Development of Species-Specific *Cichla* Species eDNA Primers for Rapid Alien Invasive Species (AIS) Monitoring

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Abstract. Locally known as top predator fishes, peacock bass or cichlids are invasive in Malaysia's freshwater ecosystem. Due to the nature of these fish, which involves hiding beneath the water's surface, detection probability for these species are often poor, especially when utilizing the traditional capture-survey method. As a result, measuring environmental DNA (eDNA) is a relatively new, non-invasive method that can be used to determine the range of these invasive fishes. Here, we outline the plan. For three selected invasive Cichla species C. kelberi, C. ocellaris, and C. monoculus it was necessary to develop small fragment (280–400 bp) specific-specific primers based on mitochondrial DNA (mtDNA) COI gene sequences. It was also necessary to determine how to extract high-quality DNA and e-DNA, as well as how to validate primers for specificity, (iv) developed and tested a new quantitative PCR (qPCR) assay to detect the presence of C. kelberi, and C. ocellaris, environmental DNA (eDNA) in water samples. The COI gene can be utilized to create species-specific primers for specific species, according to recent research. To improve the specificity of primers, a few primer-design factors must be changed. With recommendations for further references for the researcher, they were thoroughly examined. Furthermore, we discovered that e-DNA isolation must be completed within 22 days following capture in order to avoid producing false-negative results. Since just AGE (Agarose Gel Electrophoresis) analysis was required for the validation procedures, these data can be easily implemented in any fish genomics lab with a low-cost setting. Overall, eDNA analysis with species-specific primers represents a new monitoring tool in the detection of Aquatic Invasive Species (AIS) for management and conservation purpose.

Keywords: Species-specific primer · COI primer · e-DNA marker · Invasive species

M. Fadilah et al. (Eds.): IcoBioSE 2021, ABSR 32, pp. 39–50, 2023.
https://doi.org/10.2991/978-94-6463-166-1_7

1 Introduction

Being a region surrounded by water, Malaysia is not left behind in the introduction of alien species since the early 19th century [1]. Non-native or alien species is globally defined as an organism that is translocated from its natural or historical habitat, either accidentally or on purpose, and subsequently successful in residing in its new environment [2]. Most of the species introduced in Malaysia have been brought in from the Amazon River (South America) and introduced as popular game fishes usually in lakes [3]. The commonly known invasive alien fish species in the local water system are Arapaima gigas, Oreochromis mossambicus, Pygocentrus nattereri, Claria gariepinus, Belone belone, and Cichla ocellaris [4]. However, the extended invasion status of alien species in this country is still not known.

The major reason for the introduction and establishment of alien fish species in Malaysia is recreational fishing. The most prominent example is the peacock bass (Cichla sp.) which was intentionally released into rivers or lakes made from former mining areas by irresponsible anglers for sport fisheries [2]. The peacock bass is known as a good sport fish [5] but the uncontrolled spread and unintentional release of this species had caused a negative impact globally on the decline of native fish species [2]. Thus, the peacock bass was also labeled as alien invasive species (AIS) due to the threat it caused to local freshwater biodiversity. It is an urgent need to monitor AIS to preserve our native fish species but local authorities are still dependent on the common method of long-term surveys and large fish catchment. This so-called traditional field capture technique is often considered to be costly and labor-intensive [6]. Moreover, detection probabilities typically are low due to the AIS behavior that prefers to be hidden beneath the water's surface. Such fish surveillance programs also employ nets or electrofishing gear but these tools often lead to major false sightings as the target AIS species is unable to be predicted underwater [7].

Currently, an alternative and rapid new technique is known as environmental DNA (eDNA) has gained much attention by AIS researchers as it enables the detection of environmental organisms via the examination of water samples [8]. Environmental DNA (eDNA) is defined as DNA that has been released by an organism into the environment, via feces, hair, urine, skin, or, gametes [9]. This DNA can be extracted from environmental samples such as soil, water, or feces without having to isolate the target organism [10]. This DNA can be isolated from organisms that were present in a specific water sample using polymerase chain reaction (PCR) technology. eDNA also permits early detection of AIS even at very low densities, and at any life stage [10–12]. This has been proven in several studies, of which the best-known examples are on invading Asian carp in European water systems [12]. Positive feedback has been vastly reported upon the application of this method as it strongly aids in Aquatic Invasive Species (AIS) monitoring and management effort [9].

