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Phylogeographic Study of Betutu Fish (*Oxyeleotris marmorata*) Interpreted Using DNA Barcodes in Three Rivers in East Java Region, Indonesia

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Abstract— **Betutu (*Oxyeleotris marmorata*) is a freshwater fish of high economic value widely consumed in Southeast Asia. Overfishing in nature has resulted in a decline in population and genetic diversity. Genetic diversity is an important factor in the long-term survival of *O. marmorata* populations. This study aimed to analyze the genetic diversity and phylogenetic relationships of *O. marmorata* in three rivers in East Java (Bengawan Solo River, Kali Tengah River, and Bondoyudo River) using a DNA barcoding approach. Twenty-two fish specimens were collected, identified, and analyzed using PCR and DNA sequencing. The results showed that *O. marmorata* from the three locations had a low level of genetic variation, with genetic distance differences between 0.00-0.02. Phylogenetic analysis showed that specimens from East Java were closely related to populations from Thailand and Australia. This study emphasizes the importance of genetic conservation and population management with the development of hatcheries to reduce dependence on natural catch.**

Keywords— DNA barcoding, fisheries conservation, genetic diversity, phylogeography, *O. marmorata*.

I. INTRODUCTION

Betutu (*Oxyeleotris marmorata*) is one of the most popular freshwater fish in Southeast Asia due to its economic value and high market demand [1; 2; 3]. Although East Asian countries such as China, Japan, Hong Kong, and Taiwan remain the main markets, they are also gaining attention in the United States and the European Union as live or chilled consumption fish and ornamental fish. However, large-scale production still relies on larvae obtained from the wild, which are diminishing in number. In contrast, hatchery production has not been optimized due to challenges in developing effective culture techniques [4]. Environmental changes, influenced by population increase, agricultural sector expansion, and urbanization, have significantly impacted the population size of this fish in nature. In addition, overfishing practices by local communities have further exacerbated the population decline, as the exploitation rate exceeds its natural reproductive capacity. This fish is also known for its relatively slow growth [5]. This condition raises concerns about the sustainability of genetic diversity, especially in key reproductive areas such as Indonesia.

O. marmorata belongs to the Eleotridae family and is native to the Mekong River in China. It has a wide distribution, covering the Mekong and Chao Phraya basins and other areas such as Peninsular Malaysia, Indochina, Thailand, Cambodia, Vietnam, Singapore, and the Philippines. In Indonesia, this species was first introduced in 1927 [6; 7; 8]. Its spread to various aquatic ecosystems is primarily influenced by human activities, both intentionally and unintentionally. *O. marmorata* has been identified into 17 species [9]. Of these, 11 species are distributed globally. In comparison, eight species, *O. urophthalmoides, O. colasi, O. wisselensis, O. heterodon, O. paucipora, O. urophthalmus, O. marmorata*, and *O. altipinna*, are found in Indonesian waters. In Indonesia, *O. marmorata* can be found in Sumatra, Java, Kalimantan, and Papua [10]. In East Java, *O. marmorata* can be found in the Bengawan Solo River, Kali Tengah River, and Bondoyudo River.

The Bengawan Solo River is one of Java's most prominent and longest rivers, ranking second [11; 12]. The river flows across two provinces, namely Central Java and East Java, with a length of about 548.53 km and is supported by about 2,200 tributaries [13]. Bengawan Solo has two main upstream points in the Sewu Mountains, Wonogiri and Ponorogo. Meanwhile, the Bondoyudo River empties into the southern coast of East Java and flows into the waters of the Indian Ocean [14]. The river's lower reaches form the natural boundary between Jember and Lumajang districts. The primary source of the flow of the Bondoyudo River comes from the Bromo-Tengger-Semeru National Park area and the Argopuro Mountains complex, precisely on the western slope of Mount Stage. Kalitengah River is located in Banyuwangi Regency. This river crosses a densely populated urban area, resulting in an increase and accumulation of waste [15]. As the habitat of *O. marmorata*, it is important to know the diversity of *O. marmorata* in the three rivers in East Java.

