAGCCTAC TTATCCGCGCTGAACTAAGTCAACCTGGCGCACTCCTAGGAGATGACCAAATCTAT AATGTTATCGTTACCGCCCACGCGTTCGTAATAATTTTCTTTATAGTAATACCAATT ATGATTGGCGGATTTGGTAACTGACTAATCCCCTTAATGATTGGCGCCCCAGACAT GGCCTTCCCACGAATAAACAACATAAGTTTCTGACTTCTCCCGCCATCTTTCCTCCT CCTTTTAGCATCCTCTGGAGTAGAAGCAGGGGCCGGAACAGGGTGAACCGTCTAC CCGCCCCTAGCGGGCAACCTCGCCCACGCAGGCGCCTCTGTGGACCTAACAATCT TTTCACTACACCTAGCAGGGGTGTCCTCAATTCTTGGAGCAATTAATTTCATTACCA CAATTATTAACATAAAACCTCCGGCAATTTCCCAATACCAAACGCCCTTGTTCGTCT GAGCCGTTCTAATTACAGCCGTCTTATTACTATTATCCCTTCCCGTACTTGCTGCTG GCATCACAATGCTACTTACAGATCGAAATTTAAATACGACGTTCTTTGACCCGGCC GGGGGTGGGGACCCAATCTTATACCAACACCTTTTC

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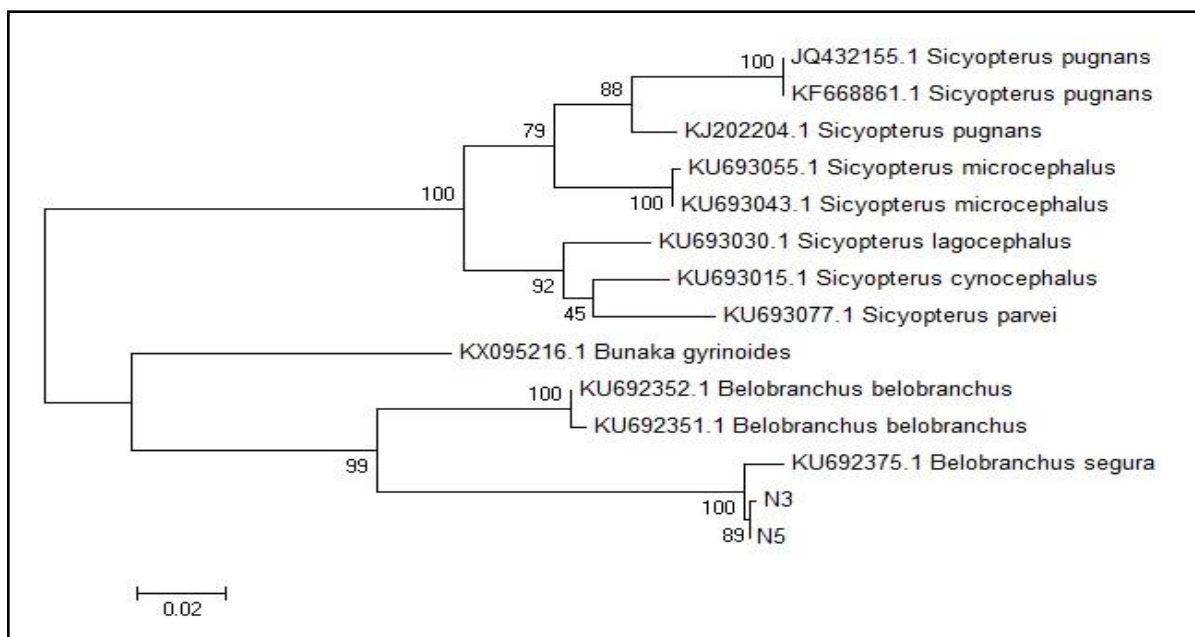
Figure 2. Phylogenetic trees of N3 and N5 samples compared with several species in the NCBI database.

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.

References

Baldwin C. C., Mounts J. H., Smith D. G., Weigt L. A., 2009 Genetic identification and color descriptions of early life-history stages of Belizean *Phaeoptyx* and *Astrapogon* (Teleostei: Apogonidae) with comments on identification of adult *Phaeoptyx*. Zootaxa 2008:1–22.

Ellien C., Werner U., Keith P., 2014 Morfological Change During the Transition from Freshwater to Sea Water in an Amphidromous Goby, *Sicyopterus lagocephalus* (Pallas 1770) (Teleostei). Ecology of Freshwater Fish 25(1):48-59.

Fitzsimons J. M., McRae M. G., Nishimoto R. T., 2007 Behavioral ecology of indigenous stream fishes in Hawaii. In: Biology of Hawaiian Streams and Estuaries. Evenhuis N. L., Fitzsimons J. M. (eds), Bishop Museum Bulletin in Cultural and Environmental Studies 3:11–21. Hobson K. A., Smith R. J. F., Sorensen P., 2007 Applications of stable isotope analysis to tracing nutrient sources to Hawaiian gobioid fishes and other stream organisms. In: Biology of Hawaiian Streams and Estuaries. Evenhuis N. L, Fitzsimons J. M. (eds), Bishop Museum Bulletin in Cultural and Environmental Studies 3:99–111.

Keith P., Hoareau T., Lord C., Ah-Yane O., Gimmoneau G., Robinet T., Valade P., 2008 Characterisation of post-larval to juvenile stages, metamorphosis, and recruitment of an amphidromous goby, *Sicyopterus lagocephalus* (Pallas, 1767) (Teleostei: Gobiidae: Sicydiinae). Marine and Freshwater Research 59(10):876-889.

Kumar A., 2017 Biodiversity conservation and management in India, Brazil and South Africa. In: Biodiversity Law, Policy and Governance. Tandon U., Parasaran M., Luthra S. (eds), Routledge India, London, 366 p.

Lucas M. C., Baras E., Thom T. J., Duncan A., Slavic O., 2001 Migration of freshwater fishes. MPG Books Ltd, Cornwall, 466 p.

