

SHORT COMMUNICATION

Molecular confirmation of the occurrence of *Anguilla interioris* (Actinopterygii: Anguilliformes) in North Maluku of Indonesia and mitochondrial DNA haplotype diversity among existing specimens

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ABSTRACT. Nineteen species of *Anguilla* Schrank, 1798 are globally distributed in the world, however knowledge on the biogeography, species diversity and ecology of the 13 species of tropical anguillids in the Indo-Pacific region is highly limited. This study examined the diversity of tropical anguillids found in North Maluku of East Indonesia, which is known to have unique and highly heterogeneous habitats, complex oceanography, high biodiversity, and representativeness of Asian and Australian fauna. By means of molecular identification, two tropical anguillid eels, *A. marmorata* Quoy & Gaimard, 1824 and *A. interioris* Whitely, 1938, were confirmed to be distributed in North Maluku. This study also examined the mitochondrial DNA haplotype diversity of *A. interioris*, as it could contribute to our understanding of the biogeography and life history of this eel species. Our molecular analyses showed the presence of the same haplotypes along the different locations in the Indo-Pacific region. Although more samples and DNA markers are required to provide more support, the results suggest that the larvae of *A. interioris* from potentially different spawning sites in the Indian and Pacific oceans could be mixed together due to the complexity of oceanic currents, and when these migrating larvae reach maturity, they would likely spawn with the local eels.

KEY WORDS. Biogeography, freshwater eels, habitat, larval transportation, migration, tropical region.

The anguillid eels of the genus *Anguilla* are widely distributed throughout the world. These eels have a catadromous life history, migrating between offshore spawning areas and continental growth habitats. Currently, nineteen species of the anguillid eels have been found, thirteen of which occur in tropical Indo-Pacific region. Of the thirteen species that occurred in tropical waters, seven species are distributed in western Pacific around Indonesia, i.e., *A. celebesensis* Kaup, 1856, *A. interioris*, *A. bengalensis bengalensis* Gray, 1831, *A. marmorata*, *A. borneensis* Popta, 1924, *A. bicolor bicolor* McClelland, 1844 and *A. bicolor pacifica* Schmidt, 1928 (Ege 1939, Watanabe et al. 2004, Arai 2016) and thus Indonesia is recognised as the highest diversity area for the anguillid eels throughout the world.

The anguillid eels are economically important fish species, and widely consumed in European and Asian countries as a delicacy food. Anguillid eels, especially temperate species, are extremely vulnerable for exploitation and overfishing since the

last decades (Arai 2014a, 2022). Currently, temperate anguillid species, *A. anguilla* Linnaeus, 1758 is listed as Critically Endangered species, whereas *A. rostrata* Lesueur, 1821, *A. japonica* Temminck & Schlegel, 1846 and *A. dieffenbachii* Gray, 1842 are listed as Endangered species, while *A. australis* Richardson, 1841 is listed as Near Threatened species in the IUCN Red List (IUCN 2022). However, at least two tropical anguillids, *A. celebesensis* and *A. interioris*, which are distributed in Indonesia, have not yet been properly assessed and thus listed with Data Deficient status (IUCN 2022). Therefore, fundamental research on the biogeography, population structure, life history and migration in those species are urgently needed for the assessment, conservation and management purposes. Several information on the life histories such as recruitment, early life history, maturation and migration is available for *A. celebesensis* (Arai et al. 2001, 2003, Aoyama et al. 2003, Arai 2014b, Watanabe et al. 2016), however such information is not available for *A. interioris* and

there is also very little information available on every aspects of its biology and ecology.

