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DNA BARCODING ASSESSMENT OF FISHES IN TASIK RABAN, LENGGONG, PERAK FOR DEVELOPMENT OF EDUCATION MANUAL

RAMIZAH BINTI ABDULL RAHMAN



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**FACULTY OF SCIENCE AND MATHEMATICS
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ABSTRACT

This study aimed to molecularly barcode fish diversity found in Tasik Raban, Lenggong Perak, using cytochrome c oxidase subunit I (COI) markers and to develop an educational manual for public reference, consisting of BOLD online data. Freshwater fish were sampled from Tasik Raban and a fin clip from each specimen was collected for molecular amplification utilizing the COI gene. Prior to creating the manual through the implementation of the ADDIE instructional learning methodology, various tasks such as morphological identification, COI divergence assessment, species delimitation, and phylogenetic analysis were undertaken. A total of 142 samples were collected encompasses eight families, 15 genera, and 16 species. Sequencing of extracted DNA revealed 77 DNA barcode with pairwise distance ranging from 0.00% to 2.00%. The application of Neighbour-Joining, Maximum Parsimony, and Maximum Likelihood in phylogenetic analysis showed consistent tree structures without any overlapping clusters. Additionally, all DNA sequences were uploaded to the BOLD online system to contribute to the creation of the educational manual. The manual received high validity and usability ratings from experts. In conclusion, the DNA barcoding assessment showed the presence of invasive fish species in Tasik Raban whilst the educational manual developed can be used to raise conservation awareness. The findings of this research provide a basis for monitoring invasive species in the local aquatic ecosystem.





ABSTRAK

PENILAIAN KOD BAR DNA DAN PEMBANGUNAN MANUAL PENDIDIKAN UNTUK IKAN DI TASIK RABAN, LENGGONG, PERAK

Kajian ini bertujuan untuk merekod secara molekul kepelbagaian ikan yang ditemui di Tasik Raban, Lenggong, Perak menggunakan penanda sitokrom c oksidase sub unit I (COI) dan membangunkan manual pendidikan bagi rujukan awam yang mengandungi data dalam talian BOLD. Persampelan ikan air tawar telah dijalankan di Tasik Raban dan setiap sirip spesimen dikumpul untuk amplifikasi molekul menggunakan gen COI. Bagi membangunkan manual yang menggunakan metodologi pembelajaran pengajaran ADDIE, pengenalpastian morfologi, penilaian perbezaan COI, pengenalan spesies, dan analisis filogenetik telah dilakukan. Sebanyak 142 sampel telah berjaya dikumpul yang terdiri daripada lapan famili, 15 genera dan 16 spesies. Penjujukan DNA yang telah diekstrak menunjukkan 77 data kod bar DNA dengan jarak berpasangan antara 0.00% hingga 2.00%. Analisis filogenetik menggunakan *Neighbour-Joining*, *Maximum Parsimony* dan *Maximum Likelihood* menunjukkan pokok filogeni yang serupa tanpa pertindihan kluster. Semua jujukan DNA juga telah dimuat naik ke sistem dalam talian BOLD untuk digunakan dalam pembangunan manual pendidikan. Manual ini mendapat penilaian kesahan dan kebolehgunaan yang tinggi daripada pakar. Kesimpulannya, penilaian kod bar DNA kajian ini mendedahkan kehadiran spesies ikan invasif di Tasik Raban dan manual pendidikan yang dibangunkan boleh digunakan untuk meningkatkan kesedaran terhadap isu pemuliharaan. Hasil kajian ini menyumbang kepada asas untuk penentuan spesis ikan invasive dalam ekosistem akuatik tempatan.



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LIST OF ABBREVIATIONS

COI	Cytochrome Oxidase 1
NJ	Neighbour Joining
ME	Minimum Evolution
MP	Maximum Parsimony
ML	Maximum Likelihood



CHAPTER 1

INTRODUCTION



1.1 Freshwater fishes

Fish consumption has experienced substantial growth in recent decades, primarily due to its recognized nutritional advantages as an integral part of a well-rounded diet. This has led to a notable upswing in the global trade of fish products (Fernandes et al., 2017). The overall output of global fisheries has seen a remarkable increase over the past decade, with an anticipated fish production ranging from 149.5 million tons in 2008 to 178.5 million tons in 2018 (FAO, 2020). Regarding human consumption, the utilization of fish as a protein source has witnessed a significant surge over the last decade. Estimated global fish consumption has risen from 129.2 million tons in 2008 to 156.4





million tons in 2018, with a corresponding value of 762.5 billion Ringgit Malaysia. Additionally, FAO (2020) highlights that over 86% of the world's fish is consumed, underscoring the pivotal role of fish as a protein source.

