Tropical Ecology (2020) 61:429–436
https://doi.org/10.1007/s42965-020-00096-4

International Society
for Tropical Ecology

SHORT COMMUNICATION

Molecular and morphological evidence for the identity of the giant mottled eel, *Anguilla marmorata* in Southeast Asia

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Received: 13 August 2019 / Revised: 11 July 2020 / Accepted: 22 July 2020 / Published online: 25 July 2020

Abstract

Tropical anguillid eels account for two-thirds of all species in the genus *Anguilla*, however information on the species diversity, biogeography, and life histories is very limited. The anguillid eels found in Southeast Asian waters, i.e. Malaysia, Thailand and Vietnam were identified using molecular genetic analysis after morphological observation. This is the first record of the occurrence of *A. marmorata* in Thailand and Vietnam that was confirmed by both morphological and molecular genetic analyses. The molecular phylogenetic tree and the haplotype network of *A. marmorata* in Malaysia, Thailand and Vietnam suggested that the eel might be transported from the western North Pacific spawning area. We also propose possible dispersion and migration of *A. marmorata* into Southeast Asian waters.

Keywords Distribution · Molecular · Species identification · Southeast Asia · Tropical anguillid eels

Introduction

The catadromous eels of the genus *Anguilla* Schrank are widely distributed throughout the world. Nineteen species of *Anguilla* have been reported worldwide, 13 of which occur in tropical regions (Ege 1939; Arai 2016). Of the thirteen species found in tropical areas, seven species or subspecies occur in the western Pacific around Indonesia and Malaysia, i.e. *A. celebesensis* Kaup 1856, *A. interioris* Whitely 1938, *A. bengalensis bengalensis* Gray 1831, *A. marmorata* Quoy & Gaimard 1824, *A. borneensis* Popta 1924, *A. bicolor bicolor* McClelland 1844 and *A. bicolor pacifica* Schmidt 1928 (Ege 1939; Castle and Williamson 1974; Arai et al. 1999). These eels have a catadromous life history, migrate between inland or coastal growth habitats, and spawn offshore, although anguillid eels do not all enter freshwater environments and that these species display a more opportunistic catadromy (e.g. Arai and Chino 2012, 2018, 2019; Arai et al. 2013, 2019).

Comprehensive studies by Ege (1939) have discussed anguillid species diversity, geographic distribution and abundance in the world and have revealed that the highest diversity of anguillids occurs in central Indonesian waters. However, there is relatively little information available on various aspects of eel biology including species composition, distribution, life history and migration in Southeast Asia. The identification of eels at the species level using solely visual observation is known to be difficult because of the similarities and overlapping morphological characteristics in eels, particularly tropical anguillids (Ege 1939; Arai 2016). To validate the identification of the tropical eel species, it is crucial to utilise both morphological and molecular genetic analyses.

The recent drastic decline of glass eel (juvenile) recruitment in temperate regions such as Europe and East Asia has caused serious problems in eel stock to sustainable levels of adult abundance (Arai 2014a). Tropical eels in Southeast Asia are currently considered a major target species for compensating for the high demand of eel resources (Arai 2014b). However, remarkably little is known about the diversity, distribution and life history of the many tropical eels in the Southeast Asia region. The lack of availability of basic life history, stock and population information on the tropical eels could lead to further serious declines in these eels.

In the present study, we collected anguillid eels in Southeast Asian waters, i.e. Malaysia, Thailand and Vietnam. These eels were subjected to identification using both morphological analyses and mitochondrial cytochrome oxidase

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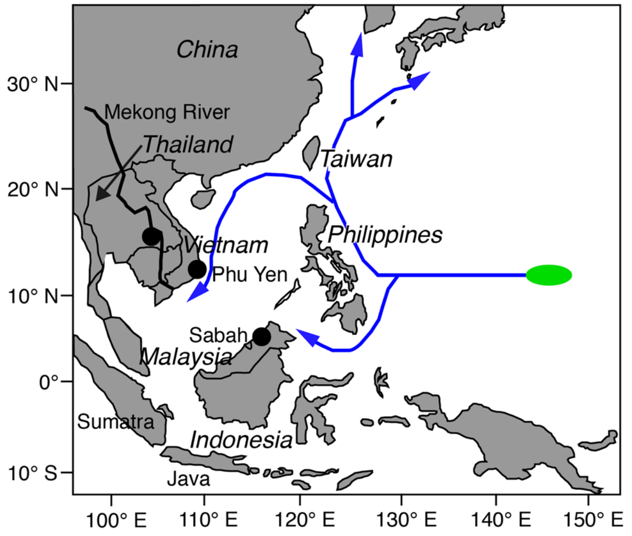