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Genetic diversity is an important factor in the long-term survival of *O. marmorata* populations, affecting adaptability to environmental changes and resistance to various stressors [16; 17]. Environmental factors and anthropogenic activities can significantly affect genetic diversity in fish populations [18; 19]. The implications of genetic diversity also play an important role in population resilience to climate change and other ecological disturbances [20; 21]. To understand genetic variation as well as population structure, mitochondrial DNA (mtDNA) marker-based analysis methods [22; 23]. This approach provides essential insights into the phylogeographic structure, population diversity, and evolutionary relationships of fish species [24; 25; 26]. The results of this study are expected to contribute to breeding efforts and genetic conservation strategies for *O. marmorata*.

DNA barcoding, which examines short and standardized gene segments, serves as a reliable approach for precise species identification [27]. This technique has been widely applied to various freshwater fish species, such as *Channa striata* and *C. punctata* [28], *Oreochromis niloticus* [29], *Cyprinus carpio* [30], and *Barbonymus gonionotus* [31]. One of its key advantages in species authentication is the ability to identify organisms at early developmental stages, which are often difficult to differentiate through conventional morphological methods [32]. To support species identification, assess genetic divergence, and explore the phylogenetic relationships of *Ophicephalus marmorata* collected from East Java waters—specifically the Bengawan Solo River, Kali Tengah River, and Bondoyudo River—researchers aimed to amplify the COI gene sequence and compare it with existing GenBank data. The use of COI genes in species identification serves as a basis for developing genetic information, which can support fish breeding selection through hybridization techniques [33; 34].

II. METHOD

Study Area

This study primarily focused on the phylogenetic analysis of O. marmorata, conducted in the waters of East Java, specifically in the Bengawan Solo, Kali Tengah, and Bondoyudo rivers (Figure 1). The research activities lasted four months, from May to August 2024, with data collection conducted every two weeks. *O. marmorata* was captured as done by Kusuma [27] using a gill net measuring 4 meters in length, 1.5 meters in height, with a 1.3 cm mesh size to capture O. marmorata. To maintain sample freshness, the dry cooling method was applied, in which ice cubes were stored in a cooler box. Upon arrival at the laboratory, the specimens were sorted by collection site and classified by sex. Local residents, experienced in catching *O. marmorata*, assisted in the sample collection process.

