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Original Article

# Distinctive color variation in *Rhinolepadichthys geminus* (Gobiesocidae: Diademichthyinae) from Alor Islands, Indonesia: morphological and molecular insights

## Variação de cor distinta em *Rhinolepadichthys geminus* (Gobiesocidae: Diademichthyinae) das Ilhas Alor, Indonésia: percepções morfológicas e moleculares

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**Abstract**

*Rhinolepadichthys geminus*, one of three recently described species in the genus *Rhinolepadichthys* (previously known as *Lepadichthys lineatus* complex), is characterized by several distinct diagnostic morphological and color features, including a pair of yellow stripes on the body ventral midline. However, one of three specimens recently collected from the Alor Islands, Indonesia, possessed a yellow circular line, instead of a pair of yellow stripes, indicating that the latter were not an invariable feature. Morphological and molecular evidence confirmed the identity of the specimen and intraspecific significance of the color variation.

**Keywords:** coloration, diagnostic characters, *Lepadicthys lineatus* complex, molecular analysis, morphology.

**Resumo**

*Rhinolepadichthys geminus*, uma das três espécies recentemente descritas no gênero *Rhinolepadichthys* (anteriormente conhecido como complexo *Lepadichthys lineatus*), é caracterizada por várias características morfológicas e de cor diagnósticas distintas, incluindo um par de listras amarelas na linha média ventral do corpo. No entanto, um dos três espécimes recentemente recolhidos nas Ilhas Alor, na Indonésia, possuía uma linha circular amarela, em vez de um par de listras amarelas, indicando que estas últimas não eram uma característica invariável. Evidências morfológicas e moleculares confirmaram a identidade do espécime e o significado intraespecífico da variação de cor.

**Palavras-chave:** coloração, caracteres diagnósticos, complexo *Lepadicthys lineatus*, análise molecular, morfologia.

## 1. Introduction

The genus *Rhinolepadichthys* (previously known as *Lepadichthys lineatus* complex; see Fujiwara and Motomura, 2021), including four valid species, e.g., *Rhinolepadichthys lineatus* (Briggs, 1966), *Rhinolepadichthys geminus* (Fujiwara and Motomura, 2021), *Rhinolepadichthys heemstraorum* (Fujiwara and Motomura, 2021), and *Rhinolepadichthys polyastrous* (Fujiwara and Motomura, 2021), was recently defined by Fujiwara et al. (2024). The genus is most similar to the genus *Discotrema* Briggs, 1976 in having the presence of a hardened cap on the surface of at least some disc papillae and the anterolateral part of the ventral postcleithrum extended anteriorly as a well-developed rod-like process (Fujiwara et al., 2024). *Rhinolepadichthys* is distinguishable from *Discotrema* by morphology of the upper-jaw lip and inner surface of both lips, condition of gill rakers on the anterior an posterior edges of ceratobranchial, and several osteological and adhesive disc features (see Fujiwara and Motomura, 2021; Fujiwara et al., 2024).

Although the four species within *Rhinolepadichthys* could be differed from one other by several morphological features, fresh coloration (i.e. striped and dotted patterns on the body) of specimens was considered a greater aid

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to their identification (Fujiwara and Motomura, 2021). *Rhinolepadichthys geminus* can be easily differed from other congeners in the genus in having the following coloration patterns: dorsal dots displaying ca. 3-5 longitudinal rows; lateral dots forming a single fragmented line, respectively, and a distinct pair of yellow stripes on ventral surface of body midline (Fujiwara and Motomura, 2021). Because the later feature was consistent in the fresh specimens examined by Fujiwara and Motomura (2021), the new species was given specific and common English names to reflect such; "geminus" (meaning "twin" or "double" in Latin) and Pacific Doubleline Clingfish, respectively.

In February 2022, as part of a marine rapid assessment project aimed at evaluating the protected marine areas in the provinces of Maluku and Nusa Tenggara Timur, Indonesia, initiated by YKCI (Yayasan Konservasi Cakrawala Indonesia) and Faculty of Fisheries and Marine Science, Pattimura University, Ambon, three specimens of *R. geminus* were collected from Alor Islands, Indonesia, at depths ranging from 12-20 m. Although the morphological, morphometric, and meristic features of the three specimens were all in accordance with the description of *R. geminus* as outlined by Fujiwara and Motomura (2021), one of the Alor specimens was unusual in having a circular pattern on the ventral body midline instead of double stripes. Consequently, the diagnostic character recognized by Fujiwara and Motomura (2021), i.e. two yellow stripes on the ventral surface of the abdomen, is now considered subject to variation, the specimen identity having been confirmed by morphological and molecular analyses.