Fig. 1 Map of sampling locations of tropical anguillid eels genus *Anguilla* in Southeast Asian countries (Malaysia, Thailand and Vietnam). The location of the offshore spawning area of *A.marmorata* in North Pacific population (green ellipse) with the oceanic currents (blue lines) from the spawning area is shown (color figure online)

subunit I (COI) sequence analysis. This study describes the first confirmed record of a tropical anguillid eel, *Anguilla marmorata*, from Thailand and Vietnam. Furthermore, the molecular phylogenetic tree and the haplotype network of *A. marmorata* proposed possible dispersion and migration of *A. marmorata* into Southeast Asian waters. *A. marmorata* is one of the most common anguillid species in the Indo-Pacific (Ege 1939; Jespersen 1942). The adults of this species reach greater than most temperate species, and range over a much more oceanographically diverse region than any temperate species. Like all anguillid species, *A. marmorata* spawns in the ocean and has a leptocephalus larva that undergoes a remarkable metamorphosis into the glass eel stage before recruitment to continental habitats. The dispersion pattern of the leptocephalus, the lengthy duration of the leptocephalus stage and the timing of metamorphosis are probably an important biological determinant of the geographical distribution of anguillid eels. Therefore, *A. marmorata* may be a key species to understand the worldwide distribution and speciation of anguillid eels.

Material and methods

Eel samples and morphological analysis

A total of seven specimens of the anguillid eels were collected from Southeast Asia, i.e. three specimens in Malaysia, one specimen in Thailand and three specimens in Vietnam through traps, hooks and lines (Fig. 1, Table 1).

Table 1 Morphometric characteristics of a tropical anguillid eel *Anguilla marmorata* collected in Southeast Asia

Country	Location	Year	Number of samples	Total length range (mm)	Body weight range (g)	FDI range(%)	Pattern of color marking of skin	Character of maxillary bands of teeth	Species by morphology	Species by molecular genetic
Malaysia	Sabah State, Borneo Island	2016	3	720–1150	812–3700	15.1–19.1	Variegated	Narrow	*A. marmorata*	*A. marmorata*
Thailand	Mekong River	2016	1	1320	7000	17.8	Variegated	Narrow	*A. marmorata*	*A. marmorata*
Vietnam	Phu Yen Province	2009	3	554–796	438–1701	15.2–21.0	Variegated	Narrow	*A. marmorata*	*A. marmorata*

FDI fin difference index

The external morphometric characteristics were measured for each sample according to the morphological description of Ege (1939) and Watanabe et al. (2004) (Table 1), and, thereafter, the dorsal fins were clipped and preserved in 95% ethanol for molecular genetic analysis. Given that the total number of vertebrae (TV) is the same in a number of anguillids, the TV may not be a useful tool for the taxonomic identification of anguillids (Ege 1939; Watanabe et al. 2004). Thus, vertebrae counting were not conducted in the present study.

Instead, another morphological characteristic was chosen for the identification of eel samples. Based on the anguillid morphological identification keys developed by Ege (1939), the fin difference index (FDI) provides the highest resolution with the least ambiguity when distinguishing eels at the species level. The FDI for the distance between the verticals from the beginning of the dorsal fin (Z) to the anus (ano-dorsal length) relative to the total length (L_T) (Ege 1939), was calculated as follows:

$$\mathrm{FDI} = 100\,\mathrm{Z}\,\mathrm{L}_{\mathrm{T}}^{-1}.$$

Anguilla has been clearly divided into four different species groups based on the external morphological characteristics of each species: the first group (four species) has variegated skin with broad maxillary bands of teeth, the second group (four species/subspecies) has variegated skin with narrow maxillary bands of teeth (Fig. 2), the third group (six species) has non-variegated skin with a long dorsal fin, and the fourth group (five species/subspecies) has

Fig. 2 *Anguilla marmorata* collected in the Mekong River, Thailand. top *Anguilla marmorata* (1320 mm in TL). bottom Narrow maxillary bands of teeth of *A. marmorata*

non-variegated skin with a short dorsal fin (Ege 1939; Watanabe et al. 2004; Arai 2016).

