

Molecular Analysis Using Cytochrome Oxidase Subunit I (COI) to Confirming New Recorded *Oryzias hubbsi* in Central East Java Ecoregion

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Abstract: *Oryzias hubbsi*'s distribution is rarely discussed, as it is believed to only occupy the western part of Java, while its conservation status is Near Threatened (NT) as of 15 October 2018 on the IUCN red list. Specimens of *O. hubbsi* were recorded from Bandung and Jakarta in the description, other reports for *O. hubbsi* are commonly from Southern Sumatra-Western Java (SWJ) ecoregion in Java. However, an unexpected discovery of the genus *Oryzias* in Semarang freshwater opens up opportunities for the hidden spread of *O. hubbsi* to Central Java, and especially to other ecoregions which is Central and East Java (CEJ). Morphological approaches with distinctive *O. hubbsi* characters, complemented by molecular approaches with *O. hubbsi* samples from the Bogor population, confirmed similarities of species between both populations. Genetic distance using p-distances between both populations was below 2%, both populations made obvious clusters with a gap of 0.5%, and differed by five nucleotide base sites only. This new distribution record of *O. hubbsi* can be a foundation for conservation and domestication planning efforts.

Keywords: Algorithm; Endemicity; Monophyletic; Mutation; Polymorphism

Introduction

Sundaland has more than 1000 freshwater fish species spread across at least 13 ecoregions, some of these species are endemic. As a global priority region, Sundaland has high endemicity, followed by high environmental degradation (Polgar & Jaafar, 2018). Sundaland has almost twice the density of species in Brazil and Congo, with endemic freshwater fishes in Sundaland estimated to be approximately 450 species (Dahrudin et al., 2021). Meanwhile, anthropogenic

pressure, especially in Java Island, has completely eliminated all preserved natural areas, this condition has been evaluated since 1993 (Verma et al., 2020). One of the endemic species on Java Island is *Oryzias hubbsi*, the distribution of this species has only been recorded in the freshwater of Jakarta and Bandung (Parenti, 2008), unpublished findings for *O. hubbsi* occurred around the western part of Java Island in Southern Sumatra-Western Java (SWJ) ecoregion. Java Island is divided into three ecoregions, namely Central and East Java (CEJ), Indian Ocean Slope of Sumatra and Java (ISJ) and

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SWJ (Chua et al., 2019). The Adrianichthyidae family that can be spotted on Java Island besides *O. hubbsi* is *Oryzias javanicus*, an euryhaline species that commonly inhabits brackish water. Both species belong to the javanicus group based on Adrianichthyidae family phylogenetics (Sudasinghe et al., 2022).

The javanicus group has high morphological similarity, leading to high potential for misidentification. All javanicus group members from phylogenetic result have recorded cases of morphological misidentification, namely *Oryzias dancena*, *Oryzias huaqiangensis*, *Oryzias uwai*, *Oryzias carnaticus*, *Oryzias minutillus*, *O. javanicus*, and *O. hubbsi* (Parenti, 2008). Furthermore, Adrianichthyidae family, especially the genus *Oryzias*, has too widely diverse members, which requires complex taxonomic revision including molecular analyses to avoid misidentification. An example of taxonomic conundrum of Adrianichthyidae based on molecular analysis occurs in the celebensis group. The celebensis group has two genera, *Adrianichthys* and *Oryzias*, while other groups are grouped in the genus *Oryzias*, including *Oryzias setnai* which differs significantly from other members both morphologically and molecularly (Sudasinghe et al., 2022). Molecular approaches using mitochondrial DNA (mtDNA) are common for various purposes, both molecular identification studies and taxonomical conundrums using the Cytochrome B (CytB) region (Duong et al., 2020), and using Cytochrome Oxidase Subunit I (COI) (Kurniawan et al., 2022; Lamadi et al., 2023). Molecular confirmation generally uses Basic Local Alignment Search Tool (BLAST) results on the blastn feature for identification using sequences (Wiadnya et al., 2023), but lack of research data on *Oryzias* in Sundaland, especially *O. hubbsi*, COI sequences of *O. hubbsi* were not found in the National Center for Biotechnology Information (NCBI) and in the Barcode of Life Data System (BOLD System), so new COI sequences of *O. hubbsi* from the common distribution is required, which is from the SWJ ecoregion.