## 2. Materials and Methods

Counts and measurements were conducted in accordance with the methodology outlined by Fujiwara and Motomura (2018). All measurements were recorded using Mitutoyo digital calipers with an accuracy of 0.01 mm. Measurement data were expressed as percentages of standard length (SL). Terminology for the adhesive disc followed description provided by Briggs (1955: Figure 1). Curatorial procedure for MZB.26660 followed Motomura and Ishikawa (2013), the other two specimens (MZB.26658 and MZB.26659) being preserved in 98% ethanol. The examined specimens were cataloged at the Museum Zoologicum Bogoriense (MZB), Bogor, Indonesia.

Two individual specimens of *R. geminus* (MZB.26658 and MZB.26659) and one individual of *Priolepis cincta* (MZB.26809) were used in the molecular study. Muscle tissue was dissected from the right pectoral fin and maintained in absolute ethanol. DNA extractions were carried out according to the instructions of the Qiagen DNeasy Blood & Tissue Kit. DNA amplification was conducted by the Polymerase Chain Reaction (PCR), targeting the cytochrome c oxidase subunit I (COI) gene, and using LCO1490-F:5'-GTCAACAAATCATAAAGATATTGG 3' and HCO2198R:5'TAAACTTCAGGGTG ACCAAAAAATCA-3' primers (Folmer et al., 1994). The PCR reaction was performed in 25 µL volumes, using 4 µL of DNA template. Each reaction contained 12.5 µL of MyTeq™ Red Mix (Bioline), 1 µL of each primer, and 6.5 µL of ddH2O. The thermocycling process began with an initial


**Figure 1.** Photographs of live individuals of *Rhinolepadichthys geminus* from Pura Island, Alor Islands, Indonesia. (a, b, c) MZB.26658, 22.4 mm SL; (d, e, f) MZB.26659, 21.1 mm SL. (a, d) lateral views, right sides, photo reversed; (b, e) dorsal views; (c, f) ventral views, photo reversed.

denaturation step at 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds, 42°C for 15 seconds, and 72°C for 30 seconds. A final extension step at 72°C for 7 minutes concluded the procedure. The PCR reactions were examined using 2% agarose gels stained with *Ethidium bromide*. Gel electrophoresis was used to visualize the results of amplification of the COI gene fragment, using a UV-transilluminator and documented with a digital camera. Amplicons were then sequenced using the Sanger Sequencing Platform.

Forward and reverse sequences were checked, cleaned, and aligned using Mega X software (Kumar et al., 2018). Subsequently, they were compared to the open database of NCBI (https://www.ncbi.nlm.nih.gov) using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The most similar sequences from NCBI, *Rhinolepadichthys lineatus* (MF123935.1 and KT883587.1) were downloaded and aligned with the sample sequences, together with *Discotrema crinophila* (KY656438.1), *Lepadichthys coccinotaenia* (MT053036.1), *Lepadichthys erythraeus* (MN560936.1), *Lepadichthys frenatus* (MK657900.1 and MK657310.1), and *Priolepis cincta* (OQ385626.1) as outgroup. *Lepadichthys coccinotaenia* later described as *Lepadichthys thishula* (Fujiwara et al., 2020a; Conway et al., 2020), and *Lepadichthys frenatus* later described as *Lepadichthys conwayi* (Fujiwara and Motomura, 2020). The phylogenetic trees were reconstructed using Neighbor Joining and Maximum Likelihood analysis, using 1000 bootstrap replicates of the Kimura 2-Parameter model to evaluate the support for clades.

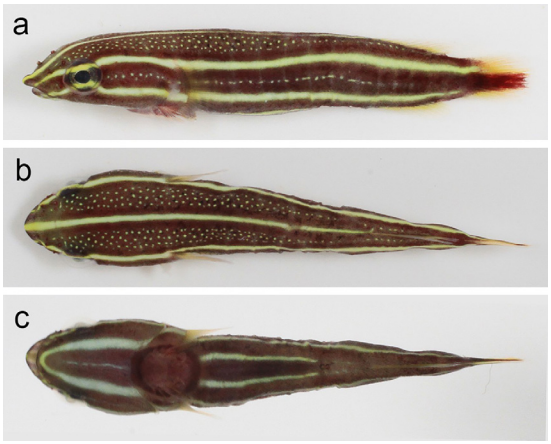


**Figure 2.** Fresh specimen of *Rhinolepadichthys geminus* from Pantar Island, Alor Islands, Indonesia, MZB.26660, 16.7 mm SL, (a) lateral view, (b) dorsal view, (c) ventral view.

