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Novel primers for mini-barcoding of seahorses for wildlife trade and seafood forensics

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Abstract. *Salamida EMM, Tabugo SRM. 2026. Novel primers for mini-barcoding of seahorses for wildlife trade and seafood forensics. Biodiversitas 27 (3): d270335. https://doi.org/10.13057/biodiv/d270335*. Seahorses are charismatic fish facing increasing threats from habitat loss and the illegal wildlife trade. They are targeted and trafficked in the global market, further threatening their status. The high levels of both interspecific and intraspecific variability are also affecting the accuracy of species definition. Thus, this study developed a systematic pipeline for designing DNA mini-barcode primers using accessible software and integrated bioinformatics tools to enhance the detection of vulnerable seahorse species in wildlife trade and seafood forensics. The overarching goal is to support conservation efforts and promote the sustainable use of marine resources. DNA mini-barcodes, short sequences under 200 bp, show promise and can be useful for identifying species from degraded or processed samples. In this study, cytochrome b gene sequences—known for combining conserved and variable regions—were the basis for designing species-specific mini-barcode primers. These primers were tailored to improve molecular identification from low-quality seahorse samples, such as dried or frozen specimens commonly encountered in trade. Novel primers were initially evaluated using in silico Polymerase Chain Reaction (PCR), yielding four top-performing primer sets, which were subsequently validated by actual PCR. A total of nine *Hippocampus* spp. samples (four dried, five frozen) were used to test primer efficacy. PCR amplification and gel electrophoresis results confirmed that all primer sets successfully amplified DNA and produced good-quality PCR products. Sequencing data revealed accurate base-calling, with high percentage identity matches to reference sequences. These novel primers show potential for species identification in seahorse monitoring and enforcement contexts.

Keywords: Bioinformatics, conservation, *Hippocampus*, illegal trade, mini-barcodes

INTRODUCTION

Seahorses are known to live in a variety of habitats, including coastal and marine environments, which are increasingly under threat from overfishing, climate change, habitat loss and global illegal trading (Lourie et al. 2016). It is estimated that the seahorse trade involves up to 70 million seahorses each year, including at least 24 million specimens from 23 species in 75 countries (Lawson et al. 2017).

Considering the complexity of the seahorse trade, it is not possible to effectively manage the trade and the exploitation of seahorse species by focusing only on the trade volumes. More effective and advanced strategies are required to monitor and regulate the trade in seahorses, and this is only possible by improving the ability to identify the species of seahorses. Improved strategies for identifying seahorse species, monitoring wild populations, and regulating the trade in seahorses are essential for effective management and conservation of seahorses (Zeng et al. 2019; Pavitt et al. 2021).

The accuracy of identifying seahorse species, which have mainly relied on traditional methods, is reduced by post-processing, poor preservation in trade, and the presence of cryptic species (Zhang et al. 2017). In addition, homogeneity in characters that often allow one to diagnose species, such as numbers of spines and fin rays, coronet shape, and broad species-to-species overlap in other characters, such as patterns or markings, has led to taxonomic confusion in seahorses (Heard et al. 2019). Meanwhile, molecular identification using sequencing technologies has been tested to provide more taxonomically accurate data on seahorse populations in both the wild and the trade (Hou et al. 2016; Zhang et al. 2017; Wang et al. 2020).

Molecular identification has been used to increase the accuracy of species identification and population assessment (Lourie et al. 2016). It has yielded positive and efficient results, aiding in conservation and sustainable development efforts (Gao et al. 2019; Tizard et al. 2019; Toha et al 2020; Trivedi et al. 2020; Hobern 2021). The most common molecular identification method used is DNA barcoding (Wang et al. 2020). It identifies an organism by utilizing short, standardized fragments of its genome (Hebert et al. 2003). However, if the amount and quality of DNA are inadequate, such that it is damaged or fragmented, full-length target region DNA barcoding may not be effective. This is a common case in which DNA is obtained from processed or improperly preserved samples. Because DNA degrades readily, numerous studies have used DNA from poorly preserved or decomposed specimens

(Barbisin and Shewale 2010; Xavier et al. 2021). The amount of DNA retrieved from these samples is typically minimal and damaged. Specifically, DNA is more fragmented and shorter. As a result, the efficacy of DNA barcoding is significantly reduced (Fields et al. 2015; Günther et al. 2018). Hence, an alternative method was created. This method utilizes shorter DNA fragments (e.g., 100-300 bp) within a full-length barcode termed. This is called "mini-barcoding," and it successfully identifies species from degraded DNA (Shokralla et al. 2015).

DNA mini-barcoding typically utilizes short mitochondrial gene fragments for species delineation. The mitochondrial gene encoding Cytochrome b (Cytb) is a commonly used DNA barcode for species identification and assessment of phylogenetic relationships at the species level. This gene contains codons that evolve slowly and rapidly. Furthermore, the Cytb gene contains both variable and conserved regions and domains, making it an excellent barcode. Moreover, data obtained from sequencing Cytochrome b (Cytb) mini-barcode fragments can be used to simultaneously identify species and determine evolutionary relationships among lower taxa (Casey et al. 2004). Studies have shown that shorter Cytb fragments, as mini-barcodes, increase the amplification success rate of damaged DNA (Modave et al. 2017).

