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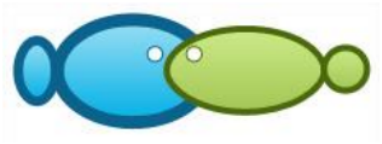

# Morphometric and molecular analysis for identification of the early stages of gobioid fishes in Poigar River estuary, North Sulawesi, Indonesia

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**Abstract**. This study aimed to describe the morphological and molecular performances of gobioid fish in Poigar River estuary. This approach was very useful in overcoming the difficulties in analyzing the morphometric and meristic aspects of the small size fish (post-larva and juvenile). To identify and study the fish species, the melanophore pattern and molecular analysis were performed using the cytochrome oxidase subunit I (COI) gene (mitochondrial gene). Sampling was conducted from February to May 2020 in the Poigar River estuary. The study was carried out on fish catches. Samples of gobioid fish were grouped based on differences in the melanophore patterns of their bodies. Seven different groups were found and they were coded as: N1-P, N7-P, N11-P, N12-P, N13-P, N16-P and N18-P. The molecular analysis using COI gene was used to identify the fish species. Genetic investigations showed that from the seven groups, six had different melanophore patterns (N1-P, N7-P, N11-P, N12-P, N13-P, N16-P and N18-P). Each of the considered species, *Sicyopterus cynocephalus, S. lagocephalus, Stiphodon semoni* and *Awaous ocellaris,* had different morphologies, particularly in the melanophore patterns, but *Sicyopterus pugnans* had three melanophore patterns.
**Key Words**: melanophore, amphidromous, *Sicyopterus*, *Stiphodon*, *Awaous*.

**Introduction**. Tracker & Roje (2011) mentioned that the Gobiidae are one of the largest acanthomorph fish groups, with approximately 1,120 species of 30 already described genera and many others that have not yet been reported. The large number of Goby fishes can allow for genetic similarity and it can enable natural copulation. In their life cycle, gobioids, as amphidromous fish, have to pass through several environments. The different conditions and topography of the waters can allow for differences in adaptation and ecology, as well as in behaviors, that may be expressed through discoloration and size. To ascertain, it is necessary to have an identification study based on morphometric and molecular characters.

The Gobi fish (the Gobiidae family) are benthic fish living in various habitats, from deep-sea environments to freshwater streams (Patzner et al 2011). Gobi species are found in large quantities in the Indo-Pacific region, including freshwater habitats from Indo-Malay Nusantara (Kottelat et al 1993). Yamasaki et al (2011) stated that the newly hatched larvae of *Awaous melanocephalus*, *Sicyopterus japonicus* and *Stenogobius* sp. can be differentiated based on the melanophore pattern. Based on the observation results, another gobioid, the nike fish (*Awaous* sp.), showed melanophore patterns, which can differentiate fish species of these assemblages (Sahami et al 2019a; Sahami et al 2019b; Pangemanan et al 2020).

Based on studies of Yamasaki et al (2011) and Pasisingi & Abdullah (2018), it is suspected that the schooling of nike fishes in Gorontalo Bay are composed of several

species which are mostly not undetermined. The several species of nike fish (*Awaous* sp.) reported by Sahami et al (2019a) in Gorontalo Bay are not the same as those reported by Tantu (2001) and Olii et al (2019). In addition, other species of nike fish are found in Sungai Leppangan, West Sulawesi (Nurjirana et al 2019).

To our knowledge, there has not been conducted any research regarding the genetics identification of the early stages of Gobioid fish from the Poigar River estuary. The present study was carried out to determine some characteristics of early stages of Gobioid fish in the Poigar River estuary, by molecular and morphetric studies. Molecular characters can provide genetic information and support morphological characters data.

