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RESEARCH ARTICLE

Analysis of mitochondrial cytochrome b gene sequences of marine leech, *Pterobdella arugamensis*

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ABSTRACT. *Pterobdella arugamensis* (Silva, 1963) is a marine leech that can be found the Indo-West Pacific region and has shown undesired effects in the aquaculture industry. In the Asia Pacific region, it has infested predominantly on the cultured hybrid groupers. Despite its known distributional range, the dispersion and transportation mechanisms of *P. arugamensis* are still poorly known. Hence, analyzing the DNA sequences of *P. arugamensis* may shed some light on this enigmatic leech, which will aid in mitigating the leech-associated issues faced by the aquaculture industry. This study analyzed the mitochondrial cytochrome b (CYTB) gene sequences of *P. arugamensis* from Brunei, China and Indonesia. The results provided further confirmation that *P. arugamensis* from these sites showed variation within the overall population but significant differences in its distribution across the region were not observed, which could be due to its introduction via the aquaculture industry or due to natural migration.

KEY WORDS. aquaculture, cytochrome b, fish leech, hybrid groupers, molecular phylogeny, population genetics.

INTRODUCTION

Pterobdella arugamensis (Silva, 1963), previously known as *Zeylanicobdella arugamensis* Silva, 1963, (Burreson 2020) is a marine ectoparasitic fish leech in Piscicolidae, which infects various teleost fish host species including cultured groupers (Cruz-Lacierda et al. 2000, Nagasawa and Uyeno 2009, Murwantoko et al. 2018, Azmey et al. 2020, 2022). The leech can be found in the Indo-West Pacific region with Brunei Darussalam showing the highest prevalence and infestation of *P. arugamensis* (Azmey et al. 2020). It has been reported that heavy infestation of this leech can result in illnesses (Noga 2010) and high mortality (Kua et al. 2014) to the fish hosts. Parasite-infected and diseased cultured groupers, and heavy mortality in the cultured groupers are the main issues in grouper aquaculture (Nagasawa and Cruz-Lacierda 2004, Liu et al. 2017), which can significantly impact the production of groupers and eventually, the sustainability of the aquaculture industry (Ravi and Yahaya 2017, Rimmer and Glamuzina 2019, Koepper et al. 2020). Therefore, *P. arugamensis* is considered to be a serious threat to the industry.

In order to mitigate the problems caused by *P. arugamensis*, information on its population genetic structure could give insight into how the leech was distributed in the Asia Pacific region. Based on a recent study using the mitochondrial cytochrome c oxidase subunit I (COI) gene, *P. arugamensis* was found to be divided into four genetically distinct populations in the Indo-West Pacific region: (1) Asia Pacific, (2) Borneo, (3) Surabaya and (4) Iran, in which it was suggested that the leeches from the Asia Pacific population had their origin in Indonesia and unintentionally introduced to other locations via the aquaculture industry (Azmey et al. 2022). Although the COI gene marker has been proven to be a powerful tool for revealing phylogeographic patterns (Tan et al. 2012, Taboada and Pérez-Portela 2016) and genetic

diversity (Lejeusne et al. 2014), mitochondrial cytochrome b (CYTB) gene can be additionally used for exploring genetic differentiation between related species and within species (Tobe et al. 2010, Jagielski et al. 2018). CYTB gene marker is also widely used in phylogenetic and genetic studies (Lejeusne et al. 2014) due to its sequence variability (Tobe et al. 2010). However, CYTB gene is rarely utilised in studies of population structure of annelids. Indeed, currently only one CYTB gene sequence of *P. arugamensis* is found in the GenBank database (accession number KY474378). Therefore, in the present study, the marine leeches from Brunei Darussalam and Indonesia were analysed using this gene. Thereafter, these sequences were examined together with the only available CYTB sequence in the GenBank database. This study aims to use CYTB gene to reveal the population structure of *P. arugamensis* in the Asia Pacific region.

