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Morphological and Molecular Characterization of Pufferfish from Lekok Coastal Area, Pasuruan Indonesia

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Abstract. Pufferfish belong to the Tetraodontidae family, which consists of 28 genera and 184 species; however, there are limited studies on the species from East Java, especially Pasuruan. This indicates the need for the collection of more accurate morphological and genetic resources as an important step for its conservation. Therefore, this study was conducted to identify pufferfish species found in the Lekok Coastal Waters, Pasuruan District, through morphological characterization and molecular analysis using DNA barcoding of the COI gene. A total of 12 samples were collected from the Lekok Coastal Waters, Pasuruan Regency. Fin samples were stored in 96% absolute ethanol. DNA extraction, polymerase chain reaction (PCR), and sequence analysis were performed using bioinformatics tools, the BOLD system, and the Automatic Barcode Gap Discovery (ABGD) web platform. Phenetic taxonomy was further applied using Ntysc to enhance the robustness of genetic analysis. The results showed that there were identified five pufferfish species were identified: *Lagocephalus spadiceus, Chelonodontops patoca, Cyclichthys orbicularis, Arothron stellatus,* and *Arothron reticularis*. High haplotype diversity (Hd) of 1 and nucleotide diversity (π) of 0.1 indicate significant genetic variation. The frequency of parsimonious informative sites was 22.6%, with 13 polymorphic sites and an overall ts/tv ratio of 2.2. The phylogenetic tree showed unambiguous branching patterns among species. These findings are supported by morphological and molecular identification results, which indicate the presence of five species of pufferfish along the Lekok Coast, Pasuruan Regency.

Keywords: Diagnostic characters; DNA barcodes; genetic composition; phenetic

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INTRODUCTION

Indonesia is the largest archipelagic country in the world, with two-thirds of its territory consisting of oceans and possessing a wide variety of biodiversity, including biological and non-biological diversity (Djunarsjah & Putra, 2021). One of the biological diversities found in Indonesian waters is the diversity of fish species, such as the pufferfish. The pufferfish belongs to the Tetraodontidae family, which consists of 28 genera and 184 species (Matsuura, 2015; Farrag et al., 2016).

In Indonesia, Pufferfish can be found in Pangandaran Regency, West Java, namely the species *Canthigaster amboinensis, Canthigaster compressa, Canthigaster valentinii,* dan *Chelonodon patoca* (Nuryanto et al., 2020), in the waters of Pati, Central Java, namely the species *Tetraodon lunaris* (Hapsara et al., 2019) in Bengkalis District, Riau Province, seven pufferfish species were found, *Tetraodon nigroviridis, Tetraodon fluviatilis, Sphoeroides lunaris, Sphoeroides oblongatus, Chonerhinos*

amabilis, Chonerhinos sillus, and *Chelonodon patoca* (Djakatara et al., 2018)In the Maluku Sea and the Thousand Islands Sea, the species *Triodon macropterus* (Tetraodontiformes: Triodontidae) was found (Wibowo et al., 2013), and in the Barito River, South Kalimantan, three species were found, namely *Chonerhinos naritus, Tetraodon fluviatilis,* and *Tetraodon nigroviridis* (Makri et al., 2021). Meanwhile, the pufferfish commonly found along the Beranta coast in East Java are *Lagocephalus spadiceus* (Noordyanto et al., 2023) and *Tetraodon lunaris* in Probolinggo (Domili, 2017).

The distribution and abundance of pufferfish are influenced by environmental factors such as temperature, pH, salinity, and turbidity (Gartanto, 2023). Pufferfish can be found in tropical and subtropical waters of the Indian Ocean and in marine, brackish, and freshwater habitats Generally, pufferfish inhabit calm waters and coral reefs (Chen & Peng, 2019). One such habitat is in Pasuruan Regency, which is suitable for pufferfish due to the presence of coral reefs, specifically the Lekok Coastal Area (Sahidin et al., 2018).

Pufferfish come in various shapes, it challenging to find morphological characteristics that distinguish some species from their close relatives. Morphology is highly similar between species, but there are still some differences, such as between *Arothron hispidus* and *Arothron reticularis*, which are so similar that they are difficult to distinguish. *Lagocephalus spadiceus and Lagocephalus lunaris* have only a few differences, namely the fine spines on their backs. The lack of scientific information encourages further research using DNA barcoding, which has a higher accuracy rate than morphological observation, to identify pufferfish species found on the Lekok Coast, Pasuruan Regency. Research conducted by Kaleshkumar et al. (2015) discovered the species *Chilomycterus retikulatus, Arothon hispidus,* and *Lagochepalus guentheri*, which show significant similarities with existing species and subspecies in India. Liu et al. (2024) revealed synonyms within Tetraodontiformes and identified the valid species *Takifugu rubripes* and *Lagocephalus spadiceus* in the South China Sea.

DNA barcoding is a molecular method that offers advantages in species identification with high accuracy compared to morphological observation (Anzani et al., 2019; Juniar et al., 2021; Liu et al., 2024). Molecular techniques aim to identify species through genetic markers that differ between taxa (Abdullah et al., 2019; Nugroho et al., 2017; Ricardo et al., 2020; Winarni et al., 2024). The COI gene (*Cytochrome Oxidase I*) is a coding gene found in the mtDNA genome. The advantage of this gene is that it rarely undergoes deletions and insertions in its sequence (Tindi et al., 2017). The COI gene can distinguish species from various animal groups, including freshwater and marine fish. Turan et al. (2017) stated that the COI gene can identify pufferfish species (Tetraodontidae) in Turkish waters. Based on this background, this study aims to identify the species of pufferfish found in the Lekok Coast, Pasuruan Regency, through morphological characterization and molecular analysis using the COI gene DNA barcoding. Additionally, this study provides deeper insights into SDG 14, which focuses on the conservation and sustainable use of marine natural resources.