Maie T., Wilson M., Schoenfuss H., Blob R., 2009 Feeding kinematics and performance of Hawaiian stream Gobies, *Awaous guamensis* and *Lentipes concolor*: linkage of functional morphology. Journal of Morphology 270(3):344-356.

Milton D. A., 2009 Living in two worlds: Diadromous fishes, and factors affecting population connectivity between tropical rivers and coasts. In: Ecological connectivity among tropical

coastal ecosystems. Nagelkerken I. (ed), Dordrecht: Springer Science+Business Media B. V., Berlin, pp. 325-355.

Nishimoto R. T., Fitzsimons J. M., 1986 Courtship, territoriality, and coloration in the endemic Hawaiian freshwater goby, *Lentipes concolor*. In: Indo-Pacific fish biology. Uyeno T., Arai R., Taniuchi T., Matsuura K. (eds.), Proceedings of the second International Conference on Indo-Pacific fishes, Ichthyological Society of Japan, Tokyo, pp. 811–817.

Olii A. H., Sahami F. M., Hamzah S. N., Pasisingi N., 2017 Preliminary findings on distribution pattern of larvae of nike fish (*Awaous* sp.) in the estuary of Bone River, Gorontalo Province, Indonesia. AACL Bioflux 10:1110-1118.

Olii A. H., Sahami F. M., Hamzah S. N., Pasisingi N., 2019 Molecular Approach to Identify Gobioid Fishes, "Nike" and "Hundala" (Local Name), from Gorontalo Waters, Indonesia. Journal of Biological Science 19(1):52-56.

Pasisingi N., Abdullah S., 2018 [Pattern of nike fish (Gobiidae) occurrence in the Gorontalo Bay, Indonesia]. Depik 7:111-118. [in Indonesian]

Purnama A. A., Mubarak J., Daruwati I., Roslim D. I., Elvyra R., 2019 First Report of Morphological and Molecular Identification of Greater Scissotail *Rasbora caudimaculata* from Rokan Hulu District, Riau Province, Indonesia. AACL Bioflux 12(1):34-41.

Tantu F., 2001 [Spatial-Temporal Abbundance of Nike (Order of Gobioidea) in the Bone Gorontalo Estuary]. Master Thesis, Manado: Program Pasca Sarjana, Universitas Sam Ratulangi, Manado, 45 p. [in Indonesian]

Teichert N., Valade P., Grondin H., Trichet E., Sardenne F., Gaudin P., 2016 Pelagic larval traits of the amphidromous goby *Sicyopterus lagocephalus* display seasonal variations related to temperature in La Réunion Island. Ecology of Freshwater Fish 25:234-247.

Thacker C. E., 2003. Moleculer Phylogeny of The Gobioid Fishes (Teleostei: Perciformes: Gobioidei). Molecular Phylogenetics and Evolution 26:354-368.

Thacker C. E., Roje D. M., 2011 Phylogeny of Gobiidae and identification of gobiid lineages. Systematics and Biodiversity 9(4):329–347.

Valade P., Lord C., Grondin H., Bosc P., Takillebois L. M. I., 2009 Early Life History and Description of Larval Stages of An Amphidromous Goby, *Sicyopterus lagocephalus* (Pallas, 1767) (Teleostei: Gobiidae: Sicydiinae). Cybium 33:309-319.

Wolok T., Fachrussyah Z. C., Yantu I., 2019 Technical And Economic Analysis Of Catching Equipment Totaluo In Nike Fishing (*Awaous melanocephalus*) In Gorontalo City. Jambura Science of Management 1(2):65-71.

Yamasaki N., Kondo M., Maeda K., Tachihara K., 2011 Reproductive biology of three amphidromous gobies, *Sicyopterus japonicus*, *Awaous melanocephalus*, and *Stenogobius* sp., on Okinawa Island. Cybium 35(4):345-359.

Authors:
Femy Mahmud Sahami, Department of Aquatic Resources Management, Gorontalo State University, Jl. Jendral Sudirman, No.6, 96128, Gorontalo City, Indonesia, e-mail: femysahami@yahoo.co.id
Rene Charles Kepel, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia, e-mail: renecharleskepel65@gmail.com
Abdul Hafidz Olii, Department of Aquatic Resources Management, Gorontalo State University, Jl. Jendral Sudirman, No.6, 96128, Gorontalo City, Indonesia, e-mail: oliihafidz@gmail.com
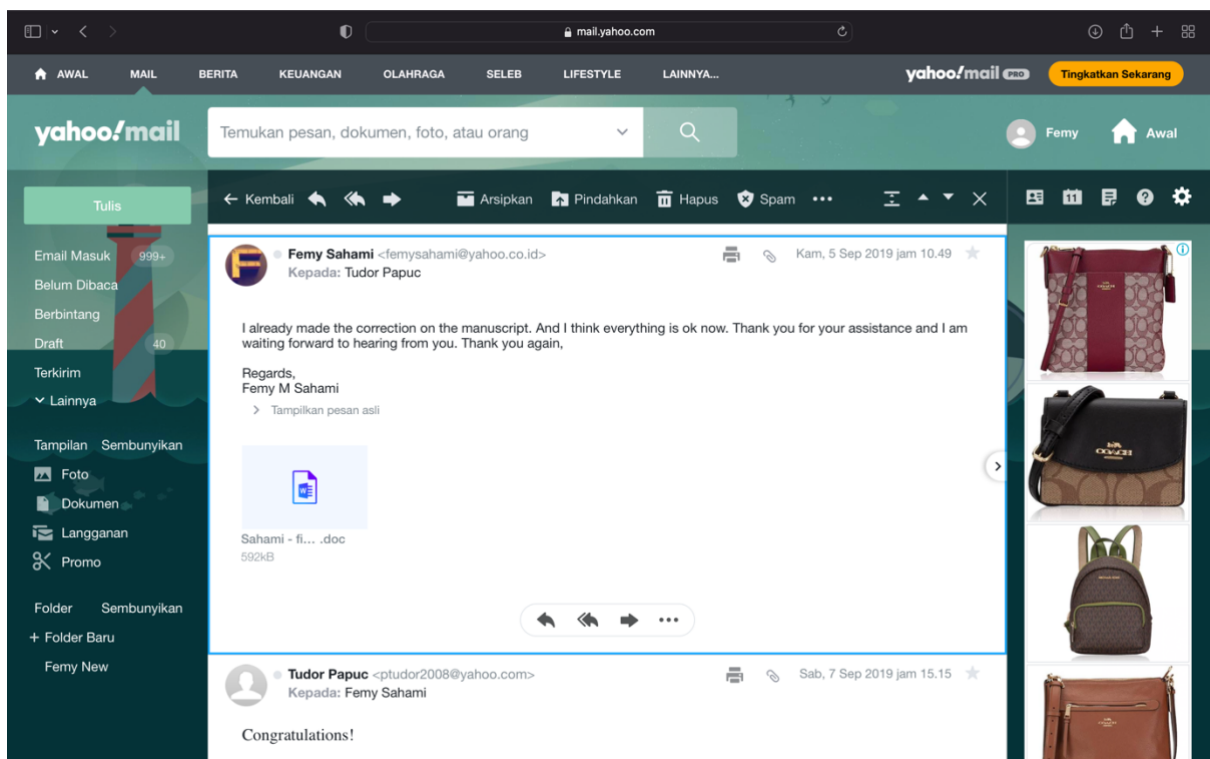Silvester Benny Pratasik, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia, e-mail: spjong07@yahoo.com

