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Distribution Map Revision of Ricefish (Adrianichthyidae: *Oryzias* spp.) based on Morphological Identification in Sundaland Indonesia

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Abstract: Members of the Adrianichthyidae family share typical distributions, as does the javanicus group, which inhabits western parts of Southeast Asia. Javanicus group members, specifically *Oryzias javanicus* and *Oryzias hubbsi*, is the only Adrianichthyidae species that can be spotted in Sundaland Indonesia. Morphological identification and the species distribution mapping of *Oryzias* specimens from Sundaland in this study were interpreted using ecoregional and dendogram tree approach. Specimens were collected from 11 areas representing five ecoregions in Sundaland Indonesia. Specimens were collected from islands of Java, Sumatra, Belitung and Kalimantan. Six individuals from each population of *Oryzias* were sampled for morphological identification. Specimens collected from estuaries were morphologically identified as *O. javanicus* and specimens from freshwater identified as *O. hubbsi*. Distribution of *O. hubbsi* from Salatiga is the first record from Central East Java (CEJ) ecoregion, which was previously only distributed in Southern Sumatra-Western Java (SWJ) ecoregion. Morphology of *O. hubbsi* from Sundaland Indonesia has mean of Standard Length (SL) 1.56 cm, Total Length (TL) 1.94 cm, Anal rays (A) 16, Pectoral rays (P) 8, and Dorsal rays (D) 6. Meanwhile, morphology of *O. javanicus* from Sundaland has mean of SL 2.07 cm, TL 2.56 cm, A 22, P 11, and D 6.

Keywords: Endemic; Isolation; Meristic; Morphometric; Speciation

Introduction

Rice fish (members of Adrianichthyidae family) have been phylogenetically divided into four major group which are javanicus, celebensis, latipes and setnai. These groups have typical distribution patterns. Members of the javanicus and celebensis groups can be spotted throughout the Indonesian Archipelago. The javanicus group inhabits Sundaland region, which includes at least three major islands of Borneo, Sumatra and Java, while the celebensis group inhabits Wallacea region, especially Sulawesi (Sudasinghe et al., 2022). Distributional records of the Adrianichthyidae family in Sundaland Indonesia are finite to only two species, *Oryzias javanicus* and *Oryzias hubbsi*. Nowadays, *O. javanicus* reported has an extensive distribution from Thailand to Sundaland Indonesia, in contrast to *O. hubbsi* which is reported to be endemic in western of Java

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Island (Parenti, 2008). Research on Adrianichthyidae family in Indonesia has been mostly focused on the celebensis group in Sulawesi, which includes 2 genera and more than 17 species. New species discovered in last two years from the family in Sulawesi are *Oryzias kalimpaaensis* (Gani et al., 2022) and *Oryzias loxolepis* (Kobayashi et al., 2023). Finite studies of the family in Sundaland Indonesia lead to numerous evolutionary possibilities that have yet to be discovered.

Oryzias habitats are generally in freshwater, so its distribution depends on geographical boundaries. *O. hubbsi* distribution have been described in Jakarta and at elevations of 700 to 1000 metres above sea level in freshwater of Bandung (Roberts, 1998). While the *O. javanicus* distribution generally occurs in estuaries, there are no records that indicate *O. javanicus* is capable to migrate across the sea, so its distribution may still be affected by geographical boundaries (Lee et al., 2020). Modern geographical physical conditions can affect fish reproduction, including elevation, humidity and topography. Differences in geographic elevation can affect several parameters such as rainfall, temperature, primary productivity, food availability, and anthrophogenic levels of human activities (He et al., 2022). Furthermore, in this case also salinity parameters.

Geographical boundaries approach in analysing distribution of fish species may use ecoregion classification. Sundaland has been divided into at least 13 ecoregions, and 10 of them belong to an administrative zone under territory of Republic Indonesia, including three major islands of Java, Sumatra and Kalimantan (Chua et al., 2019). Ecoregion classification based on three things, which are based on biodiversity patterns, ecological relationships and historical evolution including geographical history of establishment of related regions (Abell et al., 2008). Regional species pool was modulated by abiotic factors such as spatial-temporal geographic isolation, but these factors are not the only probability. Biotic factors may also influence regional species pool by the capability of certain species to be dominant in ecological niche within an ecosystem (Gomes et al., 2023).