Freshwater fish species are the most affected vertebrates by anthropogenic pressures, but conservation efforts have been extremely lacking, in contrast to other vertebrates (He et al., 2022). General factors that threaten freshwater fish populations are environmental degradation, domestic and industrial pollution, climate change, overexploitation, and introduced species (Costa et al., 2021). Introduced species in the Sundaland region come from domesticated consumption and ornamental fish commodities (Andriyono & Fitriani, 2021). Confirmation of *O. hubbsi*'s distribution in the CEJ ecoregion could expand its conservation and domestication efforts in the future, considering an endemic species that only

inhabits one ecoregion has high potential of extinction (Chua et al., 2019). The expansion of distribution records may provide a basic reference for broodstock selection, both in aquaculture and restocking efforts to avoid low genetic diversity due to environmental pressures on a population.

Method

Specimens were collected using a hand net at population gathering points, *O. hubbsi* Semarang populations inhabit small streams and irrigated rice fields with muddy stone substrates, the main population is at coordinates -7.2854714, 110.4045872. *O. hubbsi* populations can be found in irrigation streams and under *Bacapo* sp. vegetation, intermixed with *Poecilia reticulata*. According to its size and colour dominance seen from the surface, people generally identify *O. hubbsi* as similar to *P. reticulata*, which is known locally as cetul fish. The habitat of *O. hubbsi* has only been reported from freshwater, similar to the finding in Semarang (Figure 1). Local people often describe this area as Salatiga, but administratively it belongs to Semarang District.

Captured specimens were identified based on the description of *O. hubbsi* in Roberts (1998) and Parenti (2008) by taking six representative individuals. Specimens were conditioned with gradual temperature reduction until the specimens were completely euthanized (Lamadi et al., 2023). Specimens which died in the field were preserved using 70% ethanol to avoid decay. Whole individuals were chopped and extracted according to the Wizard® Genomic DNA Purification Kit protocol, then the isolates were amplified using primers that common to amplified fish specimens in general to COI region, which is fish forward 1 5' TCAACCAACCACAAAGACATTGGCAC 3' and fish Reverse 1 5' TAGACTTCTGGGTGGCCAAAGAATCA 3' (Ward et al., 2005). Amplification temperature was 94°C for denaturation (15 seconds), annealing at 55°C (1 minute) and elongation at 72°C (5 minutes), with 35 cycles. The amplicons were tested qualitatively by electrophoresis method, amplicons with good band quality were sent to Apical Scientific Sdn Bhd, Malaysia for sequencing.

Sequences data were processed using several applications such as Chromas 2.6.6, UGENE 46.0, Mesquite 3.70, DnaSP 6, Network 10.2 and MEGA 11 (Lamadi et al., 2023; Wiadnya et al., 2023). Data sets were constructed with ingroup and outgroup additions from BOLD Systems, while genetic confirmation of *O. hubbsi* species was taken from the team's private sequence using one of the *O. hubbsi* Bogor population sequence.