## 3. Results

### 3.1. Taxonomy

Order Gobiesociformes

Family Gobiesocidae Bleeker, 1859

Genus *Rhinolepadichthys* Fujiwara, Motomura, Summers, and Conway, 2024

*Rhinolepadichthys geminus* (Fujiwara and Motomura, 2021)

English name: Pacific Doubleline Clingfish

Material examined: MZB.26658, 21.1 mm SL, MZB.26659, 22.4 mm SL, east coast Pura Island, Alor Islands, Indonesia, depths of 18-20 m, hand net, coll. by K. Wibowo, 27 Feb. 2022; MZB.26660, 16.7 mm SL, east coast of Pantar Island, Alor Islands, Indonesia, depth of 12 m, hand net, coll. by K. Wibowo, 26 Feb. 2022; see Figures 1-4 and Tables 1-2.

### 3.2. Description

Morphometric data of the specimens are presented in Table 1. Body cylindrical laterally, progressively more compressed posteriorly. Mouth terminal; lip of upper jaw joined with snout membrane; oral papillae present at inner surface of upper and lower lips. Snout pointed and triangular in lateral and dorsal views, respectively; snout pointed, directed upward, its tip situated distinctly anterior to lower-jaw tip; snout tip positioned in line with lower edge of eye lens (in lateral view). Uppermost of gill opening positioned parallel to 9th or 10th pectoral-fin ray base (in lateral view). Distance from anus to anal fin origin shorter than distance from anus to posterior edge of adhesive disc. Anal fin origin positioned slightly behind to vertical through origin of dorsal fin. Dorsal and anal fins positioned posteriorly, no membrane connected their posteriormost rays to caudal fin. Tips of pectoral and caudal fins rounded. Dorsal-fin rays 9 or 10. Anal-fin rays

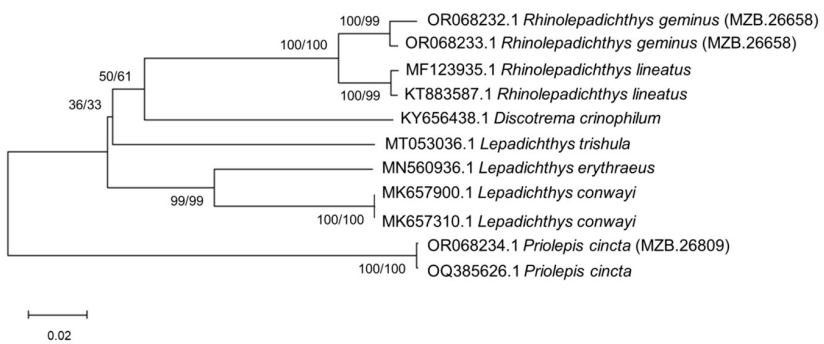


**Figure 3.** Phylogenetic tree of *Rhinolepadichthys geminus* with Maximum Likelihood (ML) topology generated from mtDNA COI gene. Numbers below major nodes represent bootstrap support for 1000 replicates using maximum likelihood and neighbor-joining, respectively.

**Table 1.** Measurement data of *Rhinolepadichthys geminus* from Alor Islands, Indonesia (expressed as percentages of SL).