How to cite this article:

[1] Department of Aquatic Resources Management, Faculty of Fisheries and Marine Science, Gorontalo State University, Jl. Jendral Sudirman. No. 6, Gorontalo City, Gorontalo Province, Indonesia; [2] Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia. Corresponding author: F. M. Sahami, femysahami@yahoo.co.id

Gambar 16. Korespondensi

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

Femy M. Sahami[1], Rene C. Kepel[2], Abdul H. Olii[1], Silvester B. Pratasik[2]

[1] Department of Aquatic Resources Management, Faculty of Fisheries and Marine Science, Gorontalo State University, Gorontalo City, Indonesia; [2] Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia. Corresponding author: F. M. Sahami, femysahami@yahoo.co.id

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) also reported that morphological characteristic of nike was shown by the small body size which only reach 3 cm and the body color is still transparent, the body has 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.
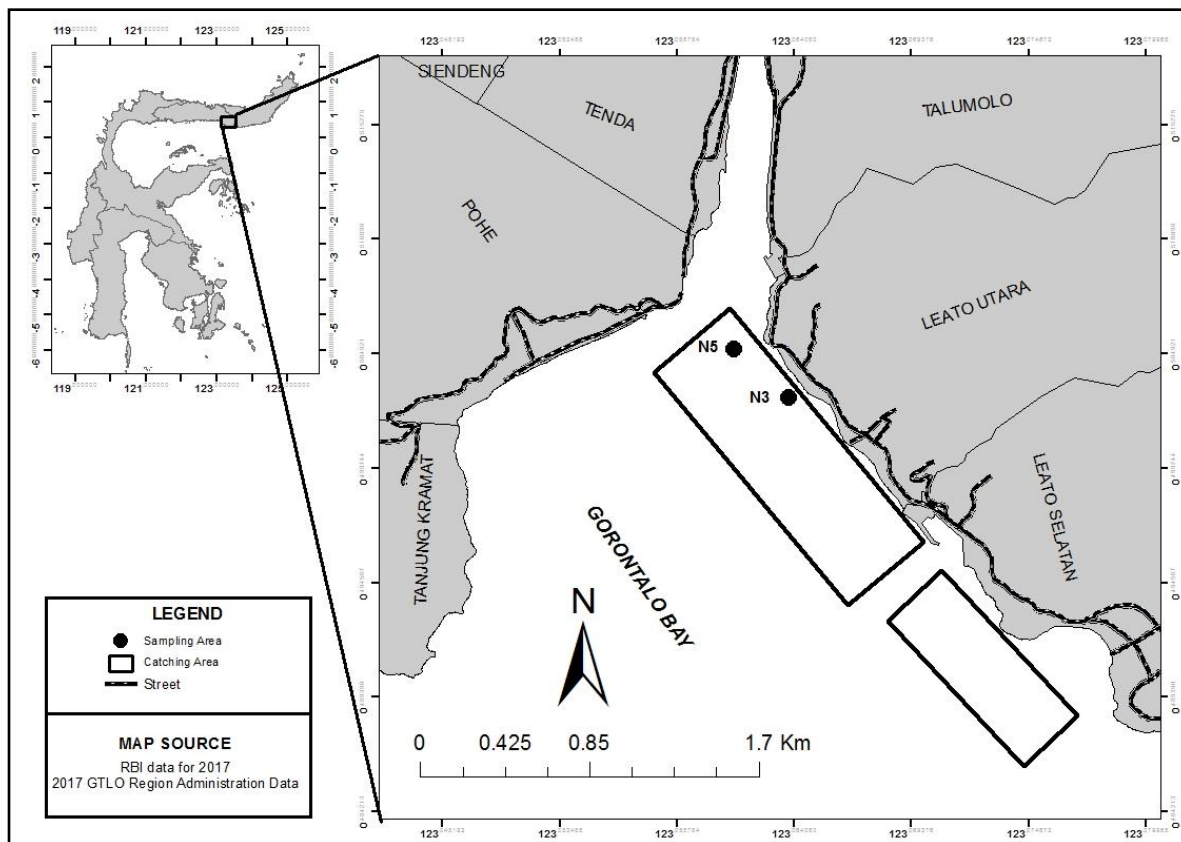
Figure 1. Map of the sampling sites in Gorontalo Bay.

