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Species Identification of Sea Bamboo (*Isis hippuris*) Using *COI*-based DNA Barcoding

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ABSTRACT

Conservation and effective management of marine ecosystems and biodiversity requires accurate species identification. This study classifies sea bamboo (*Isis hippuris*) specimens using DNA barcoding, a technique widely recognized for its speed, accuracy, and objectivity. This study examines the *cytochrome c oxidase subunit I* (*COI*) gene analysis for species identification. *Isis hippuris* was collected from two stations (coral and seagrass areas) of Tanjung Tiram Waters, South Konawe, Southeast Sulawesi. Genomic DNA was extracted from the base, main, and lateral stem of *I. hippuris*. Polymerase chain reaction (PCR) was used to amplify the mtDNA of *I. hippuris* with HCO2198 and LCO1490 primers. The highest quality PCR product based on the *COI* gene was chosen for sequencing analysis. The study revealed that *COI* gene analysis could only be performed on the base and main stem of the *I. hippuris*. Samples from coral and seagrass-coral areas on lateral stems were not further analyzed due to low concentration and purity values, which could potentially fail DNA sequencing. Each part of *I. hippuris* may have unique genetic differences. This study highlights the advantages of DNA sequencing in providing a unique genetic fingerprint for each species, enabling accurate species identification. This research provides insight into using DNA barcoding for sea bamboo species identification.

Keywords: *COI* gene analysis, conservation, DNA barcoding, management strategies, sea bamboo (*Isis hippuris*)

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INTRODUCTION

Conservation of marine biodiversity is essential for maintaining the equilibrium of the environment. Biodiversity is the variety of genes, species, and ecosystems that make life on Earth (Laxmi et al., 2015; Watson et al., 2012). Due to changing complex interactions, the environment, ecosystem degradation, and anthropogenic pressures have decreased marine species' populations, including the sea bamboo (*Isis hippuris*) (Díaz et al., 2019). It is essential to strengthen conservation management to protect sea bamboo (*Isis* spp.) as a fish species and prevent overexploitation. This effort is in line with the Convention on International Trade in Endangered Species (CITES) recommendations (Edrus & Suman, 2013), especially considering its important function in the commerce of jewelry (Cooper et al., 2011). The pace of reduction in biodiversity is equivalent to the extinction rates during the previous five big extinction events on Earth, and it is happening now (Hoegh-Guldberg & Bruno, 2010; Pimm et al., 2014).

The success of biodiversity conservation measures is inconsistent, and their implementation is not widely spread (Lunt et al., 2013). There has been a growing awareness of the significance of biodiversity and the need for conservation and efficient management of marine ecosystems in recent years. Appropriate species identification is critical in understanding marine species' diversity and distribution and formulating efficient conservation strategies. The agreement permits local and regional governments to manage environmental activities and strive for regional conservation standards. However, conventional species identification methods, such as morphological analysis of the product's identifiable portion using morphology, can be subjective and time-consuming (Posthouwer et al., 2018). DNA barcoding, a molecular method, has become increasingly popular for species identification because of its rapid, accurate, and objective approach.

Mitochondrial DNA sequencing is a highly reliable and commonly used molecular method that is a specific molecular marker for investigating parental analysis, population genetics, and species identification. It also supports long-term ecosystem sustainability, as highlighted in various studies (Ceruso et al., 2019; Ravago-Gotanco & Kim, 2019; Saad, 2019). The precision, speed, and cost-effectiveness of DNA barcoding have been highlighted in prior studies, making it a useful policy tool for species identification (Clark, 2015). Researchers in taxonomy, genetics, and evolutionary biology from all over the world are interested in this method since it is well known for its effectiveness in identifying species (Hellberg et al., 2016).

DNA sequences can be used as a reliable species identifier through DNA barcoding. While some studies have used multiple genes for species identification, a single gene is recommended for a universal animal identification system. The *COI* mitochondrial gene can be used for DNA barcoding techniques to meet global demand for animal identification.

Researchers rely on the National Center for Biotechnology Information (NCBI), Barcode of Life Data System (BOLD), and International Nucleotide Sequence Database Collaboration (INSDC) databases for short nucleotide sequences. The mitochondrial *COI* gene is the most used gene for DNA barcoding in animal groups. These findings are supported (Hebert et al., 2003; Wallace et al., 2012).

