

## Sex Differentiation of *Hyporhamphus limbatus* Based on Morphological Traits and Regression Relationships of Some Measurements

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Received: 8 January 2025, Revised: 21 February 2025, Accepted: 28 February 2025, Published: 5 May 2025

### Abstract

*Hyporhamphus limbatus* is a commercial value fish in the Mekong Delta, but little is known about its biological traits, especially sex differentiation using morphological characteristics and the regression relationship of some measurements, helping scientists avoid killing them in further studies; therefore, this study aimed to provide these data to this species. Samples were collected monthly at Phuoc Long and Dam Doi, using gillnets with a 1.0 cm mesh-size from May to July 2024. After collection, specimens were identified using morphological descriptions and preserved in 4 % formalin before transporting them to the laboratory for analysis. The morphological traits of 499 individuals were measured, including standard length (*SL*), body depth, head length, distance from the upper jaw to the dorsal fin, distance from the upper jaw to the anal fin, under jaw length, eye diameter, distance between eyes, mouth width, and tail height (*CD*). Males had a sharp-tipped upper jaw with asymmetrically dispersed black spots, while females had an arc-shaped, rounded upper jaw tip with evenly dispersed black spots. Males had a thin, orange-yellow upper jaw border and pale pink upper jaw surface, while females had a broader, greenish-yellow upper jaw border with a dark moss-green tip and 2 bright spots on their heads. The regression relationship between *SL* and *CD* could determine gender. Distinguishing fish sexes based on morphological traits helps reduce the number of specimens killed while studying the species' population biology. The study highlighted the sex differentiation of *Hyporhamphus limbatus* through morphological traits and the regression relationship of some measurements.

**Keywords:** *Hyporhamphus limbatus*, Mekong delta, Morphological traits, Sex differentiation, Regression relationship, Standard length, Tail height

### Introduction

*Hyporhamphus limbatus* belongs to the Hemiramphidae family, which contains eight recognized genera and 61 species worldwide, with the *Hyporhamphus* genus consisting of 38 species that mainly live in freshwater and brackish water estuaries [1]. *Hyporhamphus limbatus* is found throughout the Indian Ocean and Western Pacific, from the Persian Gulf to China, the Mekong River basin, and the Lake Kampuchea Sea [2], including several provinces in the Mekong Delta, particularly Bac Lieu and Ca Mau [3]. In the research area, this fish has a relatively high economic value because the fish meat is soft and

delicious, so it is very popular in people's daily meals [4]. For fresh fish caught directly from the net, the price is about 80,000 VND/kg, depending on the size of the fish. Products processed from this fish (dried fish) are sold for about 250,000 VND/kg.

*Hyporhamphus limbatus* possesses a long cylindrical body with slightly flattened sides, a narrow mouth, a short triangular upper jaw, an elongated lower jaw, and a red-tipped lower jaw. The dorsal surface is somewhat yellowish-green, the ventral surface is silvery white, and the fish's body has 2 huge stripes extending from head to tail [5]. The dorsal fin begins on the

opposite side of the anal fin, with its initial location almost equal to that of the anal fin. Males' dorsal and anal fin rays are not changed [6]. The fish has an average length of 13 cm and may reach up to 25 cm. *Hyporhamphus limbatus* lacks spines in its fins but has 13 - 16 dorsal fin rays and 13 - 16 anal fin rays. Their caudal fin is segmented, and the scales on the body are round and easily fall off [7]. *Hyporhamphus limbatus* breeds at least twice a year, with round eggs measuring 1.3 mm in diameter [8,9].

Distinguishing fish sexes offers various benefits in studying species biology and ecology. Many fish species can be identified by their exterior color. For example, male *Periophthalmodon septemradiatus* have longer and more colorful dorsal fins than females [10]. Male *Perca flavescens* urogenital papillae were round or oval and red, whereas female urogenital papillae were round at the anterior margin, pointed at the posterior margin, and frequently lacking red [11].

There are currently few investigations on the biological properties of *Hyporhamphus limbatus*, notably sex determination using morphological markers and their association. This method has been applied in studies of other fish species such as *Glossogobius giuris*

[12], *Glossogobius sparsipapillus* [13], *Mystus mysticetus* [14], *Ellochelon vaigiensis* [15], *Caragobius urolepis* [16], *Mystus albolineatus* [17]. This method improves fish sex determination while reducing sample mortality. The purpose of this study is to give sex determination information and to provide the base for future research on *Hyporhamphus limbatus*.