METHODS

Sample Collection

Pufferfish were collected from the coastal waters of Lekok Bay, Pasuruan Regency, East Java, Indonesia (Figure 1) at each sampling station. Samples were collected using a gill net assisted by fishermen. All samples were placed in bottles containing 96% absolute ethanol specifically for DNA analysis.

Procedure

Morphological Character Identification

The pufferfish found were then collected. All pufferfish species in the laboratory were sorted and washed to facilitate morphological observation and identification. Morphological measurements were performed on 17 characters using a digital caliper with a precision of 0.01 mm, following the protocol (Han et al., 2017). The pufferfish specimens were then preserved in 70% alcohol and stored at the Zoological Taxonomy Department, Surabaya State University, Indonesia. Before molecular analysis, the specimens were frozen at -20°C for DNA extraction.

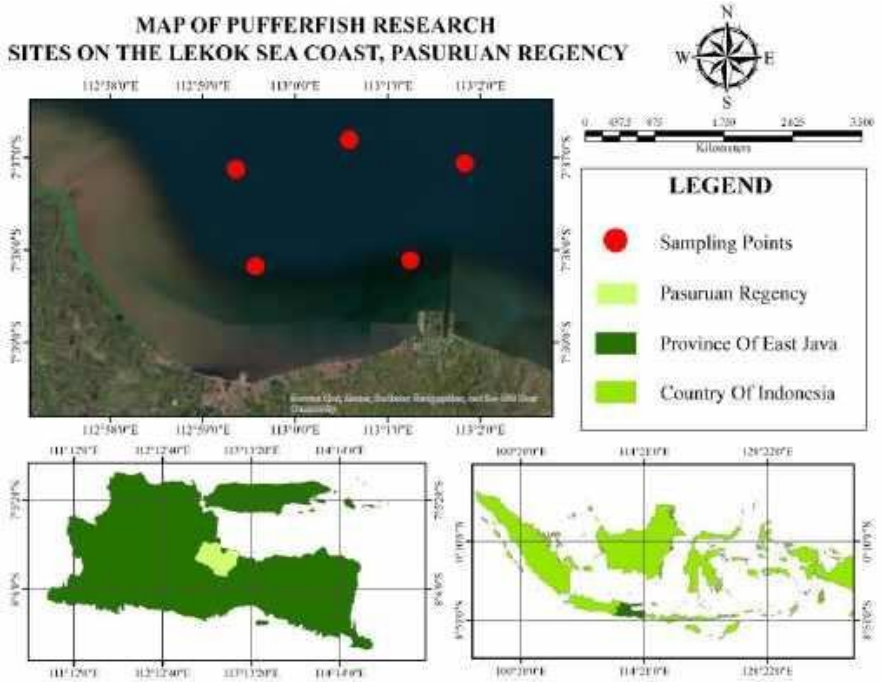

Figure 1. Map of pufferfish sampling locations on the Lekok coast, Pasuruan Regency, East Java, Indonesia.

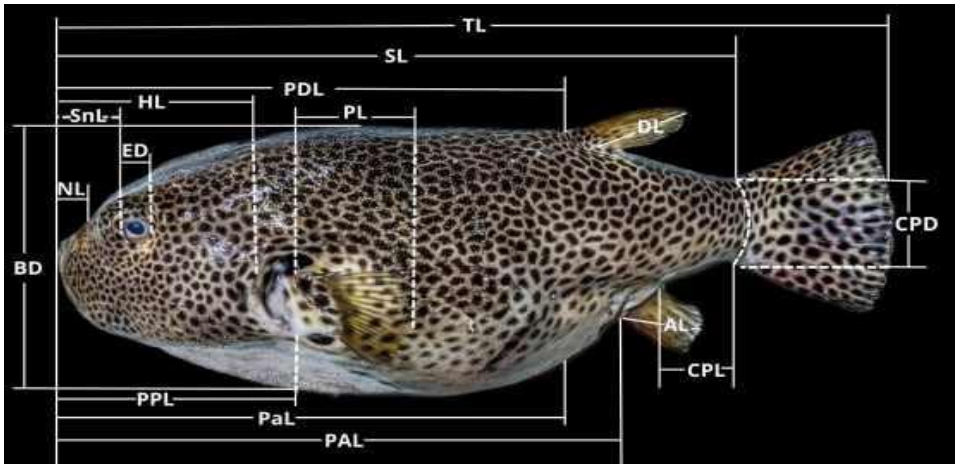

Figure 2. Morphometric characteristics of pufferfish in Lekok Coast, Pasuruan Regency, East Java, Indonesia. Notes: AL, anal fin length; BD, body depth; CPD, caudal peduncle depth; CPL, caudal peduncle length; DL, dorsal fin length; ED, eye diameter; HL, head length; NL, snout length; IW, interorbital width; PAL, preanal length; Pal, preanus length; PDL, predorsal length; PL, pectoral fin length; PPL, prepektoral length; TL, total length; SL, standard length; SnL, snout length (Han *et al*., 2017).

DNA extraction

Total DNA isolation of fin tissue samples was performed using the NexPrep Kit. The process began by weighing 20 mg of fins. The fish samples were then ground using nitrogen until smooth, then placed in a 1.5 ml tube. Store the DNA at 20°C for several days and at -70°C for long-term storage.