Because eDNA is still new and has not been implemented frequently in the tropical environment, especially in Malaysia, we report here our development strategy to produce species-specific primers that can be utilized to detect the peacock bass fishes by the eDNA-PCR and quantitative PCR (qPCR) methods to monitor the presence and relative abundance of eDNA.

Table 1. *Cichla sp.* Reference sequences for primer design in details with FASTA format downloaded from GenBank

No	Species Name	Accession number	Sequence length (bp)
1	*C. kelberi*	JN988796.1	636
2	*C. ocellaris*	NC_030272.1	16, 526
3	*C. monoculus*	JN988798.1	630

2 Methods

2.1 Construction of Species-Specific Primers

For this research, species-specific primers for Cichla kelberi, Cichla ocellaris, and Cichla monoculus were developed to target the mitochondrial cytochrome c oxidase (COI) genes. A primary survey among anglers showed that these three species are often been misidentified due to highly similar morphological features. Therefore, to design species-specific primers for each, Cichla sp. Reference sequences (Table 1) in the form of FASTA format were downloaded from GenBank and screened manually for any ambiguous nucleotide data. These sequences were aligned using MEGA X [13], and analyzed through BLAST [14] analysis. Using BLAST, the similarity and differentiation between retrieved sequences can be determined, and subsequently, The variable areas that were discovered served as possible targets for species-specific primer annealing. By focusing on those variable regions, hypothetical primers were created using the Primer-BLAST program [15]. We searched for primer sequence targets that differed from non-target species' primer sequences by no more than two base pairs in order to achieve species specificity. A thorough design analysis was conducted using five adjusted parameters; (1) primer must contain 18–23 nucleotides, (2) amplicon length must be between 280 to 400 bp, (3) a minimum of 2 mismatches at the 3' end, (4) primer melting and annealing temperature must be between 50 °C to 60 °C, and (5) primer GC content between 20% to 60%.

2.2 Validation of Designated Primer Through PRIMER BLAST and AGE (Agarose Gel Electrophoresis) Analyses

Using Primer Blast [15], each hypothetical primer was subjected to a specificity checking process. We followed Ye et al., 2012 default parameters [4] but changes were made to the formerly mentioned parameters. The protocol was modified as stringent as possible to avoid the unintended target. A primer pair is considered to be specific only if it has no amplicon on any targets other than the specified Cichla species template. Otherwise, it is considered non-specific.

Cichla specimens were collected and identified as correctly as possible to species level according to [16]. Each specimen was given a lab inventory ID and was photographed for future reference. Table 2 described details the specimen used. Fish DNA was extracted using DNEasy Blood and Tissue Kit (Qiagen, USA) according to the manufacturer's protocol with the modification described by Bakar et al., 2018 [17]. Eluted

DNA was quantified using UV spectrophotometer Q3000 (Quawell, USA). DNA concentration was expected to be between 10 and 200 ng/ml and the purity of DNA was in the range of 1.7–2.0 ratio of absorbance wavelength A260/A280. The final product was stored at -20 ˚C for future use. Each primer was amplified using PCR in a 25 μL reaction mixture containing 2X EconoTaq PLUS PCR Master Mix solution (Sigma-Aldrich, Germany), 0.4 μM for each forward and reverse primer, and DNA template (120ng -500 ng). A negative control reaction was included by replacing the DNA template with ddH20. PCR reactions were a thermal cycler (T100 Biorad, USA) with an amplification profile consisting of an initial denaturation step at 95 ˚C for 2 min, 35 cycle of denaturation step at 94 ˚C for 30 s, an annealing step at the temperature set in Table 2 for 45 s, an elongation step at 72 ˚C for 1 min 30 s and a final extension step at 72 ˚C for 5 min. Subsequently, a total of 5 μL of the PCR product were subjected for quality assessment by 2% Agarose Gel Electrophoresis (AGE) with 1ul GelRed dye (Sigma-Aldrich, Germany). The gel image was captured using GeneSnap software (Syngene, UK).

3 Results

3.1 Primer Design

Primer designs of C. ocellaris (CO), C. monoculus, and C. kelberi (CK) (CM) were designed C. ocellaris (CO), C. monoculus, and C. kelberi (CK) sequences collected from GenBank as listed in Table 1. Approximately 8 primer pairs were generated for C. kelberi while 5 pairs of primer have been designed for each C. ocellaris and C. monoculus species (Table 2). All designated primer pairs consist of 18 to 23 base, annealing temperature in a range between 59.00 ˚C to 60.55 ˚C, with predicted amplified product 284 base pair up to 345 base pair.