Oryzias morphological identification in Sundaland Indonesia and its geographical distribution mapping to test evolutionary possibilities in this study was conducted under assumption of potential need to update the description of their distribution map, especially for *O. hubbsi* as an endemic species in Java Island. Morphological differences between *O. javanicus* and *O. hubbsi*, which have high similarity, can be narrowed down to several meristic calculations (number of Anal (A), Pectoral (P) and Dorsal (D) fin rays) and morphometric measurements (Standard Length (SL) and Total Length (TL)) (Parenti, 2008; Roberts, 1998). Results of distribution mapping of the genus *Oryzias* in this study, can be used as basic specific conservation considerations both in-situ and ex-situ.

Method

Sampling was conducted randomly at 12 different locations in the islands of Java, Sumatra, Belitung and Kalimantan from May 2020 to March 2023. Sampling sites on Java Island include Lamongan, Malang, Salatiga (administratively belong to Semarang Regency, but local people usually mentioned there is Salatiga, in this article it will mentioned as Salatiga), Bogor and Serang, while on Sumatra Island include Central Lampung, Indragiri Hilir and Padang, in addition to Belitung Island. Sampling sites on Kalimantan Island included two cities, Pontianak and Balikpapan. Identification was continued on all populations considered to represent the distribution of the Oryzias genus from six different ecoregions in the Sundaland Indonesia referring to Chua et al. (2019), namely Central and East Java (CEJ), Southern Sumatra-Western Java (SWJ), Indian Ocean Slope of Sumatra and Java (ISJ), Southern Central Sumatra (SCS), Eastern Borneo (EBR), and Kapuas (KPS): CEJ is represented by Salatiga and Lamongan; SWJ is represented by Bogor, Serang, Central Lampung and Belitung Island; ISJ is represented by Malang and Padang; SCS is represented by Indragiri Hilir; EBR is represented by Balikpapan; and KPS is represented by Pontianak (Figure 1). Morphometric measurements used a caliper and meristic calculations used Celestron Handheld Pro Digital Microscope. Euthanasia was carried out according to ethics by giving a gradual temperature reduction to the specimens until the fish operculum no longer moving, according to (Wiadnya et al., 2023).

Specimens were preserved using 70% or 96% technical alcohol and freezing at around -20° C according to field conditions. Preservation attempts to not much damage the tissue and save to use in sampling field. Euthanized specimens observed in several characteristics as *O. hubbsi* and *O. javanicus* identification keys. Collected data were then processed using PAST 4.03 application to construct dendrogram trees and QGIS v.3.26.1 application to make the map. The identification data of the genus *Oryzias* were interpreted using geographical and evolutionary approaches based on literature studies such as in (Sudasinghe et al., 2022).