DNA Extraction and Sequencing

The isolated DNA was then amplified using a Biorad PCR machine in 30 µl of solution consisting of 15 µl Nexpro PCR Master Mix, 3 µl DNA Template sample (100 ng/µl), 6 µl Water, and 3 µl primer (10 pmol of each forward and reverse primer). The primers used were LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 (5'-TAAACTTCAGGGTGACCA AAAAATCA-3') (Folmer et al., 1994). Amplification was performed with the following temperature settings: pre-denaturation at 94°C for 1 minute, followed by 40 cycles consisting of denaturation at 94°C for 45 seconds, annealing at

41°C for 45 seconds, and extension at 72°C for 1 minute 30 seconds. This was followed by a post-elongation step at 72°C for 10 minutes. The PCR products were then electrophoresed on a 1% agarose gel. The PCR products were subsequently sequenced using 1st BASE Laboratories Sdn Bhd sequencing services.

Data Analysis Techniques Morphology

The descriptions of morphological observations using morphometric, meristic, and diagnostic characters.

Phylogenetic Reconstruction

Phylogenetic reconstruction based on partial COI gene sequences involved 20 sequences, including in-group and out-group accessions obtained from GenBank. Phylogenetic reconstruction or phylogenetic trees were constructed using MEGA XI version (64-bit), employing the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods. For both NJ and ML trees, Kimura 2- Parameter (K2P) substitutions were used (Nishimaki & Sato, 2019). Additionally, variation in rates among locations was modeled using a Gamma distribution, and a bootstrap consensus tree was inferred from 1.000 repetitions (Russo & Selvatti, 2018).

Species restriction using ABGD

Species delineation in fish using the COI gene was performed using the Automatic Barcode Gap Discovery (ABGD) method, as described by (Puillandre et al., 2012). This algorithm identifies suspected genetic groups or species by detecting turning points in the frequency distribution of paired genetic distances ranked between homologous sequences.

RESULTS AND DISCUSSION

Morphological Characteristics

Lagocephalus spadiceus

Material examined. Lekok Sea Coast, Pasuruan Regency, East Java, Indonesia.

Description. It has an elongated and inflatable body, a white lower head, a blunt and wide snout, a small terminal mouth, a non-prominent nose with two small nostrils, an indistinct chin, round and large eyes, four large teeth, two curved lateral lines on each eye, interorbital regions not extending beyond the pectoral and abdominal fins, smooth skin (half-smooth) on the upper and sides of the body without scales but with spines, spines with a smooth texture, short spines, spines only present on the dorsal region before the base of the dorsal fin, spines are distributed on the ventral side, no pelvic fins, with four fins: D.12-13; P.17; A.10-12; and C.10-12, the tail is single- curved (Emerginate), the area around the anus is white. (Figure 3A; Figure 4A; Figure 5A).

Coloration. It has a plain grayish-yellow color on the upper body (sides), a golden yellow gradient on the sides of the body, and white with thin spines on the lower sides.

Body Length. Standard length (SL) and total length (TL) range from 13.2–14.5 cm and 14.5–15.4 cm, respectively, with averages of 13.9±0.60 cm and 14.85±0.53 cm (n=4).

Distribution. According to Xu *et al*. (2024), the species *Lagocephalus spadiceus* is distributed along the southern coast of Africa in the Indian Ocean, eastward to Indonesia and the Philippines in the Pacific Ocean, and northward to the coast of China.

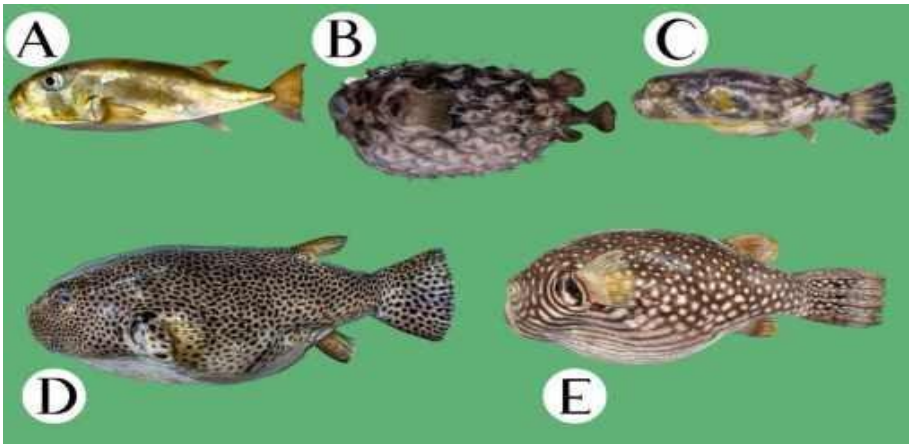

Figure 3. A. *Lagocephalus spadiceus* lateral view, B. *Cyclichthys orbicularis* lateral view, C. *Chelonodontops patoca* lateral view, D. *Arothron stellatus* lateral view, E. *Arothron reticularis* lateral view, from the Lekok Coast, Pasuruan Regency, East Java, Indonesia.