Anguilla interioris is one of the most enigmatic species in *Anguilla* because information regarding its biology and ecology including distribution, biogeography and life history is completely lacking at present. Ege (1939) first described *A. interioris* based on seven specimens collected in New Guinea. However, morphological characteristics of *A. interioris* completely overlap with two other tropical anguillid eels, *A. celebesensis* and *A. megastoma* that are distributed in the same areas (Watanabe et al. 2004). Therefore, morphological characteristics alone cannot identify these species. Based on mitochondrial DNA analysis, however, Aoyama et al. (2000) differentiated *A. interioris* from *A. celebesensis* collected from North Sulawesi Island of Indonesia and New Guinea. The study also found that *A. interioris* is widely distributed in New Guinea while *A. celebesensis* has not been detected in this location but occurs in North Sulawesi (Aoyama et al. 2000). North Maluku is located between Sulawesi Island and New Guinea and, hence, this led to the question whether each of these two species could also occur in North Maluku. It had always been thought that *A. interioris* was present in New Guinea only (Ege 1939, Aoyama et al. 2000). Surprisingly, biodiversity studies of anguillid eels by means of molecular markers found that *A. interioris* is widely distributed in Sumatra, Java, Lombok and central Sulawesi islands of Indonesia and southern Mindanao of Philippines (Sugeha et al. 2008, Fahmi et al. 2012, Shirotori 2016, Wibowo et al. 2021). Understanding species diversity and biogeography is a prerequisite for understanding the eel life history in details.

The main objective of the present study is to examine species composition and diversity of anguillid eels in North Maluku of East Indonesia. This region is known for its exceedingly high biodiversity and habitat diversity, representativeness of Asian and Australian fauna, and important role in connectivity between Papua and Sulawesi in Indonesia (Huffard et al. 2012). All eel specimens were identified using molecular markers. Consequently, we also examined the haplotype diversity of *A. interioris* and discussed its potential larval dispersion and migration mechanisms within its distribution range of Indo-Pacific region.

A total of 15 immature adult anguillid eels (yellow stage) were collected in North Maluku: six specimens in Morotai Island (2°12'43"N; 128°14'45"E) from 11 September to 23 December 2020 and nine specimens in Kayoa Island (0°1'38"N; 127°25'24"E) from 26 February to 26 March 2021 through hooks and lines (Fig. 1, Table 1). After measurement of total length (TL) in each specimen, pectoral fin was clipped for DNA extraction.

Genomic DNA was extracted by first lysing the tissue sample with TNES buffer (10 mM Tris-base pH 8, 125 mM NaCl, 10 mM EDTA, 0.5% SDS). After centrifuging the lysate, the supernatant was collected and mixed with phenol. The mixture was centrifuged and the supernatant was collected. Absolute ethanol was then added to precipitate the DNA samples which were carefully removed and transferred into a new tube. Residual ethanol was evaporated in a vacuum. Polymerase chain reaction

(PCR) was carried out to amplify the mitochondrial D-loop and cytochrome c oxidase subunit 1 (CO1) regions using the primer pair, L15774 (5'ACA TGA ATT GGA GGA ATA CCA GT3') and H16498aj (5'CCT GAA ATA GGA ACC AAA TG3') (Tanaka et al. 2014), and the primer pair, FishF1 (5'TCA ACC AAC CAC AAA GAC ATT GGC AC3') & FishR1 (5'TAG ACT TCT GGG TGG CCA AAG AAT CA3') (Ward et al. 2005), respectively. The PCR conditions were 94 °C for two minutes, 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 68 °C for 60 s, and finally 72 °C for five minutes. The PCR amplicons were sent to a service provider for sequencing using the same primers.

