

**ENVIRONMENTAL DNA (eDNA) AS A TOOL FOR
MONITORING NATIVE BELIDA SPECIES
(*Chitala lopis*) AND INTRODUCED BELIDA
SPECIES (*Chitala ornata*) IN MALAYSIAN
FRESHWATER ECOSYSTEM**

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UNIVERSITI SAINS MALAYSIA

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SPECIES (*Chitala ornata*) IN MALAYSIAN
FRESHWATER ECOSYSTEM**

by

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**Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science**

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LIST OF ABBREVIATIONS

Bp	Base pair
Ct	Threshold cycle
<i>cyt-b</i>	cytochrome <i>b</i>
ddPCR	Digital droplet PCR
DNA	Deoxyribonucleic acid
DOA	Department of Agriculture Malaysia
DOF	Department of fisheries Malaysia
eDNA	Environmental DNA
IPS	Institute of Postgraduate Studies
LOD	Limit of detection
LOQ	Limit of quantification
NGS	Next-generation sequencing
ORP	Oxidation reduction potential
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
TDS	Total dissolved solids
USM	Universiti Sains Malaysia
UV	Ultraviolet

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**DNA PERSEKITARAN (eDNA) SEBAGAI ALAT PEMANTAUAN SPESIS
BELIDA ASLI (*Chitala lopis*) DAN SPESIS BELIDA DIPERKENALKAN
(*Chitala ornata*) DALAM EKOSISTEM AIR TAWAR MALAYSIA**

ABSTRAK

Penggunaan kaedah DNA persekitaran (eDNA) menawarkan pelbagai kelebihan dari segi kos, masa, dan tenaga kerja dalam memantau spesies, sama ada spesies yang jarang ditemui atau spesies asing. Namun begitu, sebahagian besar kajian eDNA setakat ini hanya tertumpu pada persekitaran beriklim sederhana. Manakala, kajian eDNA untuk memantau organisma tropika, khususnya ikan air tawar, masih terhad dan kekurangan prosedur yang memenuhi piawaian. Dalam tesis ini, satu kaedah eDNA berasaskan qPCR telah digunakan untuk mengesan dua spesies ikan air tawar dari genus *Chitala* (famili Notopteridae) atau dikenali sebagai ‘ikan belida’ oleh masyarakat tempatan. *Chitala lopis* ialah spesies asli di Semenanjung Malaysia yang agak sukar untuk ditemui, manakala *Chitala ornata* merupakan spesies asing di rantau ini yang berkemungkinan besar boleh mengancam ekosistem tempatan. Dalam kajian ini, sepasang primer spesies-spesifik digandingkan dengan kaedah pengesanan berasaskan probe telah direka untuk mengesan *C. lopis*. Manakala, sepasang primer spesies-spesifik digandingkan dengan kaedah pengesanan berasaskan SYBR green yang dihasilkan oleh Osathanunkul & Minamoto (2020) telah digunakan untuk mengesan *C. ornata*. Beberapa langkah pengesanan telah dilaksanakan untuk menguji spesifisiti dan sensitiviti kedua-dua kaedah. Sampel air telah diambil dari dua sungai, iaitu Sungai Perak bagi mengesan *C. lopis* dan Sungai Perai bagi mengesan *C. ornata*. Di samping itu, beberapa parameter persekitaran di setiap lokasi telah direkodkan untuk mengenal pasti impak dan kesan parameter tersebut terhadap tahap pengesanan eDNA.

Secara keseluruhannya, eDNA bagi *C. lopis* dan *C. ornata* berjaya dikesan di lokasi kajian masing-masing. Akan tetapi, kadar pengesanan adalah rendah daripada apa yang dijangkakan berbanding pemerhatian secara kasar terhadap kedua-dua spesimen, manakala eDNA yang tidak dapat dikesan di beberapa tapak pensampelan air telah dihipotesiskan sebagai negatif palsu. Beberapa faktor yang boleh mempengaruhi kadar pengesanan eDNA telah dibincangkan dalam kajian ini, termasuklah sensitiviti qPCR, faktor persekitaran tropika (khususnya agen pencegah PCR, suhu air dan kadar sinaran UV), dan tingkah laku migrasi yang berkait dengan pembiakan haiwan. Kesemua faktor ini berkemungkinan menyumbang kepada pengurangan kadar pengesanan eDNA. Tambahan pula, saiz populasi bagi setiap spesies juga telah dikenal pasti sebagai faktor penting dalam mempengaruhi kadar pengesanan eDNA. Meskipun kaedah eDNA menawarkan pelbagai kelebihan, kajian ini merungkai bahawa terdapat pelbagai faktor yang boleh mempengaruhi dan menjejaskan tahap pengesanan kaedah eDNA.