Recent research stated that DNA barcoding is a highly effective technique for identifying various species, especially those with similar morphological features. One example of such a species is *I. hippuris*, where DNA barcoding has been used to distinguish between different species and identify specimens of the same species (Bineesh et al., 2017). The method can detect subtle genetic differences between closely related species, assess intraspecific genetic diversity (Verma, 2017), and identify cryptic species that are morphologically indistinguishable but genetically distinct (A. Kumar & Verma, 2017). The significance of this lies in its potential to aid conservation initiatives by safeguarding endangered species before their extinction while also enabling the detection of genetic diversity within species and identifying populations that are in danger of disappearing (Verma, 2018).

Isis hippuris can have several practical applications, including identifying areas for sampling, focusing research efforts, and developing conservation strategies. This information can be used to establish protected areas, restore habitats, and regulate the exploitation of this species. The ecological and conservation information can also be used to design long-term sustainability management strategies for *I. hippuris*.

MATERIALS AND METHODS

Sampling Site

The sample of *I. hippuris* was obtained from Tanjung Tiram Waters, South Konawe, Southeast Sulawesi, Indonesia. The sampling location was classified into two stations, including the coral area (ST 1: 122°40'8.808" E; 4°1'19.414" S) and the coral-seagrass area (ST 2: 122°40'24.070" E; 4°2'3.032" S) (Figure 1). A unique number was assigned and cataloged for each sample to ensure accurate identification. Samples were stored at recommended temperatures until DNA extraction was performed. This strict sampling protocol ensures the reliability of subsequent DNA barcoding analysis and provides valuable insights into the conservation and management of marine ecosystems. This study used three different parts of *I. hippuris*, including the base stem, main stem, and lateral stem (Figure 2), to assess the effectiveness of DNA barcoding in identifying species and their potential impacts on marine ecosystem conservation and management.

DNA Isolation and Extraction

PCR was used to investigate the genetic composition of *I. hippuris*. The genomic DNA of *I. hippuris* was extracted using gSYNC™ DNA Extraction Kit GS300