In the present study, D-loop sequences from 14 specimens were first analyzed, and thereafter CO1 sequences from four of the 14 specimens and from one additional specimen were analyzed. Limitation on available extract limited sequencing of COI from all specimens (Table 1). All DNA sequences were deposited to the GenBank with accession numbers OM738333-OM738337 (CO1) and OM818378-OM818391 (D-loop), and the sequences were compared for percentage similarity with the sequences in the GenBank by using BLAST search. In addition to these fifteen sequences, 6 D-loop sequences and 30 CO1 sequences of *A. interioris* specimens from other sites deposited in the GenBank or in the Barcode of Life Data System (BOLD) were also included in the analysis. For the D-loop, the *A. interioris* specimens were from Bengkulu in southern Sumatra of Indonesia (4 specimens; MT327750-MT327753), Papua New Guinea mainland (one specimen; AP007241) and Bougainville of Papua New Guinea (one specimen; MG977381) (Fig. 1). For the CO1, the *A. interioris* specimens were from Ambon (1 specimen; BIF5560) and Bengkulu in southern Sumatra (23 specimens; MT416654-416657, MN961249-MN961267) of Indonesia, Negros Oriental (2 specimens; MT647236, MT647239) and Sibutad (3 specimens; MT647232-MT647234) in Philippines, and Papua New Guinea mainland (one specimen; AP007241) (Fig. 1). For outgroups, the GenBank sequences of *A. bengalensis bengalensis* (AP007246) and *A. marmorata* (AP007242) were used.

MEGA X (Kumar et al. 2018) was used to edit and align the DNA sequences via ClustalW, and to carry out phylogenetic analysis using the maximum likelihood (ML) algorithm. The ML tree was constructed using the nearest neighbor interchange method and the best fitting model as determined by MEGA X. The tree was bootstrapped with 1000 replicates. DnaSP 6 (Rozas et al. 2017) was used for haplotype analysis, and Network 10 (www.fluxus-engineering.com) was used for constructing haplotype network via the median joining method.

For the 14 specimens for which D-loop was amplified and sequenced, BLAST search showed that seven specimens presented sequences similar to those identified as *A. marmorata* and another seven specimens as *A. interioris*, with high identity matches of 97 to 100% (Table 1). Out of these 14 specimens, four specimens were further submitted to BLAST using CO1, for which similarly showed high identity matches of 99 to 100% and confirmed the initial similarity using D-loop. One