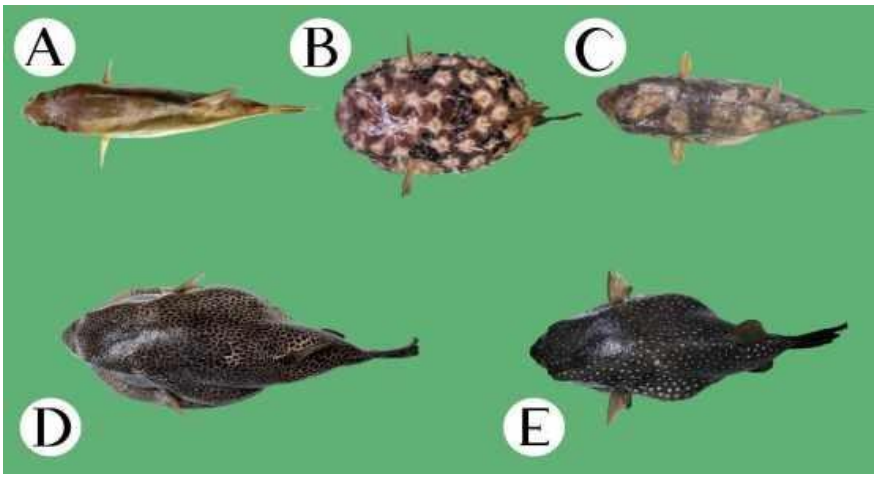

Figure 4. A. Dorsal view of *Lagocephalus spadiceus*, B. Dorsal view of *Cyclichthys orbicularis*, C. Dorsal view of *Chelonodontops patoca*, D. Dorsal view of *Arothron stellatus*, E. Dorsal view of *Arothron reticularis*, from the Lekok Coast, Pasuruan Regency, East Java, Indonesia.

Cyclichthys orbicularis

Material examined. Lekok Coast, Pasuruan Regency, East Java, Indonesia.

Description. It has a round body, a white lower head, a short snout with a small terminal mouth, a non-prominent nose, and two small nostrils. It has a flat interorbital region, large round eyes, circular patterns grouped on the dorsal and dorso-lateral regions, a hard spiny texture, long spines, spines distributed throughout the body, spines extending from the interorbital region to the dorsal fin, and spines also present on the ventral region. It lacks a caudal fin and has only four fins: D.10- 11; P.16-19; A.10-12, and C.9-10. The caudal fin is rounded. The area around the anus is white. (Figure 3B; Figure 4B; Figure 5B).

Coloration. Black spots are scattered in groups on the dorsal and dorsolateral parts of the body. Body Length. Standard length (SL) and total length (TL) range from 14.5–15.8 cm and 16.5–17.1 cm, respectively, with averages of 15.15±0.91 cm and 16.8±0.42 cm (n=2).

Distribution. Found in South Africa to the Red Sea, southern Japan, the Philippines, Australia, and New Caledonia.

Chelonodontops patoca

Material examined. Lekok Coast, Pasuruan Regency, East Java, Indonesia.

Description. *Chelonodontops patoca* has an elongated body, the lower part of the head is white, with round to oval spots on the head and dorsal body, four broad transverse stripes, a blunt snout, a small and terminal mouth, a concave nose with slightly protruding edges, a flat interorbital space, papillae on the surface of the lips, the nasal organs have two folds, the eyes are round and large with pale yellow edges, there are two lateral lines, no spines (spinulae) on the lateral body, the spine texture is smooth, the spines are short, the body is covered by short spines (spinulae), the spines extend from the interorbital region to the dorsal fin, and the spines are distributed on the ventral region. It lacks a caudal fin and has only four fins: D.9-10; P.8-10; A.8, and C.14-17. The caudal fin is flat. The area around the anus is white. (Figure 3C; Figure 4C; Figure 5C).

Coloration. The body is greenish gray to brown on the dorsal side, with round to oval white spots on the head and dorsal side of the body.

Body Length. Standard length (SL) and total length (TL) range from 9.2–11 cm and 10.7–12.3 cm, with an average of 10.1±1.27 cm and 11.5±1.13 cm (n=2).

Distribution. *Chelonodontops patoca* was discovered in brackish waters north of the Bay of Bengal (Habib et al., 2018). Several researchers have discussed *Chelonodontops patoca* in the Indo-Pacific region (Matsuura et al*.,* 2015; Sujatha and Padmavathi 2015) in the Ganges River estuary, and it is widely distributed in brackish and marine environments in the Western Indo-Pacific.

Arothron stellatus

Material examined. Lekok Sea Coast, Pasuruan Regency, East Java, Indonesia.

Description. *Arothron Stellatus* has a round body shape, the lower part of the head is white, the body has round to oval patterns, a short snout with a thick and terminal mouth, a short and slightly protruding nose with two pairs of nostrils formed from a single base branching into each nostril, a wide and flat interorbital space, wide, large, flat, and round eyes, four strong teeth, spiny skin, not

scaly, with a smooth spiny (spinal) texture, short spines, spines extending from the dorsal region between the eyes to the dorsal fin, and spines distributed on the ventral side of the body. It lacks a caudal fin and has only four fins: D.10-12; P.18-19; A.10-11, and C. 11. The caudal fin is flat, with a pattern of lines encircling the pectoral fins, an oval pattern at the base of the pectoral fins, and a round pattern at the base of the caudal fin, with the caudal fin itself being rounded. The area around the anus is black, which distinguishes it from *Arothron reticullaris*. (Figure 3D; Figure 4D; Figure 5D).

Coloration. It has many black spots, except for the white belly, all over its body. The color pattern varies according to growth. In adult fish, the head and body are brown to light brown on the upper surface of the back, while the base of the pectoral fins and the area around the anus are black.

Body Length. Standard length (SL) and total length (TL) range from 21–22.8 cm and 25.6–26.4 cm, respectively, with an average of 21.9±1.27 cm and 26±0.56 cm (n=2).

Distribution. According to Madkour et al. (2023), *Arothron Stellatus* is distributed throughout tropical and subtropical waters in the Indian Ocean, Red Sea, Polynesia, southern Japan, and the coast of Australia. It is also widely distributed along the coast of Taiwan, the South China Sea, north to southern Japan, and south to Lord Howe Island and the southeastern Atlantic, the southern coast of South Africa.

Arothron reticularis

Material examined. Lekok Sea Coast, Pasuruan Regency, East Java, Indonesia.