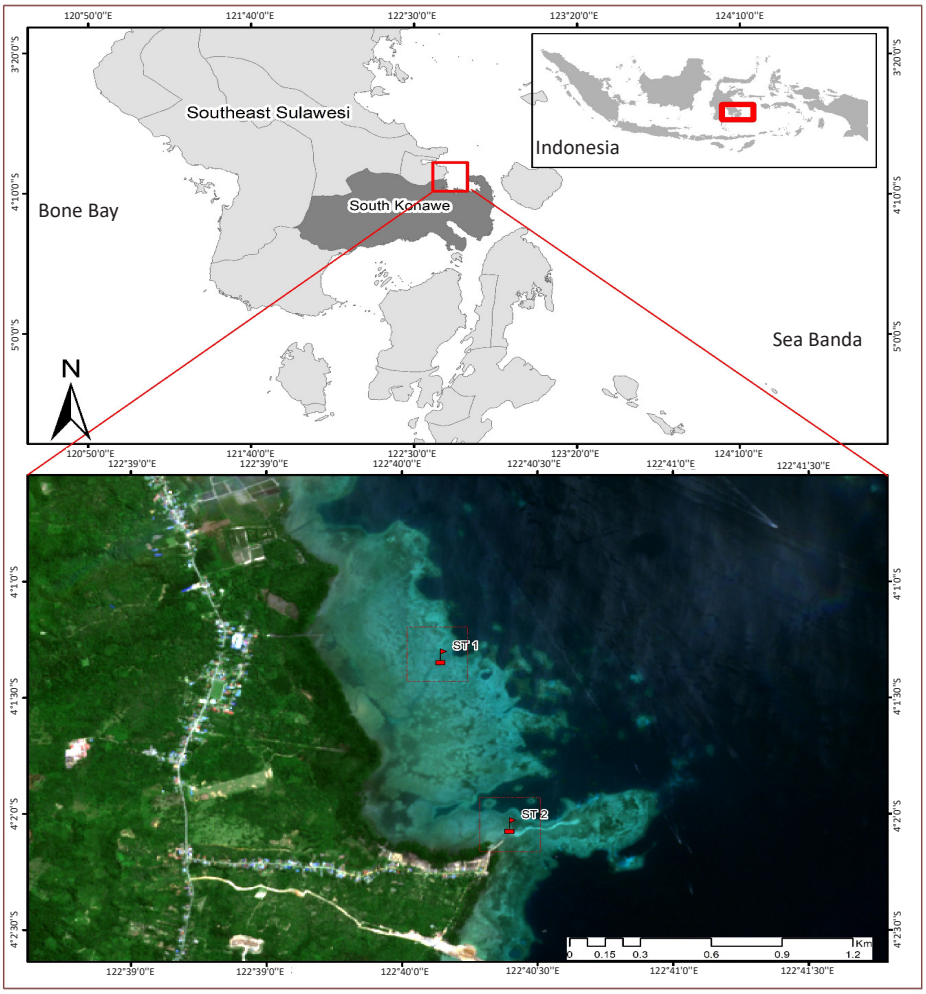

Figure 1. Sampling stations in the coral area (ST 1) and seagrass-coral area (ST 2)

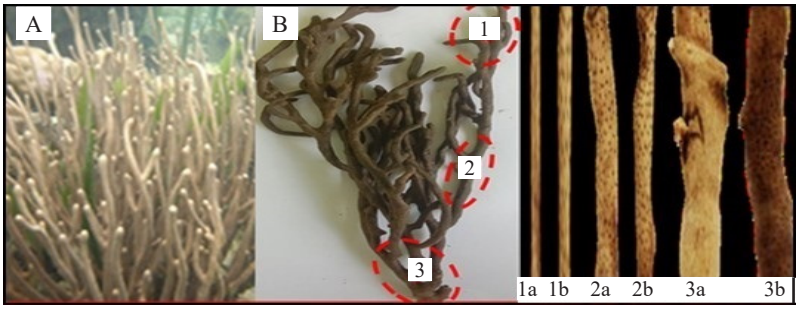

Figure 2. External morphology of soft coral specimens: (A) *Isis hippuris* colony; (B) Samples processed for DNA analysis (1. Lateral stem; 2. Main stem; 3. Stem base) to be extracted DNA
Note. 1a = Lateral stem (area seagrass – coral); 1b = Lateral stem (coral area); 2a = Main stem (area seagrass – coral); 2b = Main stem (coral area); 3a = Stem base (area seagrass – coral); 3b = Stem base (coral area)

(Geneaid Biotech Ltd., Taiwan) and then amplified using PCR with MyTaq HS Red Mix (Bioline Reagent Ltd., United Kingdom). The DNA of *I. hippuris*, preserved with absolute ethanol (EtOH, PT. SMART-LAB, Indonesia), was washed with TE buffer 2-3 times to remove the preservation. Incubation time for the lysis stage was 18 min with 205 µl GBT buffer and the DNA ligation stage using 205 µl EtOH (PT. SMART-LAB, Indonesia). The DNA purity was determined at A260/A280 nm. The obtained DNA of *I. hippuris* was categorized as high quality and quantity, as evidenced by the purity of the purified DNA was 1.8 to 2.0 and a concentration of 200 ng/L.

Amplification of Mitochondrial DNA

The PCR technique was done to amplify the mtDNA of *I. hippuris* using HCO2198 (Forward: 5'- TAA ACT TCA GGG TGA CCA AAA AAT CA 3') and LCO1490 (Reverse: 5' GGT CAA CAA ATC ATA AAG ATA TTG G 3') primers (Black et al., 1994). The following conditions were used for amplification: pre-denaturation for 3 min at 95°C, followed by 35 cycles consisting of 94°C for 45 s for denaturation, 60°C for 45 s for annealing, 1 min for extension, 6 min for post-extension, and 10 min of storage at 4°C. Then, the PCR results were visualized using a 1.5% agarose gel stained with ethidium bromide (EtBr) (Bio-Rad Laboratories, USA) and 1x TAE buffer solution (40 mM Trisacetate and 1 mM EDTA) (Invitrogen™, Thermo Fisher Scientific Inc., USA) in an electrophoretic machine under UV illumination (100 V).

DNA Sequencing

Selecting the highest quality PCR product derived from the *COI* gene is essential in the sequencing analysis. The PCR product was then used for sequencing (1st BASE DNA Sequencing Services, Singapore). The obtained sequences of *I. hippuris* have been published in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/nuccore/?term=OR165098%3AOR165101%5Baccn%5D) with accession number OR165098:OR165101[accn].