## Materials and methods

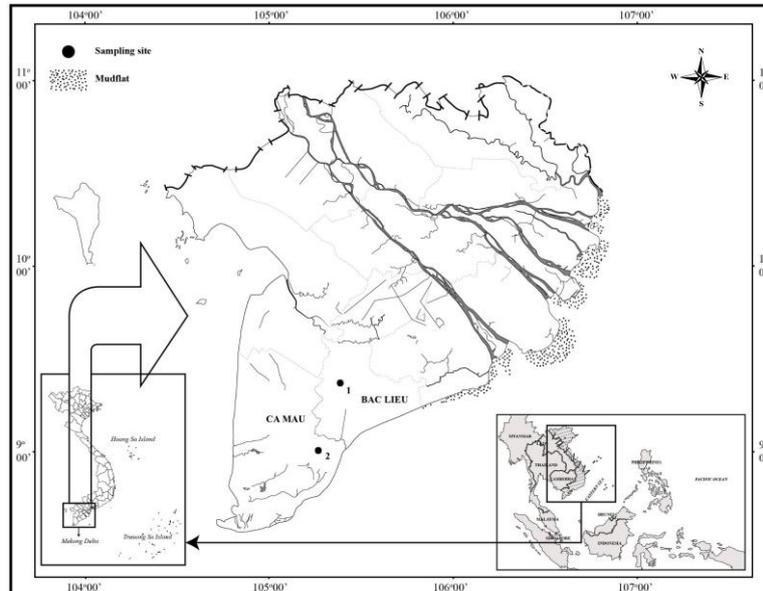
### Sample collection and analysis

Samples were collected monthly (30 per month) from 2 locations: Phuoc Long (Bac Lieu province; 9°21'50 "N 105°23'46" E) and Dam Doi (Ca Mau province; 8°59'03 "N 105°15'12" E) (**Figure 1**). Fish samples were collected directly using gillnets with a 1.0 cm mesh size from May to July 2024. Following collection, the fish samples were identified using [3] morphological descriptions and preserved in 4 % formalin before being delivered to the laboratory. **Table 1** and **Figure 2** showed the morphological parameters measured in this fish species. There was no need for an animal research permit because the study used dead fish as samples.

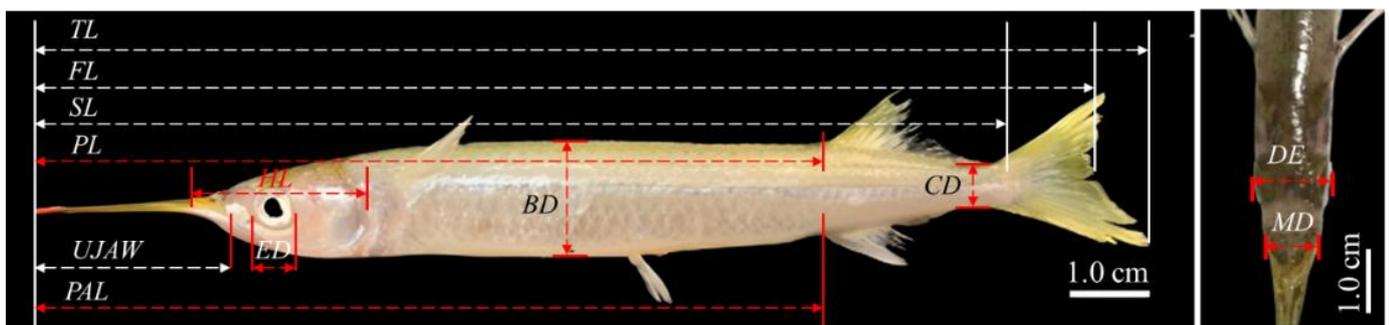
**Table 1** Morphological measurements of *Hyporhamphus limbatus* fish.

No.	Morphological measurement	Abbreviation	<i>d</i>
1	Body depth	<i>BD</i>	0.01 mm
2	Distance between 2 eyes	<i>DE</i>	0.01 mm
3	Distance from the upper jaw to the starting position of the dorsal fin	<i>PL</i>	0.1 cm
4	Distance from the upper jaw to the starting position of the anal fin	<i>PAL</i>	0.1 cm
5	Eye diameter	<i>ED</i>	0.01 mm
6	Head length	<i>HL</i>	0.01 mm
7	Mouth width	<i>MD</i>	0.01 mm
8	Standard length	<i>SL</i>	0.1 cm
9	Tail height	<i>CD</i>	0.01 mm
10	Total length	<i>TL</i>	0.1 cm
11	Under jaw length	<i>UJAW</i>	0.1 cm

\**d*: Smallest error.