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	CCTTTATCTTGTCTTCGGTGCCTGAGCCGGGATAGTGGGCACCGCTTTAAGCCTAC TTATCCGCGCTGAACTAAGTCAACCTGGCGCACTCCTAGGAGATGACCAAATCTAT AATGTTATCGTTACCGCCCACGCGTTCGTAATAATTTTCTTTATAGTAATACCAATT ATGATTGGCGGATTTGGTAACTGACTAATCCCCTTAATGATTGGCGCCCCAGACAT GGCCTTCCCACGAATAAACAACATAAGTTTCTGACTTCTCCCGCCATCTTTCCTCCT CCTTTTAGCATCCTCTGGAGTAGAAGCAGGGGCCGGAACAGGGTGAACCGTCTAC CCGCCCCTAGCGGGCAACCTCGCCCACGCAGGCGCCTCTGTGGACCTAACAATCT TTTCACTACACCTAGCAGGGGTGTCCTCAATTCTTGGAGCAATTAATTTCATTACCA CAATTATTAACATAAAACCTCCGGCAATTTCCCAATACCAAACGCCCTTGTTCGTCT GAGCCGTTCTAATTACAGCCGTCTTATTACTATTATCCCTTCCCGTACTTGCTGCTG GCATCACAATGCTACTTACAGATCGAAATTTAAATACGACGTTCTTTGACCCGGCC GGGGGTGGGGACCCAATCTTATACCAACACCTTTTC
N5	CCTTTATCTTGTCTTCGGTGCCTGAGCCGGGATAGTGGGCACAGCTTTAAGCCTAC TTATCCGCGCTGAACTAAGTCAACCTGGCGCACTCCTAGGAGATGACCAAATCTAT AATGTTATCGTTACCGCCCACGCGTTCGTAATAATTTTCTTTATAGTAATACCAATT ATGATTGGCGGATTTGGTAACTGACTAATCCCCTTAATGATTGGCGCCCCAGACAT GGCCTTCCCACGAATAAACAACATAAGTTTCTGACTTCTCCCGCCATCTTTCCTCCT CCTTTTAGCATCCTCTGGAGTAGAAGCAGGGGCCGGAACAGGGTGAACCGTCTAC CCGCCCCTAGCGGGCAACCTCGCCCACGCAGGCGCCTCTGTGGACCTAACAATCT TTTCACTACACCTAGCAGGGGTGTCCTCAATTCTTGGAGCAATTAATTTCATTACCA CAATTATTAACATAAAACCTCCGGCAATTTCCCAATACCAAACGCCCTTGTTCGTCT GAGCCGTTCTAATTACAGCCGTCTTATTACTATTATCCCTTCCCGTACTTGCTGCTG GCATCACAATGCTACTTACAGATCGAAATTTAAATACGACGTTCTTTGACCCGGCC GGGGGTGGGGACCCAATCTTATACCAACACCTTTTC

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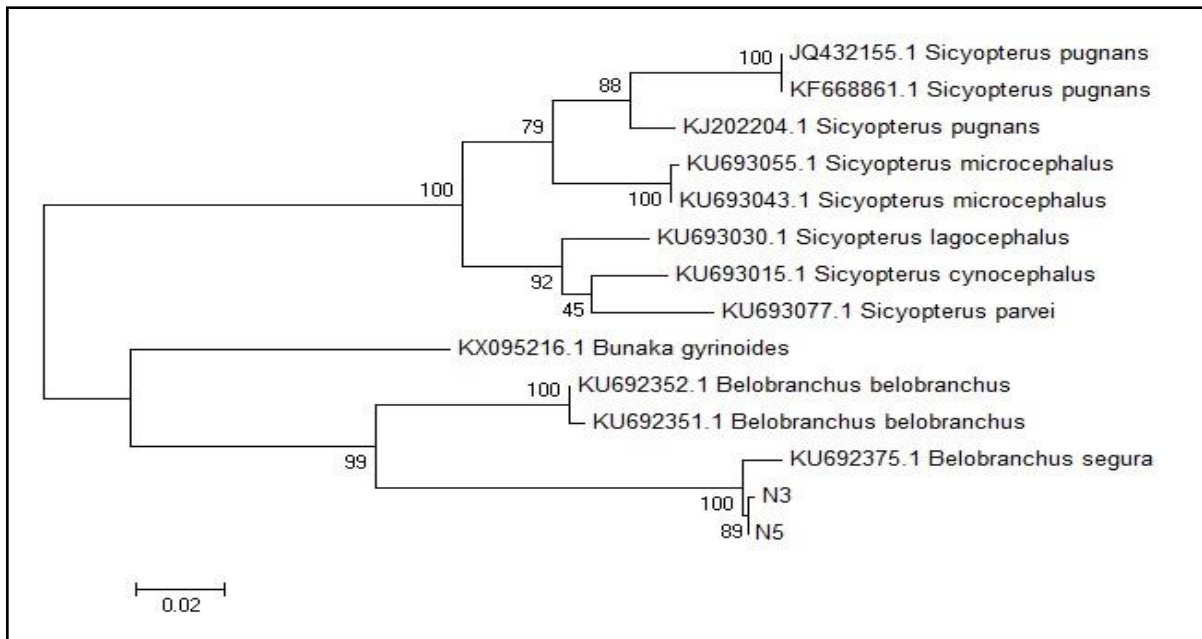
Figure 2. Phylogenetic trees of N3 and N5 samples compared with several species in the NCBI database.

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.

References

Baldwin C. C., Mounts J. H., Smith D. G., Weigt L. A., 2009 Genetic identification and color descriptions of early life-history stages of Belizean *Phaeoptyx* and *Astrapogon* (Teleostei: Apogonidae) with comments on identification of adult *Phaeoptyx*. Zootaxa 2008:1–22.