Using the Cytb gene marker to create taxon-specific mini-barcodes and primer sets would optimize species identification and population assessment. The problem of DNA barcoding being limited by DNA degradation can be solved. In addition, this will substantially aid forensic analysis of wildlife and seafood. No records of mini-barcode primers developed or tested for seahorse species employing the Cytb gene exist. Hence, this study developed a pipeline for primer design and tested novel primers for their potential to detect and distinguish seahorse species from shorter DNA fragments for seahorse species monitoring in trade and species-level delineation.

MATERIALS AND METHODS

Sample collection

Voucher specimens (seahorse samples) available at the Molecular Systematics and Conservation Genomics Laboratory of the Premier Research Institute of Science and Mathematics, Mindanao State University-Iligan Institute of Technology (MSU-IIT), were utilized in this study. The samples were collected from Lanao del Norte, Surigao del Norte, and Surigao del Sur, Philippines, with the Gratuitous Permit No. 0814-19 secured from the Department of Agriculture (DA) and Bureau of Fisheries and Aquatic Resources (BFAR) (Figure 1), through conservative sampling such that only a representative of each species was collected and deposited in the laboratory. Morphological characterization and preliminary identification of seahorses were based on illustrated keys, Guide to the Identification of Seahorses, and consultation with experts. Due to its vulnerable status, only a total of nine *Hippocampus* spp. samples (Table 1) were used for primer efficacy tests (Lourie et al. 2004). Photographs of specimens were taken for documentation purposes (Figure 2).

Table 1. Adult *Hippocampus* spp. species used in testing of primers

Location	Type	Morphological identification
Surigao del Norte	Dried	*Hippocampus barbouri* (Jordan & Richardson, 1908)
Surigao del Norte	Dried	*Hippocampus comes* (Cantor, 1849)
Lanao del Norte	Dried	*Hippocampus kuda* (Bleeker, 1852)
Sourced online	Dried	*Hippocampus kuda* (Bleeker, 1852)
Lanao del Norte	Frozen	*Hippocampus kuda* (Bleeker, 1852)
Surigao del Sur	Frozen	*Hippocampus kuda* (Bleeker, 1852)
Surigao del Norte	Frozen	*Hippocampus barbouri* (Jordan & Richardson, 1908)
Surigao del Norte	Frozen	*Hippocampus comes* (Cantor, 1849)
Surigao del Norte	Frozen	*Hippocampus kuda* (Bleeker, 1852)

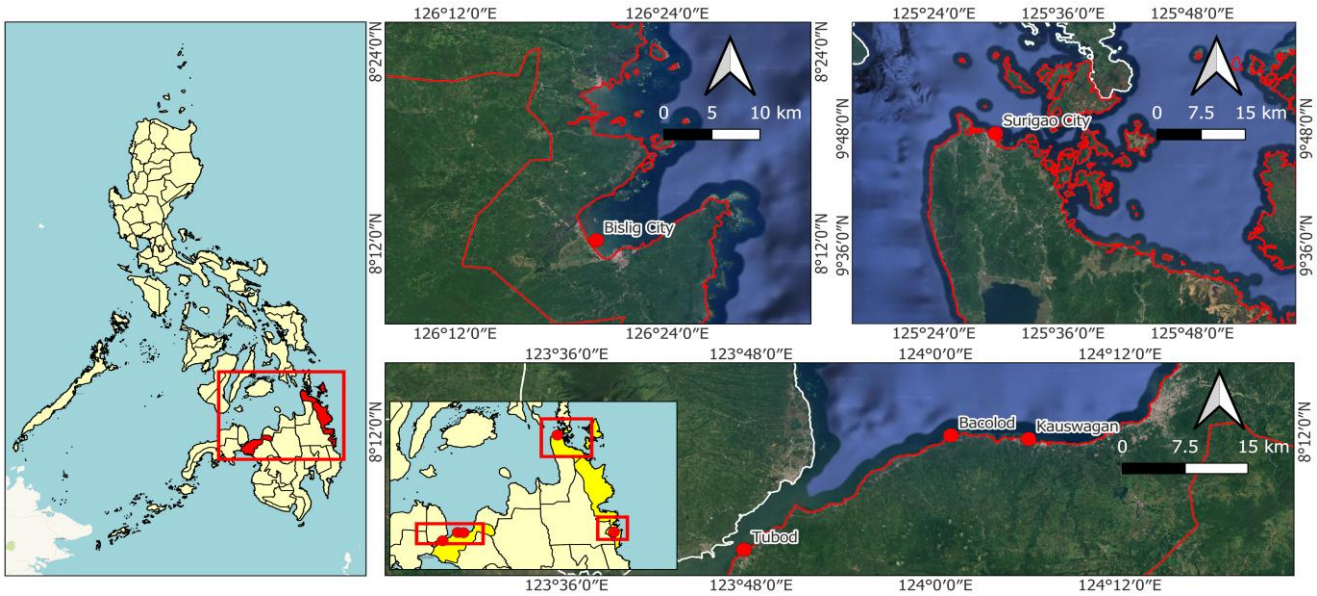

Figure 1. Geographical locations of the study sites at Lanao del Norte, Surigao del Norte, and Surigao del Sur, Philippines

DNA extraction

According to the manufacturer's protocol, the DNA of the seahorse samples was extracted using the Marine Animal Tissue Genomic DNA Extraction kit (Dongsheng Biotech). Ten milligrams of tissue from each sample were prepared to yield 3-35 µg genomic DNA.