Description. *Arothron reticularis* has a round body shape, with longitudinal stripes on the lower abdomen, round markings on the body, a convex back with a straight, slightly concave head profile, a blunt snout with a large terminal mouth, a short, slightly protruding nose with two pairs of nostrils formed by the branching of a single opening at each nostril, a wide interorbital space, wide, round eyes with a white ring around them, and four teeth, with the front teeth protruding further than the premaxilla. The entire body and head are covered with spines, except for the lips, which have a smooth spiny texture, short spines, spines on the dorsal side from the interorbital region to the dorsal fin, and spines spread on the ventral side of the body. No caudal fin, and only four fins: D.10-11; P.17-19; A.9-10; and C. 8-11, the caudal fin is flat, with a pattern of lines encircling the pectoral fins, an oval pattern at the base of the pectoral fins, and a round pattern at the base of the caudal fin, and the caudal fin is rounded (Figure 3E; Figure 4E; Figure 5E).

Coloration. The head and body are brown on top and whitish on the underside. The dorsal and lateral sides of the body, the base of the tail, and the caudal fin are white.

Body Length. Standard length (SL) and total length (TL) range from 36–37.5 cm and 46.4–47 cm, respectively, with an average of 36.75±1.06 cm and 46.7±0.42 cm (n=2).

Distribution. According to Kang et al. (2020), the genus Arothron is distributed in the tropical Indo-Pacific, an antotropical zone (Matsuura, 2016). *Arothron reticularis* can be found in the tropical waters of Okinawa, Taiwan, and the western Indo-Pacific.

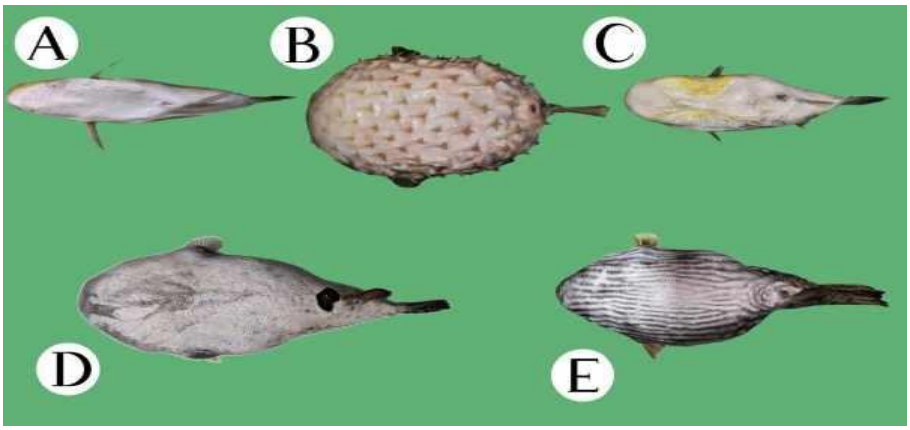

Figure 5. A. *Lagocephalus spadiceus* ventral view, B. *Cyclichthys orbicularis* ventral view, C. *Chelonodontops patoca* ventral view, D. *Arothron stellatus* ventral view, E. *Arothron reticularis* ventral view, from the Lekok Sea Coast, Pasuruan Regency, East Java, Indonesia.

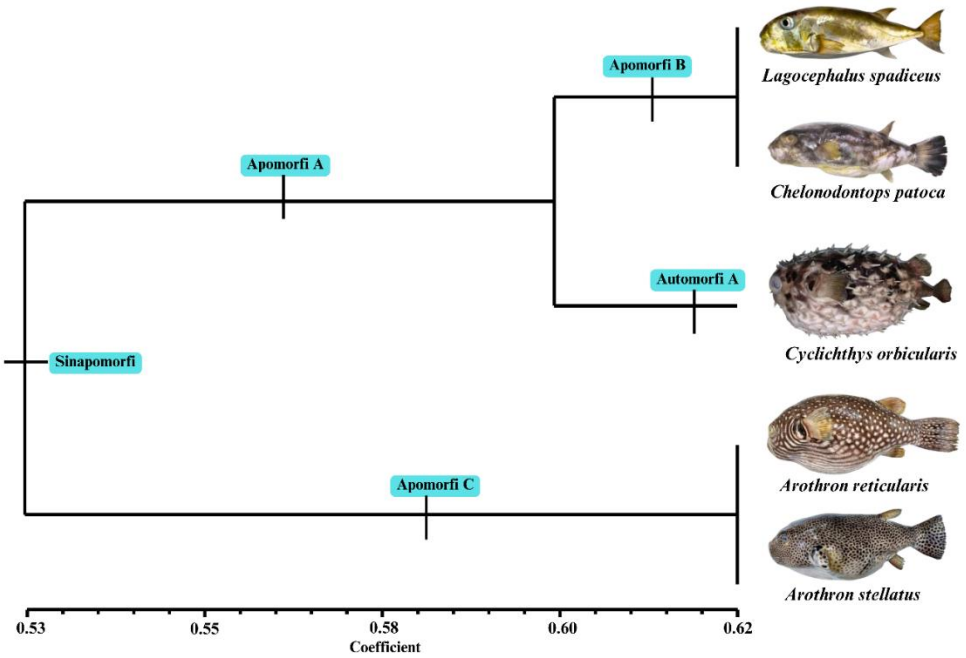

Figure 6. Dendogram of character morphology of species *Lagocephalus spadiceus, Chelonodontops patoca, Cyclichthys orbicularis, Arothron reticularis,* dan *Arothron stellatus*