Ellien C., Werner U., Keith P., 2014 Morfological Change During the Transition from Freshwater to Sea Water in an Amphidromous Goby, *Sicyopterus lagocephalus* (Pallas 1770) (Teleostei). Ecology of Freshwater Fish 25(1):48-59.

Fitzsimons J. M., McRae M. G., Nishimoto R. T., 2007 Behavioral ecology of indigenous stream fishes in Hawaii. In: Biology of Hawaiian Streams and Estuaries. Evenhuis N. L., Fitzsimons J. M. (eds), Bishop Museum Bulletin in Cultural and Environmental Studies 3:11–21. Hobson K. A., Smith R. J. F., Sorensen P., 2007 Applications of stable isotope analysis to tracing nutrient sources to Hawaiian gobioid fishes and other stream organisms. In: Biology of Hawaiian Streams and Estuaries. Evenhuis N. L, Fitzsimons J. M. (eds), Bishop Museum Bulletin in Cultural and Environmental Studies 3:99–111.

Keith P., Hoareau T., Lord C., Ah-Yane O., Gimmoneau G., Robinet T., Valade P., 2008 Characterisation of post-larval to juvenile stages, metamorphosis, and recruitment of an amphidromous goby, *Sicyopterus lagocephalus* (Pallas, 1767) (Teleostei: Gobiidae: Sicydiinae). Marine and Freshwater Research 59(10):876-889.

Kumar A., 2017 Biodiversity conservation and management in India, Brazil and South Africa. In: Biodiversity Law, Policy and Governance. Tandon U., Parasaran M., Luthra S. (eds), Routledge India, London, 366 p.

Lucas M. C., Baras E., Thom T. J., Duncan A., Slavic O., 2001 Migration of freshwater fishes. MPG Books Ltd, Cornwall, 466 p.

Maie T., Wilson M., Schoenfuss H., Blob R., 2009 Feeding kinematics and performance of Hawaiian stream Gobies, *Awaous guamensis* and *Lentipes concolor*: linkage of functional morphology. Journal of Morphology 270(3):344-356.

Milton D. A., 2009 Living in two worlds: Diadromous fishes, and factors affecting population connectivity between tropical rivers and coasts. In: Ecological connectivity among tropical

coastal ecosystems. Nagelkerken I. (ed), Dordrecht: Springer Science+Business Media B. V., Berlin, pp. 325-355.

Nishimoto R. T., Fitzsimons J. M., 1986 Courtship, territoriality, and coloration in the endemic Hawaiian freshwater goby, *Lentipes concolor*. In: Indo-Pacific fish biology. Uyeno T., Arai R., Taniuchi T., Matsuura K. (eds.), Proceedings of the second International Conference on Indo-Pacific fishes, Ichthyological Society of Japan, Tokyo, pp. 811–817.

Olii A. H., Sahami F. M., Hamzah S. N., Pasisingi N., 2017 Preliminary findings on distribution pattern of larvae of nike fish (*Awaous* sp.) in the estuary of Bone River, Gorontalo Province, Indonesia. AACL Bioflux 10:1110-1118.

Olii A. H., Sahami F. M., Hamzah S. N., Pasisingi N., 2019 Molecular Approach to Identify Gobioid Fishes, "Nike" and "Hundala" (Local Name), from Gorontalo Waters, Indonesia. Journal of Biological Science 19(1):52-56.

Pasisingi N., Abdullah S., 2018 [Pattern of nike fish (Gobiidae) occurrence in the Gorontalo Bay, Indonesia]. Depik 7:111-118. [in Indonesian]

Purnama A. A., Mubarak J., Daruwati I., Roslim D. I., Elvyra R., 2019 First Report of Morphological and Molecular Identification of Greater Scissotail *Rasbora caudimaculata* from Rokan Hulu District, Riau Province, Indonesia. AACL Bioflux 12(1):34-41.

Tantu F., 2001 [Spatial-Temporal Abbundance of Nike (Order of Gobioidea) in the Bone Gorontalo Estuary]. Master Thesis, Manado: Program Pasca Sarjana, Universitas Sam Ratulangi, Manado, 68 p. [in Indonesian]

Teichert N., Valade P., Grondin H., Trichet E., Sardenne F., Gaudin P., 2016 Pelagic larval traits of the amphidromous goby *Sicyopterus lagocephalus* display seasonal variations related to temperature in La Réunion Island. Ecology of Freshwater Fish 25:234-247.

Thacker C. E., 2003. Moleculer Phylogeny of The Gobioid Fishes (Teleostei: Perciformes: Gobioidei). Molecular Phylogenetics and Evolution 26:354-368.

Thacker C. E., Roje D. M., 2011 Phylogeny of Gobiidae and identification of gobiid lineages. Systematics and Biodiversity 9(4):329–347.

Valade P., Lord C., Grondin H., Bosc P., Takillebois L. M. I., 2009 Early Life History and Description of Larval Stages of An Amphidromous Goby, *Sicyopterus lagocephalus* (Pallas, 1767) (Teleostei: Gobiidae: Sicydiinae). Cybium 33:309-319.

Wolok T., Fachrussyah Z. C., Yantu I., 2019 Technical And Economic Analysis Of Catching Equipment Totaluo In Nike Fishing (*Awaous melanocephalus*) In Gorontalo City. Jambura Science of Management 1(2):65-71.

Yamasaki N., Kondo M., Maeda K., Tachihara K., 2011 Reproductive biology of three amphidromous gobies, *Sicyopterus japonicus*, *Awaous melanocephalus*, and *Stenogobius* sp., on Okinawa Island. Cybium 35(4):345-359.