Mini-barcode primer design and in silico testing

A total of 412 seahorse Cytochrome b (Cytb*)* barcodes/ nucleotide sequences were downloaded from GenBank (n = 27). All sequences were aligned and optimized manually. Multiple copies of identical sequences were also removed. These were done using MEGA X: Molecular Evolutionary Genetics Analysis (Kumar et al. 2018). These sequences were then used to design multiple mini-barcode primer sets to amplify partial fragments within the standard Cytb barcoding region. The primers were picked according to the availability of highly conserved priming sites in a wide range of seahorse species, with consideration of the primer stability in PCR reactions as well as the physical and structural properties of oligos (e.g., length, melting temperature, annealing temperature, GC content, GC clamp, self- and hetero-dimer formation, hairpin formation, and runs). To ensure novelty, the generated primer sets were compared with existing primer sets specific to organisms in the Syngnathidae family (seahorses, pipefishes, and seadragons) (Table 2).

Figure 2. Sample seahorses. A. *Hippocampus comes*, B. *Hippocampus barbouri*, C. *Hippocampus kuda*

Table 2. Existing primers for identifying Syngnathidae species

Primer name	Type	Primer sequence (5' to 3')	Source
SHORSE5.3L	Cytochrome b	ATATCCTTCTGAGGAGCC	Casey et al. (2004)
SHORSE5.4L	Cytochrome b	CAGGATCAAATAACCC	Casey et al. (2004)
SHORSE3.2H	Cytochrome b	GGGTGGAATGAGATTT	Casey et al. (2004)
SHORSE3.3H	Cytochrome b	GGATAGCATAGGCAAA	Casey et al. (2004)
SHORSE3.3L	Cytochrome b	TTTGCCTATGCTATCC	Casey et al. (2004)
SHORSE3.4H	Cytochrome b	CCAGATACAGGTAAAGC	Casey et al. (2004)
HIPPCYTB-R	Cytochrome b	AGGGGGTTCTACAGGCATTAC	Woodall et al. (2011)
L14725	Cytochrome b	CGAAGCTTGATATGAAAAACCATCGTTG	Kocher et al. (1989)
L15162	Cytochrome b	GCAAGCTTCTACCATGAGGACAAATATC	Taberlet et al. (1991)
H15240	Cytochrome b	TTRTCTACNGARAANCCNCCTCA	Taberlet et al. (1991)
H15915	Cytochrome b	TCATCTCCGGTTTACAAGAC	Irwin et al. (1991)
H15926	Cytochrome b	AAGGGKGGATTTAACCTCCG	Wilson et al. 2001)
shf	Cytochrome b	TACCTGCACCATCAAATATTTC	Lourie and Vincent (2004)
shf2	Cytochrome b	TTGCAACCGCATTTTCTTCAG	Lourie and Vincent (2004)
shr2	Cytochrome b	CGGAAGGTGAGTCCTCGTTG	Lourie and Vincent (2004)
SH-F	Cytochrome b	AACYAGGACYAATGRCTTGA	Chang et al. (2013)
SH-R	Cytochrome b	GCASWAGGGAGGRKTTTAAC	Chang et al. (2013)
16S_FishSyn_Short	16S rRNA	GACGAGAAGACCCTGTGGAGC	Nester et al. (2020)
16S_FishSyn_Short	16S rRNA	CCGYGGTCGCCCCAAC	Nester et al. (2020)
16S_FishSyn_Long	16S rRNA	GACGAGAAGACCCTDTGGAG	Nester et al. (2020)
16S_FishSyn_Long	16S rRNA	GRATTGCGNTGTTCCCT	Nester et al. (2020)
HIP136-F	16S rRNA	CCAGAGGGCAATGTTGTAA	Marín et al. (2021)
HIP136-R	16S rRNA	GGCGAGGCTAGTGTTATTATG	Marín et al. (2021)
HIPCURV-F	16S rRNA	TTAGTGGGCCTGAAAGCAG	Marín et al. (2021)
HIPCURV-R	16S rRNA	CAGGCGGGACCTCTTAT	Marín et al. (2021)
HCAL2	mtDNA Control Region (CR)	CACACTTTCATCGACGCTT	Teske et al. (2003)
HCAH2	mtDNA Control Region (CR)	TCTTCAGTGTTATGCTTTA	Teske et al. (2003)
HIPPCONR	mtDNA Control Region (CR)	AAGCCGAGCGTTCTCTCC	Woodall et al. (2011)

PCR primer design and in silico analysis were carried out by utilizing software (freeware) and web-based informatics tools. For web-based primer design, Primer-BLAST (Basic Local Alignment Search Tool) was used to design PCR primers, and PCR Primer Stats was then used to assess the oligos. Freeware was mainly used for the software-based PCR primer design. The Unipro UGENE v.41.0 and FastPCR 6.8.03 software were used for primer design and oligo assessment. To identify and prevent potential amplification problems across all primer sets generated by web- and software-based primer design, in silico PCR analysis was performed using UGENE v.41.0 and FastPCR 6.8.03. This software determines the presence of primer dimers (i.e., self-dimers and heterodimers) and Single Nucleotide Polymorphisms (SNPs), hairpin formation, delta G values, annealing temperatures, and amplicon lengths. Four (4) top-scoring primer sets were selected. These top-scoring primer sets were synthesized at the Integrated DNA Technologies (IDT) (Kalendar et al. 2017; Rose et al. 2018).

