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DNA barcoding of two amphidromous goby postlarvae ('penja') morphotypes from Mandar River, West Sulawesi, Indonesia

by

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Abstract. – Post-larvae of amphidromous gobies, locally known as ‘penja’ in Sulawesi, is a group of fish that are often caught by fishermen as consumption fish when migrating and has a life-cycle which begins with adults spawning in the river, the eggs and embryos are then carried by river currents, hatching before arriving at the sea, and spend their larval and postlarval phases in the sea, before returning to the river to grow and eventually reproduce. Belonging to several species, they often school together and are difficult to identify with certainty based on morphological characters. Molecular biology tools such as DNA barcoding can help overcome this challenge. This study, conducted from June 2018 to December 2019, aimed at identifying ‘penja’ post-larvae migrating upstream in the Mandar River in West Sulawesi (Indonesia) using a molecular approach. Two species were identified: the threadfin goby *Sicyopterus longifilis* de Beaufort, 1912, locally known as ‘penja mawassar’, and the blue neon goby *Stiphodon semoni* Weber, 1895, local name ‘penja alus’. Both are widespread species. Main characteristics of these species and of their fisheries are given. The determination of harvested postlarvae is a major issue for fishery management.

Résumé. – Barcoding de deux morphotypes de post-larves de gobies amphidromes (*penja*) de la rivière Mandar, Sulawesi occidentale, Indonésie.

Les post-larves des gobies amphidromes connus localement à Sulawesi sous le nom de ‘penja’, appartiennent à plusieurs espèces ; elles se regroupent souvent et sont difficiles à identifier avec certitude sur la base des seuls caractères morphologiques. Des outils de biologie moléculaire, tels que le code-barre ADN, peuvent aider à surmonter cette difficulté. Cette étude, menée de juin 2018 à décembre 2019, visait à identifier les post-larves de ‘penja’ migrant en amont dans la rivière Mandar en Sulawesi occidentale (Indonésie) en utilisant une approche moléculaire. Deux espèces ont été identifiées : le sicyoptère filamenteux, *Sicyopterus longifilis* de Beaufort, 1912, connu localement sous le nom de ‘penja mawassar’, et le *Stiphodon* de Semon, *Stiphodon semoni* Weber, 1895, nom local ‘penja alus’. Tous deux sont des espèces à large répartition géographique. Les principales caractéristiques de ces espèces ainsi que des informations sur les pêcheries sont documentées. La détermination des post-larves pêchées est un enjeu majeur pour la gestion des pêcheries.

Key words
Post-larva
Sicydiinae
Migratory fish
Mandar River
Sulawesi

In the Indonesian Province of West Sulawesi, the fisheries commodity, locally called ‘ikan penja’, is important both economically and as a source of protein for human consumption of the local community. They are often caught by fishermen during their seasonal migration from coastal waters to rivers. Preliminary research conducted by Nurjirana *et al.* (2019b) found that there were variations in the types of ‘penja’ fish postlarvae based on morphological differences from postlarvae, which indicated that there were diverse species at each migration period. Other studies related to species identification of postlarvae of amphidromous goby in Sulawesi have previously been carried out through molecular analysis including Usman (2016) identifying species of *Awaous melanocephalus* (Bleeker, 1849) and Sahami *et al.* (2019) identifying species of *Belobranchus segura* Keith,

Hadiaty & Lord, 2012 in Gorontalo and West Sulawesi waters. ‘Penja’ are in fact the postlarvae of amphidromous gobies belonging to the suborder Gobioidi which comprised four families, the Gobiidae (189 genera with 1,259 species), Butidae (10 genera with 46 species), Eleotridae (26 genera with 139 species) and Oxudercidae (86 genera with 598 species) (Nelson *et al.*, 2016; Nurjirana *et al.*, 2019a). Within the Oxudercidae, the Sicydiinae subfamily includes the most frequently fished and consumed species.