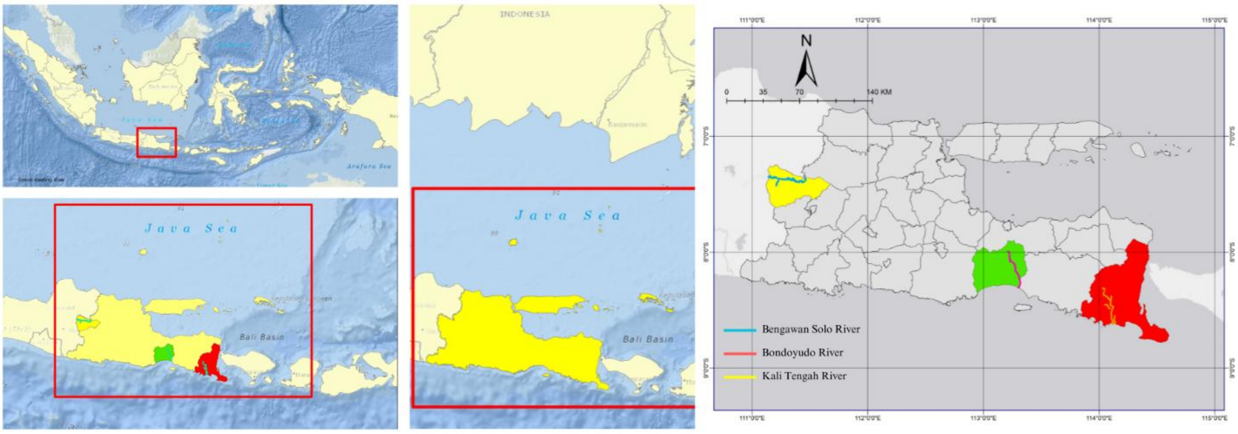

Figure 1. Research location in East Java waters (Bengawan Solo River, Kali Tengah River, and Bondoyudo River). Bengawan Solo River with coordinates: S 07o 57' 14.34 ", E 111 31' 43 22", Bondoyudo River with coordinates: S 7o 39' 20.0 ", E 112 o40' 21.5" and Kali tengah River with coordinates: S 08o 04' 34.98 ", E 112 o19'45.29".

Biological materials

Twenty-two specimens of *O. marmorata* were obtained from the wild environment of East Java waters. Local fishermen caught wild specimens that ranged from 23.67 to 210 g in weight and about 14.68 cm in length. For each fish, a 4 cm caudal fin segment was cut using a sterile knife, then preserved in a tube containing 96% ethanol, labeled, and stored at -20^{0}C until the DNA extraction stage.

DNA isolation and sequencing

Genomic DNA was extracted from *O. marmorata* tissue samples collected from three rivers in East Java using the AquaGenomic Solution Protocol Kits (MultiTarget Pharmaceuticals) and subsequently analyzed, as described by Abdullah [35]. The extraction process followed the manufacturer's protocol with slight modifications to enhance DNA yield and quality. The cytochrome oxidase subunit I (COI) gene, located in the mitochondrial genome of the studied species, was amplified using the polymerase chain reaction (PCR) method. Each 25 µL PCR reaction mixture contained 18.75 µL of ultrapure water, 2.25 µL of 10X PCR buffer, 3.0 µL of $MgCl_2$, 0.25 µL of each primer (FishF1: 5'-TCAACCAACCACAAAGACATTGGCAC-3' and FishR1: 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3') [36], 0.5 µL of dNTPs, 0.1 µL of Taq polymerase enzyme, and 1.5 µL of template DNA. The amplification process began with an initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at

72°C for 10 minutes. PCR products were analyzed via agarose gel electrophoresis, after which the most distinct DNA bands were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA). The purified DNA was then sent to PT Genetika Science Jakarta for sequencing using an ABI3730 sequencer.

Data Analysis

The three COI gene sequences of *O. marmorata*, obtained using the forward1 and reverse1 primers, were stored in FASTA format. Sequence identification was conducted using the BLAST (Basic Local Alignment Search Tool) application available on the NCBI (National Center for Biotechnology Information) platform. To assess the pairwise genetic distance, the Kimura 2-parameter (K2P) distance model [37] was applied for sequence comparison. The consensus sequences generated in this study were integrated with reference sequences from GenBank (http://www.ncbi.nlm.nih.gov/) to reconstruct the phylogenetic tree. The Neighbor-Joining (NJ) method was employed for phylogenetic tree construction using MEGA XI software [38; 39], with bootstrap analysis performed over 1000 replicates.

III. RESULTS AND DISCUSSION

The results of the study showed that the application of mechanical and chemical filters in intensive shrimp farming systems had a significant impact on water quality. Based on the results of the study, the pond without a filter (K) had a pH of 8.3, while the pond using a mechanical filter (FF) and a chemical filter (FK) showed a higher pH, which was 8.5. In addition, dissolved oxygen (DO) levels increased from 7.8 mg/L in pond K to 8.2 mg/L in FF and 8.5 mg/L in FK, indicating more optimal oxygenation conditions. The highest total organic matter (TOM) content was recorded in pond K with a value of 94.3 mg/L, while in FF, there was a decrease to 90 mg/L, and FK experienced the most significant reduction to 60.8 mg/L. This indicates that chemical filters are more effective in reducing organic matter in water. On the other hand, the total suspended solids (TSS) initially high in pond K (83–83.5 mg/L) decreased to 43–44 mg/L in FF and 50–51 mg/L in FK. This finding indicates that the mechanical filter is more efficient in filtering suspended particles (Table 1). The resulting improvement in water quality also affects the pond ecosystem, especially in reducing the potential for Vibrio bacteria growth (Table 2). Chemical filters, which are more effective in lowering TOM content, play an essential role in suppressing the population of pathogenic bacteria. Meanwhile, mechanical filters increase water clarity, thus supporting the balance of phytoplankton in the pond environment