## Material and Method

***Sample collection***. The study was conducted from February to May 2020, including the sampling of the fish from fishermen that was carried out randomly in the Poigar River estuary. 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 in the Laboratory of the Faculty of Fisheries and Marine Sciences, at the Sam Ratulangi University, and in the Integrated Laboratory of the Faculty of Fisheries and Marine Sciences, at the Gorontalo State University. Samples of early stages of gobioid fish were sorted in seven different groups, labelled N1-P, N7-P, N11-P, N12-P, N13-P, N16-P and N18-P. 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 labs of the company PT Genetika Science Indonesia, Jakarta. The small size of this fish (post-larvae/juvenile) causes difficulties in determining morphometric and meristic aspects. In order to determine the morphometric and meristic aspects in an attempt to identify these small sized fish (post-larvae/juvenile), the melanophore pattern approach and molecular analysis using the COI gene were employed.

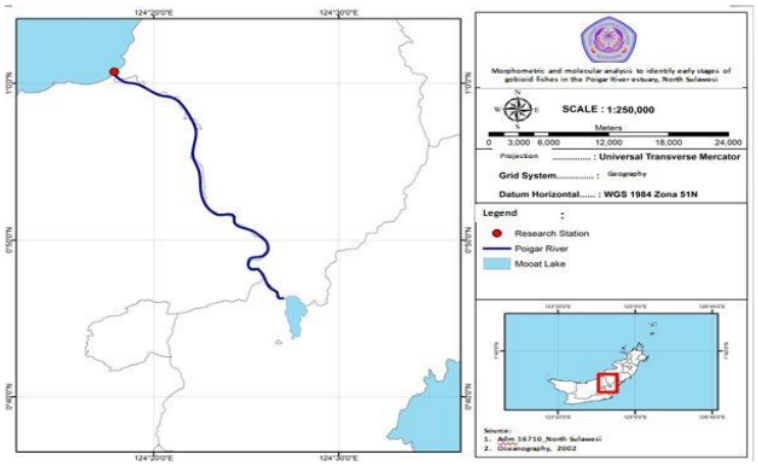

Figure 1. Map of sampling sites in the Poigar River estuary.

***Molecular analysis***. Isolation of DNA genomes from the samples was carried out using isolation kits (Genomic DNA Mini Kit Tissue). The isolation method was carried out in accordance with the standard protocol of the product. The PCR process was carried out using a mix of universal fish primers, namely: VF2_t1 (TGTAAAACGACGGCCAGT CAACCAACCACAAGACATTGGCAC), FishF2_t1 (TGTAAAACGACGGCCAGTCGACTAATCATAAAG ATATCGGCAC), FishR2_t1 (CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA), FR1d_t1 (CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA), referring to the Canadian Center for DNA Barcoding (CCDB) protocol 2006. The PCR products were

determined by electrophoresis, evaluated and photographed under ultraviolet (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 determine 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 indicated that early stages of gobioid fishes sampled consisted of 7 morphologically dissimilar groups, with different melanophore patterns and also genetically different species. The proofreading results from the forward and reserve sequences combined with the sequence of the 7 samples (N1-P, N7-P, N11-P, N12-P, N13-P, N16-P and N18-P) are presented in Table 1.