Amphidromous adult fishes spawn in rivers and, after hatching, their embryos are carried by river currents to the sea where the larvae will undergo a planktonic phase for several months. Once they enter the postlarval phase, at specific times the postlarvae will migrate back to freshwater, where they will remain to grow and reproduce in river-

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ine habitat (McDowall, 2007; Keith *et al.*, 2008; Lord *et al.*, 2010). As they enter the estuarine and then the riverine environment, the shoals of postlarvae generally encounter strong seaward currents; therefore, they tend to swim close to the river bank where the current is often weaker, and frequently crowd into gaps between rocks and pebbles offering shelter from the pressure of fast-flowing water (Tate, 1997; Keith, 2003). During their migration to and within inland freshwater habitats, the ventral sucker formed by the modified pelvic fins plays an important role, as the morphology of the mouth, enabling these sicydiine gobies to attach themselves to rocky substrate and to advance upstream against strong currents (Tate, 1997; Keith, 2003; Keith *et al.*, 2012; Lord *et al.*, 2019).

The estuary of the Mandar River, in Polewali Mandar District, West Sulawesi Province (Indonesia), is one area where 'penja' appear regularly with a predictable seasonal pattern. Local fishermen come in groups to harvest these migrating postlarvae, using a variety of fishing gear. Although the local community use different names for certain groups of 'penja', distinguished based on their size and appearance, the formal taxonomic identity of the species of 'penja' migrating regularly to the Mandar River was unknown. Therefore, research was needed to identify the amphidromous post-larvae recruiting to the Mandar River and caught by local fishermen as 'penja'.

Classical morphological approaches to the identification of these gobiid postlarvae present several challenges. There is a lack of references for the identification of amphidromous gobies at the postlarval stage, particularly in Indonesia and Sulawesi where numerous species of the two main genera of Sicydiinae (*i.e.* *Sicyopterus* and *Stiphodon*) are found. Recent research in this last region has identified four *Sicyopterus* species (*S. lagocephalus* (Pallas, 1770), *S. longifilis* de Beaufort, 1912, *S. cynocephalus* (Valenciennes, 1837), and *S. microcephalus* (Bleeker, 1855)) and six *Stiphodon* species (*S. semoni* Weber, 1895, *S. atropurpureus* (Herre, 1927), *S. rutilaureus* Watson, 1996, *S. pelewensis* Herre, 1936, *S. annieae* Keith & Hadiaty, 2015, and *S. surrufus* Watson & Kottelat, 1995) (Keith *et al.*, 2015; Nurjirana *et al.*, 2019a, 2020; Gani *et al.*, 2020, 2021). Research on the inventory of freshwater fish species in the Mandar River has never been carried out, so information regarding the diversity of freshwater fish species is not available. Furthermore, some species are known to experience changes in body shape (metamorphosis) during their growth, with many differences in characters between postlarval and adult phases, so that it is difficult to determine the morphological diagnostic characters of each species during the post-larval phase (Keith *et al.*, 2008; Nurjirana *et al.*, 2019b). Several authors have attempted to work on live colour patterns and sizes of postlarvae, but within each genus the differences are rarely sufficient to determine the species with confidence (Tate, 1997; Benbow

et al. 2004; Maeda and Tachihara, 2005; Keith *et al.*, 2006, 2015). As postlarval fisheries in the Indo-Pacific area are not sustainable under current conditions, partly because of the lack of knowledge of the biology and the ecology of these species, it is important to know what species are really concerned in each area, in order to determine their respective conservation status and establish appropriate management strategies (Keith *et al.*, 2015), in general and in particular in West Sulawesi.

DNA Barcoding is a relatively recent but well established identification method using a molecular biology approach, which can be applied to all life-stages of an organism (Hebert *et al.*, 2003; Hajibabaei *et al.*, 2006). A DNA barcode is a short nucleotide sequence from a standardized region of the genome, which varies little within species but differs enough between species to provide a biological marker and enabling species to be distinguished (Hebert *et al.*, 2003). DNA barcoding is increasingly widely used to identify specimens that are difficult to recognize, such fragments from organs or even body parts that have undergone processing, and in particular specimens in the larval phase (Hebert *et al.*, 2003; Hajibabaei *et al.*, 2006; Steinke *et al.*, 2009).

This study aimed at identifying the species of gobiid post-larvae ('penja') based on two morphotypes migrating to the Mandar River in West Sulawesi with a molecular approach, using DNA barcoding.