Morphology of *O. marmorata*

O. marmorata has rough skin with a torpedo-shaped body, stout posture, and brownish to dark or yellowish color with grayish-black patches scattered throughout it. The upper part of the body tends to be darker than the lighter lower part. The underside of the head has pink markings, while the back of the body is marked with irregular red transverse lines. This color pattern is often used to identify sex, with females having a darker body color than males. The face is concave with a flattened head and large, protruding eyes that can be moved. The mouth is broad and thick, equipped with small but sharp teeth. The position of the mouth is slightly upward (subterminal) and can be raised. There are fewer teeth in the upper jaw compared to the lower jaw (Figure 2). *O. marmorata* prefers calm current waters with a muddy bottom of about 40 cm deep. This fish often uses water areas with dense vegetation as shelter and spawning grounds. Living on the bottom, this fish often immerses itself in mud and only occasionally comes to the surface.


Figure 2. *O. marmorata* found in three East Java water areas

COI Sequence Characterization and Genetic Diversity of *O. marmorata*

The results of the molecular analysis conducted showed that the mtDNA fragment of *O. marmorata* was 612-620 bp. The base composition of the COI gene from 14 species in GenBank was compared with *O. marmorata* in this study (Table 1). The average gene base composition of *O. marmorata* in the three rivers in this study was T(U) (29.02%), C (29.49%), A (23.74%), G (17.74%), C+G (47.24%), A+T (52.76%). Species average gene base results showed T(U) (30.21%), C (29.54%), A (22.93%), G (17.32%), C+G (46.86%), A+T (53.14%).

TABLE 1.
COMPARISON OF NUCLEOTIDE COMPOSITION BETWEEN *O. MARMORATA* IN THIS STUDY USING THE COI GENE WITH DATA IN GENBANK.

Species	Country	T(U) %	C %	A %	G %	C+G %	A+T %
O. marmorata Bengawan Solo River	Indonesia	28.77	29.95	23.58	17.69	47.64	52.36
O. marmorata Kali Tengah River	Indonesia	28.61	29.31	23.88	18.20	47.52	52.48
O. marmorata Bondoyudo River	Indonesia	29.69	29.22	23.75	17.34	46.56	53.44
K448189 (*O. marmorata*)	Thailand	29.70	30.56	22.44	17.31	47.86	52.14
KU692720 (*O. marmorata*)	Indonesia	29.70	30.56	22.44	17.31	47.86	52.14
EF609424 (*O. marmorata*)	Australia	29.70	30.56	22.44	17.31	47.86	52.14
KU692718 (*O. marmorata*)	Indonesia	29.70	30.56	22.44	17.31	47.86	52.14
KJ669574 (*O. lineolata*)	Australia	29.91	30.13	23.72	16.24	46.37	53.63
KU944192 (*Girella punctata*)	Taiwan	33.55	25.43	25.64	15.38	40.81	59.19
OR082995 (*Mugil curema*)	Mexico	30.34	29.49	20.94	19.23	48.72	51.28
MF122700 (*Rhinogobius leavelli*)	China	29.91	30.56	21.15	18.38	48.93	51.07
MK448189 (*O. marmorata*)	Thailand	29.70	30.56	22.44	17.31	47.86	52.14
OK670609O (*O. marmorata*)	Indonesia	29.70	30.56	22.44	17.31	47.86	52.14
PP085180 (*O. marmorata*)	Indonesia	29.70	30.56	22.44	17.31	47.86	52.14
PV034318 (*O. marmorata*)	Indonesia	29.91	30.56	22.65	16.88	47.44	52.56
MG835686 (*Girella punctata*)	Korea	33.55	25.43	25.64	15.38	40.81	59.19
JX185196 (*Mugil curema*)	Brazil	31.41	28.21	21.79	18.59	46.79	53.21
Average		30.21	29.54	22.93	17.32	46.86	53.14