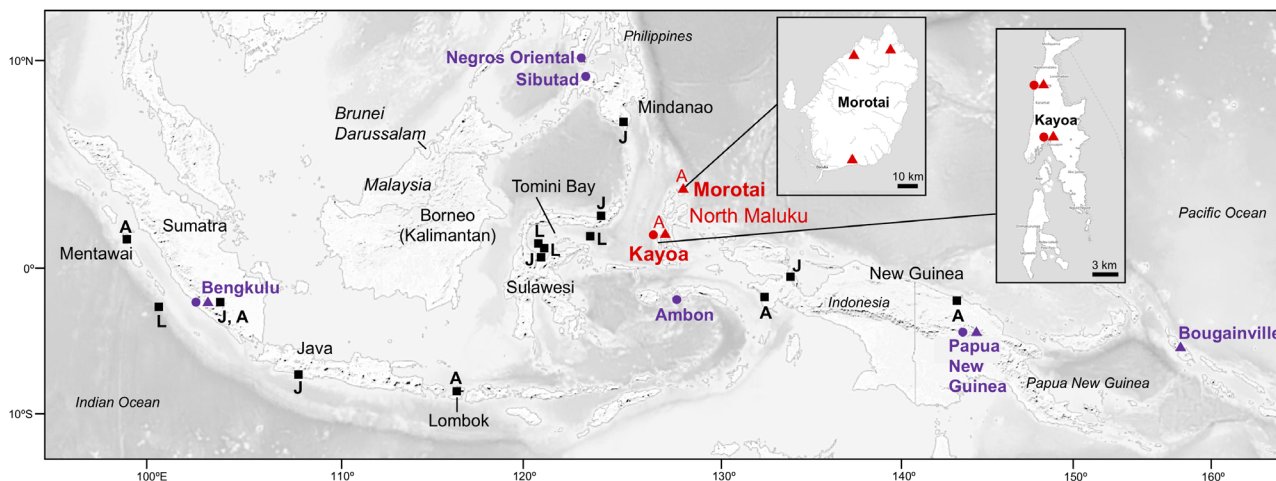


Figure 1. Sampling locations of *Anguilla interioris* in North Maluku of East Indonesia. Sampling locations are shown in red (circle: COI, triangle: D-loop). Locations of Bougainville of Papua New Guinea, Papua New Guinea mainland, Negros Oriental and Sibutad of Philippines, and Ambon and Bengkulu of Indonesia, which were used in molecular phylogenetic and haplotype network analyses, are shown in purple (circle: COI, triangle: D-loop). Specimen records are shown in square with growth stages; L [larval (leptocephalus); Kuroki et al. 2006, Wouthuyzen et al. 2009, Aoyama et al. 2018], J [juvenile (glass eel); Sugeha et al. 2008, Fahmi et al. 2012, Wibowo et al. 2021] and A (adult; Watanabe et al. 2004, Fahmi et al. 2012, Wibowo et al. 2021, this study). Base maps were downloaded from <http://viewer.nationalmap.gov/viewer> (USGS 2022) and from the OpenStreetMap at <https://www.openstreetmap.org>.

Table 1. Specimens used in molecular identification.

| Specimen | Sampling location | Total length (cm) | Identification | |
|----------|-------------------|-------------------|----------------------|----------------------|
| | | | D-loop | COI |
| M1 | Morotai Island | 56 | <i>A. marmorata</i> | nd |
| M2 | Morotai Island | 52 | <i>A. interioris</i> | nd |
| M3 | Morotai Island | 40 | <i>A. interioris</i> | nd |
| M4 | Morotai Island | 27 | <i>A. marmorata</i> | nd |
| M5 | Morotai Island | 40 | <i>A. marmorata</i> | nd |
| M6 | Morotai Island | 48 | <i>A. interioris</i> | nd |
| K1 | Kayoa Island | 78 | <i>A. marmorata</i> | <i>A. marmorata</i> |
| K2 | Kayoa Island | 89 | <i>A. marmorata</i> | nd |
| K3 | Kayoa Island | 65 | <i>A. interioris</i> | <i>A. interioris</i> |
| K4 | Kayoa Island | 66 | nd | <i>A. marmorata</i> |
| K5 | Kayoa Island | 57 | <i>A. interioris</i> | nd |
| K6 | Kayoa Island | 59 | <i>A. marmorata</i> | <i>A. marmorata</i> |
| K7 | Kayoa Island | 74 | <i>A. interioris</i> | nd |
| K8 | Kayoa Island | 80 | <i>A. interioris</i> | <i>A. interioris</i> |
| K9 | Kayoa Island | 75 | <i>A. marmorata</i> | nd |

nd: not determined.

additional specimen also depicted close similarity using COI to *A. marmorata* with a high identity match of 99% (Table 1). Therefore, by molecular analysis confirms the occurrence of *A. interioris* in North Maluku of East Indonesia. For *A. marmorata*, several studies have reported the occurrence of this eel species in East Indonesia region including North Maluku (Sugeha et al. 2008, Fahmi et al. 2012). However, the occurrence of *A. interioris* in North Maluku was never previously reported and thus, the present study is the first to find this species in this region.