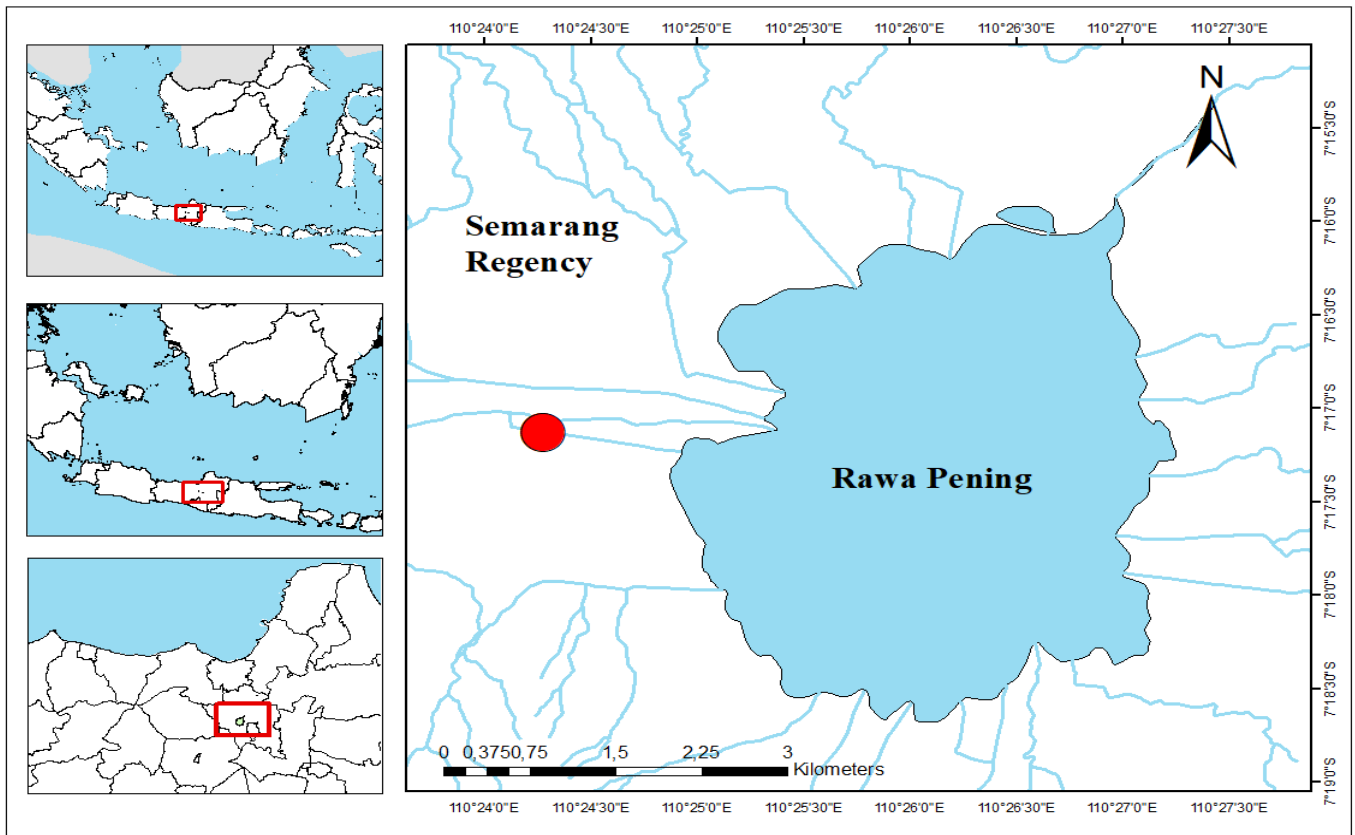


Figure 1. Sampling map of *O. hubbsi* in Semarang Regency, near Lake Rawa Pening. Sampling point spotted with red dot

Phylogenetics were processed using the Maximum Likelihood algorithm by finding the best model in MEGA 11 features. Data sets were processed using the Hasegawa-Kishino-Yano + Gama Distributed (HKY+G) model with 1000 replications. Haplotype networks were analysed using median joining in Network 10.2.

Result and Discussion

Morphological examination of *O. hubbsi* Semarang in terms of Standard Length (SL), number of Anal (A), Pectoral (P), and Dorsal (D) fin rays, similar based on the description of *O. hubbsi*. The description of *O. hubbsi* has a maximum SL of 21.3 mm, A 16-21, P 8-9, and D 5-6 (Parenti, 2008; Roberts, 1998), whereas the Semarang population has SL 13.60-18.00 mm, A 16-17, P 8-9, and D 5-6. The first molecular confirmation using p-distance, the interpopulation distance between Semarang and Bogor populations is 0.50%, while the interpopulation distance of Semarang population identified as *O. hubbsi* with Banten population identified as *O. javanicus* is 11.10%, and with *Oryzias latipes* population as outgroup is 38.00%. Furthermore, the highest intrapopulation distance was in the *O. latipes* population at 2.90%, followed by the *O. javanicus* Banten population at 1.80% (Table 1).