Molecular genetic analysis

All seven samples (Table 1) and a mitochondrial gene, cytochrome oxidase subunit I (COI), were used. DNA was extracted from a dorsal fin clip using DNeasy Blood and Tissue Kit (QIAGEN, Germany), according to the manufacturer's instructions. Mitochondrial COI gene was amplified using different combinations of universal primers to validate the species identity, which were FishF1 (5′TCA ACC AAC CAC AAA GAC ATT GGC AC3′), FishF2 (5′TCG ACT AAT CAT AAA GAT ATC GGC AC3′), FishR1 (5′TAG ACT TCT GGG TGG CCA AAG AAT CA3′), and FishR2 (5′ACT TCA GGG TGA CCG AAG AAT CAG AA3′) (Ward et al. 2005). Each PCR contained 2 µl of DNA sample, 2.5 µl of each 10 µM primer, 25 µl of 2 × *Taq* PCR Master Mix (QIAGEN, Germany) and 13 µl of distilled water. The PCR conditions were initially 95 °C for 2 min, then 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s, finally 72 °C for 10 min. PCR amplicon was purified using QIAquick Gel Extraction Kit (QIAGEN, Germany), according to the manufacturer's instructions, and sequenced bi-directionally with the same primers.

Generated sequence trace files were manually edited and assembled using MEGA version 7 (Kumar et al. 2016). The contig sequences were compared for percentage similarity with the reference sequences in the GenBank database using BLAST search. MEGA was used to construct a phylogenetic tree using the UPGMA method. The evolutionary distances were computed using the Kimura 2-parameter method. All codon positions were included, and the positions with gaps and missing data were eliminated. Bootstrap test was carried out with 1000 replicates. Haplotype analysis was conducted using DnaSP version 6 (Rozas et al. 2017), and haplotype network was constructed via the reduced median method using Network version 5 (https://www.fluxus-engineering.com). To compare the COI sequences in this study with other localities, other COI sequences that were deposited in the GenBank database were also included in the analysis.

Results

Morphological implications

All samples were assigned into the second group of the genus *Anguilla* based on their variegated skin and narrow maxillary bands of teeth (Ege 1939; Watanabe et al. 2004; Arai 2016) (Table 1). The geographical distribution of anguillids is used in combination with key morphological characteristics to determine the classification of each species into four groups. Within the second group, *A. bengalensis labiata* and *A. reinhardtii* exist in the mid-south-eastern region of Africa and eastern Australia and Tasmania, respectively (Ege 1939). Therefore, both of these species were not considered when identifying the samples in the present study. The FDIs of the other two species, *A. bengalensis bengalensis* and *A. marmorata* were studied further. According to the key morphological characteristics used for identification (Ege 1939; Watanabe et al. 2004), the FDI of *A. marmorata* is in the range of 12 to 20, higher than that of *A. bengalensis bengalensis*, which is in the range of 8 to 14 (Ege 1939; Watanabe et al. 2004). FDIs of all samples were in the range of 15 to 21, which is within or closer the FDI range of *A. marmorata* (Table 1).

Genetic implications

The ~ 700 bp mitochondrial COI genes of all 7 samples were successfully amplified and sequenced. Using BLAST, the mitochondrial sequences revealed definitive identity matches with *A. marmorata* in the range of 99–100% for all samples, indicating highly significant similarities between the samples and the GenBank reference sequences. Thus, all variegated long-finned eels from Malaysia, Thailand and Vietnam were identified as *A. marmorata* (Table 1).

The DNA sequences of *A. marmorata* collected in Malaysia, Thailand and Vietnam were deposited in the GenBank database with the accession numbers MG324010 to MG324012, MG324009 and MK818583 to MK818585, respectively.

Molecular phylogenetic tree and haplotype network

A total of 23 deposited *A. marmorata* COI sequences were obtained from the GenBank database, and analysed together with the 7 COI sequences from this study (Fig. 3). Haplotype analysis revealed a total of nine haplotypes (H1–H9) from all the analysed sequences, with the eels from Malaysia and Thailand belonged to H1, and the eels from Vietnam consisted of two haplotypes i.e. H1 and H8 (Fig. 4).