MATERIAL AND METHODS

Field survey was conducted from June 2018 to December 2019. The migration time of 'penja' fish is uncertain in the Mandar River, as well as for other rivers around it, even though it has entered the new moon phase, but the Mandar River becomes not necessarily the migration route for 'penja' fish, it could be through other rivers in West Sulawesi, so that in collecting samples at a different time, for the 'penja mawassar' it was collected in June 2018, while the 'penja alus' was collected in December 2019. Both of morphotype samples were collected once a month. Samples of goby post-larvae were collected in one container containing 750-1300 individuals obtained from the catches of fishermen operating in the Mandar River and nearby coastal waters in Polewali Mandar Regency, West Sulawesi Province, Indonesia (Fig. 1). The fishermen catch 'penja' using several types of fishing gear such as 'panesser' (seine nets), 'bunde' (scoop nets), and 'seser' or 'passambe' (another type of hand net). The postlarvae samples were divided into groups based on differences in size and amount of pigmentation on the body (morphotype) and three individuals were selected at random from each group. The selected specimens were preserved in labelled sample bottles filled with 96% ethanol and stored. Total length was measured using digital callipers at the Has-

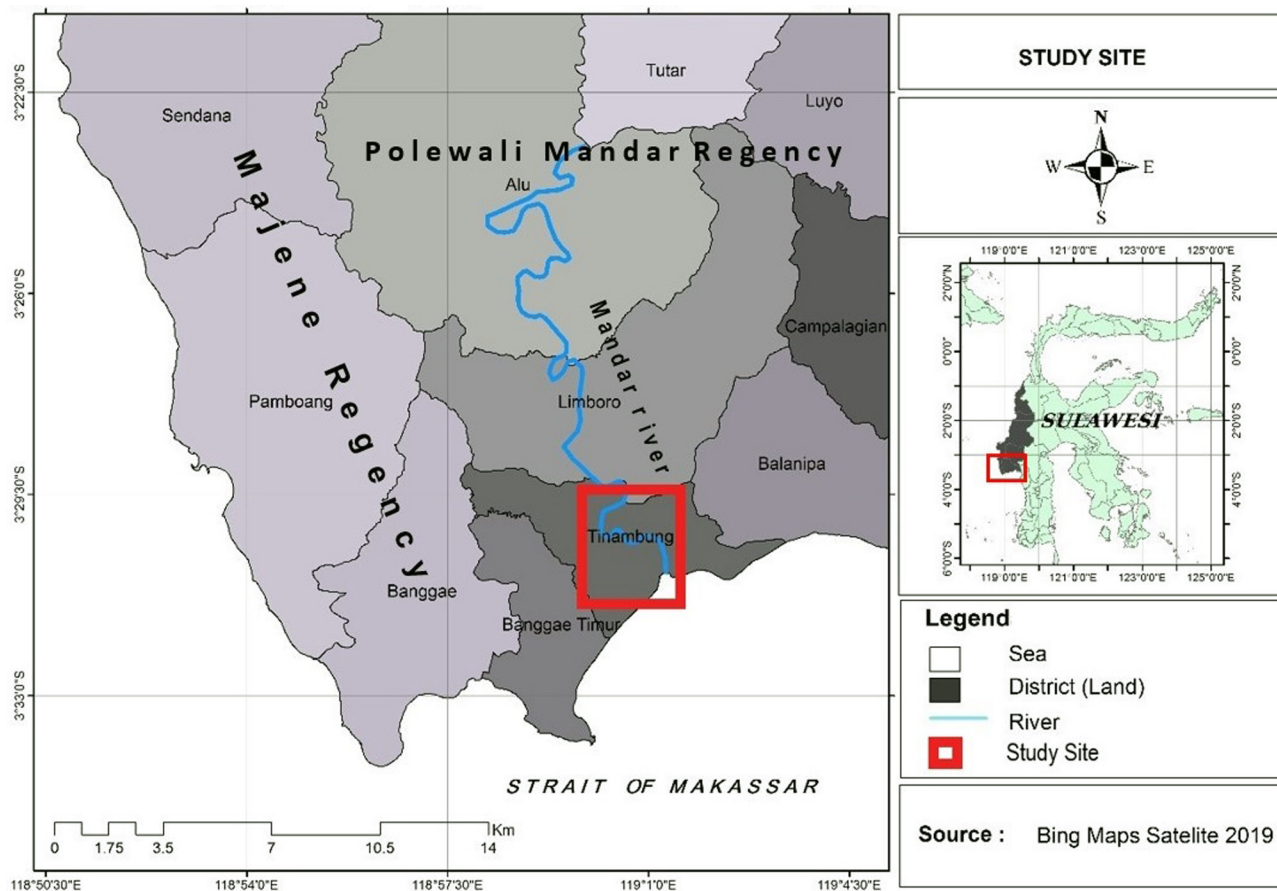


Figure 1. – Map of West Sulawesi showing the Mandar River and the study site.

anuddin University Marine Biology Laboratory in Makassar. Molecular analysis procedures (DNA Barcoding) were performed at the Indonesian Biodiversity (BIONESIA) Laboratory in Denpasar, Bali.

DNA extraction

DNA was extracted from each sample using a Chelex 10% solution referring to Walsh *et al.* (1991). Tissue samples about 2 mm³ were taken from the caudal fin of each post-larva using tweezers and inserted into a 1.5 ml Eppendorf tube containing the Chelex solution. Before and after the tissue was removed, the tweezers were sterilised by dipping in 95% ethanol and passing through the flame of a Bunsen burner. The tube containing the Chelex solution and sampled tissue was vortexed and centrifuged for around 20 seconds, then heated in a heating block at a temperature of 95°C for ± 45 minutes. After heating, the tube was vortexed again and then centrifuged for ± 20 seconds. The extracted DNA was then ready for amplification.