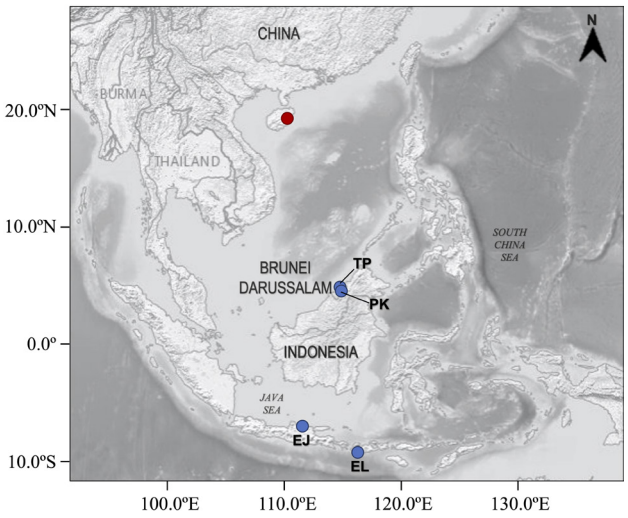

Figure 1. Locations of *P. arugamensis* used in this study. Sampling locations are shown by blue circles: Brunei (TP: Tanjong Pelumpong, PK: Pulau Kaingaran), Surabaya in Indonesia (EJ), and Lombok in Indonesia (EL). Red circle indicates the GenBank sample from Hainan, China. The map was adapted from the USGS National Map Viewer (open access) at http://viewer.nationalmap.gov/viewer/.

MATERIAL AND METHODS

Marine leech samples

A total of 22 *P. arugamensis* samples were previously collected from three sites in the Asia Pacific region (Azmey et al. 2022). Thirteen specimens were from Brunei Darussalam, specifically from Tanjong Pelumpong (TP; 5°02'01"N, 115°05'53"E) and Pulau Kaingaran (PK; 4°56'59"N, 115°01'37"E). Five specimens were from Lamongan, Surabaya in Indonesia (EJ; 6°53'22"S, 112°11'52"E). Four specimens were from Ekas Bay, East Lombok in Indonesia (EL; 8°52'156"S, 116°27'14"E). One sequence from Hainan, China was obtained the GenBank database (Fig. 1; Table 1).

Mitochondrial DNA Analysis

The genomic DNA was previously extracted (Azmey et al. 2022), and in this study, the partial CYTB gene was amplified by polymerase chain reaction (PCR) using primers that were specifically designed using the available *P. arugamensis* CYTB gene sequence (GenBank accession number KY474378), which were CYTBF (5'–TCA TGC AAA TGG AGC TTC ACT–3') and CYTBR (5'–TGC TGC AAT AAC TCC ACC AAG–3'). The PCR was conducted using the 2x Taq PCR Master Mix (QIAGEN) according to the manufacturer's instructions with the following conditions: 95 °C for three minutes; 30 cycles of 95 °C (30 seconds), 45 °C for 30 seconds, 72 °C (60 seconds); 72 °C for five minutes. The PCR product was then purified using QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions. Bidirectional sequencing using the same primers was carried out by a

Table 1. List of haplotypes of *Pterobdella arugamensis* and their locations.

Haplotype	Number of samples	Locations (sample number or GenBank accession number)
H1	5	Brunei Darussalam (3 samples) and Indonesia: Surabaya (2 samples)
H2	7	Brunei Darussalam (7 samples)
H3	1	Brunei Darussalam (1 sample)
H4	1	Brunei Darussalam (1 sample)
H5	2	Brunei Darussalam (1 sample) and Indonesia: Lombok (1 sample)
H6	3	Indonesia: Lombok (2 samples) and Indonesia: Surabaya (1 sample)
H7	2	Indonesia: Lombok (1 sample) and Indonesia: Surabaya (1 sample)
H8	1	Indonesia: Surabaya (1 sample)
H9	1	China: Hainan (KY474378)

service provider. MEGA X (20) was used to establish the contig sequence. All contig sequences were uploaded to the GenBank database with accession no. OP947674-OP947695.

MEGA X was also used to align the multiple sequences via Clustal W, in which there were no internal gaps observed in the alignment. External gaps or missing data were not analysed. Genetic distance (uncorrected p distance) was calculated using MEGA X. As previously described (Azmey et al. 2022), phylogenetic trees were constructed via MEGA X using the neighbour-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) algorithms, in which the ML tree (using T92+I model) was shown here as the representative tree. DnaSP 6 (Rozas et al. 2017) was used for haplotype and diversity analyses. Arlequin 3.5.2.2 (Excoffier and Lischer 2010) was used for the Analysis of Molecular Variance (AMOVA) and fixation index (F_{ST}). Network 10 (www.fluxus-engineering.com) was used to construct a haplotype network via the reduced-median method.