Discussion

This is the first description of the occurrence and distribution of *Anguilla marmorata* in Thailand and Vietnam as identified by both morphological and genetic analyses. *Anguilla marmorata* was found in West (Peninsular Malaysia) and East (Borneo Island) Malaysia (Arai and Wong 2016; Abdul Kadir et al. 2017; Arai and Abdul Kadir 2017; Wong et al. 2017) and Brunei Darussalam on Borneo Island (Zan et al. 2020) in our previous studies. The identification of eels at the species level using solely visual observation is known

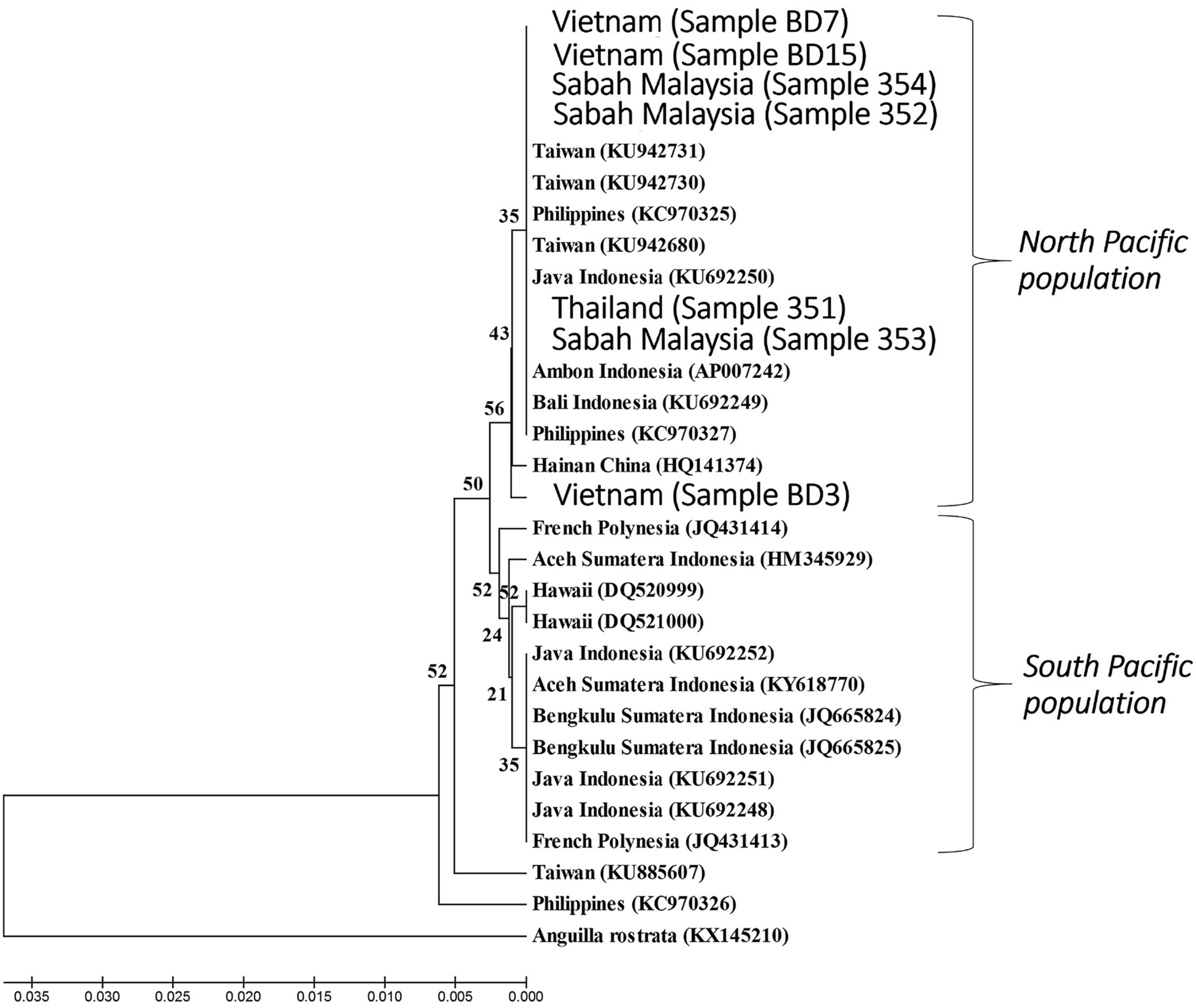

Fig. 3 Phylogenetic UPGMA tree using the mtDNA COI of *Anguilla marmorata* and added sequences from the GenBank database with indicated accession numbers. *A. rostrata* was used as outgroup. The percentages of the boostrap test are shown next to the branches. Scale refers to evolutionary distance and in the unit number of base substitutions per site

to be difficult because of the similarities and overlapping morphological characteristics in eels, particularly tropical anguillids (Ege 1939; Arai 2016). In fact, the difficulty in distinguishing both *A. marmorata* and *A. bengalensis bengalensis* is augmented by their overlapping morphological characteristics, which cause further identification ambiguities (Abdul Kadir et al. 2015; Arai et al. 2015; Arai and Wong 2016). To validate the identification of the tropical eel species, it is crucial to utilise both morphological and molecular genetic analyses for further biological and ecological studies.