Authors:
Femy Mahmud Sahami, Department of Aquatic Resources Management, Gorontalo State University, Jl. Jendral Sudirman, No.6, 96128, Gorontalo City, Indonesia, e-mail: femysahami@yahoo.co.id
Rene Charles Kepel, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia, e-mail: renecharleskepel65@gmail.com
Abdul Hafidz Olii, Department of Aquatic Resources Management, Gorontalo State University, Jl. Jendral Sudirman, No.6, 96128, Gorontalo City, Indonesia, e-mail: oliihafidz@gmail.com
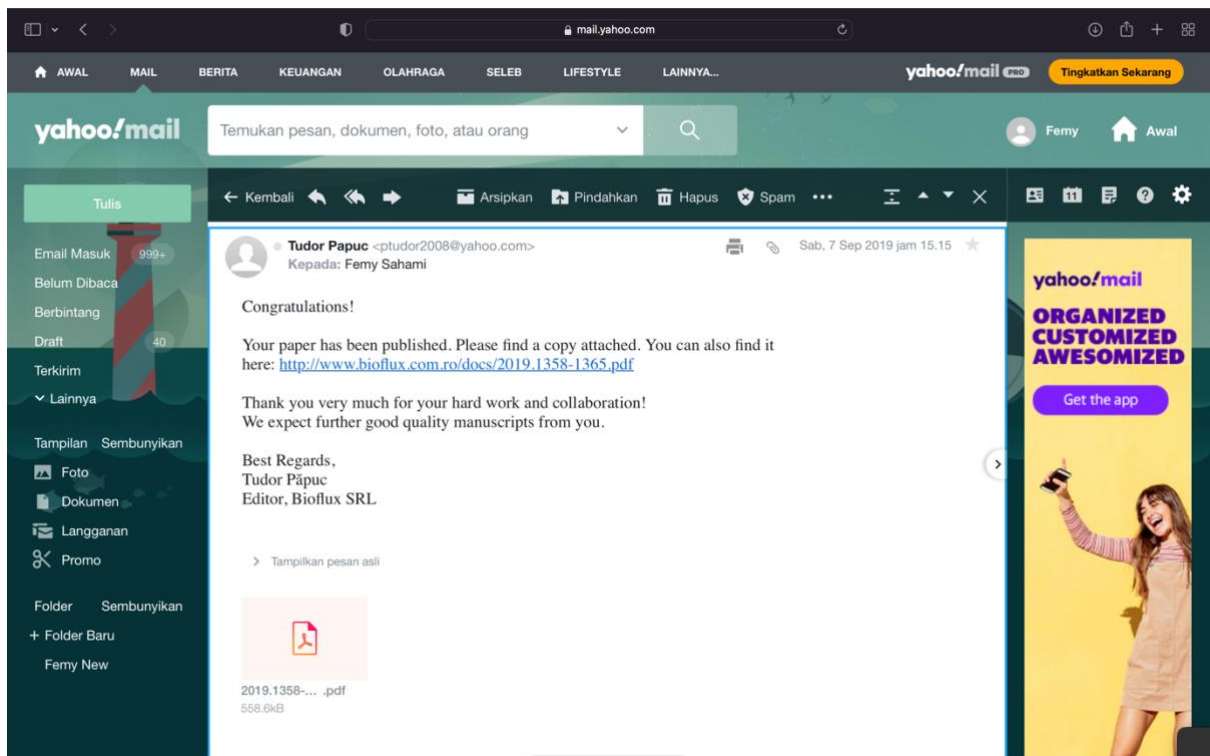Silvester Benny Pratasik, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia, e-mail: spjong07@yahoo.com

How to cite this article:

[1] Department of Aquatic Resources Management, Faculty of Fisheries and Marine Science, Gorontalo State University, Jl. Jendral Sudirman. No. 6, Gorontalo City, Gorontalo Province, Indonesia; [2] Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia. Corresponding author: F. M. Sahami, femysahami@yahoo.co.id

Gambar 17. Korespondensi
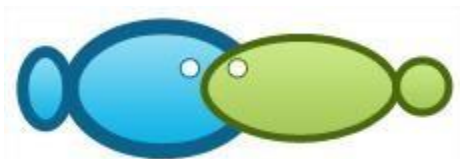
Determination of morphological alteration based on molecular analysis and melanophore pattern of the migrating Nike fish in Gorontalo Bay, Indonesia

[1]**Femy M. Sahami, ** [2]**Rene C. Kepel, ** [1]**Abdul H. Olii, ** [2]**Silvester B. Pratasik**

[1] Department of Aquatic Resources Management, Faculty of Fisheries and Marine Science, Gorontalo State University, Gorontalo City, Indonesia; [2] Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia. Corresponding author: F. M. Sahami, femysahami@yahoo.co.id