**ENVIRONMENTAL DNA (eDNA) AS A TOOL FOR MONITORING
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ABSTRACT

The use of environmental DNA (eDNA) methods offers significant advantages in terms of cost, time and labour for monitoring rare and introduced species. However, most of the eDNA studies to date have been conducted in temperate environments, while eDNA-based applications for monitoring tropical organisms, particularly freshwater fishes remain limited and lack standardised procedures. In this study, qPCR-based eDNA methods were used to detect the presence of two freshwater fish species from the genus *Chitala* (family Notopteridae), locally known as ‘ikan belida’. *Chitala lopis* is native to Peninsular Malaysia but is rare, whereas *Chitala ornata* has been introduced in this region and may represent a threat to local ecosystems. A species-specific pair of primers incorporated with a probe-based assay was designed to detect *C. lopis*, whereas a pair of primers designed by Osathanunkul & Minamoto (2020) coupled with SYBR green-based assay was used for detecting *C. ornata*. A series of validation steps were included to test the specificity and sensitivity of each method. Environmental water samples were collected from the two rivers where either species was reported, namely the Perak River for *C. lopis* and the Perai River for *C. ornata*. The possible impacts of several environmental factors on eDNA detection were evaluated by recording environmental parameters at each sampling site. Overall, eDNA of each species was successfully detected at their respective locations, but not at all sampling sites. Detection rates were lower than expected based on specimen observations, and the absence of detection at some sites was hypothesised to

represent false negatives. Several factors influencing detection are discussed, including qPCR sensitivity, tropical environmental conditions (particularly PCR inhibitors, water temperature, and UV insolation), and migratory behavior related to reproduction. These factors likely contributed to the reduced detection rates. Population size of each species is also identified as an important parameter. While eDNA methods offer distinct advantages for species detection, this study revealed that various causes influence and may jeopardize qPCR-based eDNA detections.

CHAPTER 1

GENERAL INTRODUCTION

1.1 General Introduction

Located within one of the world's biodiversity hotspots, Malaysia is mega(bio)diverse, hosting a significantly remarkable species diversity (Myers et al., 2000). For example, Malaysia is home to more than 2,000 fish species, with about 500 species inhabiting freshwater habitats, about 100 species residing only in brackish water, and around 1,500 species found in marine ecosystems (Chong et al., 2010). However, due to the rapid development in this country, many fish species face frequent threats from habitat degradation to overfishing (Chong et al., 2010; Hashim et al., 2012; Zainal Abidin et al., 2022). Freshwater fishes are particularly vulnerable, given their close proximity to human activities (Chong et al., 2010). Furthermore, the spread of invasive alien species (IAS) has also become a growing concern for native fish populations in Malaysia. Over 20 species of alien freshwater fishes are classified as invasive, most of which were introduced through the aquarium trade and aquaculture sector (Chong et al., 2010; DOA, 2021). The presence of invasive species threatening the native ecosystems either through predation, competition for resources, and introduction of pathogens (Pyšek et al., 2020). Consequently, these factors lead to the declining population of several native species (Chong et al., 2010).

Given the current situation in Malaysia, robust monitoring efforts and management strategies are necessary for addressing the declining populations of native species, which are becoming increasingly rare. Simultaneously, early intervention strategies are needed to assess the spread of IAS across the country. However, monitoring both native and alien species using conventional methods, such as seine netting, electrofishing or trapping, can be difficult, particularly for elusive species that

inhabit complex aquatic systems (Ficetola et al., 2008; Pilliod et al., 2013; Smart et al., 2015; Takahara et al., 2012). Conventional methods are often invasive, labour-intensive and time-consuming, making them of limited interest for rapid and large-scale monitoring efforts (Ficetola et al., 2008; Pilliod et al., 2013; Smart et al., 2015; Takahara et al., 2012).

Such limitations of conventional methods can be addressed with the advancement of molecular techniques, particularly through environmental DNA (eDNA) methods. eDNA methods involve the collection and analysis of DNA traces shed by organisms into their surrounding environment, allowing species detection without the need for physical capture or direct observation (Ficetola et al., 2008; Pilliod et al., 2013; Takahara et al., 2012; Thomsen et al., 2012). These techniques are gaining popularity in biodiversity monitoring as a promising tool for detecting elusive or rare species due to their higher sensitivity, non-invasive and reduced labour requirements, making them ideal tools for supporting effective conservation strategies and rapid invasive species management (Ficetola et al., 2008; Pilliod et al., 2013; Takahara et al., 2012; Thomsen et al., 2012).

However, the vast majority of eDNA studies are concentrated in temperate regions, leaving tropical regions underexplored and lacking standard procedures for using eDNA methods (Huerlimann et al., 2020). To date, only a few studies have demonstrated the potential of eDNA methods for species detection in Southeast Asia, most of them focused on endangered species, such as the Mekong giant catfish in Cambodia, Lao PDR and Thailand (Eva et al., 2016; Osathanunkul, 2022), and the roughback whipray in Peninsular Malaysia (Lim & Then, 2022). These studies have shown that eDNA methods are promising for detecting endangered species. Regardless

of the species status, further research is needed to evaluate the potential of eDNA methods in tropical environments, particularly in Peninsular Malaysia.

In Peninsular Malaysia, the giant featherback (Teleostei; Notopteridae; *Chitala lopis*), locally known as ‘belida’, is one of the largest freshwater fish in Peninsular Malaysia (Fricke et al., 2024; Robert, 1992). This species is primarily found in major river basins in the central and northern regions of Peninsular Malaysia, particularly in the largest basins, the Perak and Pahang Rivers (Hashim et al., 2012; Rashid et al., 2015). Sadly, the populations of *C. lopis* are under threat likely due to habitat degradation and overfishing (Amang, 2020a). Monitoring elusive species using conventional methods can be challenging, especially in remote locations that are logistically difficult to access (Ficetola et al., 2008; Pilliod et al., 2013; Takahara et al., 2012; Thomsen et al., 2012). Therefore, eDNA methods have the potential to serve as effective monitoring tools for *C. lopis* in Malaysia.