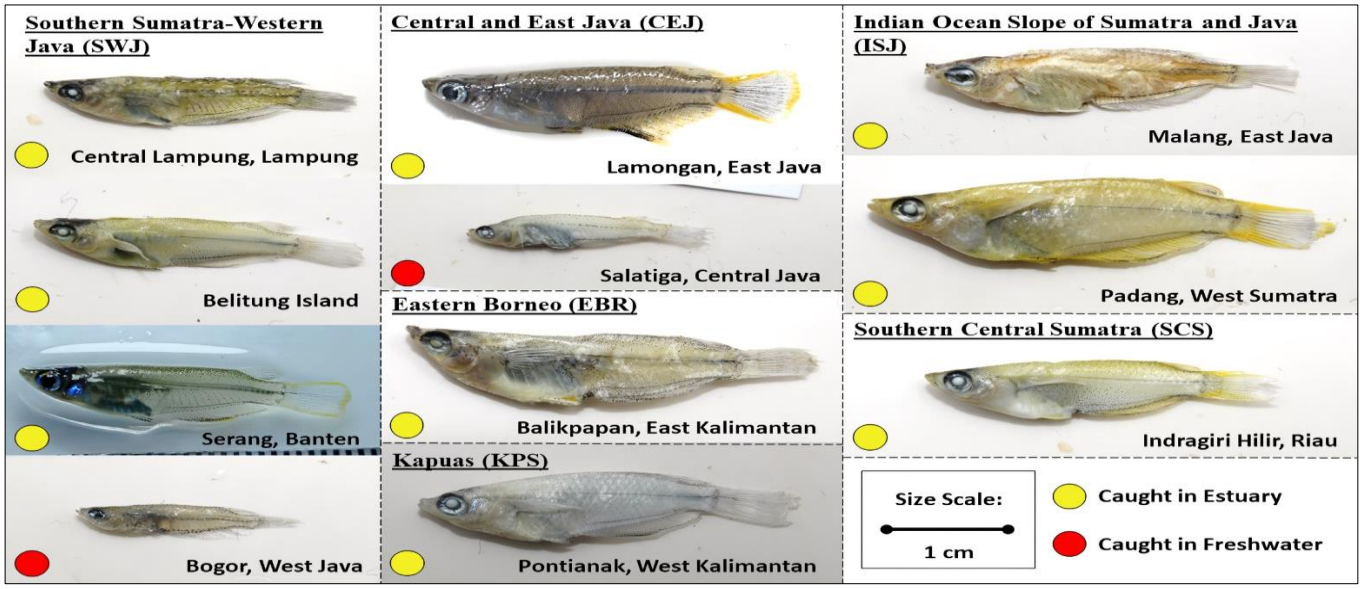

Figure 1. *Oryzias* specimens documentation from 11 population on six ecoregion of Sundaland Indonesia. Two population collected from freshwater that far from estuary, and nine population collected from estuary habitats.

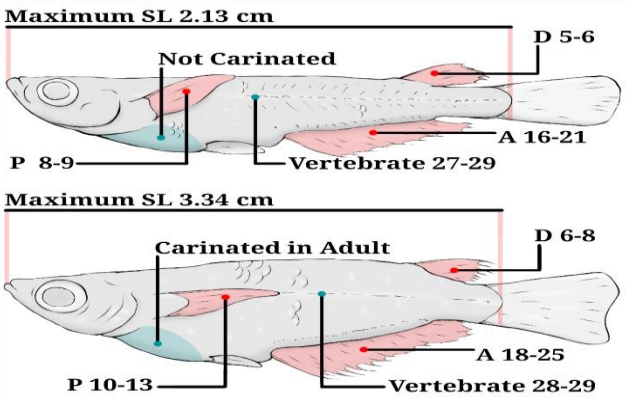

Figure 2. Identification keys to distinguishing *O. hubbsi* (above) and *O. javanicus* (below). Red color is assessed characters in this study and cyan color stands for unassessed characters demand to specific reasons.

Unweighted Pair Group Method with Arithmatic Mean (UPGMA) classical analysis is capable of grouping morphological characteristics into a dendrogram, although it will be slightly different from phylogenetics in its interpretation methods. Morphological characteristics measurement will be very influential in clustering, especially on species distinguishing characters (Rahman, Azmir & Hussin, 2022). High similarity in *O. javanicus* and *O. hubbsi* as mentioned in the introduction, can be narrowed down to several distingishing characters, namely body length both SL and TL, fin rays number of A, P and D (Figure 2). Identification references used were based on Roberts (1998) and Parenti (2008). Two of the distinguishing characters between the two species that not measured was the tendency of abdominal shape during adulthood and vertebrate number. The abdomen of *O. javanicus* has a tendency to develop into carinate shape demand to specimens ontogeny, while *O. hubbsi* in contrast does not (Parenti, 2008). These variables were not assessed because the specimens used were taken randomly without necessarily entering adulthood for make sure the abdominal shape tendency (also correlated to specimens body depth), and to assess vertebrate number potentially damaging the morphologic preserved specimens. Six specimens were used in each population, resulting in a total of 66 specimens analysed from 11 populations to mapping *Oryzias* distribution by its identification keys.

Result and Discussion

Oryzias specimens were separated by collection locality and grouped according to the literature. The population collected from freshwater is suspected to be *O. hubbsi* and the population from estuary is suspected to be *O. javanicus*. Morphological identification by comparing each species' description in Roberts (1998) and Parenti (2008) with the previously mentioned variables, found no abnormal characteristics that lead to unrecognized species in one taxonomical name (Table 1). The Bogor and Salatiga populations were identified as *O. hubbsi*, while the remaining populations which is Central Lampung, Belitung Island, Serang, Lamongan, Padang, Balikpapan, Pontianak, Malang, and Indragiri Hilir were identified as *O. javanicus*. The average measurement results of *O. hubbsi* with 12 samples in total were SL 1.6 cm; TL 1.9 cm; A 16; P 8; and D 6. The average measurement results of *O. javanicus* identified specimens from 54 specimens were SL 2.1 cm; TL 2.6 cm;

A 22; P 11; and D 6. The results of morphological identification confirm that grouping based on salinity is effective so far to separate *O. javanicus* and *O. hubbsi* in Sundaland Indonesia.