During the year-round survey, Wong et al. (2017) could collect only two specimens of *A. marmorata* in Sabah, Borneo Island of Malaysia. We could collect further three *A. marmorata* in Sabah in this study (Table 1). However, this limited number of *A. marmorata* specimens suggests that the study site might be out of the species main distribution region. A previous study on the population structure of the giant mottled eel, *A. marmorata*, suggested that this species has four genetically different populations (North Pacific, South Pacific, Indian Ocean, Guam region) (Minegishi et al. 2008). The molecular phylogenetic tree and the haplotype network found that *A. marmorata* from Sabah (Malaysia), Thailand and Vietnam were in the same cluster and population together with *A. marmorata* from China, Philippines and Taiwan which belong to the North Pacific population (Figs. 3, 4). The western North Pacific, which is the spawning ground for the Japanese eel, *A. japonica*, is suggested to be a possible spawning area for *A. marmorata* of the North Pacific population (Arai et al. 2002a; Tsukamoto et al. 2011)

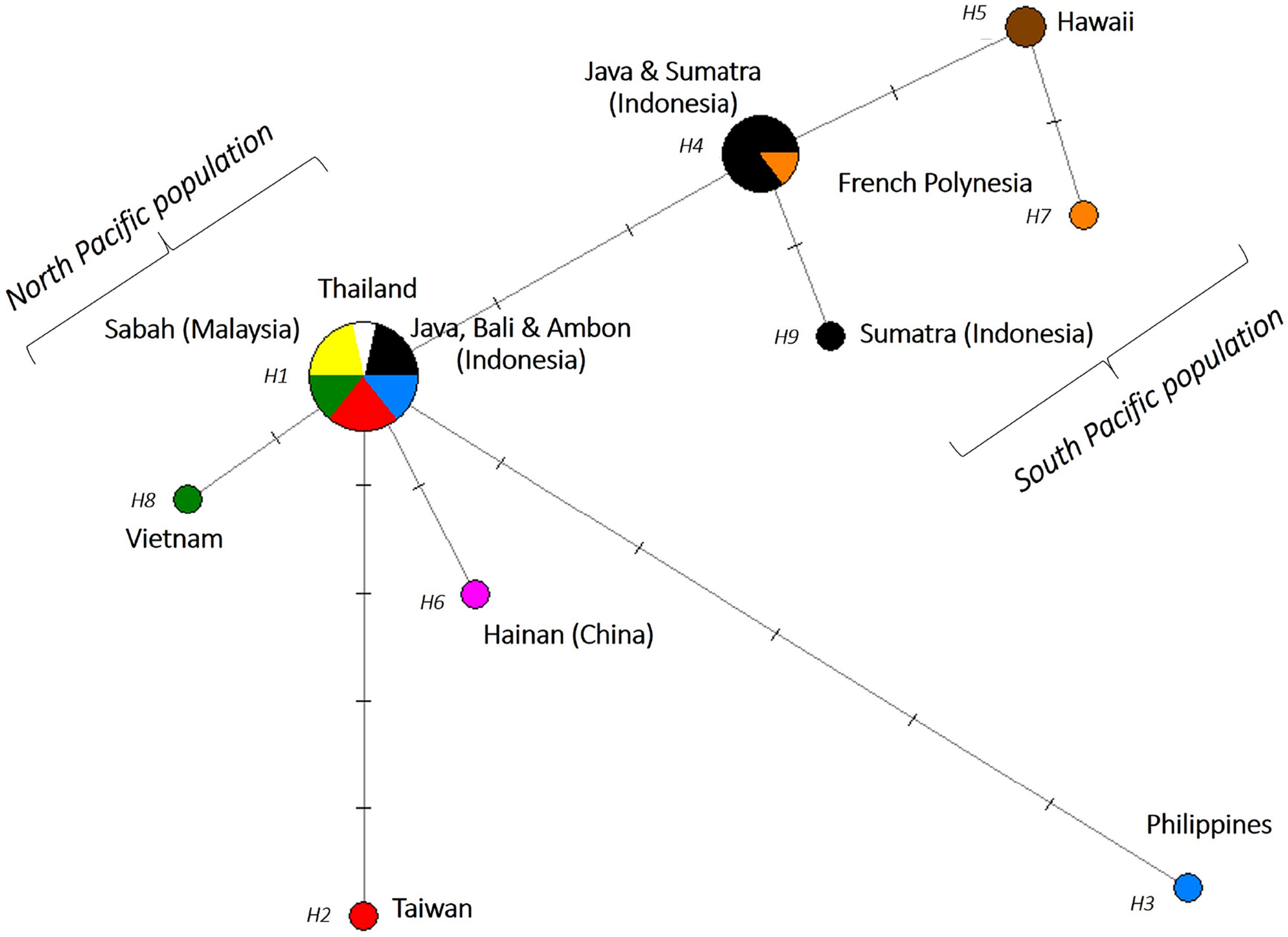

Fig. 4 Haplotype network constructed with *Anguilla marmorata* mtDNA COI sequences. Each color represents a sample site. The size of the circle is proportional to the number of samples that belong to each haplotype. Each dash, which appears on the line that connects two haplotypes together, symbolizes one mutational step (color figure online)

(Fig. 2). However, only one spawning site of this species has been discovered and estimated, which is located offshore in the western North Pacific to the west of Guam (Arai et al. 2001a, b, 2002a; Tsukamoto et al. 2011; Arai 2014c) among four populations. The age at recruitment of the species ranged from 4 to 6 months by means of otolith analyses (Arai et al. 2001a, b, 2002a; b). These results suggest that *A. marmorata* would be transported to Southeast Asian countries not more than six months after hatching from the western North Pacific spawning area. Further intensive sampling should be undertaken, along with molecular genetic studies, in order to better understand the details of the distribution and migration in the species.