Table 1

Proofreading results of forward and reserve sequence of samples

| *Sample code* | *Proof reading results* |
|---|---|
| N1-P | GGCACCCTATACCTTGTTTTCGGTGCCTGAGCAGGAATAGTAGGGACTGCCCTCAGCC<br>TG61CTCATCCGAGCTGAATTAAGTCAACCTGGAGCTCTTCTAGGGGACGACCAAATTT<br>ACAATGTAATTGTTACTGCACATGCCTTTGTAATAATTTTCTTTATAGTAATACCAATCAT<br>GATTGGAGGCTTTGGGAACTGACTTATTCCCCTAATGATCGGTGCCCCTGATATGGCCT<br>TTCCTCGAATAAATAACATAAGCTTTTGACTTCTCCCCCCTTCATTCCTTCTCCTCCTAG<br>CATCTTCTGGTGTTGAAGCAGGGGCCGGAACTGGCTGAACAGTGTATCCTCCTCTGGC<br>AGGAAACCTTGCACATGCAGGAGCTTCTGTTGACTTAACTATTTTCTCCCTCCACCTGG<br>CAGGTATTTCATCAATTCTTGGGGCAATTAATTTCATTACAACCATCCTAAACATGAAAC<br>CCCCTGCAATTTCACAATATCAAACACCTCTATTTGTATGAGCTGTTCTTATTACAGCAG<br>TCCTCCTACTTCTCTCCCTCCCTGTCCTTGCAGCTGGCATTACAATGCTACTAACAGACC<br>GAAACCTTAACACAACCTTCTTTGACCCATCAGGAGGAGGTGACCCAATTCTCTACCAA<br>CATCTATTCTGATTCTTCGGACACCCTGAGGTGTCATA |
| N7-P | GGCACCCTATACCTTGTTTTCGGTGCCTGAGCAGGAATAGTAGGGACTGCCCTCAGCC<br>TACTCATCCGAGCTGAATTAAGTCAACCTGGGGCTCTTCTAGGGGACGACCAAATTTAC<br>AATGTAATTGTTACTGCACATGCCTTTGTAATAATTTTCTTTATAGTAATACCAATCATGA<br>TTGGAGGCTTTGGGAACTGACTTATTCCCCTAATGATCGGTGCCCCTGATATGGCCTTT<br>CCTCGAATAAATAACATAAGCTTTTGACTTCTCCCCCCTTCATTCCTTCTCCTCCTAGCA<br>TCTTCTGGTGTTGAAGCAGGGGCCGGAACTGGCTGAACAGTATATCCTCCTCTGGCAG<br>GAAACCTTGCACATGCAGGAGCTTCTGTTGACTTAACTATTTTCTCCCTCCACCTGGCA<br>GGTATTTCATCAATTCTTGGGGCAATTAATTTCATTACAACCATCCTAAACATGAAACCC<br>CCTGCAATTTCACAATATCAAACACCTCTATTTGTATGAGCTGTTCTTATTACAGCAGTC<br>CTCCTACTTCTCTCCCTCCCTGTCCTTGCAGCTGGCATTACAATGCTACTAACAGACCG<br>AAACCTTAACACAACCTTCTTTGACCCATCAGGAGGGGGTGACCCAATTCTCTACCAAC<br>ATCTATTCTGATTCTTCGGACACCCTGAGGTGTCAT |
| N11-P | TATGACACCTCAGGGTGTCCGAAGAATCAGAATAGGTGTTGGTAAAGAATTGGGTCAC<br>CACCTCCTGAGGGGTCAAAGAAGGTTGTGTTTAGATTTCGATCTGTTAGTAGCATTGTA<br>ATGCCAGCTGCAAGAACAGGTAGAGAAAGAAGCAGTAGAACTGCTGTAATAAGGACAG<br>CTCAGACAAACAGGGGTGTCTGGTATTGTGAGATTGCAGGGGGTTTCATGTTTAGAAT<br>GGTTGTAATAAAATTAATTGCACCTAAAATTGAAGAAATTCCTGCTAAGTGTAGGGAGA<br>AAATTGTAAGGTCAACAGAAGCTCCTGCATGGGCAAGGTTTCCTGCTAGTGGGGGGTA |