Freshwater fishes have been statistically proven to be economically and nutritionally significant, as exemplified in the Mekong River basin, the Brazilian Amazon, and Ayeryarwadi, where these fishes serve as a crucial source of livelihood and sustenance for millions of people (Baran et al., 2015; FAO, 2020). Malaysia's fisheries sector has played a vital role as a substantial economic contributor and source of animal protein for its citizens for many years. Holding 2% of global fish production, Malaysia ranks sixteenth worldwide—a noteworthy improvement from the 1980s when its global output share was merely 0.76% (FAO, 2020). In 2017, Malaysia's total fisheries production reached 1.7 million tons (FAO, 2019), with 1.5 million tons from capture and 0.2 million tons from aquaculture, amounting to 1.7 billion Ringgit in total. Moreover, freshwater fish are estimated to contribute 6% to the world's annual supply of animal protein (FAO, 2007).

Nevertheless, recent years have witnessed a decline in the number of freshwater fish species globally. A third of all known freshwater fish species are now on the brink of extinction (IUCN, 2020). Consequently, it is imperative to closely monitor and analyze freshwater species to prevent further extinctions. Addressing the root causes of habitat loss, pollution, and overfishing is crucial in halting this alarming trend.





1.2 Freshwater fish diversity

Fish constitute more than half of all vertebrate species, establishing them as the most diverse group among vertebrate animals, with approximately 54,711 recognized species (Wiens, 2015). Nelson (2006), reports that there are roughly 32,500 species of freshwater and marine life, classified into 515 groups. Froese and Pauly (2022) estimate the global count at around 35,000 fish species. Among these, 18,000 are freshwater fish, categorized into more than 47 orders, 224 families, and 2,375 genera (Van der Laan, 2022). Unsurprisingly, Malaysia, with its rich aquatic ecosystems, harbors a significant diversity of freshwater and marine fish species, distributed among more than 186 families and 704 genera (Chong, Lee & Lau, 2010). As reported by the World Conservation Monitoring Centre of the United Nations Environment Program, Malaysia holds the 12th position globally and the 4th in Asia concerning biodiversity (Williams et al., 2001). Ng, Wong and Khoo (2017) further specify that Malaysia boasts 634 species of freshwater fish, spanning 68 families. This abundance underscores the country's remarkable contribution to the overall diversity of fish species, both regionally and globally.

Helfman, Collette, Facey and Bowson (2009) estimate that nearly 40% of the world's fish species, totaling 11,952, are exclusively or predominantly found in freshwater environments. Malaysia, situated in the Oriental tropical ecoregion of Asia encompassing the eastern, southern, and southeastern regions, is recognized for its rich biodiversity. The global diversity of freshwater fish is thought to reach 3,500 species, with tropical regions like Malaysia, Indonesia, India, and Indochina exhibiting the





highest levels of diversity. According to Ng et al. (2017), Tropical Asia's inland waters harbor 121 different fish families, with cyprinids being particularly prevalent.

Fish residing in inland streams, rivers, lakes, and brackish water environments are commonly referred to as freshwater species (Ng et al., 2017). Malaysia boasts a diverse collection of lakes, comprising both natural and artificial bodies of water. Natural lakes are relatively scarce, with a total of 51 man-made lakes distributed across the country. Among these, 46 are situated in Peninsular Malaysia, three in Sabah, and two in Sarawak (Yusoff, Shariff & Nagaraj, 2006). Notable examples include Tasik Bera and Tasik Chini in the state of Pahang, Tasik Dayang Bunting on Langkawi Island in the province of Kedah, as well as Lake Chenderoh and Batang Ai in Sarawak. Tasik Bera attained the status of Malaysia's first Wetland of International Importance in 1995 (Prentice, 2018).

Chong et al. (2010) report that 48% of all known species are under threat, comprising 133 species across 25 families and 28 genera. Freshwater fishes are particularly vulnerable, accounting for the highest percentage at 87%. Various studies, including those by Bakar (2009), Chong et al. (2010), and Reid, Conteras and Csatádi (2013), consistently highlight the significant vulnerability of freshwater species. The primary causes contributing to this vulnerability are identified as habitat loss (76%), overfishing (27%), and by-catch (23%).