Molecular analysis techniques are scientific approaches to examining evolutionary relationships between organisms by analyzing molecular data, such as DNA or protein sequences. This approach is based on the principle that organisms with similar genetic makeup tend to come from a common ancestor. This concept aligns with the biological evolution theory, which explains a population's genetic changes over time. The main factors involved in creating genetic variation include mutation and recombination. In this study, species such as *O. lineolata, Girella punctata, Mugil curema*, and *Rhinogobius leavelli* were compared to evaluate the evolutionary relationships of *O. marmorata* found in Three Rivers in the East Java Region.

Genetic Distance and Phylogenetic Genetics

Genetic distance indicates genetic differentiation between species and populations [40]. Thus, the smaller the genetic distance value, the more difficult it is to distinguish partial sequences of the CO1 gene between two species [41]. Based on the data available in GenBank, the genetic distance of *O. marmorata* from three rivers in the East Java region (Table 2) showed variations between 0.00 and 0.02. Meanwhile, the level of genetic divergence between *O. marmorata* and *O. lineolata* is in the range of 0.00 to 0.15. Comparisons with other species show the following variations: *O. marmorata* with *Girella punctata* (0.00-0.22), *Mugil curema* (0.00-0.22), and *Rhinogobius leavelli* (0.00-0.23). In genetic studies, a species is generally considered identical if it has a genetic difference of less than 2%. A divergence above 2% indicates that the species is classified as different [10].

Phylogenetic analysis of *O. marmorata* from three rivers in East Java showed that this species can be clearly distinguished from other species. This result is based on research conducted by Syaifudin et al. [10] in South Sumatra and on the sequences of *O. marmorata* in the GenBank database. In addition, the results of this study indicate that *O. marmorata* has a close genetic relationship with *O. lineolata*. As shown in Figure 3, *O. marmorata* of the three rivers in the East Java region is identical to *O. marmorata* in Thai and Australian waters. Phylogenetic tree analysis (Figure 3) also shows that, at the family level, *O. marmorata* has the highest genetic similarity with *Rhinogobius leavelli*. The results of studies conducted by Chen [42] and Zang [43] also showed that *O. marmorata* is very close to *O. lineolatus*. Hilman [44] also showed that *O. marmorata* is closely related to *O. porocephala* and *O. lineolata*. Pan [17] also showed that *O. marmorata* has a close evolutionary relationship with other species in the Eleotridae family.

The spread of genetic material between regions is influenced by various factors, such as species migration and larval movement [45; 46]. Many other aquatic organisms migrate seasonally, contributing to genetic exchange between populations while maintaining genetic diversity [47]. In addition, larval dispersal plays an important role, as many aquatic organisms release larvae that are carried by ocean currents or river flow, often traveling considerable distances before settling. The extent of this dispersal depends on the species' reproductive strategy, the larval phase's length, and the individual's swimming ability. Environmental factors such as ocean currents, tides, and water flow patterns also significantly influence the transportation of genetic material between regions. Research in Thua Thien Hue, Vietnam also showed multiple genetic groups within *O. marmorata*, indicating a complex metapopulation structure. This structure is influenced by ecological factors and geographical barriers that limit genetic dispersal [48]. This finding is consistent with a broader-scale study showing that *O. marmorata* has a wide distribution spanning the Indian Ocean and Southeast Asia, with evidence of gene flow resulting from long-distance migration [1; 2]. Studies have also shown that various factors, including water salinity and environmental stress, play a role in determining the growth rate and survival of *O. marmorata*. These impacts can ultimately affect its reproductive success and level of genetic diversity [4; 49].