Numerical taxonomy classifies species based on morphological similarities. This study examined five pufferfish species: *Lagocephalus spadiceus*, *Chelonodontops patoca*, *Cyclichthys orbicularis*, *Arothron reticularis*, and *Arothron stellatus*. Morphological traits were coded (0 for ancestral, 1 for derived) and analyzed using 66 characters via UPGMA and NTSYSpcV2.02i software. The resulting dendrogram identified three apomorphies: A (*L. spadiceus* and *C. patoca*), B, and C (*A. reticularis* and *A. stellatus*), with *C. orbicularis* forming its own branch. No prior morphological classification of these species exists. The phenetic taxonomic analysis highlights the morphological traits of five taxa: *Lagocephalus spadiceus*, *Chelonodontops patoca*, *Cyclichthys orbicularis*, *Arothron reticularis*, and *Arothron stellatus*. Shared traits among taxa in a branch are synapomorphies, while unique traits in a single taxon are automorphies. Apomorphy A forms two subclades: *L. spadiceus* and *C. patoca*, with a similarity of 61.54%. Subclade variations often arise due to different taxonomic markers. A more comprehensive morphological analysis helps clarify pufferfish taxonomy, especially along the Lekok Coast, Indonesia. The more shared traits, the closer the relationship (Rahayu et al., 2019). *A. reticularis* and *A. stellatus* show the highest similarity at 66%. Overall, the classification based on 65 morphological characters confirms the distinction among the five species. Related OTU traits are shown in Figure 6.

Sequence Composition and Genetic Diversity

The amplification of the COI target gene was successfully verified by the appearance of clear DNA bands and the absence of any smear, as visualized at approximately 639 base pairs (Table 4). The appearance of clear DNA bands indicates the success of genomic DNA isolation and COI gene amplification (Peloa et al., 2015). These results indicate that the amplification process accurately of the functional mitochondrial COI sequence, as evidenced by the absence of stop codons. Therefore, this analysis did not detect nuclear DNA sequences derived from mitochondrial DNA (NUMT), which typically have a length shorter than 639 base pairs (Ricardo et al., 2020) The COI gene is widely recognized as a tool capable of identifying species due to the presence of pseudogenes that were eliminated during protein translation. Various studies have also shown that COI sequences effectively distinguish species across different taxa. For example, recent biodiversity studies have generated new COI sequences for most species (Venera-Pontón et al., 2020).

DNA barcoding is a highly effective method for distinguishing pufferfish species across different regions, including Turkey, the United States, and East Belitung (Turan et al., 2017). In this study, we have compiled partial COI gene sequence profiles for *Lagocephalus spadiceus, Chelonodontops patoca, Cyclichthys orbicularis, Arothron stellatus, and Arothron reticularis*, five

marine fish species found along the Lekok Coast, Pasuruan Regency, Indonesia. This finding confirms the efficacy of DNA barcoding in accurately identifying species. No insertions/deletions or codon stop codons were observed during nucleotide sequence translation. These results demonstrate the accuracy of DNA barcoding as an effective tool for species identification and biodiversity assessment in marine fish populations.

Base composition analysis of partial COI gene sequences revealed a higher AT content (58.6%) than GC content (40.4%), a significant finding consistent with similar observations in various fish species across different regions (Modeel et al., 2024). in the Beas River and (Bingpeng et al., 2018). in the Taiwan Strait, reported similar COI gene base composition analysis trends, showing a pattern of AT dominance in fish species. Lower GC and G compositions were observed, which are characteristic of fish mitochondrial DNA. The GC content at the first codon position was significantly higher than at the other two positions, with the lowest base usage bias observed at the second codon position.

Table 4. Partial COI gene sequences used for phylogenetic tree reconstruction and genetic distance analysis include sequences from research samples and GenBank/BOLD systems (in group and out group).

Parameters	Position at codon			
	1st	2nd	3rd	Total
Thyrosine frequency	21.54%	40.1%	21%	639 bp
Cytosine frequency	35.40%	28.0%	36.2%	639 bp
Adenine frequency	20.65%	18.5%	32.1%	639 bp
Guanine frequency	22.41%	12.4%	12.7%	639 bp
Frequency of invariant sites	57.32%			
Frequency of parsimony informative sites	22.66%			
Nucleotide diversity (Pi)	0.15270			
Haplotype diversity	0.9778			
Number of haplotypes	24			
Polymorphic sites	132			
Variance of Haplotype diversity	0.09864			
ts/tv ratio (R)	2.167			
Gamma discrete distribution	0.18941			
Mean of evolutionary rate	0.01, 0.06, 0.11, 0.22, 0.25, 0.30, 0.41, 0.56, 0.63, 0.93, 1.20, 1.53, 1.98, and 2.74 substitutions per site			

Table 5. Identification Using the BOLD System

Species	Highest BOLD Identifikasi	Similarity (%)	Status
PV570132 *Lagocephalus spadiceus*	*Lagocephalus spadiceus*	97.23	Published
PV571720 *Lagocephalus spadiceus*	*Lagocephalus spadiceus*	96.84	Published
PV576000 *Lagocephalus spadiceus*	*Lagocephalus spadiceus*	97.23	Published
PV576001 *Lagocephalus spadiceus*	*Lagocephalus spadiceus*	96.44	Published
PV570028 *Chelonodontops patoca*	*Chelonodontops patoca*	99.58	Published
PV570125 *Chelonodontops patoca*	*Chelonodontops patoca*	99.37	Published
PV570127 *Cyclichthys orbicularis*	*Cyclichthys orbicularis*	99.49	Published
PV570128 *Cyclichthys orbicularis*	*Cyclichthys orbicularis*	99.32	Published
PV569962 *Arothron stellatus*	*Arothron stellatus*	99.83	Published
PV569965 *Arothron stellatus*	*Arothron stellatus*	99.5	Published
PV569960 *Arothron reticullaris*	*Arothron reticullaris*	99.84	Published
PV569961 *Arothron reticullaris*	*Arothron reticullaris*	99.52	Published