1.3 DNA barcoding for sustainability of freshwater fishes

DNA barcoding is a taxonomic approach employing a concise genetic marker from the complete genome for the identification of organisms or species (Barcaccia, Lucchin & Cassandro, 2016; Bhattacharya et al., 2016; Kress & Erickson, 2012). According to Hebert and Gregory (2005), DNA barcoding is a tool extensively used by taxonomists for species identification. The concept gained prominence in 2003 when Paul Hebert's research group at the University of Guelph published the study titled "Biological Identifications through DNA Barcodes" (Kaur, 2015).

The primary goal of DNA barcoding is to identify or categorize unknown samples at the species level. This method proves particularly valuable for species with intricate or hard-to-access morphologies, such as larvae, invasive species, cryptic species, new species, those involved in the illegal trade of protected species, and for purposes like stock management, biodiversity assessment, ecosystem monitoring, revising certain taxa, inferring phylogenetic relationships, and studying phylogeographic and speciation patterns (En et al., 2019; W. Kress, 2017; Kaur, 2015; Hubert et al., 2012; DiBattista, Wilcox, Craig, Rocha & Bowen, 2010; Zemplak, Ward, Connell, Holmes & Hebert, 2009; Concepcion, Crepeau, Wagner, Kahng & Toonen, 2008; Barber & Boyce, 2006).

Given its expert-validated verification system and notable accuracy, molecular biology approaches centered on sequencing, particularly the DNA barcoding method,





have garnered significant attention as promising tools for fish species identification (Clark, 2015). Importantly, the DNA barcoding method is not designed to substitute morphological studies with a wholly molecular identification system (Will & Rubinoff, 2004). Instead, the overarching objective is to establish a collaborative framework between molecular and morphological taxonomists, aiming to achieve efficient species identification (Summerbell et al., 2005).

The DNA barcoding approach has experienced a notable increase in Malaysia, with a predominant focus on marine species in various research studies such as those by Jaafar (2012) and Bakar et al. (2018). Notably, there has been a significant rise in freshwater fish DNA barcoding research, as evidenced by studies conducted by Fogelström (2015), Azmir, Esa, Amin, Yasin and Yusof (2017), and Seth, Kawamura, Abdul Kadar and Manjaji (2019). This surge in research suggests that there is a growing recognition of the urgent need for a fast and reliable monitoring procedure to assess the current diversity and distribution of freshwater fish species in Malaysia.

1.4 Research background

Malaysia boasts an impressive natural landscape featuring 150 rivers, over 95 lakes of both natural and man-made origin, and abundant freshwater swamps that harbor a diverse array of plant and animal species (Yusoff et al., 2006). Lenggong in Perak, inclusive of Tasik Raban, holds UNESCO designation as one of Malaysia's four





heritage sites (UNESCO, 2012). Tasik Raban has further been acknowledged as a strategic location for the largest breeding ground for freshwater fish, a recognition put forth by then minister Datuk Seri Ahmad Shabery Cheek in 2017 (Dolasoh, 2017). Desa and Aidi (2013) note a rising trend in developments in the area, encompassing housing projects, park construction, and modernization efforts, which may significantly impact the fish population within Tasik Raban. Traditionally, Tasik Raban has been a vital source of sustenance and economic significance for the local community.

To the best of our knowledge, two research studies have been conducted to determine the fish diversity in Tasik Raban. The initial study, carried out by Khaw Hui Leng in 2008, utilized a morphological approach for the identification of freshwater fish in Tasik Raban. In this research, a total of 10 families, 17 genera, and 20 species were successfully identified. Subsequently, Desa and Aidi (2013) revisited and expanded upon Khaw Hui Leng's work by conducting another study on Tasik Raban's fish diversity. Desa and Aidi (2013) continued to employ a morphological approach, focusing primarily on three main features: body shape, caudal fin type, and type of mouth. During their investigation, they identified a total of nine families and 19 species.

Comparing the data from both studies, it became evident that there was a significant loss of species within the lake. Furthermore, several irregularities and identification mistakes were noted in both studies. These included the use of synonyms, spelling errors, and instances of misidentification. The findings highlight the importance of rigorous verification and consistency in taxonomic studies for accurate and reliable assessments of biodiversity.