| | MZB.26658 | MZB.26659 | MZB.26660 | Means |
|---|---|---|---|---|
| Standard length (mm) | 21.1 | 22.4 | 16.7 | |
| Head length | 30.6 | 30.4 | 30.3 | 30.4 |
| Post-orbital length | 15.1 | 14.8 | 15.4 | 15.1 |
| Head depth | 10.1 | 9.9 | 11.3 | 10.4 |
| Head width | 18.0 | 17.8 | 18.3 | 18.0 |
| Body depth | 13.7 | 13.8 | 12.9 | 13.5 |
| Body width | 16.4 | 16.9 | 17.5 | 16.9 |
| Gill opening depth | 5.7 | 5.1 | 6.5 | 5.8 |
| Snout length | 7.8 | 8.8 | 7.1 | 7.9 |
| Snout depth | 7.2 | 6.7 | 8.5 | 7.5 |
| Upper-jaw length | 8.0 | 8.1 | 7.0 | 7.7 |
| Orbit diameter | 8.6 | 8.1 | 8.0 | 8.2 |
| Anterior interorbital width | 11.3 | 12.1 | 12.8 | 12.1 |
| Posterior interorbital width | 16.4 | 16.3 | 18.4 | 17.1 |
| Least interorbital width | 7.8 | 7.0 | 6.9 | 7.2 |
| Disc length | 15.1 | 15.3 | 17.1 | 15.8 |
| Disc width | 12.9 | 13.0 | 14.1 | 13.3 |
| Caudal-peduncle length | 7.4 | 7.2 | 7.8 | 7.5 |
| Caudal-peduncle depth | 7.5 | 7.3 | 8.5 | 7.7 |
| Pre-disc length | 26.2 | 25.0 | 23.2 | 24.8 |
| Pre-anus length | 68.8 | 68.8 | 67.5 | 68.4 |
| Disc to anal-fin origin length | 43.0 | 42.1 | 38.4 | 41.2 |
| Disc to anus length | 29.8 | 29.2 | 25.8 | 28.3 |
| Pre-dorsal-fin length | 78.2 | 73.9 | 74.4 | 75.5 |
| Pre-anal-fin length | 79.3 | 74.9 | 75.7 | 76.6 |
| Dorsal-caudal length | 24.2 | 24.0 | 26.5 | 24.9 |
| Post-dorsal-caudal length | 7.6 | 8.4 | 7.2 | 7.8 |
| Anal-caudal length | 23.7 | 23.2 | 24.2 | 23.7 |
| Dorsal-fin base length | 17.6 | 16.4 | 19.5 | 17.8 |
| Anal-fin base length | 15.0 | 15.4 | 17.2 | 15.9 |
| Pectoral-fin length | 13.6 | 13.8 | 13.1 | 13.5 |
| Caudal-fin length | broken | 15.7 | 17.2 | 16.5 |

**Table 2.** Average interspecific pairwise genetic distance matrix for the mtDNA COI sequences in the genus *Rhinolepadichthys*, *Lepadichthys*, *Discotrema* and *Priolepis cincta* as an outgroup.

| *Species* | *R. geminus* | *P. cincta* | *L. conwayi* | *R. lineatus* | *L. trishula* | *L. erythraeus* | *D. crinophila* |
|---|---|---|---|---|---|---|---|
| *R. geminus* | 0.000 | | | | | | |
| *P. cincta* | 0.272 | 0.000 | | | | | |
| *L. conwayi* | 0.200 | 0.263 | 0.000 | | | | |
| *R. lineatus* | 0.045 | 0.276 | 0.179 | 0.000 | | | |
| *L. trishula* | 0.199 | 0.267 | 0.183 | 0.191 | 0.000 | | |
| *L. erythraeus* | 0.194 | 0.269 | 0.109 | 0.191 | 0.182 | 0.000 | |
| *D. crinophila* | 0.172 | 0.275 | 0.202 | 0.177 | 0.181 | 0.182 | 0.000 |

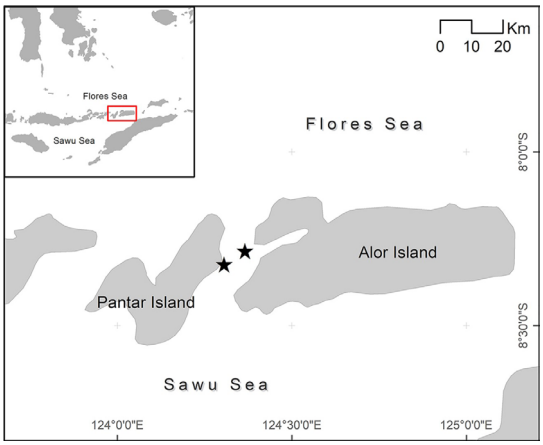


**Figure 4.** Collection site (stars) of the specimens of *Rhinolepadichthys geminus* examined in this study.

9 (not counted in MZB.26659). Pectoral-fin rays 25 (only counted in MZB.26658); upper and lowermost rays minute. Single ventral adhesive disc; flattened papillae present on disc regions A and B; disc region C without a cavity or papillae. Disc region A with ca. 3 rows of papillae across center, disc region B with ca. 4 rows; inner papillae rows greater than outer rows.