Another conservation concern comes from the introduction of the clown featherback (Teleostei; Notopteridae; *Chitala ornata*), a non-native featherback, into Peninsular Malaysia (Chong et al., 2010). This introduced species has been recognised as a threat in various countries, including North America (Invasive.org, 2018), Sri Lanka (Kumudinie & Wijeyaratne, 2005), and the Philippines (Cagauan, 2008). Such reputation was given to *C. ornata* because of their voracious behaviours, preying on native species and disturbing the ecosystems in these countries. Given these ecological threats, early detection and monitoring of *C. ornata* in Peninsular Malaysia is vital for assessing its spread and mitigating its potential impacts on native ecosystems. Still, using conventional methods might be impractical for the rapid detection of *C. ornata* (Ficetola et al., 2008; Pilliod et al., 2013; Takahara et al., 2012; Thomsen et al., 2012).

In this context, eDNA methods offer a promising alternative for detecting *C. ornata* where it has been introduced in Malaysia.;

1.2 Problem Statement

The population of native fish species in Malaysia is vulnerable due to many factors, including habitat degradation, overfishing, and the presence of invasive species. Among the native species, the giant featherback (Teleostei; Notopteridae; *Chitala lopis*) is one of the largest freshwater fish in Peninsular Malaysia that are under threat (Amang, 2020a; Fricke et al., 2024; Robert, 1992). *Chitala lopis* is primarily found in the largest and complex river basins in the central and northern regions of Peninsular Malaysia, particularly in the Perak and Pahang Rivers (Hashim et al., 2012; Rashid et al., 2015). Monitoring efforts are necessary to preserve this species but it can be challenging to execute, particularly in remote and complex ecosystems, such in the Perak and Pahang Rivers. Additionally, monitoring using conventional methods, such as seine netting, electrofishing or trapping, can be challenging (Ficetola et al., 2008; Pilliod et al., 2013; Smart et al., 2015; Takahara et al., 2012).

Another conservation concern comes from the introduction of the clown featherback (Teleostei; Notopteridae; *Chitala ornata*), a non-native featherback, into Peninsular Malaysia (Chong et al., 2010). This introduced species has been recognised as a threat in various countries, including North America (Invasive.org, 2018), Sri Lanka (Kumudinie & Wijeyaratne, 2005), and the Philippines (Cagauan, 2008). Given the impacts that had arisen in different countries, early detection and monitoring of *Chitala ornata* is crucial for assessing its spread, and simultaneously mitigating its potential threats on native ecosystems in Peninsular Malaysia. Since time play a crucial role in the spread of *Chitala ornata*, a rapid and practical tool is necessary for detecting

the distribution of this invasive species. However, similar to *Chitala lopis*, using conventional methods might be impractical for rapid detection of *Chitala ornata*.

Given the context of both species, monitoring both native and alien species using conventional methods, such as seine netting, electrofishing or trapping, can be challenging, especially for species that inhabit complex ecosystems (Ficetola et al., 2008; Pilliod et al., 2013; Smart et al., 2015; Takahara et al., 2012). These limitations can be addressed with the advancement of molecular techniques, particularly through environmental DNA (eDNA) methods. eDNA methods involve the collection and analysis of DNA traces shed by organisms into their surrounding environment, allowing species detection without the need for physical capture or direct observation (Ficetola et al., 2008; Pilliod et al., 2013; Takahara et al., 2012; Thomsen et al., 2012). These techniques are gaining popularity in biodiversity monitoring as a promising tool for detecting elusive or rare species due to their higher sensitivity, non-invasive and reduced labour requirements, making them ideal tools for effective conservation strategies and rapid invasive species management (Ficetola et al., 2008; Pilliod et al., 2013; Takahara et al., 2012; Thomsen et al., 2012).

1.3 Objectives

The primary aim of this study is to utilise eDNA methods as detection tools for native and introduced fish species in Malaysia. Two species from the genus *Chitala*, namely *Chitala lopis* and *Chitala ornata*, were selected as focal species due to their respective statuses as native and introduced species in Malaysia. Locally, these species are known as ‘belida’. The precise objectives of this thesis are:

1. To develop and test a species-specific eDNA method incorporating with a qPCR-based approach for detecting the rare native belida, the giant featherback (*Chitala lopis*), in Peninsular Malaysia.
2. To apply a standard species-specific eDNA method coupled with a qPCR-based approach for early detection of the introduced belida, the clown featherback (*Chitala ornata*) in Peninsular Malaysia.

1.4 Thesis Outline

This thesis is composed of five chapters. Chapter 1 is the general introduction, providing a brief overview and outlining the objectives of the study.

Chapter 2 is the literature review, providing comprehensive information on both previous and current research related to environmental DNA methods and the focal species of this study.

Chapter 3 addresses the first objective, focusing on the development and testing of a species-specific eDNA method to detect the giant featherback (*Chitala lopis*) in Peninsular Malaysia. A species-specific eDNA method incorporated with a qPCR-based approach was designed and validated through a series of tests, including *in silico*, *in vitro* and aquarium experiments. Then, field testing of the eDNA method was conducted at the Perak River, Peninsular Malaysia, a basin where *C. lopis* is known to inhabit. Several environmental parameters were measured to assess their impacts on eDNA detection. The outcomes are discussed in this chapter.

Chapter 4 addresses the second objective, focusing on the application of an eDNA method for early detection of the clown featherback (*Chitala ornata*) in Peninsular Malaysia. A standard species-specific eDNA method coupled with a qPCR-

based approach was adapted from a previous study to detect *C. ornata*. A series of tests of *in silico* and *in vitro* validations were performed on the method. Then, the method was used to monitor *C. ornata* in the Perai River, Peninsular Malaysia. This river was selected based on information of *C. ornata* sightings. Several environmental parameters were also measured to evaluate their impacts on eDNA detection. The outcomes of the method are further discussed in this chapter.