| | |
|---|---|
| | AACTGTTCAGCCAGTCCCAGCTCCAGCTTCAACTCCTGAGGAGGCTAGGAGAAGAAGG |
| | AATGAGGGAGGAAGCAGTCAGAAGCTCATGTTATTTATTCGGGGAAAGGCCATGTCAG |
| | GGGCGCCGATCATTAGGGGAATTAGTCAGTTCCCAAAGCCTCCAATCATGATTGGTAT |
| | TACTATAAAGAAAATTATTACAAAAGCATGTGCAGTAACAATTACATTATAAATTTGGTC |
| | GTCACCTAGAAGAGCCCCAGGTTGGCTTAGTTCAGCTCGGATGAGTAGGCTAAGGGCT |
| | GTGCCTACCATTCCTGCTCAGGCACCGAAAACAAGGTATAGGGTGC |
| N12-P | TATGACACCTCAGGGTGTCCGAAGAATCAGAACAAGTGTTGGTAAAGGATTGGGTCTC |
| | CTCCACCTGACGGGTCAAAGAAGGTCGTGTTCAGGTTGCGGTCTGTTAGTAGCATTGT |
| | AATGCCGGCAGCAAGGACTGGTAGGGAAAGAAGTAGTAGGACTGCTGTGATTAGAAC |
| | GGCTCAAACGAACAGGGGTGTTTGGTATTGTGAAATTGCAGGGGGTTTCATATTTAGG |
| | ATGGTTGTAATGAAGTTAATTGCACCTAAAATTGATGAGACACCTGCCAGGTGAAGTGA |
| | GAAAATTGTTAGGTCTACAGAAGCTCCAGCATGGGCAAGGTTCCCTGCTAGTGGGGGG |
| | TAGACAGTTCAACCAGTCCCTGCTCCAGCTTCAACCCCTGATGATGCTAGGAGAAGCA |
| | GGAATGAAGGAGGGAGAAGTCAAAAGCTCATGTTATTCATTCGAGGGAAGGCCATGTC |
| | AGGGGCACCAATCATTAGGGGGATTAGTCAGTTCCAAAGCCACCAATCATAATTGGTA |
| | TTACTATAAAGAAAATTATTACAAATGCATGTGCTGTTACAATGACATTATAGATTTGGT |
| | CGTCTCCTAAAAGAGCCCCAGGTTGGCTAAGTTCAGCTCGGATTAGAAGGCTAAGAGC |
| | TGTGCCTACTATTCCAGCTCAGGCACCGAAAACCAAATATAGGGTGCC |
| N13-P | ATGACACCTCAGGGTGTCCGAAGAATCAGAATAGATGTTGGTAGAGAATTGGGTCACC |
| | TCCTCCTGATGGGTCAAAGAAGGTTGTGTTAAGGTTTCGGTCTGTTAGTAGCATTGTAA |
| | TGCCAGCTGCAAGGACAGGGAGGGAGAGAAGTAGGAGGACTGCTGTAATAAGAACAG |
| | CTCATACAAATAGAGGTGTTTGATATTGTGAAATTGCAGGGGGTTTCATGTTTAGGATG |
| | GTTGTAATGAAATTAATTGCCCCAAGAATTGATGAAATACCTGCCAGGTGGAGGGAGA |
| | AAATAGTTAAGTCAACAGAAGCTCCTGCATGTGCAAGGTTTCCTGCCAGAGGAGGATA |
| | TACTGTTCAGCCAGTTCCGGCCCCTGCTTCAACACCAGAAGATGCTAGGAGGAGAAGG |
| | AATGAAGGGGGGAGAAGTCAAAAGCTTATGTTATTTATTCGAGGAAAGGCCATATCAG |
| | GGGCACCGATCATTAGGGGAATAAGTCAGTTCCCAAAGCCTCCAATCATGATTGGTATT |
| | ACTATAAAGAAAATTATTACAAAGGCATGTGCAGTAACAATTACATTGTAAATTTGGTC |
| | GTCCCCTAGAAGAGCTCCAGGTTGACTTAATTCAGCTCGGATGAGTAGGCTGAGGGCA |
| | GTCCCTACTATTCCTGCTCAGGCACCGAAAACAAGGTATAGGGTGCC |
| N16-P | ATGACACCTCAGGGTGTCCGAAGAATCAGAATAGATGTTGGTAGAGAATTGGGTCACC |
| | TCCTCCTGATGGGTCAAAGAAGGTTGTGTTGAGGTTTCGGTCTGTCAGTAGCATTGTG |
| | ATGCCAGCTGCAAGAACTGGGAGAGAAAGAAGTAGTAGGACTGCCGTAATAAGAACA |
| | GCTCAGACGAAGAGAGGTGTCTGATATTGTGAAATTGCAGGAGGTTTCATATTTAGAAT |
| | AGTTGTAATGAAATTAATTGCACCAAGAATTGATGAAATACCTGCTAGGTGGAGGGAG |
| | AAAATAGTTAGATCAACAGAAGCCCCTGCATGGGCAAGGTTTCCTGCTAGAGGGGGGT |
| | AGACTGTTCAGCCAGTCCCAGCCCCTGCTTCAACACCTGAAGATGCCAGGAGGAGAAG |
| | GAATGAAGGGGGGAGGAGTCAAAAGCTTATGTTGTTCATTCGGGGGAAGGCCATATCT |
| | GGGGCACCGATCATTAGGGGGATGAGTCAGTTTCCAAATCCTCCAATCATGATTGGCA |
| | TTACTATAAAGAAAATTATTACAAAGGCATGTGCAGTAACAATTACATTGTAAATCTGGT |
| | CGTCTCCTAGAAGAGCCCCAGGTTGGCTTAGTTCAGCTCGGATAAGTAGGCTTAGGGC |
| | AGTTCCTACTATTCCTGCTCAGGCACCGAAAACAAGGTATAGGGTGCC |
| N18-P | ATGACACCTCAGGGTGTCCGAAGAATCAGAATAGATGTTGGTAAGAAGAATTGGGTCA |
| | CCTCCTCCTGATGGGTCAAAGAAGGTTGTGTTGAGGTTTCGGTCTGTCAGTAGCATTGT |
| | GATGCCAGCTGCAAGAACTGGGAGAGAAAGAAGTAGTAGGACTGCCGTAATAAGAAC |
| | AGCTCAGACGAAGAGAGGTGTCTGATATTGTGAAATTGCAGGAGGTTTCATATTTAGA |
| | ATAGTTGTAATGAAATTAATTGCACCAAGAATTGATGAAATACCTGCTAGGTGGAGGGA |
| | GAAAATAGTTAGATCAACAGAAGCCCCTGCATGGGCAAGGTTTCCTGCTAGAGGGGG |
| | GTAGACTGTTCAGCCAGTCCCAGCCCCTGCTTCAACACCTGAAGATGCCAGGAGGAGA |
| | AGGAATGAAGGGGGGAGGAGTCAAAAGCTTATGTTGTTCATTCGGGGGAAGGCCATA |
| | TCTGGGGCACCGATCATTAGGGGGATGAGTCAGTTTCCAAATCCTCCAATCATGATTG |
| | GCATTACTATAAAGAAAATTATTACAAAGGCATGTGCAGTAACAATTACATTGTAAATCT |
| | GGTCGTCTCCTAGAAGAGCCCCAGGTTGGCTTAGTTCAGCTCGGATAAGTAGGCTTAG |
| | GGCAGTTCCTACCTATTCCTGCTCAGGCACCGAAAACAAGGTATAGGGTGCC |