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 lowfrequency 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 fishare 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 theexistence 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 forpreserving 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.
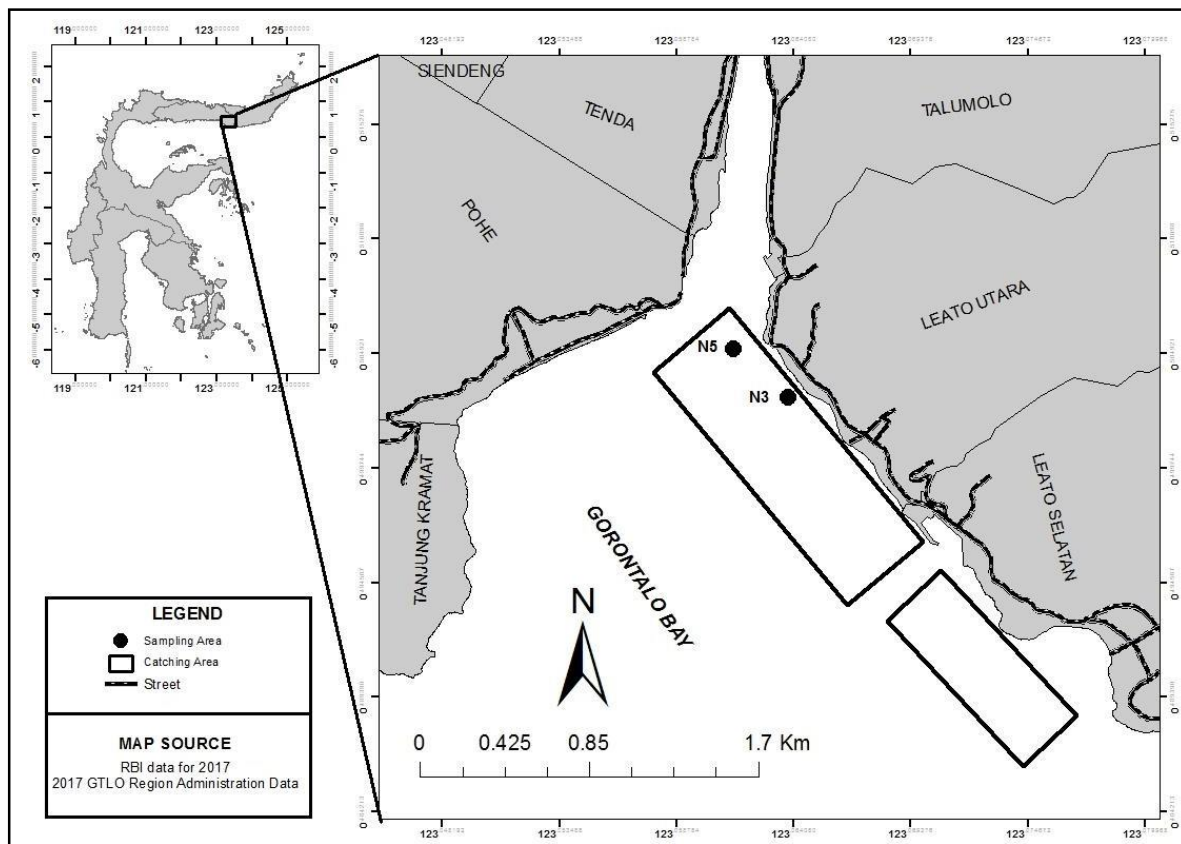
Figure 1. Map of the sampling sites in Gorontalo Bay.

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 GTTCTTTGACCCGGCCGGGGGTGGGGACCCAATCTTATACCAACACCTTTTC
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 thekinship relations between samples (N3 and N5) and several species available in the NCBI database (Figure 2).
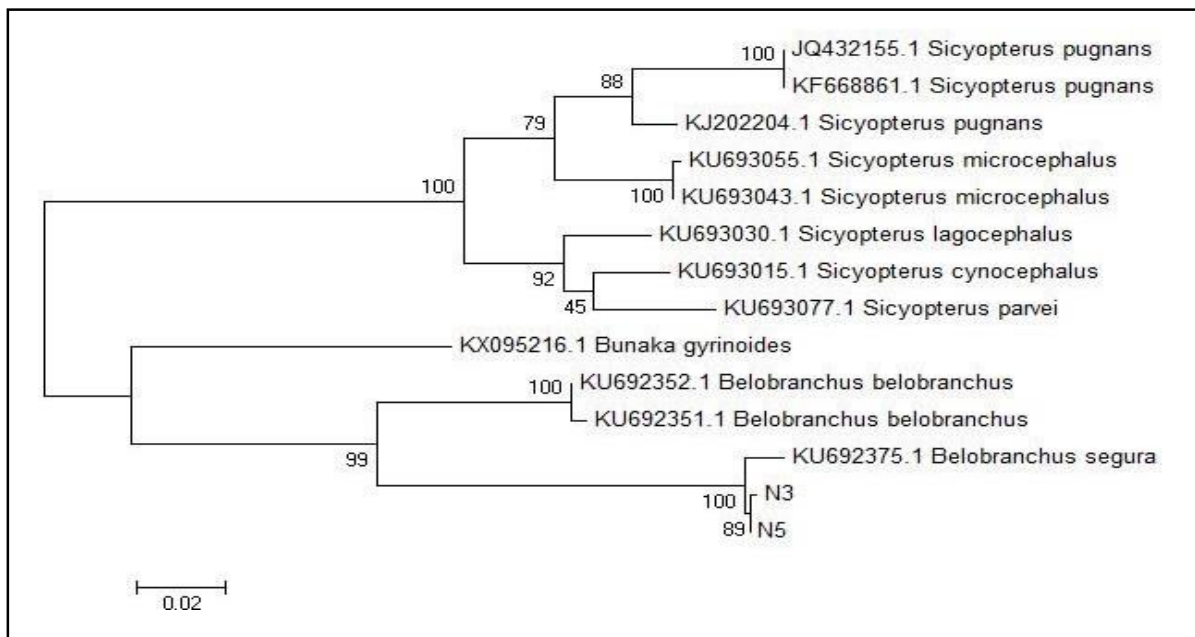
Figure 2. Phylogenetic trees of N3 and N5 samples compared with several species in the NCBI database.

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 yetdispersed, 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 backto freshwater. *Sicyopterus lagocephalus* as an amphidromous goby species, meets drasticchanges of habitat to fulfil its life cycle. Adults live and spawn in rivers, where eggs hatchinto 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.

References

Baldwin C. C., Mounts J. H., Smith D. G., Weigt L. A., 2009 Genetic identification and color descriptions of early life-history stages of Belizean *Phaeoptyx* and *Astrapogon* (Teleostei: Apogonidae) with comments on identification of adult *Phaeoptyx*. Zootaxa 2008:1–22.

Ellien C., Werner U., Keith P., 2014 Morfological Change During the Transition from Freshwater to Sea Water in an Amphidromous Goby, *Sicyopterus lagocephalus* (Pallas 1770) (Teleostei). Ecology of Freshwater Fish 25(1):48-59.

Fitzsimons J. M., McRae M. G., Nishimoto R. T., 2007 Behavioral ecology of indigenous stream fishes in Hawaii. In: Biology of Hawaiian Streams and Estuaries. Evenhuis N. L., Fitzsimons J. M. (eds), Bishop Museum Bulletin in Cultural and Environmental Studies 3:11–21. Hobson K. A., Smith R. J. F., Sorensen P., 2007 Applications of stable isotope analysis to tracing nutrient sources to Hawaiian gobioid fishes and other stream organisms. In: Biology of Hawaiian Streams and Estuaries. Evenhuis N. L, Fitzsimons J. M. (eds), Bishop Museum Bulletin in Cultural and Environmental Studies 3:99–111.