Table 1. Genetic Distance Using P-Distance, Bold Value Stands for Intrapopulation Distances

	(1)	(2)	(3)	(4)
(1) Semarang	0.00%			
(2) Bogor	0.50%	n/c		
(3) Banten	11.10%	11.50%	1.80%	
(4) Outgroup	38.00%	38.40%	32.90%	2.90%

Estimation result of phylogenetic tree using Maximum Likelihood algorithm showed that Semarang population formed monophyletic relation with *O. hubbsi* Bogor population, separated from its closest species, *O. javanicus*, and outgroup, *O. latipes*. Haplotype network result indicates the haplotype of Semarang population and Bogor population are only separated by three nucleotide base sites, whereas with *O. javanicus* Banten.

Separated by more than 60 sites, and with *O. latipes* separated by more than 180 sites (Figure 2). Second molecular confirmation through phylogenetics and haplotype networks, in parallel with p-distance confirmation showing that the Semarang population belongs to the same species as *O. hubbsi* Bogor with high bootstraps of 99, means that the shape of the monophyletic tree of *O. hubbsi* Semarang and Bogor populations is less likely to be estimated differently with an identical data set.

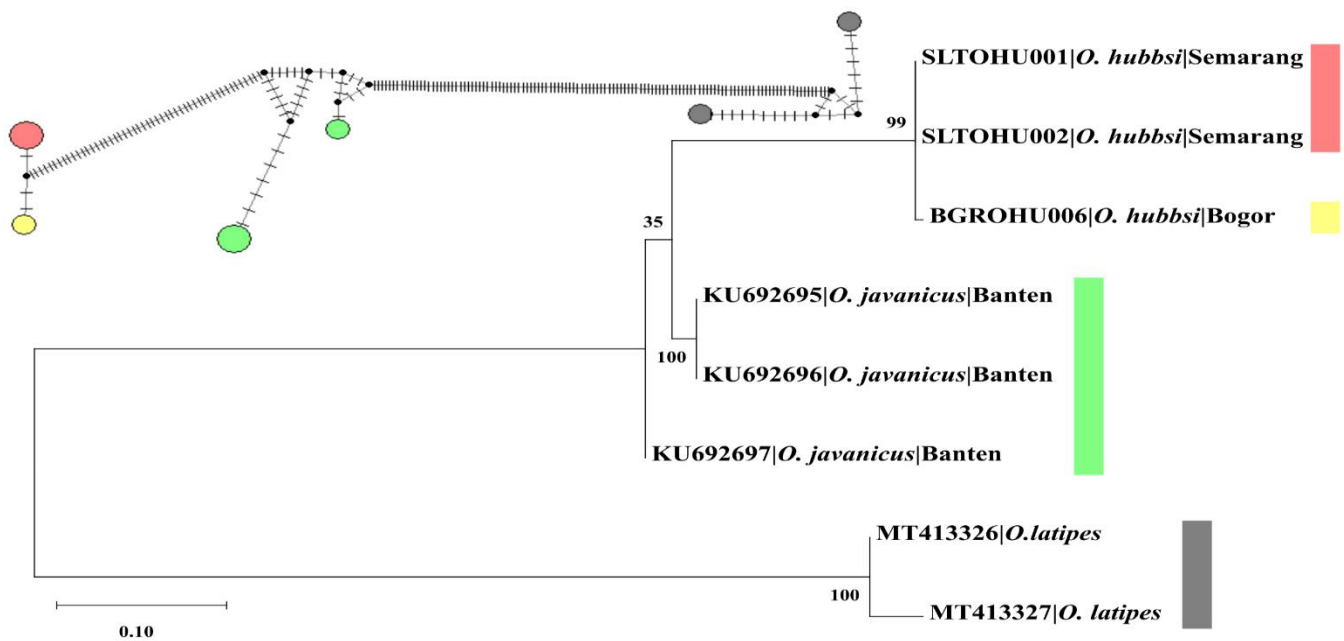


Figure 2. Phylogenetic and haplotype network of *O. hubbsi* Semarang with several comparison data, different colors for each population stands specifically similar for phylogenetic and haplotype network