RESULTS

A total of 22 samples, each comprising of a CYTB sequence length of 527 bp, were successfully amplified, sequenced and analysed. Although the sample sizes were small, the nucleotide diversity (π) for the samples from Brunei Darussalam, Lombok and Surabaya were 0.005, 0.012 and 0.012, respectively. The within-group mean genetic distance values for Brunei Darussalam, Lombok and Surabaya were 0.5, 1.2 and 1.1%, respectively. The between-group mean genetic distance values were lower: 0.09% (Brunei Darussalam vs. Surabaya) and 0.10% (Brunei Darussalam vs. Lombok; Surabaya vs. Lombok). When compared with the single sample from China, the between-group mean genetic distance values were even lower, ranging from 0.3 to 0.8%. These suggest that the samples from all sites were genetically similar. Furthermore, for the three studied sites, AMOVA showed that the within-group variation was 88.75%, whereas the between-group variation was only 11.25% with the fixation index (F_{ST}) of 0.11 ($p > 0.05$; not significant). This indicates that the leech samples from Brunei Darussalam, Lombok and Surabaya were not significantly different.

A total of nine different haplotypes (labelled H1 to H9) were identified in this study (Table 1). The haplotype diversity were 0.69, 0.83 and 0.90 for Brunei Darussalam, Lombok and Surabaya, respectively, although these values might be affected by the small sample numbers. The phylogenetic trees and haplotype network of the nine haplotypes were examined for the presence of population structure. ML tree showed no distinct structure (Fig. 2) and similarly, both NJ and MP trees also did not show any distinct structure (see Supplementary Material 1 Fig. S1). This suggests that the *P. arugamensis* samples from all sites were from one genetic stock. Similarly, the constructed haplotype network (Fig. 3) also did not show any spatial genetic structure. Some haplotypes could be found in two different locations. For example, H1 was found in both Brunei Darussalam and Surabaya even though these sites are geographically distant from each other. This further supported that the samples were of one genetically homogenous population.

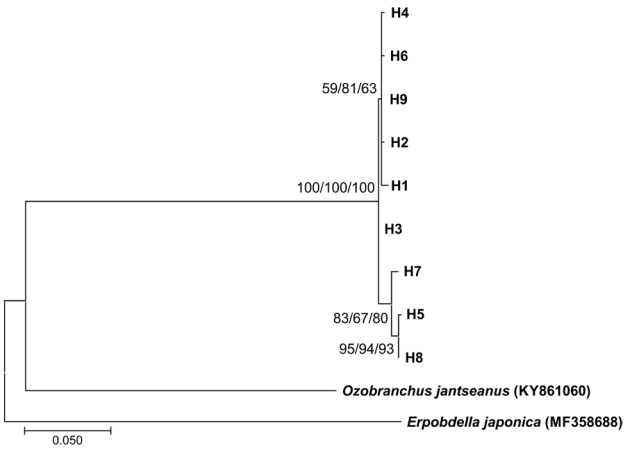

Figure 2. Representative maximum-likelihood tree showing nine haplotypes of *P. arugamensis* based on CYTB gene sequences. *Ozobranchus jantseanus* and *Erpobdella japonica* from the GenBank database were used as outgroups. The bootstrap percentages (1000 replicates) for maximum likelihood/maximum parsimony/neighbour joining trees are shown.

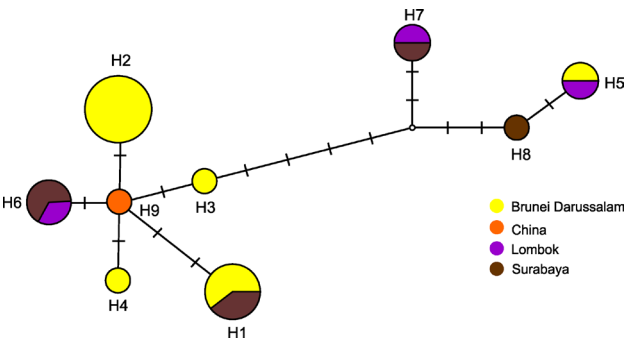

Figure 3. Haplotype network of *P. arugamensis* CYTB gene sequences. Different colours represent different locations. The circle size is proportional to the sample number. Each dash on the line symbolises one mutational event. Tiny white circle indicates median vector.