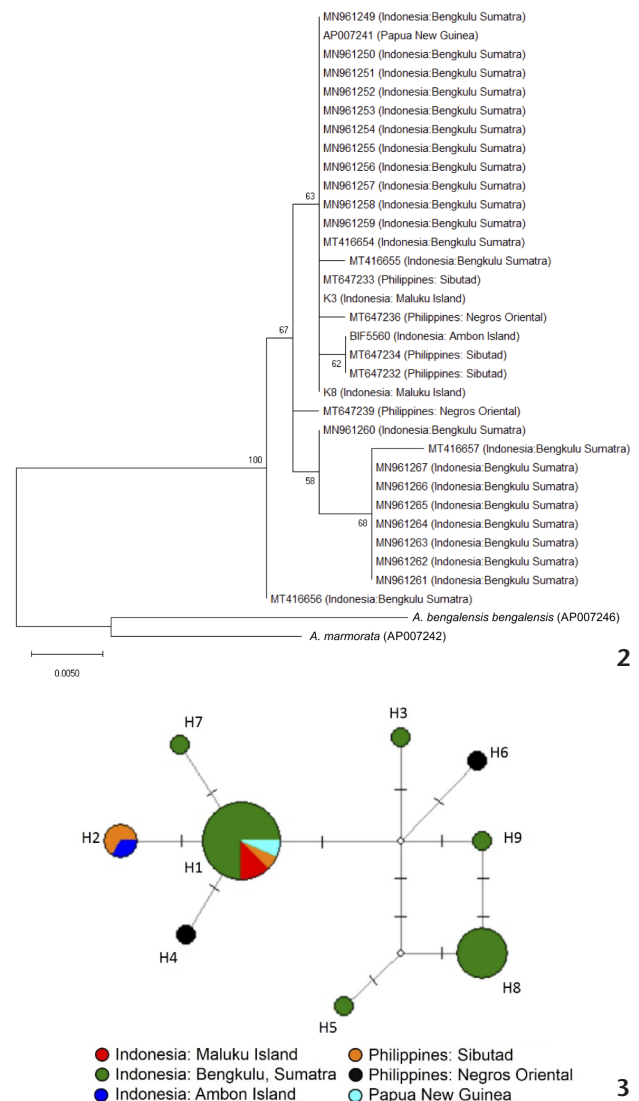
Among 19 anguillid eel species, *A. interioris* is the most enigmatic species because its biology and ecology are mostly unknown. Several sporadic studies on eel biogeography and biodiversity found that adult *A. interioris* was mainly distributed in New Guinea which is located further east of North Maluku (Ege 1939, Watanabe et al. 2004) (Fig. 1), whereas juvenile (glass eel) *A. interioris* was found to recruit to the vicinity of North Maluku, as well as to North and Central Sulawesi of Indonesia (Sugeha et al. 2008, Fahmi et al. 2012) and southern Mindanao of Philippines (Shirotori et al. 2016), although no adult *A. interioris* was ever recorded in those sites. Tropical anguillid eels are found to spawn throughout the year (Arai et al. 2001, 2016, Arai and Abdul-Kadir 2017). Annual recruitment of glass eels for three tropical anguillids, *A. celebesensis*, *A. marmorata* and *A. bicolor pacifica* were observed for three years in North Sulawesi of Indonesia which is located to the west of North Maluku (Fig. 1), however no *A. interioris* was recruited to the estuary (Arai et al. 1999, 2001, 2020, Sugeha et al. 2001). These results suggest that the distribution of adult *A. interioris* would be highly restricted and the recruitment of juvenile *A. interioris* would be seasonal in East Indonesia.

Juvenile and adult *A. interioris* were also found in other Indonesian areas: Java, Sumatra and Lombok in West Indonesia of the Indian Ocean region (Sugeha et al. 2008, Fahmi et al. 2012, Wibowo et al. 2021), which are geographically distant from North Maluku and New Guinea of the Pacific Ocean region (Fig. 1). However, no *A. interioris* has been reported in Borneo (Kalimantan) by previous studies (Sugeha et al. 2008, Fahmi

et al. 2012, Wong et al. 2017, Zan et al. 2020, 2021). Borneo is located between East and West Indonesia (Fig. 1), suggesting that the distribution of *A. interioris* would be disconnected between East and West Indonesia, although *A. marmorata* and *A. bicolor* are widely distributed throughout Indonesia including Borneo (Sugeha et al. 2008, Fahmi et al. 2012, Wong et al. 2017, Zan et al. 2020, 2021). Interestingly, both juvenile and adult *A. interioris* were found in Sumatra with a larger number of specimens (35 adults and 15 glass eels) (Wibowo et al. 2021) compared to the number of *A. interioris* specimens found in New Guinea and North Maluku (seven adults in New Guinea (Ege 1939), eight adults in New Guinea (Watanabe et al. 2004) and seven adults in the present study). These results suggest that *A. interioris* might have at least two populations and/or spawning areas in Indian and Pacific oceans.