DNA amplification (PCR)

The genome region amplified was the *Cytochrome Oxidase I (COI)* locus of the mitochondrial genome (mtDNA).

Amplification of the target DNA sequence (barcode) was done using Hot-start PCR (*Polymerase Chain Reaction*). The primer pair used was the jgLCO1490 forward primer (TITCIACIAAYCAYAARGAYATTGG) and jgHCO2198 reverse primer (TAIACYTCIGGRTGICCRARAAYCA) (Geller *et al.*, 2013). Each reaction tube contained 13 µL PCR mix Hotstart of 4.5 µL ddH₂O, 1.5 µL buffer, 2.5 µL dNTP, 2.0 µL MgCl₂, PE Amplitaq 0.72 µL, and 1.25 µL of each the two primers. The parameters of the PCR cycling process were as follows: denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 30 seconds, with 38 cycles (Barber *et al.*, 2006). The PCR product from each sample (3 samples from each morphotype) was verified using electrophoresis and then sent to a sequencing service for Sanger Sequencing at PT. Genetic Science Indonesia (<https://ptgenetika.com>) to obtain the *COI* barcode nucleotide sequences.

Data analysis

The *COI* mtDNA sequences obtained were trimmed and aligned and subsequent phylogenetic analyses were performed in MEGA 5.2 (Tamura *et al.*, 2011). The Clustal W alignment routine was used with default parameters. DNA

Table I. – GenBank BLAST™ query result for *COI* barcode sequences of six goby postlarvae from two morphotypes.

Sample Code	Accession Number	Closest Relative Description	Query Coverage	Identity	GenBank Accession Number
MSFUH199	MN812961	<i>Sicyopterus longifilis</i> isolate lon2 mitochondrion, complete genome	100%	99.10%	MK496957.1 (Lord <i>et al.</i> , 2019)
MSFUH218	MN812973				
MSFUH216	MN812971				
MSFUH212	MN812978	<i>Stiphodon semoni</i> voucher BIF1709 cytochrome oxidase subunit 1 (<i>COI</i>) gene, partial cds; mitochondrial	95%	99.54%	KU693172.1 (Dahrudin <i>et al.</i> , 2016)
MSFUH211	MN812977				
MSFUH213	MN812979				

sequences were then compared with DNA sequences in the NCBI GenBank database using the BLAST™ routine (Altschul *et al.*, 1990; Ramsay *et al.*, 2000) to find the closest matching sequences in the database and determine the species identity of each sample. A phylogenetic tree was computed based on genetic distance using the Neighbour-Joining method (Tamura *et al.*, 2004) with the Kimura-2-parameter substitution model (Kimura, 1980).