Generally, p-distance values for Teleost fishes are considered to be a potentially distinct species if they are over 2.00% (Bañón et al., 2022), therefore the distance between Semarang and *O. hubbsi* Bogor populations can be considered to be the same species, which is 0.50%. However, the p-distance value is not a fixed estimation, as the mutation rate of each species may be highly diverse. Molecular confirmation in this study is dominated by using MEGA 11 application, MEGA 11 application can be used to estimate evolutionary distance and divergence, predict ancestor sequences, diagnose mutations in disease examination, phylogenetic reconstruction and more (Tamura et al., 2021). Several algorithms used in MEGA include UPGMA Maximum Parsimony, Bayesian Inference, Neighbor Joining, and Maximum Likelihood. Maximum likelihood has circular order algorithm, so it is commonly used for the accuracy of estimation. Bootstraps above 70 indicate that the branches in phylogenetics are robust and reliable, below 70 are questionable (Hall, 2013).

The International Union for Conservation of Nature's (IUCN) Red List of Threatened Species categorised *O. hubbsi* as a Near Threatened (NT) species with decreasing population trend. The distribution of *O. hubbsi* has only occurred in SWJ, but IUCN reported a distribution range map up to the Indian Ocean Slope of Sumatra and Java (ISJ) ecoregion through Tasikmalaya, but this distribution was not found in scientific articles and still doubtful due to its high potential for misidentification in the javanicus group. *O. hubbsi*'s Bogor population was confirmed by IUCN in 2017, and

that record became the Extent of Occurrence (EOO) of *O. hubbsi*. Wider distribution of *O. hubbsi* to other ecoregions and islands will greatly enhance their conservation, including their dispersal to ISJ and or to parts of Sumatra Island that also contain parts of the SWJ and ISJ ecoregions, and or to another hidden spots in Java Island as in East Java. *Oryzias* distribution in the freshwater of Sundaland Indonesia is suspected as *O. hubbsi* distribution nowadays, which also underlies the initial suspicion of its distribution in Semarang. Suspicious distribution was also found in Padang freshwater recently, but this scientifically unrecorded finding requires further investigation, but may be accurate due to the ecoregion similarity between Padang and *O. hubbsi*'s initial distribution record which is in the SWJ ecoregion. Similar cases of freshwater fish species occurring in the western part of Java and also spread to Sumatra that have been recorded, is as in the distributional case of *Nemacheilus fasciatus* (Hubert et al., 2019). Occurrence confirmation of *O. hubbsi* in CEJ ecoregion makes *O. hubbsi* no longer an endemic species within a single ecoregion, considering that ecoregion-specific endemic species are more threatened by extinction (Chua et al., 2019). Collecting broodstock from other populations, especially other ecoregions, may avoid inbreeding possibilities and increase polymorphism, both for domestication and conservation efforts such as restocking in wild. Polymorphism in a population can reduce extinction potential from population genetics standpoint (Phadphong et al., 2019), furthermore, genetic diversity studies should be conducted in the Semarang population, considering that

both COI sequences are identical to each other and belong to the same haplotype.