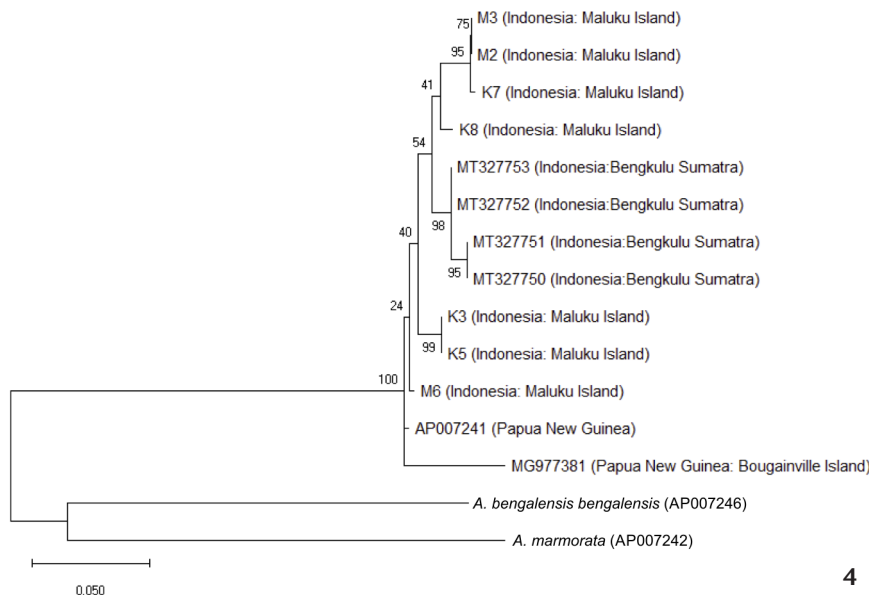
There is very little information on the spawning area(s) of *A. interioris*, similar to other tropical anguillids. A small larva (leptocephalus) with 12.4 mm in TL and 19 days in age was discovered off Sumatra in Indian Ocean (Kuroki et al. 2006) (Fig. 1). This suggests that one spawning area might be located off Sumatra because the larva was quite small and young so it would be just after hatching. Glass eel recruitment into Sumatra and Java coasts supports the possible spawning area off Sumatra. In East Indonesia and New Guinea in Pacific Ocean, however such small larva of *A. interioris* has never been collected. Large larvae (33.1 to 49.5 mm in TL) of several tropical eels including three *A. interioris* larvae were collected from within and beyond Tomini Bay (Wouthyzen et al. (2009) (Fig. 1). Tomini Bay in Sulawesi (Fig. 1) is assumed to be a spawning area of *A. interioris* based on the collection of a large larva (43.5 mm in TL) and two small larvae (9.6 and 28.2 mm) of anguillid eels (Aoyama et al. 2018). However, this assumption is highly speculative because the spawning area was hypothesized based on the unknown *Anguilla* species. Further oceanic research is necessary to estimate the possible spawning area through collections of small larvae, eggs and spawning adults in the area.

The molecular analyses based on CO1 sequences (Figs 2, 3) showed the presence of the same haplotypes in different locations. For example, haplotype 1 (H1) was found both in the Indian Ocean (Sumatra) and Pacific Ocean (Maluku, Papua New Guinea and Philippines). This suggests that the Indian Ocean samples were not genetically distinct from the Pacific Ocean samples, meaning one homogenous population. Similar analyses using D-loop sequences (Figs 4, 5) produced an ambiguous haplotype network, perhaps due to the low number of samples being analyzed. The results seems to be inconsistent with the hypothesis of two possible populations based on the distribution and recruitment patterns of the adults and juveniles of *A. interioris* as mentioned above. North Maluku is located along the flow of Pacific and Indian Ocean water mass current (Sahetapy 2018). The Indonesian Throughflow (ITF) always flows from the Pacific Ocean to the Indian Ocean (Hao et al. 2021). Anguillid eel larvae are passively drifted and transported through prevail-