RESULTS

COI analysis

The BLAST™ nucleotide base matching with sequences deposited in the NCBI GenBank identified the goby postlarvae samples from two groups of ‘penja’ as *Sicyopterus longifilis* (Lord *et al.*, 2019) with 99.10% similarity and *Stiphodon semoni* (Dahrudin *et al.*, 2016) with 99.54% similarity (Tab. I). The phylogenetic tree (Fig. 2) shows two distinct clades corresponding to these species.

Morphology

The postlarvae of *S. longifilis* and *S. semoni* differed in overall appearance and some morphological characteristics (Fig. 3A, B). Firstly, they differed in size, with *S. longifilis* postlarvae measuring 28.88 mm to 29.94 mm in total length, and *S. semoni* postlarvae from 16.68 mm to 18.58 mm. Secondly, postlarva of *S. longifilis* had five vertical black

stripes stretching from the dorsal to ventral region spread out along the body between the head and the caudal peduncle. When schooling, these postlarvae are easy to see as they cause a change in the apparent water colour. *S. semoni* postlarvae generally have three vertical stripes spread along the body between the head and the caudal peduncle with a reddish-coloured pigmentation; not only are there fewer stripes, but sometimes the postlarvae are virtually transparent (light stripes or only faintly visible). Schools of migrating *S. semoni* postlarvae are much less visible.

DISCUSSION

Several Sicydiinae are targeted in fisheries around Indo-Pacific islands, especially in the genus *Sicyopterus* (Bell, 1999). Postlarvae are captured as they recruit back to rivers. However, as it is difficult to morphologically tell different Sicydiinae species apart, postlarvae from different species and genera may also be captured, such as endemic or endangered species (Keith *et al.*, 2015; Ellien *et al.*, 2016). Our research provides the identity of two types of ‘penja’ migrating in the Mandar River: they are in fact the postlarvae of amphidromous sicydiine gobies living as adult in the river. Many people, especially in West Sulawesi, consider that ‘penja’ are adult fish of a small species similar to anchovies (Engraulidae, locally known as *ikan teri*) and which also occur in the waters around Sulawesi and are fished in a similar manner, but that is not the case, they are goby postlarvae.

The two ‘penja’ species identified both belong to the Sicydiinae subfamily. Keith *et al.* (2015) listed 26 species of the genus *Sicyopterus* and 30 species of the genus *Stiphodon* in the Indo-Pacific region. The distribution of *S. longifilis* includes Indonesia, Papua New Guinea, the Philippines, and Solomon Islands (Allen, 1991; Boseto *et al.*, 2007; Keith *et al.*, 2015). Specific characters of *S. longifilis* include colouration and length of dorsal fins (de Beaufort, 1912; Keith *et al.*, 2015). Males are described as generally blue to green, with 5-6 dark (grey or black) saddle bars often present between the head and the caudal region, which sometimes join together on the belly. The cheeks, head and back are greyish

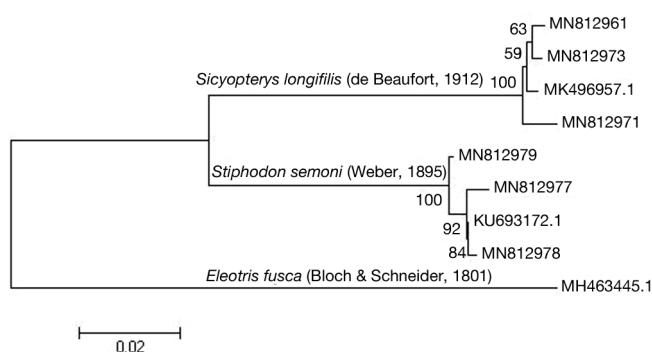


Figure 2. – Neighbour-joining phylogenetic tree of amphidromous gobiid post-larvae (‘penja’) from the Mandar River based on *Cytochrome oxidase I (COI)* sequences.



Figure 3. – *Penja* gobiid post-larvae from the Mandar River: A. ‘penja alus’ (*Stiphodon semoni*), B. ‘penja mawassar’ (*Sicyopterus longifilis*).

to blackish, while the belly is bluish to blackish. The first dorsal fin is greyish to translucent, and a diffuse black spot is often visible (Fig. 3).