Mini-barcoding PCR optimization strategy

Mini-barcode amplification was carried out on all seahorse specimens using each of the four mini-barcode primer sets. A gradient PCR approach was used to test the amplification conditions for all primer sets at annealing temperatures ranging from 50 to 62°C. PCR amplifications were done in a 50 µL reaction: 25 µL 2× PCR MasterMix (BIORAD, iTaq Universal SYBR Green SuperMix), 2.5 µL FP, 2.5 µL RP, 15 µL ultrapure water or sterilized distilled water, 5 µL template DNA with the following conditions: initial denaturation: 94°C for 4 min; followed by 35 cycles of denaturation at 94°C for 30s; annealing at 50-62°C for 30s; extension at 72°C for 90 s and final extension at 72°C for 7 min. PCR products were assessed by gel electrophoresis in 1.5% (w/v) agarose gel in 1× TBE buffer using BlueGel system (by MiniPCR) with built-in power supply (AC 100-240V, 50-60hz). Gels were dyed with GelGreen (Ca, USA) (10,000× in water). PCR products were then sent to Macrogen Inc., Seoul, Korea, for purification and standard sequencing (Sanger sequencing).

RESULTS AND DISCUSSION

Mini-barcode PCR primer design and in silico testing

Primer design is generally regarded as one of the key determinants for successful PCR reactions, given that it affects specificity and efficiency directly. In this study, the selection of nucleotide templates was pivotal in ensuring that primer pairs performed successfully. By aligning 412 seahorse cytochrome *b* sequences, it was possible to identify key areas where primer pairs could amplify different species while ensuring specificity. This demonstrates the importance of including nucleotide variability in primer pair development for improved success in amplification reactions for closely related species (Thaenkham et al. 2022).

In this study, integrating several bioinformatic tools strengthened the primer design and validation process. Web-based tools like Primer-BLAST and PCR Primer Stats helped validate the primers by analyzing their binding sites and amplification efficiency. While bioinformatic tools such as Unipro UGENE and FastPCR facilitated the in silico analysis of the primers. This ensured that the best primers were selected, thus strengthening the primer design process (Kalendar et al. 2017; Rose et al. 2018; Delghandi et al. 2022). Developing a robust and reproducible primer design pipeline is essential.

The first successful primer pair could also be attributed to strict adherence to established guidelines for oligonucleotide primer pair development. Various parameters, including primer pair length, melting temperature, and structural stability, influence primer pair binding and amplification efficiency. For example, primer pair length within the optimum range (18-22 bp) could have improved specificity without compromising amplification efficiency. Similarly, appropriate ranges for melting and annealing temperatures could have improved amplification specificity, minimize non-specific products, and maximize yield. This is in line with previous studies that have emphasized that primer pairs that deviate from optimum thermodynamic conditions may result in reduced amplification efficiency or increased mismatched nucleotide incorporation (Bustin et al. 2019).

GC content and the inclusion of the GC clamp at the 3' end also contributed to the primers' stability and binding capacity. However, if the GC content is too high or there are clusters of G/C nucleotides at the 3' end of the primers, there is also the possibility of nonspecific binding, which can adversely affect the outcome of the PCR. The absence of nonspecific secondary structures such as hairpin, self-dimer, and cross-dimer in the designed primers also indicates that primer-template binding was not compromised, thereby ensuring the success of the process. Furthermore, the evaluation of repeats, runs, and the stability of the 3' end of the primers also ensured the reduction of the possibility of false binding, which has also been identified as one of the major causes of PCR artifacts in the past (Maddocks and Jenkins 2017; Guo et al. 2020; Sharma 2021).

In total, this consistency between theoretical parameters and primer efficacy highlights the value of a comprehensive and criterion-driven method for primer development. The results show that a combined method including sequence alignment, thermodynamic calculation, and in silico verification can greatly improve the reliability of PCR, especially for mini-barcode assays in genetically complex species such as seahorses. Figure 3 shows the pipeline for the mini-barcode PCR primer design done in this study.

A thorough evaluation of all the primers generated by the software and web-based bioinformatic tools was conducted. The properties of oligos and in silico analysis were strictly considered before proceeding with the synthesis primers (Biñas 2000; Maddocks and Jenkins 2017). The top four primer sets after the assessment of oligos are shown in Table 3.