Chapter 5 summarises the outcomes from the previous chapters. Key findings related the study were highlighted, and several recommendations for future research on species-specific eDNA method for detecting fish species in tropical environments are suggested.

CHAPTER 2

LITERATURE REVIEW

2.1 The Environmental DNA (eDNA) Methods

2.1.1 Definition and description of environmental DNA (eDNA)

Environmental DNA, abbreviated as eDNA, is the complex mixture of deoxyribonucleic acid (DNA) fragments that persists in the environment, released from organisms living there, either through cell shedding, cell lysis, faeces, mucous or by cellular secretion and excretion (Ficetola et al., 2008; Jerde et al., 2011; Pilliod et al., 2013; Taberlet et al., 2012; Thomsen et al., 2012). eDNA may reside in different states (Kirtane et al., 2023; Mauvisseau et al., 2022): either in the states of (1) dissolved DNA which is a highly soluble extraorganismal DNA in aqueous solution; (2) as a particle-absorbed DNA which is DNA bounded to suspended particles; (3) as an intracellular DNA which is encapsulated DNA in cells; or (4) in the state of intraorganellar DNA which is encapsulated DNA in organelles (i.e., mitochondria or nucleus). eDNA can persist for a certain period of time in the environment depending on various factors, such as the size of the DNA fragments (Suter et al., 2023), the different states of the eDNA (Kirtane et al., 2023; Mauvisseau et al., 2022), the possibility of transportation outside the ecosystem (Goldberg et al., 2016; Ramírez-Amaro et al., 2022; Wacker et al., 2019), environmental physicochemical conditions, and some biological factors such as microbiological activities (Buxton et al., 2017; Doi et al., 2017; Eichmiller et al., 2016; Huerlimann et al., 2020; Itakura et al., 2019; Kirtane et al., 2022; Loeza-Quintana et al., 2021; McCartin et al., 2022; Seymour et al., 2018; Strickler et al., 2015; Tarof et al., 2021). With the advancement of molecular techniques, eDNA, even in low quantity, can be extracted and detected using specific methods.

2.1.2 eDNA methods

Environmental DNA (eDNA) methods involve the use of DNA traces extracted from environmental samples (e.g., water or soils) to detect single species or to assess species diversity within ecosystems (Ficetola et al., 2008; Pilliod et al., 2013; Takahara et al., 2012; Thomsen et al., 2012). The general framework for eDNA methods typically includes identifying the target organism(s), designing primer/probe, and conducting field validation (Bruce et al., 2021; Harper et al., 2021; Langlois et al., 2021; Ramírez-Amaro et al., 2022; Wilson et al., 2014) (Figure 2.1).

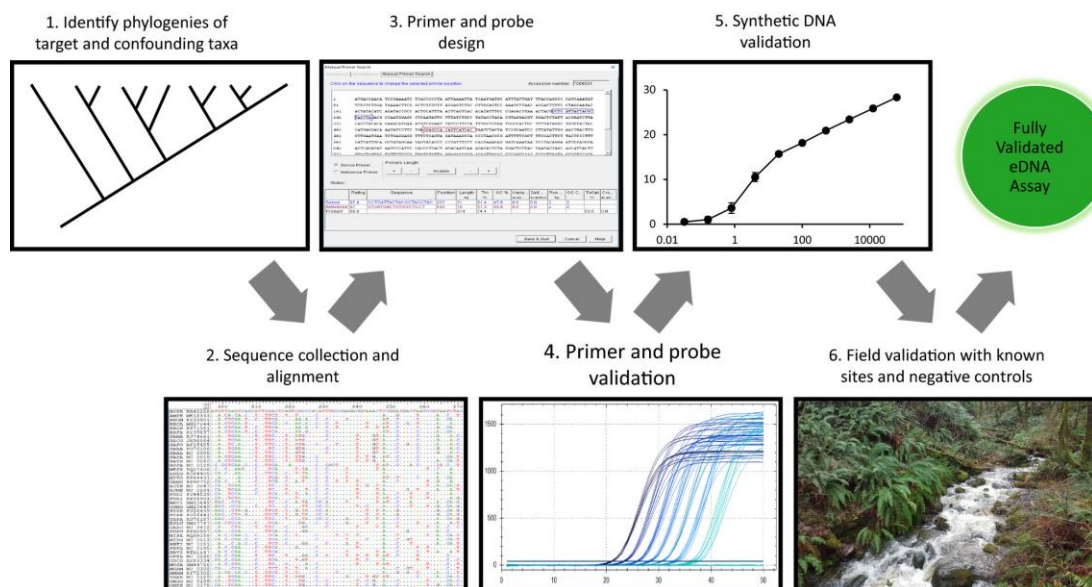


Figure 2.1 An example of a general workflow for eDNA assay design and validation (from Langlois et al., 2021).

Current eDNA methods are divided into two categories (Deiner et al., 2017; Minamoto, 2022; Ramírez-Amaro et al., 2022; Tsuji et al., 2019): (1) species-specific eDNA methods, which typically employ Polymerase Chain Reaction (PCR)-based techniques (i.e., end-point PCR, quantitative Polymerase Chain Reaction (qPCR) techniques or ddPCR), and (2) eDNA metabarcoding, also referred to as ‘multi-species

or -taxa eDNA methods', which implements New Generation Sequencing (NGS)-based techniques. The differences among these eDNA methods are listed Table 2.1.