Stiphodon semoni is widely distributed in Indonesia, and is reported from Ambon, Maluku (Watson, 1996; Keith *et al.*, 2015), Java and Bali (Dahruddin *et al.*, 2016) as well as Papua New Guinea, East Timor, Solomon Islands, Vanuatu, New Caledonia and Australia (Watson, 1996; Watson *et al.*, 1998; Keith *et al.*, 2010, 2015; Ebner *et al.*, 2011; Maeda and Tan, 2013; Keith and Hadiaty, 2014). This species exhibits sexual dimorphism, with males and females differing in several characteristics, particularly colouration (Watson, 1996; Keith *et al.*, 2015). Male *S. semoni* have a pale blue-green longitudinal band extending dorso-laterally from the snout to the upper part of the caudal peduncle, which becomes brilliant blue or blue green during courtship, when the dorsum also tends to become darker or even blackish. The pale grey first and second dorsal fins often have alternating faint black and white spots on the rays. The anal fin is also pale grey, often has a bluish white margin and a dusky submarginal band. The mostly grey caudal fin has alternating blackish and whitish vertical stripes with a faint, narrow bluish white margin. There are no black spots on the pectoral fin rays (Keith *et al.*, 2015). Females have two black longitudinal bands, one of which is mid-lateral and usually serrated; they also often have 4–6 dusky blotches, which are spaced at irregular intervals (Fig. 3).

Local communities along the coast of West Sulawesi, especially in Polewali Mandar Regency, routinely catch ‘penja’ from every migration period. The local fishermen observe the phases of the moon as a sign that the time for

‘penja’ migration has arrived. In Reunion Island, in the Indian Ocean, the recruitment in rivers is thought to be linked to the new moon period and to heavy rainfalls for *S. lagocephalus* (Hoareau, 2005). Thus, in West Sulawesi, the migration occurs every new moon and lasts for approximately 3–4 days; sometimes schools of migrating *penja* are still found 2–3 days after the new moon phase. When the migration time has arrived, schools of ‘penja’ approach the coastal area before entering the estuary. Sometimes, the ‘penja’ schools, along the coast or in and around the estuary, can be so abundant that they change the colour of the surrounding waters, which turn a blackish brown.

Fishing for ‘penja’ generally begins in the coastal waters in the vicinity of the Mandar River and other rivers, then in the estuaries and lower reaches close to the coast. A few days later, fishing may take place further inland; in the case of the Mandar River, ‘penja’ are caught far upstream as the Alu District, in the foothills of the mountainous area of Polewali Mandar Regency, at an elevation of around 100 metres above sea level.

Local people generally have a special designation for certain types of schooling ‘penja’. The name ‘penja mawassar’ appears to include several *Sicyopterus* species (not found in our study here), including *S. longifilis*. The local people describe ‘penja mawassar’ as being with a fairly large size and these ‘penja’ are the dominant postlarvae at the height of the migration period in West Sulawesi. The name ‘penja alus’, which means smooth or fine or petite, is used for schools of the smaller migrating postlarvae, in particular *S. semoni*, because of their relatively smaller size and thinner bodies. Within a genus, it is sometimes difficult to find

differences in body patterns of postlarvae, because sicydiine gobies experience colour and morphological changes during the migration process from the sea to the river due to changes in osmotic pressure according to salinity (Keith *et al.*, 2006, 2008; Ellien *et al.*, 2016). Thus, in this case, DNA Barcoding is a good identification method. However, among genera, the differences in size of the postlarvae reflect the differences in adult size. For the species in our study, FishBase reported maximum total lengths of 9.7 cm for *S. longifilis* and 4.6 cm for *S. semoni* (Froese and Pauly 2020).

Postlarval Sicydiinae often play a very important socio-economic role, but no long-term monitoring measure has ever been taken, particularly in Sulawesi. Nevertheless, their conservation is crucial for sustaining unique freshwater fish assemblages on this island. In the future, it will be also important to determine the other species caught as postlarvae in Sulawesi, particularly under the name of 'penja mawas-sar'.

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