Human activities such as aquaculture practices, ship ballast water discharge, and alien species' introduction can also further affect the dynamics of genetic movement and, in some cases, cause unwanted hybridization. Some environmental barriers, such as temperature changes, salinity levels, and habitat fragmentation, can also inhibit gene flow and lead to genetic isolation between populations. These factors shape aquatic species' genetic structure, ultimately affecting their adaptability, resilience, and long-term survival. Research conducted by Zhao [26] also showed a loss of genetic diversity of *O. marmorata* in China caused by the founder effect. The founder effect can arise in artificial propagation when only a few parent individuals are used or when no wild individuals are introduced into the population.

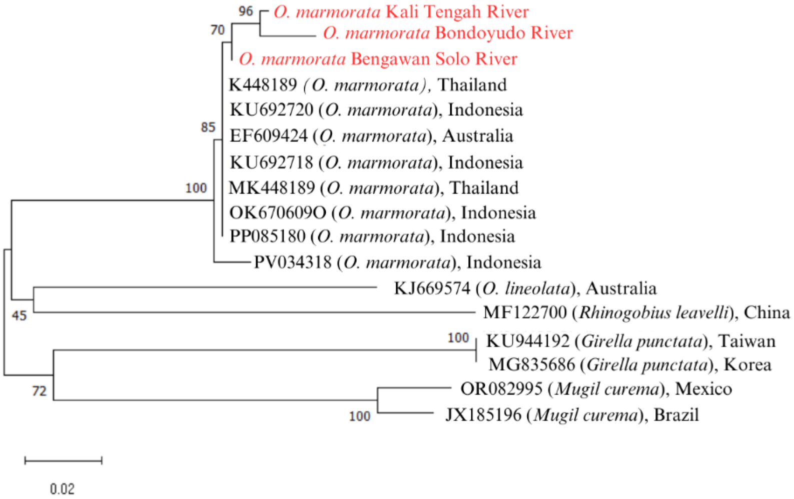

Figure 3. Phylogenetic tree of *O. marmorata* from three waters in the East Java region

Distribution of *O. marmorata*

O. marmorata is widely distributed in Southeast Asia [50; 51]. Its range extends from southern China to Indonesia, including Thailand, Cambodia, Laos, Vietnam, Malaysia, and the Philippines [10] (Figure 4). *O. marmorata* is usually found in freshwater and brackish water habitats, such as rivers, lakes, and estuaries [52; 53]. It prefers dense vegetation and soft substrates, where it can burrow and hide [54; 55]. Environmental factors, including salinity levels and habitat availability, play a role in determining its distribution pattern [56]. The distribution of *O. marmorata* tends to be influenced by its tolerance to temperature, with a preference for warm tropical and subtropical waters [57].