DISCUSSION

The present study suggests that the *P. arugamensis* samples from the Asia Pacific region (specifically Brunei Darussalam, Lombok and Surabaya in Indonesia, and Hainan in China) were from one genetic stock despite their geographical localities. This observation is consistent with the previous study that found the Asia Pacific population of *P. arugamensis* was genetically homogenous (Azmey et al. 2022) even though the present study used CYTB gene while the previous study used COI gene. Thus, this means that CYTB gene is an effective gene marker for phylogenetic research despite the marker rarely being utilised in the population studies of annelida.

The genetically homogenous population of *P. arugamensis* found in this study suggests the existence of possible dispersal origin within the Asia Pacific region, as similarly suggested by the previous study (Azmey et al. 2022). It is known that *P. arugamensis* can be distributed within the Indo-Pacific region via several teleost fish host species including groupers (Cruz-Lacierda et al. 2000, Nagasawa and Cruz-Lacierda 2004, Murwantoko et al. 2018, Azmey et al. 2020, 2022). The rise in the grouper aquaculture industry that has spread throughout the Southeast Asian region (Seng 1998, Sadovy 2000, Hamid 2001, Pudadera et al. 2002, Koepper et al. 2020) might be responsible in further dispersing the leech in this region. Indonesia is known as a major distributor of grouper fingerlings (Rimmer and Glamuzina 2019), and the grouper fingerlings are produced from hatcheries in Bali, East Java and Sumatra in Indonesia (Sugama et al. 2008, Kongkeo et al. 2010). The groupers were then exported to other countries including Brunei Darussalam (Hamid 2001, Pudadera et al. 2002, Rimmer and Glamuzina 2019) and especially to China, Japan and Singapore (Afero et al. 2010). This suggests that *P. arugamensis* from these hatcheries or grouper farms might be unintentionally exported to other countries that imported groupers from Indonesia. According to Azmey et al. (2022), an outbreak of marine leeches was only observed in 2017 onwards in Brunei Darussalam although the aquaculture of groupers already had started in 1993 in the country. It could be possible that the outbreak had its origin in Indonesia. Since Brunei Darussalam imported from Indonesia, it would be expected that there would be more genetic variation in the source population but a reduced genetic variation in the introduced population. This study showed that the nucleotide diversity and within-group mean genetic distance values were lower in Brunei Darussalam compared to the two studied sites (Lombok and Surabaya) in Indonesia.

Final remarks

This is the first study that used mitochondrial CYTB gene as a molecular marker for population study of marine leech, *P. arugamensis*. This study showed the presence of a genetically homogenous population of *P. arugamensis* in the Asia Pacific region. The results suggest that the population might originate from Indonesia and spread to other localities via the aquaculture industry, or it could be due to natural migration such as via migrating teleost fish hosts. Although the sample sizes of this study were considered small and not covering all localities in the Asia Pacific region, the results of this study were consistent with the results from a previous study. However, more samples and comprehensive research involving other genetic markers (such as microsatellites or single nucleotide polymorphisms) and involving various localities are needed to further understand this enigmatic leech not only in the Asia Pacific region but across its whole distribution range. This would eventually lead to a full understanding of its dispersion mechanism and to implementation measures for mitigating the prevalence and infestation of *P. arugamensis* in the grouper aquaculture.

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Author Contributions

SA: Conceptualization, Methodology, Formal Analysis, Investigation, Resources, Writing-original draft. TA: Validation, Resources, Writing-review & editing, Supervision, Funding Acquisition. HT: Validation, Formal Analysis, Writing-review & editing, Supervision. GM and MA: Resources.

Competing Interests

The authors have declared that no competing interests exist.

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Supplementary material 1

Figure S1. Phylogenetic trees of *P. arugamensis* based on CYTB gene sequences with *O. jantseanus* and *E. japonica* used as outgroups. A: Neighbor joining (NJ) tree, B: Maximum parsimony (MP) tree, C: Maximum likelihood (ML) tree.

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