Table 2.1 Differences among qPCR- and NGS-based eDNA methods (from Huerlimann et al., 2020; Langlois et al., 2021; Tsuji et al., 2019).

Feature	Quantitative PCR (qPCR) eDNA-based methods	Next Generation Sequencing (NGS) eDNA-based methods
Target	Single species or small group of species.	Generally multiple species within a taxonomic group simultaneously (although a single species is targetable as well).
Output	Detects presence/absence and provides a relative measure of DNA quantity by indicating the cycle number (Cq) at which fluorescence is detectable.	Species richness and community composition. Provides an estimate of the total biodiversity from a single sample.
Sensitivity	High sensitivity but can be susceptible to inhibitors in the environmental sample that reduce accuracy.	Can be less sensitive than species-specific PCR for detecting rare or low-abundance species due to competition among primers.
Cost	Less expensive per sample than metabarcoding.	More expensive than qPCR (high-throughput sequencing costs and bioinformatics).
Interpretation	Requires a standard curve for quantification and can be affected by inhibitors, making comparisons between different studies difficult.	Results are qualitative (presence/absence) or semi-quantitative (relative read counts), and can be influenced by primer bias, making abundance estimates complex.
Use Case	Monitoring a specific species of interest (e.g., invasive or endangered).	Broad biodiversity assessments, discovering cryptic species, and understanding whole-community structure.
Limitations	Environmental factors can affect results. Provides relative quantification.	Results are affected by primer bias and incomplete reference databases. Expensive and complex analysis.

While both eDNA approaches can provide valuable insights into the presence of species within a habitat, qPCR-based methods are often more practical and efficient in detecting individual species compared to NGS-based methods. This is because qPCR-based approaches are typically more-cost efficient, primarily due to the relatively affordable consumables, accessible equipment and the less technological complexity needed to operate (Harper et al., 2018; Minamoto, 2022; Tsuji et al., 2019; Westfall et al., 2022). Furthermore, qPCR-based approaches exhibit higher sensitivity in quantifying low amounts of specific target DNA, making them particularly effective for species-specific monitoring compared to NGS-based approaches (Harper et al., 2018; Minamoto, 2022; Westfall et al., 2022; Yu et al., 2022).

2.1.3 Application of species-specific eDNA methods

The applications of species-specific eDNA methods (either using end-point PCR or qPCR techniques) are rapidly increasing, especially in the field of conservation biology. These applications include detecting endangered and rare species, such as angel shark (Faure et al., 2023), European weather loach (Brys et al., 2021), freshwater Murray cod and euryhaline mullet (Wilkes Walburn et al., 2022), as well as monitoring invasive species like guppy (Smith et al., 2022), armoured catfish (Dubreuil et al., 2022), chain pickerel and smallmouth bass (Loeza-Quintana et al., 2021).

Besides, the rapid increase in species-specific eDNA applications is also driven by the effectiveness of the methods in detecting target species compared to conventional methods (e.g., seine netting, electrofishing, trapping and visual observation) (Ficetola et al., 2008; Pilliod et al., 2013; Smart et al., 2015; Takahara et al., 2012). Species-specific eDNA methods, particularly the qPCR-based approaches,

are often preferred because they are more sensitive, time-saving and less-labour than conventional methods, especially for surveying aquatic species in a complex riverine system. For instance, a study on Japanese eels (*Anguilla japonica*) in Japan river basins demonstrated that qPCR-based eDNA method is more sensitive than electrofishing method in detecting this endangered eel species (Itakura et al., 2019). The qPCR-based eDNA method has also proven effective for monitoring aquatic invasive species (AIS), such as the armoured catfish (*Hypostomus robinii*), outperforming traditional trapping method (Dubreuil et al., 2022). Additional, study on invasive European newt (*Lissotriton vulgaris*) demonstrated that qPCR-based eDNA method requires significantly less sampling efforts than trapping method (Smart et al., 2015).

Despite numerous applications in all world regions, the majority of eDNA studies have predominantly concentrated in temperate regions (Huerlimann et al., 2020). In contrast, such studies remain underexplored in tropical regions, possibly due to various reasons including 1) the synergistic effects of environmental conditions specific to tropical ecosystems that might influence eDNA detection, 2) a lack of comprehensive species assemblage database, 3) logistical issues in accessing sampling locations, as well as 4) insufficient capacity and resources for research (Cantera et al., 2019; Huerlimann et al., 2020; Zainal Abidin et al., 2022). For instance, environmental conditions of tropical ecosystems, such as its temperature, pH, or UV exposure may influence eDNA persistence, either directly or indirectly (see subchapter 2.2.2). Furthermore, a poor database on the species diversity in the tropical ecosystems may prevent a comprehensive development of eDNA method, especially when it comes to elusive or rare species that require one to use a specific genetic marker or method for detection these species. Besides that, the equipment and tools for an eDNA research expedition might become a logistic issue, particularly in a remote and challenging

location in a tropical region. Additionally, some research institutions in tropical regions lack the capacity and resources to perform eDNA study, in which some of them may not have a proper lab to perform a basic molecular work, let alone a lab specifically for eDNA research.

Despite these situations, a few studies in Southeast Asia have demonstrated the potential of using species-specific eDNA methods to detect endangered species, such as the Mekong giant catfish (Eva et al., 2016; Osathanunkul, 2022) and the roughback whipray (Lim & Then, 2022), as well as to monitor economically important fish like the clown featherback in Thailand (Osathanunkul & Minamoto, 2020). Although such studies are significant contributions to the field of eDNA research in tropical Asia, most of them did not assess potential factors that could affect the eDNA detectability. Therefore, there is an urgent need for research tailored to tropical ecosystems, focusing on protocol optimisation and the development of standardise guidelines that account for local environmental variability and species diversity. Addressing these challenges would enhance the applicability and robustness of eDNA methods in tropical biodiversity monitoring and conservation.