Table 3. The top four mini-barcode PCR primer sets were generated and used in this study

Primer set	Primer name	Direction	Primer sequence (5'- 3')	Length (bp)	GC (%)	Product length (bp)	Melting temperature (°C)	Annealing temperature (°C)
SHRS_mini1	shrs14f	Forward	CACACTTTCATCGACGCTT	22	50.0	234	56.7	52.99
	shrs14r	Reverse	TCTTCAGTGTTATGCTTTA	21	52.4		56.8	
SHRS_mini2	shrs16f	Forward	CACACTTTCATCGACGCTT	20	50.0	300	54.7	56.99
	shrs16r	Reverse	TCTTCAGTGTTATGCTTTA	20	50.0		56.3	
SHRS_mini3	shrs_u1f	Forward	GTGTGAGGAGGATTTTCCGT	20	45.0	233	53.0	57.10
	shrs_u1r	Reverse	TGACTAAAGGGTTGGCTGGT	20	55.0		57.8	
SHRS_mini4	shrs_f1f	Forward	TTTCCGTAGACAATGCTACTCT	22	40.9	126	53.4	54.53
	shrs_f1r	Reverse	CGGCAGGGTTATTTGATCCT	20	50.0		54.9	

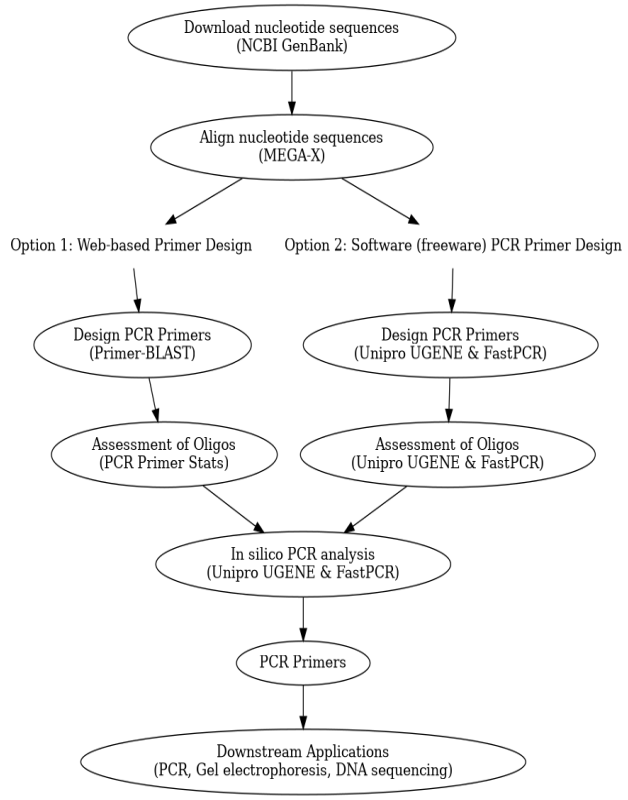

Figure 3. Pipeline for PCR design and in silico PCR analysis utilizing integrative software and web-based bioinformatic tools

The top four mini-barcode primer sets were SHRS_mini1, SHRS_mini2, SHRS_mini3, and SHRS_mini4. These primers yield PCR products of no more than 300 bp, a mini-barcode characteristic. All primers fall within the optimal primer length range of 18-22 bp, with the shortest at 20 bp (shrs16f, shrs16r, shrsh_u1f, shrs_u1r, and shrs_f1r) and the longest at 22 bp (shrs14f). The desired 40-60% GC content was observed among the primers, with 40.9% (shrs_f1f) as the lowest and 55% (shrsh_u1r) as the highest. The primers also have melting temperatures in the range of 52-58°C, which yield the best results. Regarding annealing temperature, which depends on the primers' melting temperatures (within 5°C of each other), all primers fall within the acceptable range (Lorenz 2012). No runs were found in the primers. In addition, most primer pairs do not exhibit secondary structures that would disrupt PCR reactions. Of the 4 primer sets, only one (SHRS_mini4) forms a 4-bp cross-dimer.

PCR optimization

Annealing temperatures were varied to determine the optimal annealing temperature for each primer set. The average melting temperature of the component forward and reverse primers in each set was used to guide targeting the closest optimal annealing temperature. Gradient PCR optimization was performed for each species, with increments of 1° from 50-62°C. Figure 4 shows the gel electrophoresis results of the primer sets SHRS_mini1, SHRS_mini2, SHRS_mini3, and SHRS_mini4 on frozen (A1-A12, C1-C12, E1-E12, and G1-G12) and dried (B1-12, D1-D12, F1-F12, and H1-H12) *Hippocampus* spp. samples at varying annealing temperatures (50-62°C).

The optimal annealing temperature of the primer sets SHRS_mini1, SHRS_mini2, SHRS_mini3, and SHRS_mini4 are 55.5°C (A6 and B6), 55.6°C (C6 and D6), 56.6°C (E7 and F7), and 57.6°C (G8 and H8), respectively. The sharpest bands are observed in both frozen and dried *Hippocampus* sp. samples at these annealing temperatures, indicating the highest yield of PCR product. These temperatures were within the generally accepted 5°C range from the melting temperatures of each primer set's forward and reverse primers. We observed that the optimal annealing temperatures for the primer sets were lower than their melting points, as higher or very low annealing temperatures would yield lower-quality products due to mismatches and faulty priming (Maddocks and Jenkins 2017; Green and Sambrook 2019). Moreover, the optimal annealing temperatures were very close to the average melting temperatures of the forward and reverse primers in the primer sets.