Data Analysis

Molecular Evolutionary Genetics Analysis (MEGA X) version 11.0 software was used to analyze the nucleotide sequences, including reading and editing the individual sequence results, conducting bioinformatics analysis, and examining homology (S. Kumar et al., 2018). Nucleotide sequence alignment was performed using the CLUSTALX application (Thompson, 1997), and nucleotide sequence homology analysis was carried out via the Basic Local Alignment Search Tool-Nucleotide (BLAST-N) program on the NCBI website (www.ncbi.nlm.nih.gov). This analysis provided valuable information on the genetic variations and similarities between the samples, which the evolutionary relationships and genetic diversity of the species can conclude.

RESULTS AND DISCUSSION

The *COI* gene of three different parts of *I. hippuris* (stem base, main stem, and

lateral stem) was amplified using the PCR. The electrophoresis findings of the PCR product with LCO1490/HCO2198 primers are shown in Figure 3. The results significantly influenced the quality of the DNA samples and the efficacy of the PCR procedure. The use of diverse sections and distinct regions enabled a comprehensive exploration of the genetic diversity of the studied species, which can provide vital insights into the ecological and evolutionary processes of the species. These findings have important implications for understanding the species' population structure, geographic distribution, and potential for adaptation to changing environments.

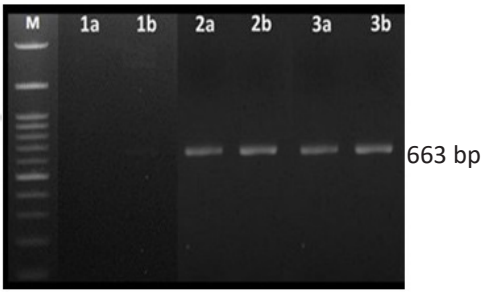

Figure 3. Electrophoresis of *COI* gene mtDNA PCR products primer LCO1490/HCO2198 *Isis hippuris*

Note. M = Marker size 1 kb; 1a = Lateral stem (area seagrass – coral); 1b = Lateral stem (coral area); 2a = Main stem (area seagrass – coral); 2b = Main stem (coral area); 3a = Stem base (area seagrass – coral); 3b = Stem base (coral area)

The genetic diversity of the *COI* genes in multiple samples from various locations and populations has been examined. The DNA sequence data acquired from these samples has provided valuable insights into the variation in *COI* gene sequences and genetic distinctions among populations. This investigation authenticated the species identity of the samples, which was accomplished through BLAST analysis. By contrasting the DNA sequences of the samples with those of reference sequences in the NCBI database, the most closely matching species and their level of similarity were determined (Table 1). The BLAST analysis entailed comparing the DNA sequences of samples with those of numerous species in the NCBI database to confirm their species identity, which is crucial for comprehending their biological characteristics and behaviors.

Table 1
BLAST analysis result based on NCBI for species identification

No.	Sample ID	Query cover (%)	Identification (%)	Species identified
1	1a	-	-	-
2	1b	-	-	-
3	2a	97	99.55	*Isis hippuris*
4	2b	99	99.26	*Isis hippuris*
5	3a	97	99.40	*Isis hippuris*
6	3b	96	99.70	*Isis hippuris*

Note. 1a = Lateral stem (area seagrass – coral); 1b = Lateral stem (coral area); 2a = Main stem (area seagrass – coral); 2b = Main stem (coral area); 3a = Stem base (area seagrass – coral); 3b = Stem base (coral area)

Analysis of population structure and genetic diversity are critical components of effective conservation and management strategies of marine biodiversity. However, morphology-based identification of marine species is challenging due to the high degree of morphological similarity, which makes DNA barcoding and mitochondrial DNA sequencing very important tools for accurate species identification and phylogenetic analysis. The use of *COI* genes has been widely recognized as valuable genetic markers. Several studies have emphasized their importance for conservation management decisions, including fishing site design and regulation.

Bingpeng et al. (2018), Cooke et al. (2016), and Wang et al. (2017) have highlighted the need to analyze population structure and genetic diversity using *COI* genes to promote resource recovery, population zoning, sustainable harvesting and utilization, biodiversity conservation, and fisheries management (Han et al., 2008; Palumbi, 2003; Thai et al., 2006). However, it is critical to carefully select DNA extraction sites to ensure accurate specimen identification, as *COI* gene expression may vary depending on the sampling site (Ceruso et al., 2019; Meriam et al., 2015; Saad, 2019; Sari et al., 2015). This study underscores this point, suggesting that additional markers or sequencing methods may be needed to assess genetic diversity and fully characterize the species, especially in environmental factors affecting its distribution.