The sequences produced were 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

| *Species code* | *Accession number* | Species | *Query cover* | *Identity* |
|---|---|---|---|---|
| N1-P | MT227831.1 | *Sicyopterus pugnans* | 100% | 99.70% |
| N7-P | MT227831.1 | *Sicyopterus pugnans* | 100% | 99.70% |
| N11-P | KU693172.1 | *Stiphodon semoni* | 98% | 100% |
| N12-P | KC959856.1 | *Awaous ocellaris* | 100% | 99.54% |
| N13-P | MT227831.1 | *Sicyopterus pugnans* | 100% | 100% |
| N16-P | MK496936.1 | *Sicyopterus cynocephalus* | 100% | 99.70% |
| N18-P | MK496948.1 | *Sicyopterus lagocephalus* | 100% | 99.85% |

The 7 samples of *S. pugnans, S. cynochepalus, S. lagocephalus*, *S. semoni, A. ocellaris* were based on genetic analysis with mitochondrial COI. Since previous studies never reported about the larval, post-larval and juvenile of the early stages of gobioid fishes from Sulawesi Sea, the current study in the Poigar River estuary is a pioneering work. Further analysis was conducted with the phylogenetic tree to show the kindship relations between the samples (N1-P, N7-P, N11-P, N12-P, N13-P, N16-P and N18-P) and several species available in the NCBI database (Figure 2).