Figure 5 shows the amplification of cytochrome b sequences for sample specimens: dried and frozen seahorse samples (*H. kuda*, *H. comes*, *H. barbouri*). The primer sets SHRS_mini2, SHRS_mini3, and SHRS_mini4 were able to amplify DNA from frozen and dried seahorse samples, but SHRS_mini1 showed faint to no bands for dried samples and good-quality bands for frozen samples. The primer set SHRS_mini2 seems to be very efficient for both frozen and dried samples. The optimal annealing temperature for SHRS_mini1 is 55.5°C, SHRS_mini2 is 55.6°C, SHRS_mini3 is 56.6°C and SHRS_mini4 is 57.6°C respectively.

Based on the luminescence and sharpness of bands, which determine the presence and quality of amplified DNA, the SHRS_mini2 primer set performs best at amplifying the three seahorse species tested in this study among the four primer sets. It is then followed by SHRS_mini3, SHRS_mini1, and lastly, SHRS_mini4 (Table 4).

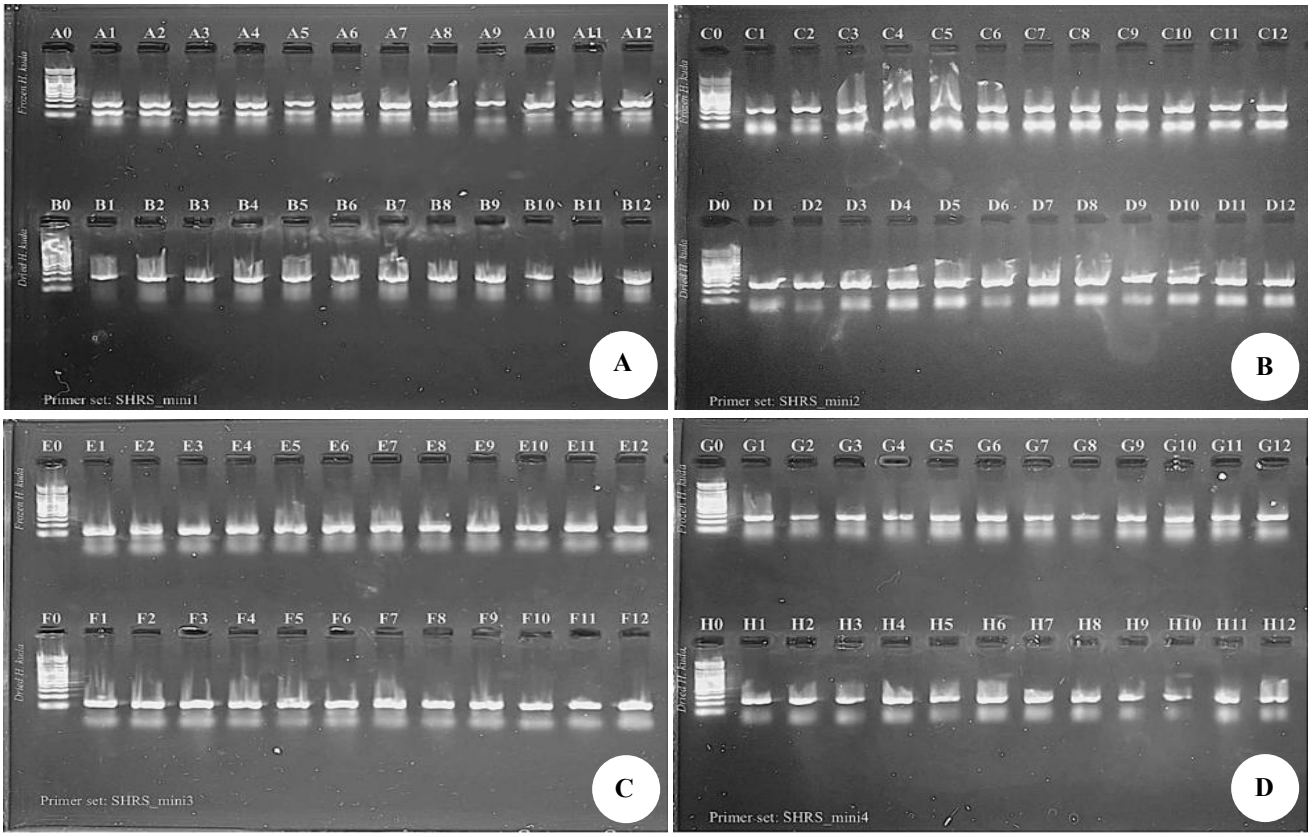

Figure 4. Representative image for gradient PCR optimization of four different primer sets designed in this study: A. Amplification of cytb primer set SHRS_mini1, B. SHRS_mini2, C. SHRS_mini3, D. SHRS_mini4; for frozen and dried *Hippocampus* spp. A0, B0, C0, D0, E0, F0, G0, and H0 are 100bp plus molecular weight ladder (Dongsheng Biotech)