The molecular phylogenetic tree and the haplotype network used in the present and previous studies suggested the divergence of *A. marmorata* in Southeast Asia. There were two distinctive haplotypes, H1 and H4, of *A. marmorata* in Southeast Asia that belong to the North Pacific and the South Pacific populations, respectively. Although *A. marmorata* from Philippines and Taiwan (East Asia) belong to the North Pacific population, some of them showed greater distances from main haplotype (H1, Fig. 4). Their population divergences were probably not a recent event due to the higher number of mutational steps observed between the haplotypes (5 and 4 mutational steps for Philippines and Taiwan, respectively) (Fig. 4). Therefore, the results might suggest the occurrence of sub-populations in the North Pacific population. Interestingly, *A. marmorata* from Java Island of Indonesia belonged to both the North Pacific and the South Pacific populations, and *A. marmorata* from Sumatra island of Indonesia belonged to the South Pacific population (Fig. 4), although these were previously considered to belong to the Indian Ocean population (Minegishi et al. 2008; Gagnaire et al. 2009). There is no ecological evidence on the plausible spawning sites for *A. marmorata* of Java and Sumatra islands of Indonesia. Gagnaire et al. (2009) hypothesized the existence of two geographically and/or temporally separated spawning areas between southwestern Indian Ocean and Sumatra populations. However, our results did not support the hypothesis. *A. bicolor bicolor* occurred dominantly, constituted of 85% in Java and Sumatra regions (Jespersen 1942), and thus these regions might be out of the main distribution of *A. marmorata*. The evidence suggests that *A. marmorata* larvae (leptocephali) might be transported through vagrant routes (ocean currents). We found *A. marmorata* from Sabah, Borneo Island of Malaysia where is close to Java and Sumatra islands. There are the North Equatorial Current from the North Pacific and the South Equatorial Current form the South Pacific flowing to Java and Sumatra islands through Makassar Strait off eastern Borneo Island and through off northern Australia, respectively (Wyrtki 1961). Therefore, *A. marmorata* in Java and Sumatra islands might be transported from spawning areas of the North Pacific and/or the South Pacific populations. To validate the transportation mechanisms, further study is needed to reveal details of population structure and phylogeny.

Acknowledgements We thank Azie Azri for her assistance in the laboratory work. This work was supported by Universiti Brunei Darussalam under the Competitive Research Grant Scheme (no. UBD/OVACRI/CRGWG(003)).

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