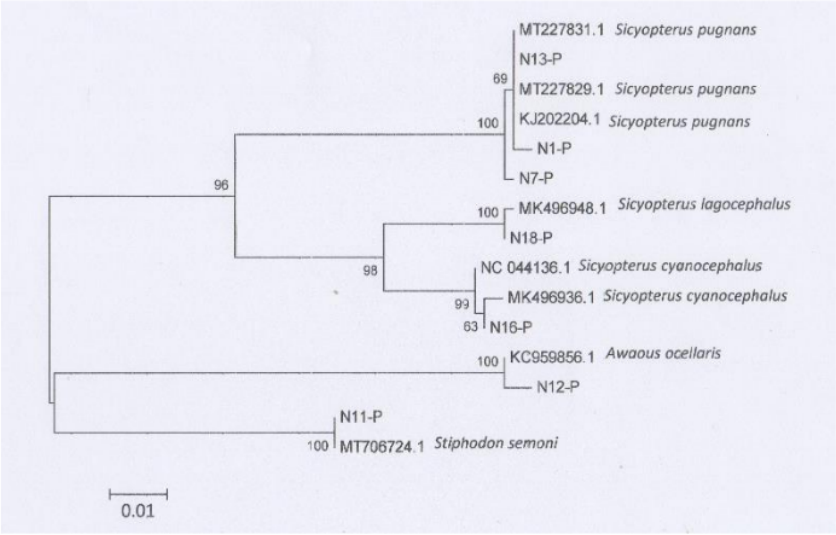


Figure 2. Phylogenetic trees of early stages of gobioid fishes (samples N1-P, N7-P, N11-P, N12-P, N13-P, N16-P and N18-P) compared with several species in the NCBI database.

The genetic analysis illustrates that the 7 samples consist of five species. Each species of *S. cynochepalus, S. lagocephalus, S. semoni* and *A. ocellaris* has different morphologies, particularly in the melanophore patterns, but *S. pugnans* has three melanophore patterns. The differences in melanophore patterns from samples N1-P, N7-P, N11-P, N12-P, N13-P, N16-P and N18-P is schematically presented in Figure 3.

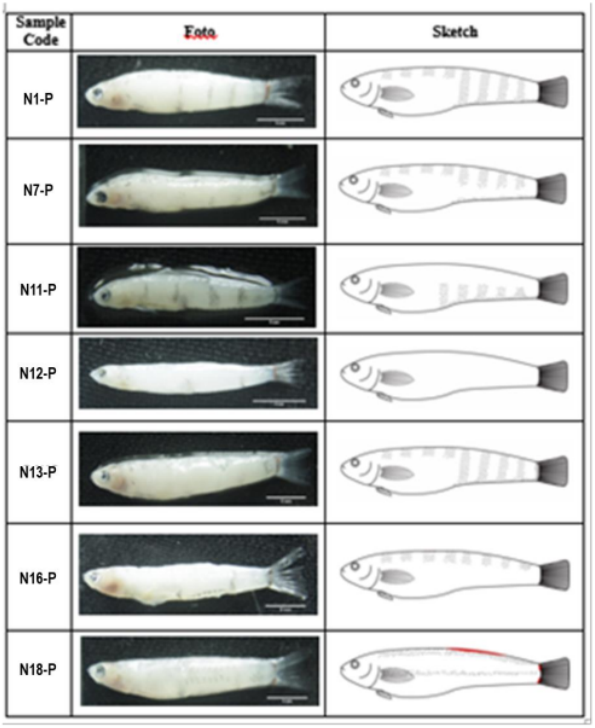


Figure 3. Schematic morphological alterations in samples, based on the melanophore pattern.