**Figure 1** Map of sampling sites (Source: Modified from [18]).



**Figure 2** Morphological measurements of fish *Hyporhamphus limbatus*.

### Data analysis

Minos *et al.* [19] used the following formula  $Y = a \times SL^b$  to determine the relationship between standard length ( $SL$ ) and fish morphological statistics, where  $Y$  represents the morphological size ( $TL$ ,  $BD$ ,  $HL$ ,  $PL$ ,  $PAL$ ,  $UJAW$ ,  $DE$ ,  $ED$ ,  $MD$ , and  $CD$ ), and  $a$  and  $b$  are the regression coefficients. Variables were determined using a t-test to compare a variable's slope and isometric value. Morphological variables were defined as dominantly correlated (A+) if the significant slope exceeded 1, less dominantly correlated (A-) if the slope was less than 1, or isometric (I) if the slope was equal to 1. The data was analyzed using SPSS v21, with all tests set at a significance level of 5 %.

### Results and discussion

#### Results

#### *Sex differentiation based on external morphological traits*

The research results proposed a new method of sex differentiation similar to using external morphological traits. The technique followed these steps:

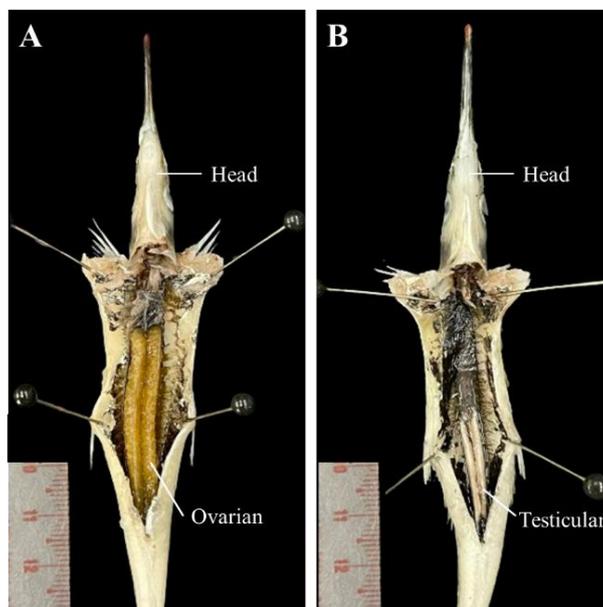
Step 1: 30 samples were randomly selected to determine males and females through the gonads. The ovarian of *Hyporhamphus limbatus* is long and thick with a rounded tip. In the mature stage of many individuals, the egg sac can be observed inside, while the testis is thin and thin with a pointed tip and is opaque white (**Figure 3**).

Step 2: Observed the morphological characteristics of these 30 samples before measuring the morphological indicators.

Step 3: Distinguish sex through the morphological characteristics of the head and upper jaw.

Step 4: Dissected to re-determine the sex of the fish through the testes and ovaries.

Step 5: Compare the results of differentiating males and females through morphological characteristics and gonads.



**Figure 3** Actual gonads photographed from the abdomen (other internal organs removed) of *Hyporhamphus limbatus* ((A) - female, (B) - male).

The sex differentiation method in this study was carried out in 5 steps in 2 parts. Part 1, experimental design to test the morphological differences of *Hyporhamphus limbatus*. First, 30 random fish samples were selected for dissection to obtain gonads. Next, the characteristics of each individual were recorded, and the different characteristics were synthesized. The characteristics that can maintain the sex differentiation of the species are the differences in shape and color of the upper jaw and head. Part 2, control experiment design. Data was compared after 2 stages: Before and after dissection. Before dissection, we performed sex prediction through the upper jaw and head morphological characteristics. After dissection, we determined the sex of the fish through the gonads. Finally, the results before and after dissection were compared.