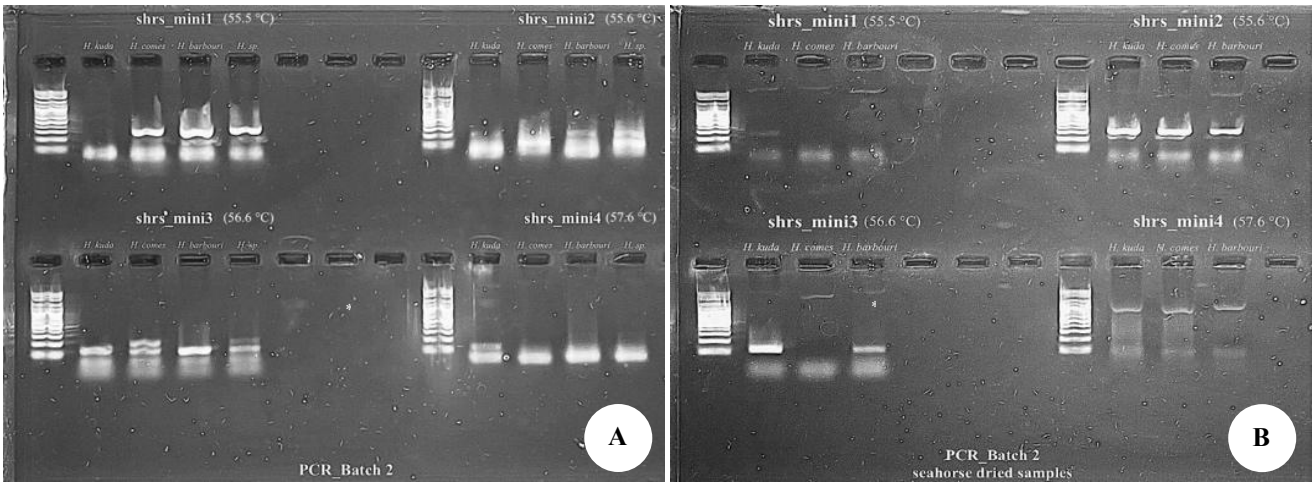

Figure 5. Testing of PCR primer sets on: A. Frozen, and B. Dried *Hippocampus* spp. samples for SHRS_mini1-SHRS_mini4. Lane 1: DNA ladder (100bp)

Moreover, sequence quality assessment confirmed the reliability of the generated data based on the evaluation of chromatograms and corresponding quality scores. Quality scores, which reflect the confidence in base calling, are widely used indicators of sequencing accuracy, with values ≥20 considered acceptable (Bell and Kramvis 2015). In this study, results show evidently low-quality bases in the beginning and end of the sequences, a pattern obviously associated with inherent limitations of sequencing reactions. But it should be noted that, these regions do not compromise data integrity, as they are routinely trimmed prior to downstream analyses.

Most importantly, however, the central regions of the sequence displayed sharp, well-defined peaks with little overlap, indicating high confidence in the base calls for the cytochrome b fragments. This is also corroborated by the consistently high quality scores obtained, which reached a maximum of 62. Out of all the primer sets that were used in this investigation, SHRS_mini1 and SHRS_mini2 displayed the highest consistency and quality of chromatograms, indicating that there is a high degree of confidence in the base calls. This indicates that these primers have high potential in amplifying DNA fragments.

In addition, what remains challenging is species delineation in illegal trade/seafood forensics and conservation, particularly for smooth seahorses, which are often broadly identified as *H. kuda*. However, accumulating evidence suggests that *H. kuda* does not refer to a single species but rather a species complex composed of multiple genetically distinct haplotypes (Lourie et al. 2016). This taxonomic ambiguity complicates accurate identification, thereby posing difficulties for effective conservation management and enforcement in wildlife trade monitoring. Thus, to evaluate primer efficacy in species delineation, some of the generated sequences were used to investigate the *H. kuda* species complex through phylogenetic analysis. BLAST results showed that these sequences matched known haplotypes of the cytochrome b gene deposited in the NCBI (National Center for Biotechnology Information) GenBank database, confirming the authenticity and accuracy of the amplified fragments. These matches indicate that the sequences correspond to established mitochondrial haplotypes and are reliable for species-level identification.

Table 4. Ranking of generated primer sets based on amplification results

Rank no.	Primer set	Primers	Direction
1	SHRS_mini2	shrs16f	Forward
		shrs16r	Reverse
2	SHRS_mini3	shrs_u1f	Forward
		shrs_u1r	Reverse
3	SHRS_mini1	shrs14f	Forward
		shrs14r	Reverse
4	SHRS_mini4	shrs_f1f	Forward
		shrs_f1r	Reverse