O. hubbsi is threatened by introduced fish based on the IUCN Red List of Threatened Species report regarding its disappearance in the Botanical Gardens, Jakarta. Some of the introduced fish families are Cichlidae (tilapia and flowerhorn cichlid), Loricariidae and Gobiidae. Common introduction species in Indonesia of Cichlidae, especially tilapia, belongs to the genus *Oreochromis* which is often known as nila and mujair in Indonesia, while from Loricariidae is suckermouth pleco or sapu-sapu fish from the genus *Hypostomus* and Gobiidae from genus *Rhinogobius* (Aqmal-Naser & Ahmad, 2018). The habitat of *O. hubbsi* in Semarang, connected to Lake Rawa Pening, and indigenous fish species that can be spotted in the habitat of *O. hubbsi* and its surroundings include *Aplocheilichthys armatus*, *Dermogenys pusilla*, *Anabas testudineus*, *Rasbora* sp., *Barbodes binotatus*, *Lepidocephalichthys hasselti*, *Trichopsis vittata*, *Trichopterus trichopterus*, *Betta picta*, *Trichopodus pectoralis*, *Channa striata*, *Monopterus albus* and *Gobiopterus chuno*. Introduced fish species that can be spotted in this habitat include *Oxyeleotris marmorata*, *Channa micropeltes*, *Oreochromis* spp., *P. reticulata*, *Rhinogobius* sp., *Hypostomus plecostomus*, *Ancistrus temminckii*, and *Clarias gariepinus*. Common consequences of introduced species in a habitat include competition, introduced species can become disease agents or parasites, modify natural habitat conditions, predation, and can interbreed with nearby taxa and produce new introduced hybrids (Pastorino et al., 2020). Furthermore, the finding of *O. hubbsi* mixed with *P. reticulata* populations indicates similar ecological niche between both species, so interspecies competition is quite likely to depress *O. hubbsi* populations in Semarang, requiring conservation efforts in the near future.

Oryzias is well known as a model organism besides *Danio* and *Takifugu* genera (Kirchmaier et al., 2015; Zaucker et al., 2014). The most common species for model organisms in the genus is *O. latipes*, whereas *O. hubbsi* has been recorded to be artificially cross-bred with *O. latipes* (Sakai et al., 2007). The high genetic distance between both species that has been discussed previously, consistent with the phylogenetic grouping of Adrianichthyidae species, where *O. hubbsi* belongs to the javanicus group and *O. latipes* separated belongs to the latipes group (Sudasinghe et al., 2022). As a model organism, conservation of *Oryzias* can be conducted ex-situ, where conservation is conducted outside of its natural habitat (Prihantini, 2023), including with domestication. Domestication efforts of fish species are generally categorised into three stages: place adaptation, reproductive adaptation and commercial feed adaptation (Shiguemoto et al., 2021). The genus *Oryzias*

is commonly used as a model organism because of its relatively small size, transparent embryos, short generation time and many others (Takehana et al., 2020). Reproduction behaviour of species in the genus *Oryzias* has been widely studied, such as on *Oryzias melastigma* (Chen et al., 2016; Lee et al., 2020), *Oryzias wawaroe* (Firmansyah et al., 2021), *O. javanicus* (Salleh et al., 2017), and *O. latipes* (Li et al., 2018). Reproduction behaviour research on other *Oryzias* species may provide the basics knowledge for sustainable domestication and conservation approaches for *O. hubbsi*.

Conclusion

New *O. hubbsi* distribution record in Semarang, which belongs to the CEJ ecoregion, have been confirmed morphologically and molecularly, and can be stated to be similar in species to the Bogor *O. hubbsi* population. Molecular analysis base on COI sequences, Semarang and *O. hubbsi* Bogor only have 0.5% p-distance, as well as their monophyletic relationship in the phylogenetics result. These evidences can be new EOO to predicting other *O. hubbsi* hidden distribution. High threat of introduced species within Semarang population's habitat requires further studies and conservation efforts in the near future.

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Author Contributions

Conceptualization, AFP, APWM and ARF; methodology, AFP and KNAK; software, AFP and RRLS; validation, AFP, DGRW, APWM and ARF; format analysis, AFP 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 DGRW; supervision, APWM, DGRW and ARF; project administration, AFP; funding acquisition, AFP, KNAK, DGRW, 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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