The isolation of DNA plays an important role in the successful extraction, purification, and quantification of DNA. DNA isolation involves cell lysis, extraction, and precipitation of lipids, proteins, polysaccharides, and inorganic and organic compounds. These contaminants can reduce DNA quality and interfere with subsequent analyses' success (Muhammad et al. 2016). Secondary structures in primers can inhibit the PCR process and decrease PCR product yield; thus, these secondary structures should be avoided (Ozturk & Can, 2017). Primer dimers can be caused by amplifying them, leading to non-specific PCR products (Dieffenbach et al., 1993). The species-specific primers for *I. hippuris* with a target base length of ~663 bp have been applied to all six samples. The absence of DNA bands in some samples may be due to suboptimal PCR conditions or primer design.

The identification of species in *I. hippuris* was performed on six samples, three from coral and three from seagrass-coral areas. Samples 1a and 1b (samples from coral and seagrass-coral areas on lateral stem) were not further analyzed due to low concentration and purity values, as shown in Figure 3, which could potentially fail DNA sequencing. Samples 2a, 2b, 3a, and 3b were amplified using the DNA LCO1490/HCO2198 primers. Each part of *I. hippuris* may have unique genetic differences. However, only two parts could be identified from the results, including the main and base stems. It can be used as a benchmark for time efficiency in conservation activities. The same gene

may be expressed with different intensities in different body parts, which can provide insight into gene regulation and phenotypic differences between sites. Furthermore, research on the genetics of *I. hippuris* has not been carried out.

The PCR products were sequenced and aligned to obtain nucleotide base sequences, and species identification was carried out based on the Gen Bank reference database. The species identification was performed on samples using the LCO1490/HCO2198 primers. Based on the BLAST sequence analysis, the detected species in the samples were identified as *I. hippuris*. Homology sequence analysis and comparison with Gen Bank using NCBI BLAST showed consistent percentage values. Drancourt et al. (2000) explained that homology values greater than or equal to 99% indicate the same species, while values greater than or equal to 97% indicate species within the same genus. Data on closely related species are important to analyze to determine the relationship between the sample sequence data and Gen Bank (Kuske et al., 2006).

The identification of *I. hippuris* could only be performed on certain parts, including the base of the stem and main stem, due to unique morphological characteristics or physical characteristics found on those parts. There was noisy data with weak signals in sequencing analysis results, resulting in short sequences because the DNA template in the PCR reaction was too much or too little, degradation of the DNA template, contaminated DNA, and no priming site. Therefore, accurate identification of specimens requires sampling from appropriate sections. In addition, this study highlights the importance of genetic diversity for the growth, development, and regeneration of species and the potential for genetic diversity to determine the ability of corals to adapt to changes in environment, climate, and disease (Perwati, 2009). The results of this study increase the understanding of genetic diversity within *I. hippuris* populations and emphasize the need to carefully select DNA extraction sites when investigating the genetic diversity of marine organisms.

In addition, environmental variables such as ocean currents, tides, and geological changes can affect marine life's genetic makeup, making understanding population structure and genetic diversity even more important. Lind et al. (2009) and Ovenden et al. (2013) have shown that environmental variables can alter a species's distribution, affecting its genetic makeup. DNA barcoding has immense potential in conserving and managing marine ecosystems by facilitating species identification, particularly in cases where morphological differences are challenging to discern. This advanced technology enables precise monitoring and management of marine species and their habitats and can also aid in identifying endangered or vulnerable species. However, the application of *COI* genetic analysis may be restricted to specific subgroups of *I. hippuris*, which can limit its usefulness in precise species identification. Various physical conditions can also affect the genetic makeup of marine species.

CONCLUSION

COI gene analysis could only be performed on the base and main stem of the *I. hippuris*. The lateral stem of *I. hippuris* from coral and seagrass-coral areas was not further analyzed due to low concentration and purity values, which could potentially fail DNA sequencing. Each part of *I. hippuris* may have unique genetic differences. This study highlights the advantages of DNA sequencing in providing a unique genetic fingerprint for each species, enabling accurate species identification. This research provides insight into using DNA barcoding for sea bamboo species identification.

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