Dendrograms resulted the *O. javanicus* and *O. hubbsi* group separately (Figure 3). *O. javanicus* populations from Balikpapan formed a small group with Lamongan and Serang populations, Malang populations formed a small group with Belitung populations and the other population namely Lampung, Padang, Pontianak and Indragiri Hilir formed another separate group. The group formation does not develop any special pattern by geographic spatial such as ecoregion. The ecoregion similarity of *O. javanicus* Belitung, Serang and Lampung populations, does not produce special dendrogram group results, although the Belitung population grouped with the Malang population, as well as Serang grouped with Balikpapan and Lamongan. The Belitung-Malang and Serang-Balikpapan-Lamongan population groups are separated by ecoregion boundaries and even by far apart island boundaries within those groups. The Malang and Belitung population group tends to have more A numbers than the other populations with a range of not less than 21 and up to 24. Variation in number of A for Balikpapan population is wider with a minimum value of 19 and a maximum value of 24, as well as being the widest range for the number of A in this study, so it is reasonable not to be grouped with Malang and Belitung even though the number of A reaches 24. On the other hand, the Balikpapan, Lamongan and Serang population groups have a tendency to have fewer number of P compared to other populations with no number of P greater than 11. Morphological variations in SL, TL and the number of D do not show separating populations into independent group based on the dendrogram results.

Table 1. Morphological data of *Oryzias* Sundaland Indonesia were measured on five variables: standard length (SL), total length (TL), fin rays number of anal (A), pectoral (P) and dorsal (D).

Specimen Locality	n	SL (cm)	TL (cm)	A	P	D
Description of *O. hubbsi* [a]	-	≤ 2.13	-	17-21	8-9	5-6
Description of *O. hubbsi* [b]	-	≤ 2.13	-	16-19	8-9	5-6
Bogor, West Java	6	1.40-1.75	1.80-2.05	16-18	8	5-6
Salatiga, Central Java	6	1.36-1.80	1.63-2.16	16-17	8-9	5-6
Description of *O. javanicus* [a]	-	≤ 3	-	20-25	11	6-7
Description of *O. javanicus* [b]	-	≤ 3.36	-	18-25	10-13	6-8
Balikpapan, East Kalimantan	6	1.75-2.30	2.4-2.95	19-24	10-11	6
Lamongan, East Java	6	1.95-2.20	2.65-2.75	20-23	10-11	6-7
Central Lampung, Lampung	6	1.35-2.05	1.65-2.55	20-23	10-12	5-7
Belitung Island	6	1.63-2.00	2.05-2.46	21-24	10-12	5-7
Pontianak, West Kalimantant	6	1.49-2.40	1.85-3.1	21-23	12	6-7
Malang, East Java	6	2.00-2.73	2.44-3.34	21-24	10-12	5-7
Padang, West Sumatra	6	2.04-2.90	2.46-3.63	20-23	11-13	5-6
Indragiri Hilir, Riau	6	1.69-2.40	2.13-2.99	20-23	11-12	6
Serang, Banten	6	1.90-2.36	2.34-2.94	21-22	9-10	6-7

Note: '[a]' based on Roberts (1998), '[b]' based on Parenti (2008)