DNA barcoding has demonstrated its robust, rapid, and reliable nature in the identification of sample species. Given the absence of prior checklists before Khaw Hui Leng's study in 2008 and Desa and Aidi's work in 2013, the understanding of freshwater fish species diversity within Tasik Raban remains relatively unclear. This study specifically targets native fish in Raban Lake, Lenggong, Perak. Samples were collected and subjected to DNA barcoding to elucidate the diversity of native fish species in Lake Raban. The primary aim of this investigation is to establish a checklist for native fishes residing in Tasik Raban. In conclusion, a comprehensive approach combining both morphological and genetic analyses will be employed to develop an exhaustive checklist outlining the freshwater fish diversity in Tasik Raban.

1.5 Problem statement

Fish production in Malaysia, particularly freshwater fishes, has seen an increase, reaching 1.8 million tons in 2019 (SEAFDEC., 2021). Despite this growth, concerns have arisen about the decline in fish species, especially in freshwater. According to Ng et al. (2017), the absence of a national repository and a shortage of ichthyologists have impeded the process of effective monitoring. Consequently, there has been a reliance on traditional and labor-intensive methods for monitoring fishes, which are often perceived as costly (Siegenthaler, Mastin, Dufaut, Mondal & Benvenuto, 2018; Thomsen et al., 2012; Lapointe, Corkum & Mandrak 2006; Cotter et al., 2004). The proliferation of invasive fish species adds to the challenges (Saba et al., 2020). The UNESCO world heritage site Tasik Raban encounters comparable challenges, marked



by insufficient studies and significant inaccuracies in fish identification. An evaluation is considered essential to oversee fish diversity for the purpose of conservation management.

In the education sector, there is a waning interest in STEM (Science, Technology, Engineering, and Mathematics) subjects among secondary school students in Malaysia (Shanmugam & Balakrishnan, 2017). Contributing factors to this decline include the perception of science as challenging, apprehension about low academic achievement, influence from parents and peers, and a lack of exposure to science-related careers (Nordin & Lin, 2010; Saleh, 2014). This decreasing interest poses a challenge to Malaysia's ambition of becoming a high-income developed country. The Malaysia Education Development Plan (PPPM), under the Ministry of Education (KPM), aims for a 60:40 ratio between pure science and social science students by 2025. However, achieving this goal may be hindered by the declining number of students opting for science majors.

To address the waning interest in STEM, citizen science programs are suggested, as they have proven to enhance student engagement through hands-on experiences. However, the concept of citizen science is relatively new in Malaysia, and the absence of suitable manual hampers program implementation. Current manual are criticized for being text-heavy with limited visuals and generalized techniques for sample gathering. The text underscores the need for improved strategies and modules to promote STEM education in Malaysia.





1.6 Research objectives

1. To identify and molecularly barcode fish diversity found in Tasik Raban, Lenggong, Perak using Cytochrome c Oxidase subunit I (COI) markers.
2. To molecularly catalogue fishes found in Tasik Raban, Lenggong, Perak using BOLD online system and to preserve the specimens in the Zoology Museum, Faculty Science and Mathematics, UPSI for public references.
3. To develop and validate a DNA barcoding manual for educational purposes

1.7 Research question



1. Does molecularly barcode fish diversity in Tasik Raban using Cytochrome c oxidase subunit I (COI) marker plausible?
2. Will BOLD system be a good online platform to molecularly catalogue native fishes in Tasik Raban for public references and is it important to preserve native fish's specimen found in Tasik Raban for public references in Museum Zoology, UPSI?
3. How a DNA Barcoding manual could be implemented for citizen science program?





1.8 Significance of the study

The global initiative for barcoding projects has been expanding rapidly (Kaur, 2015), yet in Malaysia, particularly for freshwater fishes, the field remains underexplored. Existing research, such as Jaafar (2012), Bakar et al. (2018), Fogelström (2015), Azmir et al. (2017), and Seth et al. (2019), supports DNA barcoding as a more robust and accurate method for sample identification. This study aims to contribute to the advancement of DNA barcoding projects, focusing on KEGA 9, which emphasizes the importance of coastal and maritime economies, given the high value of freshwater fishes to the local population. Monitoring the variety of freshwater fish in Tasik Raban is particularly crucial, as it serves as a foundation for the local socio-economic system. The proposed research addresses the challenge of limited ichthyologists for accurate fish species identification by employing DNA barcoding, which does not rely on morphological characteristics perceived as challenging to learn.

The project also plans to establish online databases showcasing fish species diversity in Tasik Raban for public reference, aiming to raise awareness about local fish diversity management. The information on taxonomy and molecular characterization gathered can further guide conservation programs, especially for depleted, unrecognized, and cryptic native species.