Keith P., Hoareau T., Lord C., Ah-Yane O., Gimmoneau G., Robinet T., Valade P., 2008 Characterisation of post-larval to juvenile stages, metamorphosis, and recruitment of an amphidromous goby, *Sicyopterus lagocephalus* (Pallas, 1767) (Teleostei: Gobiidae: Sicydiinae). Marine and Freshwater Research 59(10):876-889.

Kumar A., 2017 Biodiversity conservation and management in India, Brazil and South Africa. In: Biodiversity Law, Policy and Governance. Tandon U., Parasaran M., Luthra S. (eds), Routledge India, London, 366 p.

Lucas M. C., Baras E., Thom T. J., Duncan A., Slavic O., 2001 Migration of freshwater fishes. MPG Books Ltd, Cornwall, 466 p.

Maie T., Wilson M., Schoenfuss H., Blob R., 2009 Feeding kinematics and performance of Hawaiian stream Gobies, *Awaous guamensis* and *Lentipes concolor*: linkage of functional morphology. Journal of Morphology 270(3):344-356.

Milton D. A., 2009 Living in two worlds: Diadromous fishes, and factors affecting population connectivity between tropical rivers and coasts. In: Ecologicalconnectivity among tropical coastal ecosystems. Nagelkerken I. (ed), Dordrecht: Springer Science+Business Media B. V., Berlin, pp. 325-355.

Nishimoto R. T., Fitzsimons J. M., 1986 Courtship, territoriality, and coloration in the endemic Hawaiian freshwater goby, *Lentipes concolor*. In: Indo-Pacific fish biology. Uyeno T., Arai R., Taniuchi T., Matsuura K. (eds.), Proceedings of the second International Conference on Indo-Pacific fishes, Ichthyological Society of Japan, Tokyo, pp. 811–817.

Olii A. H., Sahami F. M., Hamzah S. N., Pasisingi N., 2017 Preliminary findings on distribution pattern of larvae of Nike fish (*Awaous* sp.) in the estuary of Bone River, Gorontalo Province, Indonesia. AACL Bioflux 10:1110-1118.

Olii A. H., Sahami F. M., Hamzah S. N., Pasisingi N., 2019 Molecular Approach to Identify Gobioid Fishes, "Nike" and "Hundala" (Local Name), from Gorontalo Waters, Indonesia. Journal of Biological Science 19(1):52-56.

Pasisingi N., Abdullah S., 2018 [Pattern of Nike fish (Gobiidae) occurrence in the Gorontalo Bay, Indonesia]. Depik 7:111-118. [in Indonesian]

Purnama A. A., Mubarak J., Daruwati I., Roslim D. I., Elvyra R., 2019 First Report of Morphological and Molecular Identification of Greater Scissotail *Rasbora caudimaculata* from Rokan Hulu District, Riau Province, Indonesia. AACL Bioflux 12(1):34-41.

Tantu F., 2001 [Spatial-Temporal Abbundance of Nike (Order of Gobioidea) in the Bone Gorontalo Estuary]. Master Thesis, Manado: Program Pasca Sarjana, Universitas Sam Ratulangi, Manado, 68 p. [in Indonesian]

Teichert N., Valade P., Grondin H., Trichet E., Sardenne F., Gaudin P., 2016 Pelagic larval traits of the amphidromous goby *Sicyopterus lagocephalus* display seasonal variations related to temperature in La Réunion Island. Ecology of Freshwater Fish 25:234-247.

Thacker C. E., 2003. Moleculer Phylogeny of The Gobioid Fishes (Teleostei: Perciformes: Gobioidei). Molecular Phylogenetics and Evolution 26:354-368.

Thacker C. E., Roje D. M., 2011 Phylogeny of Gobiidae and identification of gobiid lineages. Systematics and Biodiversity 9(4):329–347.

Valade P., Lord C., Grondin H., Bosc P., Takillebois L. M. I., 2009 Early Life History and Description of Larval Stages of An Amphidromous Goby, *Sicyopterus lagocephalus* (Pallas, 1767) (Teleostei: Gobiidae: Sicydiinae). Cybium 33:309-319.

Wolok T., Fachrussyah Z. C., Yantu I., 2019 Technical And Economic Analysis Of Catching Equipment Totaluo In Nike Fishing (*Awaous melanocephalus*) In Gorontalo City. Jambura Science of Management 1(2):65-71.

Yamasaki N., Kondo M., Maeda K., Tachihara K., 2011 Reproductive biology of three amphidromous gobies, *Sicyopterus japonicus*, *Awaous melanocephalus*, and *Stenogobius* sp., on Okinawa Island. Cybium 35(4):345-359.

Authors:
Femy Mahmud Sahami, Department of Aquatic Resources Management, Gorontalo State University, Jl. JendralSudirman, No. 6, 96128, Gorontalo City, Indonesia, e-mail: femysahami@yahoo.co.id
Rene Charles Kepel, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City, Indonesia,e-mail: renecharleskepel65@gmail.com
Abdul Hafidz Olii, Department of Aquatic Resources Management, Gorontalo State University, Jl. JendralSudirman, No.6, 96128, Gorontalo City, Indonesia, e-mail: oliihafidz@gmail.com
Silvester Benny Pratasik, Faculty of Fisheries and Marine Science, Sam Ratulangi University, Manado City,Indonesia, e-mail: spjong07@yahoo.com

How to cite this article:
Sahami F. M., Kepel R. C., Olii A. H., Pratasik S. B., 2019 Determination of morphological alteration based onmolecular analysis and melanophore pattern of the migrating Nike fish in Gorontalo Bay, Indonesia. AACL Bioflux 12(4):1358-1365.