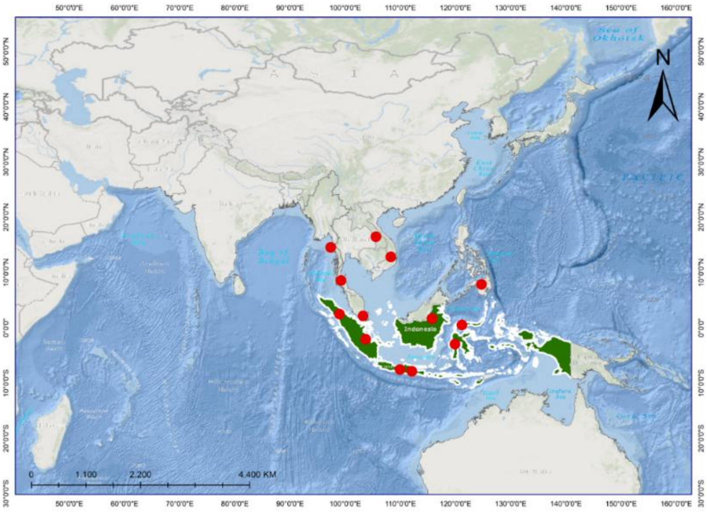

Figure 4. Distribution of *O. marmorata*

IV. CONCLUSION

The amplification results show that *O. marmorata* from the Bengawan Solo River, Kali Tengah River, and Bondoyudo River have a high genetic similarity with species documented in GenBank. The low genetic distance indicates that the populations in the three rivers are closely related and come from a common ancestor. Phylogenetic analysis showed that the species is closely related to *O. lineolate* and Rhinogobius levels and suggests gene flow between populations due to migration and human activities. The genetic variation found could play a role in the conservation and breeding of *O. marmorata* to support the sustainability of fisheries resources. Thus, this study provides important insights for managing and conserving *O. marmorata* in East Java waters.

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TABLE 2.
GENETIC DISTANCE OF *O. MARMORATA* IN THREE EAST JAVA RIVERS USING COI GENES WITH DATA IN GENBANK

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Bengawan Solo **River**	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Kali Tengah **River**	0.01	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Bondoyudo **River**	0.02	0.02	*	*	*	*	*	*	*	*	*	*	*	*	*
K448189 (*O. marmorata*), Thailand	0.00	0.01	0.02	*	*	*	*	*	*	*	*	*	*	*	*
KU692720 (*O. marmorata*), Indonesia	0.00	0.01	0.02	0.00	*	*	*	*	*	*	*	*	*	*	*
EF609424 (*O. marmorata*), Australia	0.00	0.01	0.02	0.00	0.00	*	*	*	*	*	*	*	*	*	*
KU692718 (*O. marmorata*), Indonesia	0.00	0.01	0.02	0.00	0.00	*	*	*	*	*	*	*	*	*	*
KJ669574 (*O. lineolata*), Australia	0.16	0.17	0.18	0.15	0.15	0.15	*	*	*	*	*	*	*	*	*
KU944192 (*Girella punctata*), Taiwan	0.18	0.20	0.21	0.18	0.18	0.18	0.22	*	*	*	*	*	*	*	*
OR082995 (*Mugil curema*), Mexico	0.18	0.19	0.20	0.17	0.17	0.17	0.21	0.22	*	*	*	*	*	*	*
MF122700 (*Rhinogobius leavelli*), China	0.18	0.19	0.21	0.18	0.18	0.18	0.21	0.25	0.23	*	*	*	*	*	*
MK448189 (*O. marmorata*), Thailand	0.00	0.01	0.02	0.00	0.00	0.00	0.15	0.18	0.17	0.18	*	*	*	*	*
OK6706090 (*O. marmorata*), Indonesia	0.00	0.01	0.02	0.00	0.00	0.00	0.15	0.18	0.17	0.18	0.00	*	*	*	*
PP085180 (*O. marmorata*), Indonesia	0.00	0.01	0.02	0.00	0.00	0.00	0.15	0.18	0.17	0.18	0.00	0.00	*	*	*
PV034318 (*O. marmorata*), Indonesia	0.01	0.02	0.04	0.01	0.01	0.01	0.15	0.19	0.19	0.19	0.01	0.01	0.01	*	*
MG835686 (*Girella punctata*), Korea	0.18	0.20	0.21	0.18	0.18	0.18	0.22	0.00	0.22	0.25	0.18	0.18	0.18	0.19	*
JX185196 (*Mugil curema*), Brazil	0.17	0.18	0.19	0.17	0.17	0.17	0.21	0.21	0.03	0.25	0.17	0.17	0.17	0.18	0.21

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