3.2 Extraction of DNA and eDNA

DNA of collected thirteen (13) specimens as listed in Table 2 were extracted. The quality and quantity of the isolated DNA were assessed by Agarose Gel Electrophoresis (AGE). All specimens showed the clear formation of the band with a size of 23 Kbp. For eDNA, Fig. 1 represents the result from AGE analysis showing the intensity of the isolated eDNA from water samples that have been filtered and extractat by 3 days intervals with an approximate size of 23 Kbp. Lesser intensity of the band was observed for samples filtered at day 22 and after.

3.3 Validation of Primer

The designated primer sets were validated using thirteen (13) collected specimens as listed in Table 3. Initially, we amplified all developed primers with C. kelberi and C. ocellaris specimens. No C. monoculus specimens were found throughout sampling CK1 and CO1 primers showed intense single-band for respective species while CM1 showed no amplification to any specimens. The latter was expected as there is no C. monoculus found. This clarified that CM1 might be a potential species-specific marker for C.

Table 2. Details of developed primer pairs for *C. kelberi* (CK), *C. monoculus* (CM) and *C. ocellaris* (CO) species

No.	Primer	Sequence Forward (F) and Reverse (R) 5'- 3'	Tm (°C)	GC Content (%)	Product size (bp)
1	CK 1	F- G C T T C T G G C TT TC TT TC C C C TT/R-A A T G G A G C G C G C A A T A A T C C C C	59.96 60.03	55.0 50.0	323
2	CK 2	F- A T T G G T G C C C C A G A C A T A G C/R- A A A G G A G A A G A A G G A C G G C G	60.11 60.04	55.0 55.0	334
3	CK 3	F- G A G C A GG A G C A G C T G A G C C A A C C T/R- C T T G C C A G T G G G G G A T A G A C	59.96 59.82	55.0 60.0	289
4	CK 4	F- T G T C T A T C C C C C C A C T G G C A A/R- C T C C C T C C T G C A G G T C A A A G	59.55 60.04	55.0 60.0	296
5	CK 5	F-/R- G C A G C A A C C C T G A C A T A G C C T T T C C C CG A A C T G G A A G G G A	59.82 60.25	60.0 55.0	345
6	CK 6	F- A C T A A G C C/R- C A A C C A G G C T C T A G G T G A A G T G A A G G T G A	59.38 59.68	55.0 55.0	344
7	CK 7	F- T C G G A G G C T T T G G G A A At T G A/R- T A T T G G G A G A G C C G G G G G	59.30 60.55	50.0 60.0	313
8	CK 8	F- C G A G C A G A A C T A A G C C A A C/R- A G C G G A G A G G G T A A C A G T T C A	59.92 60.25	55.0 55.0	284
9	CM1	F- A G T G G G A A C T G C A C T A A G C C/R- T T C C C G C T A G G G G T A T G G G G	59.96 60.40	55.00 60.00	320

(*continued*)

Table 2. (*continued*)

No.	Primer	Sequence Forward (F) and Reverse (R) 5'- 3'	Tm (°C)	GC Content (%)	Product size (bp)
10	CM2	F- T T G G T G C T T G A G C C G G A A T A/R- C C C G C T A G T G G A G G G T A G A	59.67 59.77	50.00 63.16	337
11	CM3	F- T A T T T G G T G C C G G A/R- T C C C G C T A G T G G A G G G T A G	59.67 59.77	50.00 63.16	341
12	CM4	F- T G A G C C G G A A T A G T G G G A A C/R- T C C C G C T A G T G G A	59.46 61.28	55.00 60.00	330
13	CM5	F- A G C C G G A T T T C C C G Ct A G T A G A T A G T G G G	59.46 60.96	55.00 57.14	330
14	CO1	F- G T C C T C A A T C C T T G G G G C A A/R- C C A A A T C C G	59.96 59.89	55.00 60.00	291
15	CO2	F- C T G A T C C T C C C C G G A T T T G G/R- T G T C C T C C T A C G G G A	59.89 59.96	60.00 55.00	327
16	CO3	F- C C G G T G T G T G T C C T C A A T C T T/R- A T C A T G C C A A A T C C G G G G A G	59.68 60.18	55.00 55.00	304
17	CO4	F- C T C C C C R- T C C T G T T A G G C C T C C T A G G A T T T G G C A T G A T C G G	60.18 60.40	55.00 60.00	324
18	CO5	F- T C C C C G G A T T T G G C A T G A T/R- C T G T T A G G C C T C C T C G G T G	59.74 59.54	50.00 60.00	321