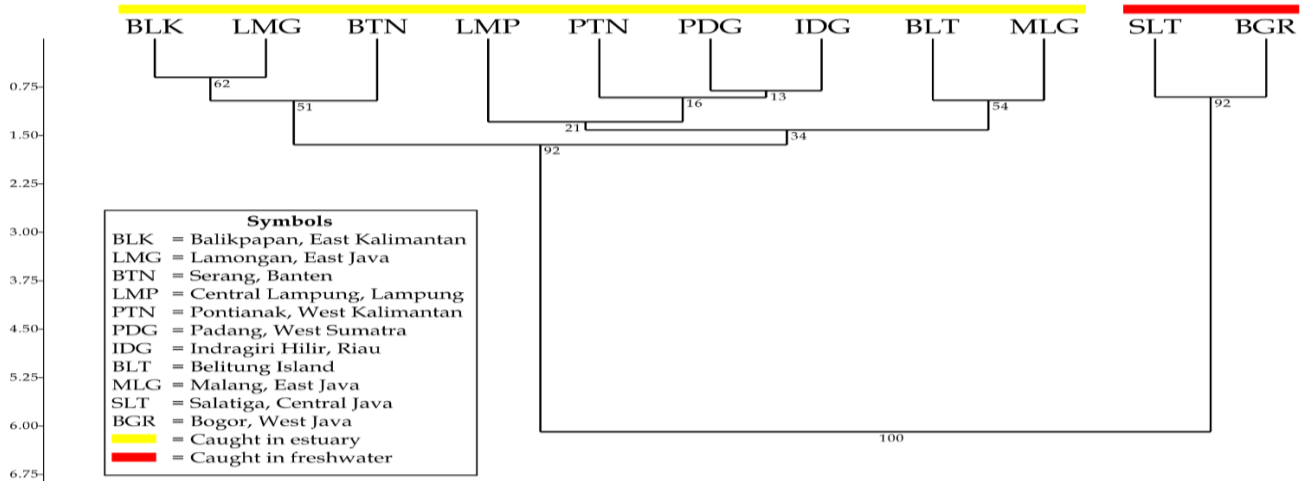

Figure 3. UPGMA dendogram tree from five distingishing characters of *O. hubbsi* and *O. javanicus*. Specimens that caught in estuary habitats grouping into same major group, also specimens caught in freshwater was grouping to another group indicating those habitats correlated to species ditinguishing characters.

Morphological variation among the 11 populations is still within the range of the identification keys from five distinguishing characters to *O. javanicus* and *O. hubbsi* with no abnormalities, but the *O. hubbsi* distribution that is limited to the SWJ ecoregion in western part of Java Island does not exactly correspond with the findings of the Salatiga population. Distribution map of Adrianichthyidae, especially *Oryzias* general using ecoregion approach illustrates the habitat separation of *O. javanicus* which tends to inhabit estuaries that close to the shores, while *O. hubbsi* inhabits freshwater sites that far from the shores (Figure 4). The Java Island has both Adrianichthyidae species of *Oryzias*, in contrast to the other two major islands namely Sumatra and Kalimantan that only have a single species, *O. javanicus*. *O. hubbsi* population from Salatiga is belong to the CEJ ecoregion, which is closer to the ISJ ecoregion than the SWJ ecoregion. *O. hubbsi* distributions around and between these two populations (Salatiga and Bogor) are very possible to be found, including in the ISJ ecoregion.

The distribution of *Oryzias* that inhabiting the estuary habitat was identified as *O. javanicus* in this study, but speciation potentially still wide open. The dendrogram tree results of nine *O. javanicus* populations were divided into three groups with each group's trend as described in the previous summary. Meristic differences such as the number of fin rays also occur in Singapore population of *O. javanicus* which has fewer A with 21-22, while Java population has wider range with 21-25 (Roberts, 1998). The number of A is almost similar to the results of *O. javanicus* Banten which has A of 21-22, but geographical reasons could not yet map specific trends as mentioned.

Further meristic and morphometric analyses can be conducted in future study with homogenous specimen quality by adding various morphological parameters. In this study, specimens from different regions were preserved with non-uniform methods due to field conditions, resulting damages for several morphological parameters in several populations, especially morphometrics, which may not be ideal to be compare to each other between populations. Preservation using ethanol on fish specimens can significantly alter fish morphology, especially eye size and body size including body depth in less than a year (Sotola et al., 2019). Several species in the javanicus group were identified as *O. javanicus* due to high morphological similarity between members of the group, namely *O. hubbsi* in 1981 and *Oryzias huagiangensis* in 1996. Misidentification within the javanicus group also occurred in *Oryzias carnaticus* and *Oryzias dancena* which were previously identified as *Oryzias melastigma* and even identified as *Horaichthys setnai* (revised as *Oryzias setnai*), and *Oryzias uwai* which was previously identified as *Oryzias minutillus* (Parenti, 2008). All members of the javanicus group in the phylogenetic analysis from Sudasinghe et al. (2022), which includes 7 species (*O. javanicus*, *O. hubbsi*, *O. huagiangensis*, *O. carnaticus*, *O. dancena*, *O. uwai*, and *O. minutillus*) have misidentification histories, opening up the potential for other misidentifications within this group. Furthermore, taxonomical problems in Adrianichthyidae related to the occurrence of two genera in the celebensis group, which is *Oryzias* and *Adrianichthys*, open up opportunities for taxonomical revisions to the celebensis group or even to all groups formed in the phylogenetic of Adrianichthyidae.