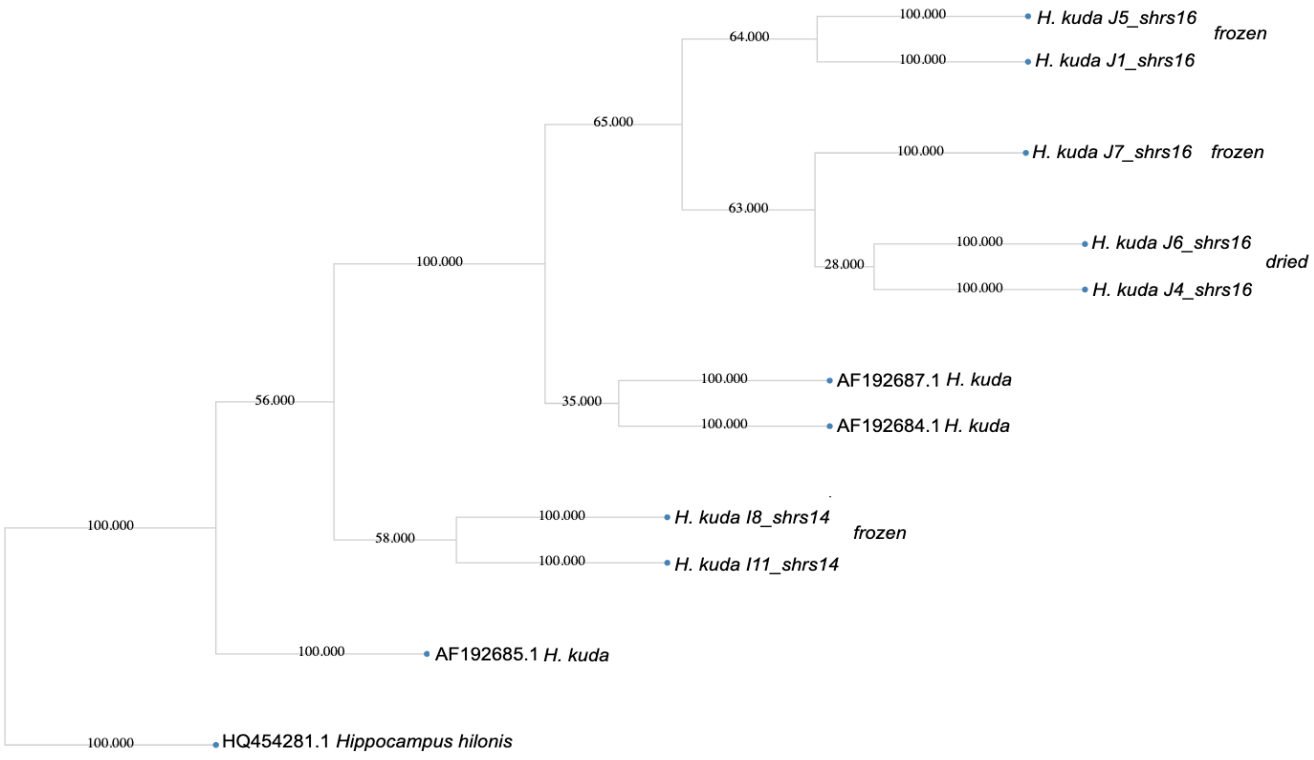

Figure 6. A sample Neighbor-Joining (NJ) phylogenetic tree was constructed for the two primer sets: SHRS_mini1 and SHRS_mini2, with *Hippocampus hilonis* designated as the outgroup, and AF12685.1, AF192684.1, AF192687.1 *Hippocampus kuda* serves as reference sequences, while other samples are sequences generated from both frozen and dried samples; bootstrap values are also shown

Based on some of the output sequences, a phylogenetic tree was generated using the Neighbor-Joining (NJ) method in the PHYLogeny Inference Package (PHYLIP) in DNA Subway, with *Hippocampus hilonis* as the outgroup (Figure 6). All ingroup sequences grouped with the reference sequences for *H. kuda*, indicating successful species identification. This also demonstrates the primers' efficacy in obtaining phylogenetically informative sequences.

It is interesting to note that *H. kuda*, is a species complex according to previous studies (Lourie et al. 2016). This could explain the high genetic similarity among the samples despite the potential genetic diversity. However, the optimized primers have strong potential and can be used effectively for accurate identification and phylogenetic reconstruction of seahorses.

Overall, despite coverage and sampling limitations, the results suggest that the primers generated in this study show potential and can produce high-quality PCR products from frozen and dried seahorse samples. Seahorse samples that have undergone processing, such as drying and freezing, which cause DNA fragmentation, can be amplified and identified by sequencing using the primer sets SHRS_mini1, SHRS_mini2, SHRS_mini3, and SHRS_mini4. We recommend using the generated primers for other species to broaden species coverage and increase sample size to assess primer generalizability. Nevertheless, the results of this study will be useful as baseline data for future studies.

In conclusion, four mini-barcode PCR primer sets capable of amplifying shorter fragments of the cytochrome b gene were designed and tested in this study to provide better primers for molecular identification of seahorses, whether fresh, frozen, or dried. These primers target shorter sequences to accommodate fragmented DNA sequences of processed seahorse samples in the global seahorse trade. All the primer sets amplified high-quality sequences, suggesting that they have potential and can be used for the identification of seahorses. Thus, this will enhance seahorse species monitoring, trade, and species-level delineation. Moreover, this may help address the data gap in seahorse biodiversity and conservation status, and reduce the exploitation and illegal trade of seahorses. However, additional sampling across different seahorse species is needed, as none are included in this study.

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