monoculus. Other primers showed multiple bands or no amplification after 3 replicate PCR trials. Subsequently, the three primers (CK1, CO1 and CM1) were amplified together with five (5) different species of native fishes (local fishes that were found in the same area where a specimen of Cichla species and eDNA were collected) as a negative control. The native fish used were: Puntioplites bulu (N1), Channa miropeltes (N2),

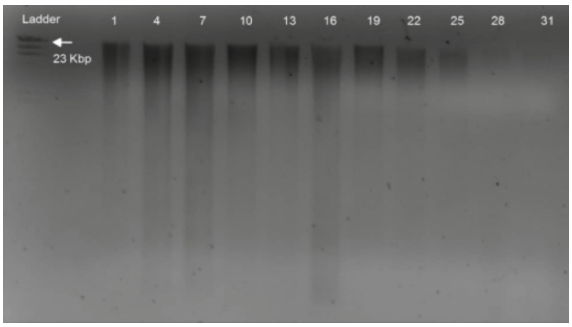


Fig. 1. The intensity of the isolated eDNA from water samples extracted at 3 days interval with an approximate size of 23 Kbp.

Thynnichthys thynnoides (N3), Osteochilus hasseltii (N4), and Notopterus notopterus (N5). As shown in Fig. 2, amplification of C.

4 Discussion

4.1 Selection of Mitochondrial Gene for Species-Specific Primer Development

The use of mitochondrial gene, as a molecular tool to aid in the discrimination of species, has long been applied [18–21]. However, the most commonly utilized and available genes for Cichla species in public sequence platforms (GenBank and BOLD) are mitochondrial cytochrome c oxidase (COI), Cytochrome B (Cyt B), 16s ribosomal RNA (rRNA), and Nicotinamide Adenine Dinucleotide (NAD) genes. In the current study, we found that only the COI gene produced species-specific primers for selected Cichla species. Cyt B, 16 rRNA, and NAD genes were found to be highly conserved for Cichla species resulting in easy cross-amplification between sister-species DNA. Therefore, these three primers might be valuable as universal primers but for eDNA analysis which focuses on species-specific monitoring, such primers can cause false-positive results. Therefore, the results of this study showed that the genetic distances of the COI barcoding region are highly divergent among the Cichla species.

4.2 Isolation of DNA and eDNA

Preserving both high-quality DNA and eDNA are challenging processes, especially for the later as eDNA is usually found to be easily degraded and fragmented. In this experiment, the fin tissues were used to obtain high- the quality of isolated DNA and this method is widely applied as it will not damage the specimen [22]. For eDNA, time of extraction and filtration, contribute to the variation of DNA yield (Fig. 1). It is suggested to extract and filter eDNA not more than 22 days after collection. The extracted eDNA then needs to handle and store properly to preserve high-quality template DNA before PCR. The poor handling technique and storage method may be contributed to the degradation of eDNA as DNA degrades over time. The smear band on the AGE test usuallyindicatese the degradation of DNA as shown in Fig. 1.

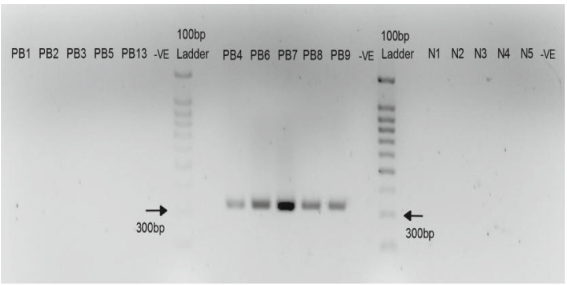


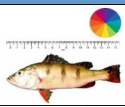
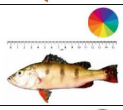
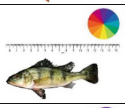
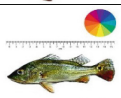
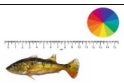
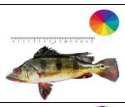
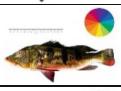
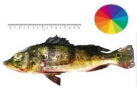
Fig. 2. AGE result for PCR using CK1 amplify PB4, PB6, PB7, PB8, PB9 (*C. kelberi*) specimen size with estimated size 300 base pair while no band formation for other PB species (PB1, PB2, PB3, PB5, PB13) and another native fish specimen (N1, N2, N3, N4, N5) where –VE is a negative control.