2.2 Potential factors influencing eDNA detection

eDNA methods are said to be useful for detecting elusive and/or rare species that are challenging to detect via conventional methods. However, the detectability of eDNA can be attributed to several key factors including: (1) the sensitivity of the methods, (2) the effects of environmental parameters on eDNA persistence, and (3) the role of biological aspects on eDNA production and degradation. Understanding the impact of these potential factors is crucial for accurate and reliable interpretation of the results.

2.2.1 Sensitivity and efficiency of eDNA methods

The sensitivity and reliability of eDNA methods are among the primary factors that significantly influence eDNA detectability (Goldberg et al., 2016; Thalinger et al., 2021). Detection thresholds, specifically the limit of detection (LOD) and limit of quantification (LOQ), are often used for ensuring the reliability and sensibility of assays of eDNA methods, such as qPCR, when monitoring rare species presence (Klymus et al., 2020; Mauvisseau et al., 2019). The LOD refers to the lowest concentration of eDNA that can be confidently distinguished from absence, whereas the LOQ represents the lowest concentration that can be measured with acceptable accuracy and precision. A common way to determine these two thresholds is by establishing a standard curve (also referred to as a calibration curve). Typically, this curve can be generated through a serial dilution of known amounts of target DNA (e.g., genomic DNA or synthetic DNA), which is then quantified using qPCR-based techniques (see Bustin et al., 2009). The components of the standard curve, such as the linear regression line, amplification plot, and coefficient of variation are some of criteria that can be used to define the detection thresholds (Bustin et al., 2009; Klymus et al., 2020; Mauvisseau et al., 2019).

However, it is important to note that the definitions of LOD and LOQ may vary among eDNA studies depending on the implemented methods. For instance, Mauvisseau et al. (2019) defined their LOD as the last standard dilution that yielded positive detection of the targeted DNA in at least one qPCR replicate with a threshold cycle (also referred to as quantification cycle) below 45, and they defined their LOQ as the last standard dilution that yielded positive detection of the targeted DNA in at least 90% of qPCR replicates with a threshold cycle below 45. In contrast, Klymus et al. (2020) defined the LOD as the lowest standard concentration that yielded at least 95%

positive replicates, and the LOQ as the lowest standard concentration that can be quantified with a coefficient of variation below 35%. Another differences between these two studies lies in how they measured the LOD and LOQ, in which Mauvisseau et al. (2019) reported their results in terms of ng/ μ L, whereas Klymus et al. (2020) reported in copies per reaction. Regardless of the specific definitions, establishing detection thresholds is essential for assessing the potentials and limitations of eDNA method.

2.2.2 Environmental factors

Environmental parameters may influence eDNA persistence either directly, by catalysing the eDNA degradation or indirectly, by reducing the efficiency of the eDNA methods (Eichmiller et al., 2016; Loeza-Quintana et al., 2021; McCartin et al., 2022; Seymour et al., 2018; Strickler et al., 2015; Tarof et al., 2021). Ambient temperature and UV (ultraviolet) exposure are among the important parameters that can affect eDNA persistence. According to Strickler et al. (2015), warmer temperature ($> 25\text{ }^{\circ}\text{C}$) can accelerate eDNA degradation, thereby reducing eDNA detectability. This finding was further corroborated by subsequent studies in a lake environment (Eichmiller et al., 2016) and seawater (McCartin et al., 2022). Strickler et al. (2015) also showed that high exposure of UV irradiance, especially the UV-B radiation is positively correlated with eDNA degradation. In the context of tropical regions, these factors are particularly influential due to consistent warm temperature and high UV irradiation throughout the year (Huerlimann et al., 2020).

The eDNA persistence and degradation dynamics can be influenced by the pH variation. Through hydrolysis processes, eDNA is more prone to destabilisation and degradation in acidic conditions compared to alkaline or neutral conditions (Seymour et al., 2018; Strickler et al., 2015). Furthermore, pH variations can influence microbial composition in the environment, with microbial activity indirectly affecting eDNA

persistence dynamics through biochemical mechanisms as microbes consume environmental particulates (Mauvisseau et al., 2022; Seymour et al., 2018; Strickler et al., 2015).

Natural environments often contain various substances, such as organic compounds, sediments, or other pollutants (Schrader et al., 2012). These substances may reduce the efficiency of eDNA capture and inhibit the downstream PCR reactions, thereby affecting eDNA detection reactions (Takasaki et al., 2021; Troth et al., 2020; Uchii et al., 2019). In aquatic environments, the levels of these substances are usually measured in terms of total dissolved solids (TDS). High levels of TDS can cause filter clogging which reduces eDNA capture efficiency, and may introduce various PCR inhibitors that interfere with PCR reactions (Buxton et al., 2017; Tarof et al., 2021).

2.2.3 Biological factors

eDNA detection is also strongly influenced by biological factors, including the quantity of DNA released into the environment and the distribution dynamics of eDNA (Doi et al., 2017; Itakura et al., 2019; Kirtane et al., 2022; Nordstrom et al., 2023). Detection probability is found to be positively correlated with the concentration of eDNA that persists in the environment (Doi et al., 2017; Itakura et al., 2019; Kirtane et al., 2022; Nordstrom et al., 2023). The amounts of eDNA released into the environment is dependent on the biomass, physiological traits and metabolic patterns of a species (Nordstrom et al., 2023). Species that are more abundant and have larger biomass typically release more eDNA, thereby resulting in greater eDNA detection rates (Doi et al., 2017; Itakura et al., 2019; Kirtane et al., 2022). Species that are metabolically active also tend to release more DNA into the environment (Nordstrom et al., 2023).