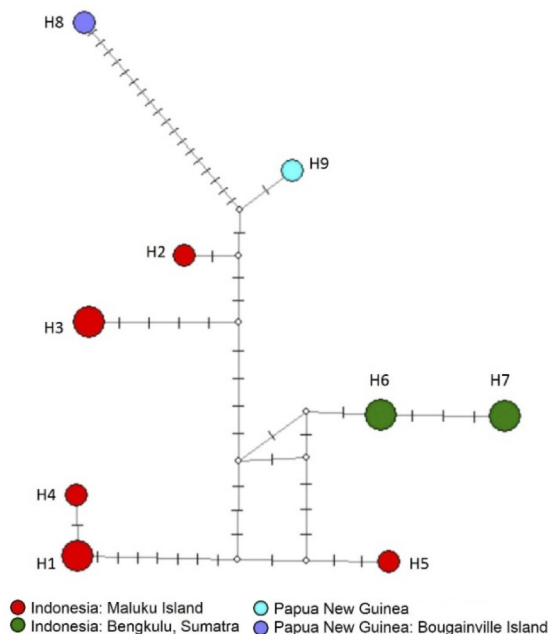


Figures 2–3. Mitochondrial cytochrome c oxidase subunit 1 (CO1) 551 bp sequence analyses. (2) Phylogenetic analysis based on maximum likelihood algorithm with the sample codes, GenBank or BOLD accession numbers and sample sites shown. Bootstrap percentages are shown at the tree nodes. (3) Haplotype network with the haplotypes labelled as H1 to H9. The circle size is proportional to the number of samples, and different sample sites are represented by different colours. Small white circle represents median vector which is the hypothesized or missing haplotype. Each dash on the line symbolizes one mutational step.

ing oceanic currents. If there were two spawning sites, the *A. interioris* larvae from the potential spawning site in the Pacific Ocean (Tomini Bay) could be transported to the Indian Ocean. Similarly, the larvae from the potential spawning site in the



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Figures 4–5. Mitochondrial D-loop 474 bp sequence analyses. (4) Phylogenetic analysis based on maximum likelihood algorithm with the sample codes, GenBank accession numbers and sample sites shown. Bootstrap percentages are shown at the tree nodes. (5) Haplotype network with the haplotypes labelled as H1 to H9. The circle size is proportional to the number of samples, and different sample sites are represented by different colours. Small white circle represents median vector which is the hypothesized or missing haplotype. Each dash on the line symbolizes one mutational step.

Indian Ocean (off Sumatra) could be transported to the Pacific Ocean. When these migrating larvae reach maturity, they could spawn at the local spawning site as it is closer geographically. A continuous migration would eventually lead to a homogenous

population. Recently, Arai and Taha (2021) found a panmictic population structure in *A. marmorata* from the Indo-Pacific region including East and West Indonesia. Therefore, like *A. marmorata*, the potential presence of only one spawning site in

either Indian Ocean or Pacific Ocean would ensure a homogeneous population of *A. interioris* within its distribution range. However, further study with more samples is required in order to confirm this preliminary finding. Nuclear DNA analysis such as microsatellites should also be carried out to validate the mitochondrial DNA findings.

The population size of *A. interioris* might be quite small compared to other tropical anguillid eels that are distributed in the Indo-Pacific region. Therefore, the species would be more susceptible and vulnerable to overexploitation and global climate change. The findings in this study have important implications for sustainable management and conservation of tropical anguillid eels including *A. interioris* as trade in eels has shifted from temperate eels towards tropical species.

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TA designed the experiment; NA and MJA conducted the experiments; NDZ, HT and TA contributed to analyzing data and writing the paper.

Competing Interests

The authors have declared that no competing interests exist.

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