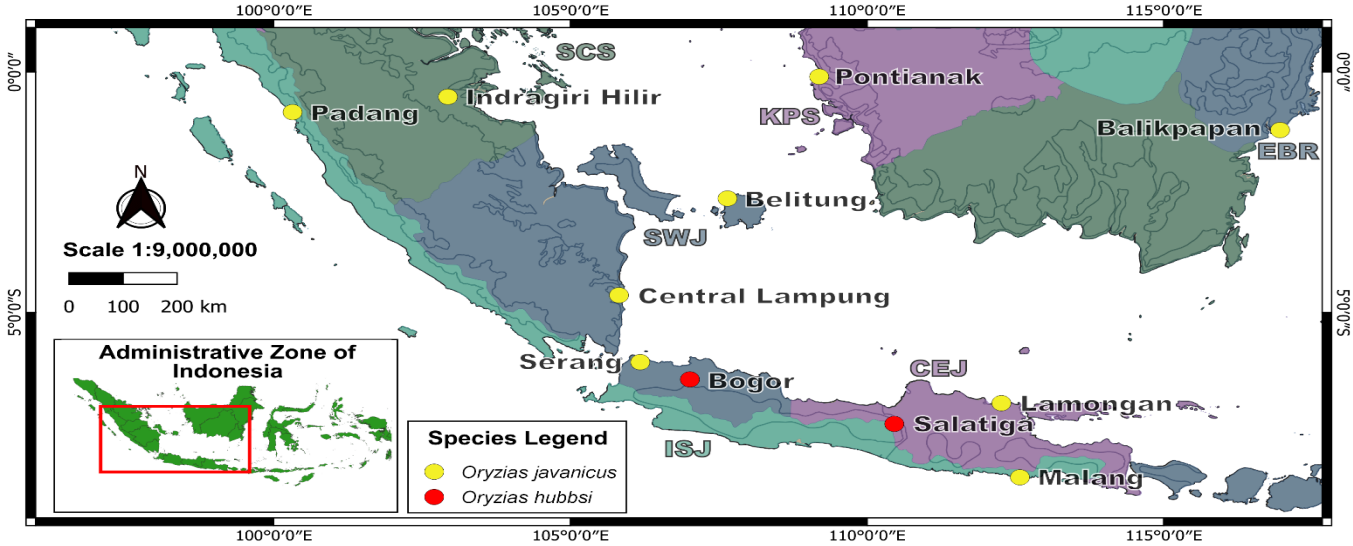

Figure 4. Distribution map of Adrianichtyidae, especially *Oryzias* genera members which is *O.* javanicus and O. *hubbsi* in Sundaland Indonesia. Different color that surrounding one region indicating different ecoregion, may used same color as SWJ and EBR, or KPS and CEJ ecoregions, but those colors does not represent similarity or something else in both regions. Spots colors also representing habitat, which red spots stands for freshwater and yellow spots stands for brackish or estuaries.

Abnormal distribution of *O. javanicus* in Indonesia occurs in the Wallacea region, which is recorded in Sulawesi and Lasser Sunda, Lombok (Parenti, 2008). Some possible cross-zoogeographical distributions of *O. javanicus* could be due to misidentification, introduction cases and migration potential. Introductions of Sundaland species to Wallacea are highly probable, as in the case of *Aplocheilus* sp., *Barbonymus gonionotus*, *Channa striata*, *Anabas testudineus*, *Clarias* sp., and *Osteochilus vittatus* (Bandjolu et al., 2021). Another possibility is the migration potential that still needs to be assessed, Lombok Island and Sulawesi Island is separated by an impenetrable trough between them to Sundaland except for fish that possible to migrating through the sea. Members of the family Adriaichtyidae commonly inhabit freshwater, but *O. javanicus* is one of the few members with euryhaline capabilities (Takehana et al., 2020). There are a few other species of Adrianichyidae that can be found in brackish or estuarine waters besides *O. javanicus*, namely *O. dancena*, *O. carnaticus*, *O. curvinotus*, *O. setnai* and *O. latipes* (Parenti, 2008). The migratory ability study of the Adrianichthyidae family from the six species that can be found in saline environments has not been established, but evolutionary and speciation records may strengthen the hypothesis of this issue. The javanicus group has shared a common ancestor, and was distributed in western Indo-Burman and eastern Indo-Burman, but *O. javanicus* only occurs in eastern Indo-Burman. Speciation due to geographic isolation between western Indo-Burman and eastern Indo-Burman is particularly significant for organisms that cannot migrate by sea and has raised much discussion among researchers such as in several genera as *Channa* (Conte-Grand et al., 2017) and *Notopterus* (Lavoué et al., 2020). Therefore, the most likely distribution of *O. javanicus* in Lombok and Sulawesi is due to the misidentification of *O. javanicus*-like species from different phylogenetic groups, or by the introduction of *O. javanicus* to those places.