The eDNA distribution dynamics can be significantly influenced by the behavioural patterns of target species, such as their reproductive behaviours and habitat preferences (Doi et al., 2017; Itakura et al., 2019; Nordstrom et al., 2023). For example, Doi et al. (2017) recorded the highest eDNA concentration of ayu fish (*Plecoglossus altivelis*) at a site with active spawning behaviours. They speculated that the elevated eDNA readings were due to the release of sperm and eggs into the water during reproductive activities, suggesting that spawning behaviours influence eDNA detection. Moreover, Itakura et al. (2019) reported that higher eDNA concentrations of Japanese eels (*Anguilla japonica*) were recorded near the river mouth, locations where the eels are more abundant and prefer to inhabit. This indicates habitat preferences of a target species can also significantly influence eDNA detection rates.

2.3 General descriptions and information of studied species

2.3.1 Characteristics and classification of *Chitala lopis* and *Chitala ornata*

Chitala lopis (Bleeker, 1851a) and *Chitala ornata* (Gray, 1831) are large freshwater fishes that belong to the family Notopteridae Bleeker, 1851b (Teleostei: Order Osteoglossiformes) (Fricke et al., 2024) (Figure 2.2). The family Notopteridae consists of four valid genera, namely *Chitala*, *Notopterus*, *Papyrocranus*, and *Xenomystus*, with currently 11 valid species (Barby et al., 2019; Fricke et al., 2024; Hilton & Lavou  , 2018; Nelson et al., 2016; Robert, 1992). Morphologically, notopterids are characterised by their extremely laterally compressed body and long anal fin that confluent seamlessly with the caudal fin (Hilton & Lavou  , 2018). Among the genera, adult individuals of the genus *Chitala* Fowler, 1934 are distinctly recognised by their prominent dorsal concavity above the head, a morphological feature that developed from ontogenetic changes (Hilton & Lavou  , 2018).

Within the genus *Chitala*, the species *Chitala lopis* and *Chitala ornata* can be distinguished from one another by their distinct marking pattern (Figure 2.2). *Chitala lopis*, known as the giant featherback, lacks prominent marking except, sometimes, for one dark spot at the base of its pectoral fins (Robert, 1992). In contrast, *Chitala ornata*, commonly known as the clown featherback has distinct markings of large ocellated black spots above the anal fins on each side of its body (Kottelat & Widjanarti, 2005; Robert, 1992). In terms of size, *Chitala lopis* is considered as the largest species within the genus *Chitala*, reaching up to 1.5 m in standard length and 15 kg in weight, whereas *Chitala ornata* attains a maximum size of 1 m (Kottelat & Widjanarti, 2005; Robert, 1992).

Table 2.2 The Taxonomic classification of *Chitala lopis* and *Chitala ornata* based on Fricke et al. (2024).

Kingdom	: Animalia
Phylum	: Chordata
Class	: Actinopterygii
Infraclass	: Teleostei
Order	: Osteoglossiformes
Family	: Notopteridae Bleeker, 1851b (featherbacks knifefishes)
Subfamily	: Notopterinae Bleeker, 1851b (Asian featherbacks)
Genus	: <i>Chitala</i> Fowler, 1934
Species	: <i>Chitala lopis</i> Bleeker, 1851a (Giant featherbacks) <i>Chitala ornata</i> Gray, 1831 (Clown featherbacks)



Figure 2.2 Photo of specimens of *Chitala lopis* (top) and *Chitala ornata* (bottom). Retrieved from Ruzman et al. (2024).

2.3.2 Habitat distribution and conservation status of *Chitala lopis*

Chitala lopis is widely distributed across major river basins in Southeast Asia. Its range includes the Musi and Kampar Rivers in Indonesia (Anjarsari et al., 2021; Robert, 1992; Wibowo et al., 2010), the Mekong and Chao Phraya Rivers in Thailand (Musikasinthorn & Ngamtampong, 2022), as well as the Perak and Pahang Rivers in Peninsular Malaysia (Hashim et al., 2012; Rashid et al., 2015). This species holds significant value, particularly in Indonesia, where it is categorised as an expensive fish due to its protected status, delicious taste and high protein content (Wibowo et al., 2010). In Peninsular Malaysia, the market price of *Chitala lopis* can reach up to

RM20.00 per kilogram, which is about RM100.00 to 300.00 (approximately 22 to 67 USD, according to the current currency) for a single adult fish (DOF, 2023).

Despite its wide distribution and large body size, information on the population of *Chitala lopis* in this region remains limited, especially in Malaysia. According to the International Union for Conservation of Nature Red List (IUCN 2019), *Chitala lopis* is extinct (Ng, 2020) but Ruzman et al. (2024) demonstrated this is not correct. Nonetheless, populations of *Chitala lopis* are reported to be critically declining in Sumatra, Indonesia (Anjarsari et al., 2021). Habitat degradation and overfishing are among the major factors contributing to declining and local extinction of *Chitala lopis* in Indonesia (Anjarsari et al., 2021; Wibowo et al., 2010). In Peninsular Malaysia, although the population status of this species has not been fully assessed, similar trends may be occurring in major rivers where the species has been sighted, raising significant conservation concerns.