## 4. Discussion

The Alor Island specimens (Figures 1, 2) were confirmed as *R. geminus*, closely matching with the diagnosis of that species provided by Fujiwara and Motomura (2021), e.g. body depth 12.9-13.8% of SL; adhesive disc circular, with length 15.1-17.1% of SL and width 12.9-14.1% of SL, proportion of length to width 1.17-1.21; anus situated posteriorly, length of pre-anus 67.5-68.8% of SL, distance from anus to posterior edge of adhesive disc 25.8-29.8% of SL; snout directed upward, pointed, its tip in line with ventral edge of eye lens (in lateral view); ventral surface of abdomen with a pair of yellow stripes (except MZB.26658, a yellow circular line; Figure 1c); body with yellowish dots dorsally, forming approximately three longitudinal rows; lateral side with a single yellowish fragmented line (indistinct in MZB.26659; Figure 1d, e).

The MZB.26658 specimen (21.1 mm SL; Figure 1ac) clearly differed from the diagnostic character that had inspired the species name *R. geminus* in having a yellow circular line, with a short mid-line extension posteriorly [vs. two parallel yellow stripes in the latter (see Fujiwara and Motomura, 2021: figures 2c, 5f; see Fujiwara et al., 2020b)]. However, all other morphological characters, including meristic and morphometric data, of the specimen agreed with those of the type and non-type specimens of *R. geminus* given by Fujiwara and Motomura (2021), in addition to the two other specimens in the present study. In addition, the specimen showed the typical diagnostic color patterns on the dorsal and lateral body surfaces (Figure 1a, b; see Kuiter and Deberius, 2006: 113, Allen and Erdmann, 2013: 840, Fujiwara and Motomura, 2017: figure 1, and Fujiwara et al., 2020b: figure 2).

The gene sequences from two *R. geminus* samples included 550 and 604 base pairs (bp), the BLAST analysis showing them to be 99% identical with *R. lineatus.* This is the first report on the COI gene of *R. geminus*, the distance between the two samples being 0.5%. The sample from MZB.26809 was 99% identical with *P. cincta* (accession number OQ385626.1). The distance between *R. geminus* and *R. lineatus* was 4%, between *R. geminus* and *D. crinophila* was 17%, between *R. geminus* and *L. conwayi* 20%, between *R. geminus* and *L. trishula* 20%, between *R. geminus* and *L. erythraeus* 19%, and between *R. geminus* and *P. cincta* 27% (Figure 3; Table 2). All sequences of two *R. geminus* and one *P. cincta* samples were submitted to NCBI with Accession Number OR068232, OR068233, and OR068234, respectively.

Both the morphological and molecular studies clearly indicated that MZB.26658 was an example of *R. geminus*, the difference in the ventral midline color pattern being attributable to intraspecific variation. Because this is the first instance of such, despite the many reports of *R. geminus* based on fresh specimens, the condition of two yellow stripes on the ventral surface of abdomen remains a reasonable diagnostic character of the species.

The ground body color of *R. geminus* seems to vary from reddish to dark brown or black (see Fujiwara and Motomura, 2021: figures 2, 5, 6; Fujiwara et al., 2020b: figure 2; this study: Figures 1, 2), suggesting that such coloration is dependent upon the habitat of the crinoids commensal with the former. Additionally, *R. geminus* has been observed to inhabit rocky substrates in association with sea urchins (Fujiwara and Motomura, 2021: figure 6c).

*Rhinolepadichthys* is widely distributed in the Indo-West Pacific region: *R. geminus* in the western Pacific, including southern Japan and Indonesia, *R. heemstraorum* and *R. polyastrous* in the southwestern Indian Ocean, and *R. lineatus* in the north Indian Ocean (Fujiwara and Motomura, 2021). In Indonesian waters, *R. geminus* has previously been reported from Ambon Bay, Ambon (Fujiwara and Motomura, 2021) and the Alor Islands (Allen and Erdmann, 2013). The specimens examined in this study were also collected from the Alor Islands (Pura and Pantar Islands; Figure 4), from a commensal crinoid habitat at depths of 12-20 m.

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