The results demonstrated that the method had a 97.80 % accuracy rate in sex determination, indicating its potential for use with fresh samples. The study identified previously unmentioned morphological differences in this fish species, described as follows:

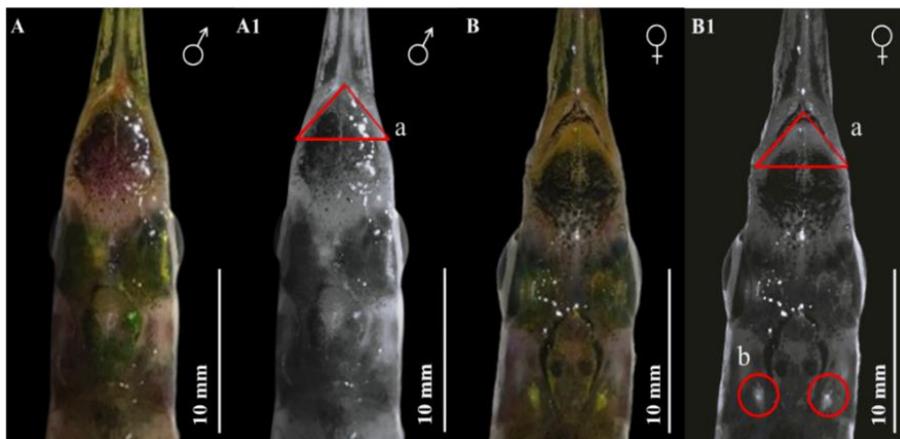
#### *Upper jaw shape*

In males, the tip of the upper jaw was pointed, and the black spots were distributed asymmetrically and almost randomly (**Figure 4(A1a)**). In females, the tip of the upper jaw was rounded, with black spots almost symmetrically distributed between the left and right sides (**Figure 1(B1a)**).

#### *Color differences*

Males had an orange-yellow edge on the upper jaw and a light pink color on the upper part of the jaw (**Figure 4(A)**). Females had a thicker edge at the tip of the upper jaw with a greenish-yellow color, and the upper part of the jaw had a dark moss-green color with 2 small bright spots on the upper part of the head (**Figure 4(B1b)**).

The statistical analysis showed that the sex of *Hyporhamphus limbatus* could be determined with a 97.80 % accuracy rate based on morphological characteristics. This accuracy was calculated from the deviation between initial predictions and results based on gonadal analysis, with 11 incorrect identifications out of 499 individuals.



**Figure 4** The head and the upper jaw of *Hyporhamphus limbatus*.

**Sex differentiation through regression relationship among some measurements**

The findings based on the measurement of morphological indicators in 499 individuals (155 males and 344 females) indicated that the sex of this fish species could be determined using the tail height (*CD*) rather than other morphological indices such as distance from the upper jaw to the starting position of the dorsal fin (*PL*), distance from the upper jaw to the starting position of the anal fin (*PAL*), Under jaw length

(*UJAW*), eye diameter (*ED*), distance between 2 eyes (*DE*), mouth width (*MD*), body depth (*BD*), head length (*HL*).

The *SL-CD* regression relationship in males was classified as I, indicating that the tail height of males developed proportionately with the standard length. Conversely, the *SL-CD* regression relationship in females was classified as A-, indicating that the tail height growth rate in females tended to be slower than the standard length (**Table 2**).

**Table 2** Regression relationship of standard length (*SL*) and body depth (*BD*), head length (*HL*), distance from the upper jaw to the starting position of the dorsal fin (*PL*), distance from the upper jaw to the starting position of the anal fin (*PAL*), Under jaw length (*UJAW*), eye diameter (*ED*), distance between 2 eyes (*DE*), mouth width (*MD*), and tail height (*CD*) of *Hyporhamphus limbatus*.

Morphometrics	<i>b</i>	SE	<i>a</i>	SE	N	<i>R</i> <sup>2</sup>	Type	<i>ts</i>	<i>P</i>
<b>Female</b>									
<i>PL</i>	0.94	0.02	0.00	0.86	343	0.83	A-	-2.58	0.01
<i>PAL</i>	0.97	0.02	0.00	0.80	343	0.85	I	-1.13	0.26
<i>UJAW</i>	0.33	0.06	0.00	1.07	343	0.09	A-	-11.17	0.00
<i>ED</i>	0.14	0.09	0.10	3.30	343	0.01	A-	-9.85	0.00
<i>DE</i>	0.68	0.04	0.00	1.16	343	0.49	A-	-8.31	0.00
<i>BD</i>	0.97	0.06	0.00	1.25	343	0.49	I	-0.63	0.53
<i>HL</i>	0.71	0.03	0.00	4.02	343	0.67	A-	-10.36	0.00
<i>MD</i>	0.96	0.05	0.00	0.57	343	0.52	I	-0.79	0.43
<i>CD</i>	0.90	0.05	0.00	0.59	343	0.56	A-	-2.13	0.03
<b>Male</b>									
<i>PL</i>	0.89	0.04	0.00	0.96	155	0.78	A-	-2.66	0.01
<i>PAL</i>	0.94	0.04	0.00	0.86	155	0.84	I	-1.77	0.08
<i>UJAW</i>	0.37	0.09	0.00	1.00	155	0.10	A-	-6.82	0.00