Adrianichtyidae, especially *Oryzias*, is one of several Actynopterygii fish model organisms that have been commonly used in various studies, along with several genera as *Takifugu* and *Danio* (Kirchmaier et al., 2015; Zaucker et al., 2014). At least 35 species of *Oryzias* are characterised by distinctive adaptations in morphology, physiology and ecology. *Oryzias* has several superiorities as model organisms with short generation time, transparent embryos, small size, and etc (Takehana et al., 2020). Research using *Oryzias* as model organisms is used in ecotoxicology, comparative anatomy, endocrinology, embryology and transgenic research (Amal et al., 2019; Cui et al., 2020; Kamarudin et al., 2019; Kowalska et al., 2020; Serdiati et al., 2020; Zhang et al., 2022). Although indicated to be unable to migrate through the sea, *O. javanicus* has a superiority to be used as model organism for marine environments with the ability to tolerate salinity (Takehana et al., 2020). Meanwhile, a study using *O. hubbsi* could produce hybrid offspring from crossing this species with a distant phylogenetic group within Adrianichtyidae, *O. latipes*, which is the most general species to be used as model organism with complete genomic data (Sakai et al., 2007). With those few examples of *Oryzias* high potential values, and finite domestication of *Oryzias* that inhabits Sundaland Indonesia, this distribution map may can be based for further research, domestication and conservation efforts in future.

Community education, including at certain academic levels, requires comprehensive evaluation (Manalu & Suhartini, 2023), one of which is the distribution evaluation and mapping of specific taxa as in this study. Anthrophogenic threats, sustainability and conservation issues have complexities in overcoming them (Irwanto, Rubini & Pursitasari, 2022), including community comprehension of the biodiversity that surrounds them. Conservation efforts can be carried out in-situ and ex-situ, in other words, indigenous or endemic species can be protected in their natural habitat or outside their natural habitat (Prihantini, 2023). In-situ and ex-situ conservation efforts on Oryzias javanicus in Banten have been carried out by studying the morphology of specimens, eggs, and habitat (Herjayanto et al., 2022). In-situ and ex-situ conservation efforts are hopefully conducted on other Oryzias populations, including the 10 localities in this study.

Conclusion

The distribution of *Oryzias* in Sundaland Indonesia is still divided into two species, freshwater habitat inhabited by *O. hubbsi* and brackish or estuary habitat inhabited by *O. javanicus*. The ecoregional approach to the distribution of *O. hubbsi* indicates a potentially wider distribution, the distribution of *O. hubbsi* is confirmed to only inhabit one ecoregion in the Java Island, namely SWJ, but there are two ecoregions in Java Island, namely SWJ and CEJ. The dendrogram tree approach produces three small groups in *O. javanicus* with specific morphological character trends, namely the number of A and P. Although ecoregional interpretation cannot make specific trends from the results of the *O. javanicus* dendrogram tree, but speciation potential is still wide open in this species.

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Author Contributions
Conceptualization, AFP, APWM and ARF; methodology, AFP and KNAK; software, AFP, IS and RRLS; validation, AFP, APWM and ARF; format analysis, AFP, IS and RRLS; investigation, AFP, KNAK and MH; data curation, AFP, KNAK and MH; writing—original draft preparation, AFP; writing—review and editing, AFP; visualization, AFP and IS; supervision, APWM and ARF; project administration, AFP; funding acquisition, AFP, KNAK, RRLS and MH. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest
The authors declare no conflict of interest.

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