4.3 PCR for Primers Validation

Specificity for PCR primer design is dependable on the length of the amplicon and the annealing temperature. As the main target for the designated primer sets is eDNA which are fragmented, hence, a short amplicon size between 280 to 400 bp was targeted. COI gene is a highly conserved region of the mitochondria genome, which provide high specificity region used as a reference sequence for the species-specific primer design in this study. Short amplicon on highly conserved region produces high- specificity of primer as shown in Fig. 2 as C. kelberi primer, CK1 only amplified C. kelberi specimen.

However, C. ocellaris primer, labeled as CO1, managed to amplify all Cichla species. Although we have used the program of PrimerBlast to help identify the specificity of primers developed, in reality, CO1 primer is only usable for identification at the genus level. We hypothesized that either i) the region of COI used was too conserved, ii) the specimen used by Lin et al., 2016, which we retrieved from GenBank is misidentified, or iii) maybe the specimen used is a hybrid as Cichla species is also known to have the ability to naturally hybridize between sister species. As for CM1, we assumed our primer can amplify only C. monoculus. No amplification has been found for other sister species during this study. Our limitation is that there is no C.monoculus yet found although many specimens in Malaysia are mostly known as C.monoculus by researchers and local anglers.

Table 3. Description and details of the *Cichla sp.* used in the study

No	Species	Sample ID	Specimen collection	Location GPS coordinate
1.	*C. ocellaris*	PB 1		3.076472, 101.491916
2.	*C. ocellaris*	PB 2		3.076472, 101.491916
3.	*C. ocellaris*	PB3		3.708263, 101.482125
4.	*C. ocellaris*	PB 5		3.708263, 101.482125
5.	*C. ocellaris*	PB 10		5.016356, 00.987212
6.	*C. ocellaris*	PB 11		4.995440, 100.952879
7.	*C. ocellaris*	PB 12		5.016356, 00.987212
8.	*C. kelberi*	PB 4		3.708263, 101.482125
9.	*C. kelberi*	PB 6		3.708263, 101.482125
10	*C. kelberi*	PB 7		3.708263, 101.482125
11.	*C. kelberi*	PB 8		3.708263, 101.482125
12.	*C. kelberi*	PB 9		3.708263, 101.482125
14.	*C. piquiti*	PB 13	No picture as only fin were supplied	Local fish shop

5 Conclusion

Issues of AIS invasion have become a worldwide concern, especially in regards to the impact it has caused on national biodiversity and ecology. Due to the slow effect of the

traditional method, it is timely that a rapid alternative strategy must be implemented such as eDNA monitoring. We hope the information on the strategy for developing species-specific eDNA primers for invasive Cichla species in current research will benefit researchers and AIS (alien invasive species) monitoring authorities as it permits early detection. These species-specific eDNA primers could be utilized for rapid assessment of ecological studies, including local biodiversity inventories, and determining AIS distributions. The fact that only AGE (Agarose Gel Electrophoresis) analysis was required for the validation methods means that this data can be implemented with ease in any fish genomics lab with a low-cost setting. However, it can also be used further in capture probe assays for continuous detection, such RT-PCR. For future recommendations, several precautions must be undertaken by AIS researchers to improve the detection of AIS using species-specific primers. It is compulsory to identify as correct as possible unknown specimen firstly by morphology. Photographs of the specimen must be saved while meristic and morphometric measurements must be analyzed in detail. Any discrepancy must be recorded for future reference. Thoroughly check retrieved sequence from public databases (eg: GenBank or BOLD) for any unknown or ambiguous base. Align the sequence with as many sequences as possible to determine variations in consensus alignment. Avoid using sequences with < than 95% and do not have information on reference specimens. During in-house lab procedures, make sure PCR amplification showed high intensity and sharp, clear band at the expected product size. Avoid sequencing products with low and unclear intensity as well as producing the double band. Re-amplify at least three times to check for reproducibility. It is advised to maintain a similar chemical brand to be used throughout the research period. Before deposition in a public reference database (eg: GenBank or BOLD), for protein-coding gene, make sure the sequence submitted does not contain a stop codon within the sequence when translated into protein sequence.

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