2.3.3 Habitat distribution and invasion status of *Chitala ornata*

Chitala ornata is native to mainland Southeast Asia, with its distribution ranging from Mekong River basin to Mae Klong River basin (Poulsen et al., 2004; Robert, 1992). This species is well known for its commercial value, especially in Thailand, where it serves as a food products (Osathanunkul & Minamoto, 2020; Poulsen et al., 2004; Robert, 1992). Additionally, *Chitala ornata* is a popular ornamental fish in the aquarium trade because of its unique ocellated black spots on each side of the body (Chong et al., 2010; Poulsen et al., 2004; Robert, 1992). Due to its commercial importance, this species is frequently bred in aquaculture farms and sell in aquarium stores, catering to both local and global markets (Chong et al., 2010; Osathanunkul & Minamoto, 2020; Poulsen et al., 2004; Robert, 1992). Unintentional escapes from aquaculture farms and intentional releases from private tanks (when specimens become

too large) are likely the sources of the introduction of *Chitala ornata* into Malaysia (Chong et al., 2010; Poulsen et al., 2004; Robert, 1992).

Currently, *Chitala ornata* has been introduced in many regions of the world, including North America (Invasive.org, 2018), Sri Lanka (Kumudinie & Wijeyaratne, 2005), the Philippines (Cagauan, 2008), Singapore (Tan et al., 2020), and Malaysia (Chong et al., 2010). Among these countries, North America, Sri Lanka and the Philippines have categorised *Chitala ornata* as an invasive species due to its voracious piscivorous behaviour, which poses a significant threat to local species. In Peninsular Malaysia, although the impact of the introduction of this species has not yet been thoroughly assessed, given the documented impacts elsewhere, similar negative consequences are expected if populations of *Chitala ornata* are not properly monitored and managed.

CHAPTER 3

DEVELOPMENT OF A QPCR-BASED ENVIRONMENTAL DNA (eDNA) METHOD TO DETECT THE DECLINING POPULATION OF BELIDA, THE GIANT FEATHERBACK (NOTOPTERIDAE; *Chitala lopis*) IN MALAYSIA

3.1 Introduction

Chitala lopis (Teleostei: Osteoglossomorpha: Notopteridae) commonly known as the giant featherback or in Malay as ‘ikan belida’ is the largest species of Asian notopterids, with adult individuals reaching up to 1.5 m in standard length (Kottelat & Widjanarti, 2005; Robert, 1992). Compared to other Asian notopterids, *C. lopis* did not have distinct markings on its body except for a dark spot located at the base of the pectoral fin (Robert, 1992). This giant species is widely distributed in major river basins in Southeast Asia, including the Mekong and Chao Phraya Rivers in Thailand, the Musi and Kampar Rivers in Sumatra, Indonesia, the Kapuas, Barito and Mahakam Rivers in Kalimantan, Indonesia, as well as the Perak and Pahang Rivers in Malaysia (Anjarsari et al., 2021; Hashim et al., 2012; Musikasinthorn & Ngamtampong, 2022; Rashid et al., 2015; Robert, 1992; Wibowo et al., 2010).

Despite its large size and wide distribution, information regarding the conservation status of *C. lopis* population in Southeast Asia is very limited. To date, their population is critically declining in Sumatra, Indonesia (Anjarsari et al., 2021), and the species is even (but incorrectly) declared extinct by the International Union for Conservation of Nature (IUCN) (Ng, 2020; Ruzman et al., 2024). In Malaysia, although proper distribution of *C. lopis* populations is lacking, the dwindling catches of this species reported by local fishermen are alarming (Amang, 2020a, 2020b). Habitat degradation and overfishing are likely contributing to the decline of freshwater fish population in Malaysia (Chong et al., 2010). Robust monitoring and management strategies are necessary to sustain the population of *C. lopis* and to conserve this species

from extinction. However, monitoring aquatic species using conventional methods, such as seine netting, electrofishing or trapping can be invasive, time-consuming and laborious, particularly for rapid and intensive surveys of dwindling species that inhabit complex riverine systems (Ficetola et al., 2008; Pilliod et al., 2013; Smart et al., 2015; Takahara et al., 2012).

Such limitations of conventional methods can be addressed by using an eDNA method, which involves the use of DNA traces in the environment to detect the species of interest (Ficetola et al., 2008; Pilliod et al., 2013; Takahara et al., 2012; Thomsen et al., 2012). In Southeast Asia, only few studies have been conducted for detecting rare and endangered aquatic fish species from their eDNA, such as the Mekong giant catfish in Thailand (Eva et al., 2016; Osathanunkul, 2022), and the roughback whipray in Malaysia (Lim & Then, 2022). Collectively, these case studies have shown the possibility to use eDNA methods for detecting rare tropical freshwater fish species in Southeast Asia. However, it is still unclear whether these methods are universally (relative to taxa and environments) applicable. eDNA methods are fast and sensitive compared to conventional methods in detecting species, but the outcome may also be influenced by environmental factors such temperature, pH, total dissolved solids, and the presence of pollutants (Goldberg et al., 2016; Huerlimann et al., 2020; Minamoto, 2022; Ramírez-Amaro et al., 2022). So far, most eDNA studies in tropical regions have not evaluated the impact of such parameters on eDNA detection (Huerlimann et al., 2020).

In this working chapter, a species-specific eDNA method incorporated with qPCR-based approach was developed to detect *C. lopis* in Perak River, Peninsular Malaysia. The eDNA method was validated through a series of tests, including *in silico*,