Identification Using the Bold System

Identification using BOLD (Barcode of Life Data System) refers to a web-based platform used to identify species through DNA data analysis based on the principle of *DNA barcoding*, which identifies species based on short DNA sequences from standard parts of the genome. In this study, the highest identification accuracy achieved for the five species of pufferfish from the Lekok Coast, Pasuruan Regency, ranged from 96.44 to 99.83%, demonstrating the effectiveness of DNA barcoding in identifying species. Accuracy is crucial in understanding the complexity and diversity of pufferfish species. These species often exhibit morphological differences that are not significantly distinct, making traditional taxonomic identification challenging. This study supports using the BOLD system as a superior tool for fish identification compared to other databases, aligning to achieve high identification accuracy, consistent with findings by (Modeel et al., 2024). The results of this study indicate that there are no differences in BOLD identification between the species observed in *Lagocephalus spadiceus, Chelonodontops patoca, Cyclichthys orbicularis, Arothron stellatus, and Arothron reticularis*.

Nucleotide Base Genetic Mutations

Analysis of nucleotide base mutations in pufferfish showed different results between species, where the species *Lagocephalus spadiceus* experienced a nucleotide base change at 157 bp from Adenine to Guanine, called a Purine Transition. *Chelonodontops patoca* exhibits a nucleotide base substitution at 277 bp and 553 bp from Cytosine to Thymine, known as a Pyrimidine Transition. The species *Cynoglossus cynoglossus* serves as an outgroup in this study and exhibits a nucleotide base transition at 358 bp from cytosine to thymine, known as a pyrimidine transition, and from cytosine to adenine at 485 bp, referred to as a transversion. The *Arothron reticularis* undergoes a nucleotide base change at 381 bp from adenine to guanine, known as a purine transition. The *Arothron stellatus* undergoes a nucleotide base change at 423 bp from adenine to guanine, known as a purine transition. The species *Cyclichthys orbicularis* experienced a nucleotide base change at 543 bp and 600 bp from thymine to cytosine, known as a pyrimidine transition, and from cytosine to adenine, known as a transversion, at 609 bp.

Phylogenetic Reconstruction

Phylogenetic reconstruction analysis produced a Neighbor-Joining (NJ) phylogenetic tree, as shown in Figure 7. Each pufferfish species was linked to a different DNA Barcoding cluster, making it easier to describe the clear phylogenetic relationships between species. The Neighbor-Joining (NJ) tree shows five distinct clusters divided into two branches. The Tetraodontidae family forms one branch, while the Diodontidae family forms another branch with a bootstrap value exceeding 99%, indicating strong relationships among the species. The phylogenetic tree shows an unambiguous branching pattern in determining the species *Lagocephalus spadiceus, Chelonodontops patoca, Cyclichthys orbicularis, Arothron stellatus, and Arothron reticularis*, which form their monophyletic branches. However, the closeness among these five species indicates a close evolutionary relationship. This distance indicates the most significant genetic separation among the species.

In addition, the ABGD method identified 5 groups of specimens with the initial approach and the barcode gap threshold calculated by the ABGD analysis of the COI dataset (see Figures 8. A and 8.B). Initial Partition with prior maximal distance P= 1.074363e-02; Barcode gap distance

= 0.001345. distance K80 Kimura =1.50. The ABGD method also identified a barcode gap centered around 1.8% of divergence between the available COI sequences. The analysis defined the existence of 5 to 9 hypothetical species in all recursive partitions with prior intraspecific genetic divergence values between 0.17% and 0.28%, a result we considered more likely than 3 or more species with intraspecific divergence values below 0.28% or as a single species with intraspecific divergence values greater than 2.15%.