Sample N1-P, N7-P, N11-P, N12-P, N13-P, N16-P and N18-P have undergone alterations in the melanophore structure and body size. The melanophore pattern is formed from a plain fish body and spreads throughout the body. Furthermore, after melanophore spreads proportionally throughout the body, there is a wider distance between melanophore because the fish body becomes large.

The research of Valade et al (2009) showed a change in the appearance of chromatophores in the body of *S. lagocephalus* 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 gobioid fish when becoming more mature. When still in larval stage, at 1.5 cm, the melanophore distribution is not yet noticeable, but at post-larval stage (1.7 to 2.5 cm) and juvenile stage (4.5 cm), the melanophoreproportionally spreads along the body (Figure 3).

Other studies on gobioid fish in Gorontalo Bay show the discovery of 7 species, *S. pugnans*, *S. cynocephalus*, *S. lagocephalus*, *Belobranchus belobranchus*, *B. segura*, *S. semoni*, and *Bunaka gyrinoides* (Sahami et al 2019a; Sahami et al 2019b; Sahami 2020). In this study, 5 species were discovered, namely *S. pugnans*, *S. cynocephalus, S. lagocepahlus*, *S. semoni*, and *A. ocellaris.* Among species unequal in number, but present in Gorontalo Bay, there is no *A. ocellaris*, while the in results of this research there are no *B. belobranchus, B. segura* and *Bunaka gyrinoides*. Moreover, the results of the studies on penja fish (amphidromous goby) in Leppangan River, West Sulawesi show 9 species, *S. lagocephalus*, *S. longifilis*, *S. semoni*, *S. atropurpureus*, *Sicyopus zosterophorus*, *Smilosicyopus leprurus*, *Schismatogobius* sp., *Eleotris fusca* and *Eleotris* sp. (Kottelat & Whitten 1996; Patzner et al 2011; Nurjirana et al 2019; Gani et al 2020). Thenumber of species is considerably higher than the results of this study. However, the result has 3 species of *S. pugnans*, *S. cynocephalus*, and *A. ocellaris* not available in Leppangan River. Differences in the species assemblage composition suggest that water specificities are determinant for the habitat, at each location.

The 5 species of fish in this study were all caught both in Poigar River estuary and in the river which demonstrates an appropriate salinity of the water measured at the time of sampling (around 2‰). *S. pugnans* was found in the Verde Island Passages, Philippines (Thomas et al 2013), and Gorontalo Bay (Sahami et al 2019a; Sahami et al 2019b). *S. cynocephalus* was found in Gorontalo Bay (Sahami et al 2019a; Sahami et al 2019b) and in Luwuk Banggai area, Central Sulawesi Province (Gani et al 2020). *S. lagocephalus* was found in Gorontalo Bay (Sahami et al 2019a; Sahami et al 2019b; Sahami 2020) and in Leppangan River, West Sulawesi (Nurjirana et al 2019). *S. semoni* was found in Gorontalo Bay (Sahami et al 2019a; Sahami et al 2019b), in Leppangan River, West Sulawesi (Nurjirana et al 2019) and in Luwuk Banggai area, Central Sulawesi Province (Gani et al 2020). *A. ocellaris* was only found in the Poigar River estuary, but Gani et al (2020) only found *Awaous grammepomus* in the Luwuk Banggai area, Central Sulawesi Province.

**Conclusions**. According to the current study, samples N1-P, N7-P, N11-P, N12-P, N13-P, N16-P and N18-P, with different melanophore patterns, are species of *S. cynocephalus, S. lagocephalus, S. semoni* and *A. ocellaris* that have different morphologies, particularly in the melanophore patterns, but *S. pugnans* had three melanophore patterns.

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