Morphometrics	<i>b</i>	SE	<i>a</i>	SE	N	<i>R</i> <sup>2</sup>	Type	<i>ts</i>	<i>P</i>
<i>ED</i>	0.28	0.10	0.01	2.51	155	0.05	A–	–6.98	0.00
<i>DE</i>	0.72	0.07	0.00	1.04	155	0.45	A–	–4.06	0.00
<i>BD</i>	1.01	0.08	0.00	1.12	155	0.52	I	0.13	0.89
<i>HL</i>	0.64	0.05	0.00	4.76	155	0.60	A–	–7.93	0.00
<i>MD</i>	0.98	0.11	0.00	0.53	155	0.37	I	–0.18	0.86
<i>CD</i>	0.88	0.09	0.00	0.62	155	0.43	I	–1.33	0.18

This study highlighted the potential of using morphological measurements for accurate sex differentiation in *Hyporhamphus limbatus*, contributing valuable insights into the species' morphological differences.

## Discussion

The sex ratio is one of the 7 fundamental characteristics of a population, making the determination of each individual's sex crucial. Accurate sex determination, achieved through measurement criteria and morphological characteristics, helps minimize the number of fish killed, thereby upholding the ethical standards of scientific research. This study has shown 2 methods of sex determination in *Hyporhamphus limbatus* based on upper jaw and head morphology and the regression relationship between *SL* and *CD*. This has not been addressed in any previous studies, which may help limit the number of fish killed in future studies.

In this study, *Hyporhamphus limbatus* was identified with a unique difference in the head and upper jaw used for sex determination, a finding not previously reported. The results of this research introduce a novel method for sex determination for this species, both in Vietnam and globally. Each fish species requires distinct sex determination methods. For example, *Mystus albolineatus* [20] is identified using secondary genitalia, a method also recorded in several species from the Mekong Delta region, such as *Butis butis* [21], *Periophthalmodon septemradiatus* [22], *Periophthalmus chrysospilos* [23].

Globally, there is a preference for non-lethal sex discrimination methods. This is evident in studies that enhance extra-diurnal morphological characteristics for sex differentiation. For instance, in the species *Kryptolebias marmoratus*, males can be distinguished by a gradual color change to orange and eye-shaped spots on the tail [24]. Similarly, sex can be identified by examining the scales of 2 species of the genus *Mugil*: *Mugil cephalus* and *Mugil curema* [25]. Most recently,

Cos and Tyler [26] demonstrated sex discrimination in *Hemichromis bimaculatus* by recognizing facial and eye colors.

Their behavior and reproductive processes influence the growth rate of fish. During growth, individuals may exhibit differences in external morphological characteristics [27]. Recent studies indicate that the growth of these characteristics also depends on the sex of the fish. For example, research on the species *Mystus albolineatus* [20] found that females have a regression relationship between total length (*TL*) and standard length (*SL*) classified as group A+, which is significantly different from males classified as group A–. Other fish species studied for sex determination using the *TL-SL* relationship include *Mystus mysticetus* [14]; *Ellochelon vaigiensis* [15], *Zacco koreanus* [27], *Heterotis niloticus* [28], *Glossogobius sparsipapillus* [13], *Glossogobius giuris* [12], *Caragobius urolepis* [16].

## Conclusions

This study proposed a method for sex determination through morphological characterization with a high accuracy rate. Additionally, statistical analysis revealed that *Hyporhamphus limbatus* exhibits a difference in the regression correlation between standard length (*SL*) and tail height (*CD*) for sex discrimination. These findings provide a basis for future management and research of *Hyporhamphus limbatus*.

## Acknowledgements

This study is funded in part by the Can Tho University, Vietnam, Code: TSV2024-92.

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