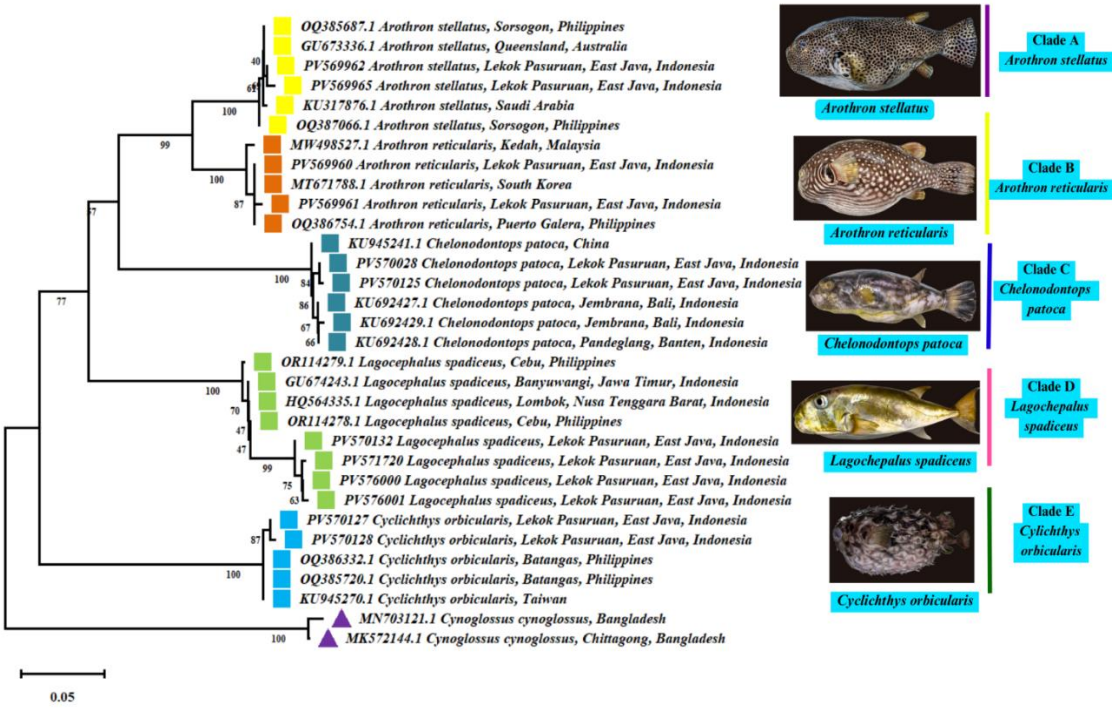

Figure 7. Neighbor Joining (NJ) phylogenetic tree of Pufferfish based on partial COI gene sequences. Clade symbols indicate the same species, and *Cynoglossus cynoglossus* is derived from the outgroup

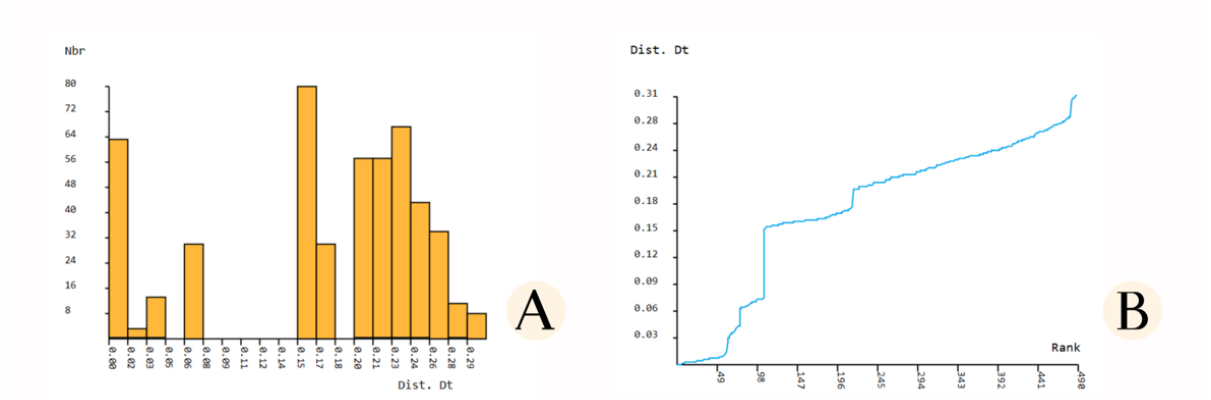

Figure 8. Analysis of Gap Barcodes of pufferfish. species generated by Automatic Barcode Discovery Gap Discovery. Distribution of K2P distances between each pair of specimens for the COI gene; A=Distance histogram; and B=Rank distance.

This study represents the first integrated morphological and molecular identification of pufferfish species from the Lekok Coastal Waters in Pasuruan, East Java—a region with limited prior taxonomic data. By combining traditional morphological characterization with DNA barcoding (COI gene) and advanced genetic analysis tools such as BOLD, ABGD, and NTSYSpc, the research offers a comprehensive and robust approach to species identification. The discovery of five distinct pufferfish species, along with evidence of high genetic diversity, provides valuable baseline data for future taxonomic, ecological, and conservation efforts in the region. The integration of phenetic taxonomy with molecular phylogenetics enhances the precision of species delineation, making this a novel contribution to both regional biodiversity records and methodological approaches in pufferfish taxonomy.

This research contributes significantly to scientific knowledge by enriching the taxonomic database of pufferfish species in a previously understudied region—Lekok Coastal Waters, Pasuruan, East Java. The integration of morphological and molecular (DNA barcoding)

methods enhances the accuracy of species identification, providing a reliable reference for future studies in marine biodiversity, systematics, and evolutionary biology. From a societal perspective, the findings support sustainable fisheries management and marine conservation efforts. Accurate species identification is essential for monitoring biodiversity and detecting local fish species. Additionally, understanding genetic diversity helps assess population health, which is crucial for protecting marine ecosystems and ensuring long-term food security for coastal communities dependent on fisheries.

CONCLUSION

This study successfully identified five pufferfish species: *Lagocephalus spadiceus*, *Chelonodontops patoca*, *Cyclichthys orbicularis*, *Arothron stellatus*, and *Arothron reticularis* from the Lekok Coastal Waters, Pasuruan Regency, through an integrated approach combining morphological characterization and molecular analysis using partial COI gene sequences. These findings provide a valuable foundation for future taxonomic, ecological, and conservation efforts. Further research is recommended to expand genetic sampling and integrate ecological data to support the sustainable use and conservation of pufferfish, particularly in relation to their ecological role and potential health applications.

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AUTHOR CONTRIBUTION STATEMENT

All authors contributed significantly to the work reported in this manuscript. VAEP, ADR, CAVTS, and DAR conceived and designed the study, conducted the data analysis, data collection, and led the manuscript writing. FK was responsible for data collection, contributed to data interpretation, and critically reviewed the manuscript for important intellectual content. EDN, RR, and NBM were assisted in the literature review, supported data visualization, and provided substantial feedback during the manuscript drafting process. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work.

INFORMED CONSENT STATEMENT

Informed consent was obtained from all subjects involved in the study. Prior to participation, each subject was provided with a detailed explanation of the study's objectives, procedures, potential risks, and benefits. All participants voluntarily agreed to participate and signed a written informed consent form.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this paper.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that no artificial intelligence (AI) tools were used in the generation, analysis, or writing of this manuscript. All aspects of the research, including data collection, interpretation, and manuscript preparation, were carried out entirely by the authors without the assistance of AI-based technologies.

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