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First Molecular Identification of Sunfish in North Bali Water

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Abstract Stranded Sunfish was found in North Bali, and we collected some part that was used for molecular identification. Samples were amplified at the d-loop locus (control region) using the PCR (Polymerase Chain Reaction) method. Primers used in PCR were H16498 as primary front (forward) and L15812 as reverse primer. Similarity value of 95% after alignment with Mola ramsayi (accession number accession AY940824) on GenBank wa obtained, with the gaps of the nucleotide was 1%. The stranded sunfish identified using partial sequence mtDNA was the same species as the species *Mola ramsayi*.

Keywords: stranded fish, *Mola ramsayi,* partial sequence.

I. INTRODUCTION

Molidae is the family with large number of species, primarily pelagic members of the Tetraodontiformes. Commonly known as ocean sunfish, and have a distinctive laterally compressed shape and "chopped off" appearance [1-3]. The median fins is primarily for swimming, they lack of ribs, caudal bones, pelvic fins, spines and have fewer vertebrae than any other fish [4]. Unlike most fish, sunfish has two distinct larva phases. A typical Tetraodon-like larval and another highly transformative stage resulting in the complete absorption of the tail [1].

Members of Molidae distributed in both temperate and tropical waters. These coasltal regions include the Central and Southern of California , Malaga of Spain, Cape Town of South Africa, Kamogawa of Japan, Hua Lien of Taiwan's, Camogli of Italy and Galapagos Islands of Ecuador's. In Indonesia, sunfish are found in southern coastal region of Bali, particularly in the area of Lembongan and Penida Islands, which those islands are belong to Bali region [5]. It has never been recorded to occur in Northern coastal region of Bali.. The species of sunfish found in coastal regions of Lembongan and Penida is *Mola ramsayi*, with density of 1 individual per 6.8 km [6]. In 2017 a sunfish was found stranded in the North Bali. Rescue process have been tried out repeatedly, but the fish was suffered and unbalance when swimming and were finally declared died on location. The fish was brought to the Biology Laboratory of Ganesha Education University for identification. The present study aimed to identify the species using molecular identification.

II. RESEARCH METHOD

Sample Collection and preparation

The samples were obtained from stranded fish found in northern coastal of Bali, Singaraja. Sample were transported to the laboratory and the morphometrics such as the weight and length were measured. Some muscle tissues were collected for molecular identification.

Extraction method

DNA extraction of samples was carried out using the 10% chelex method [7]. Sample of muscle tissue was cut with the size of ±2mm, immersed in 10% chelek solution, vented for 20 seconds and centrifuged for 20 seconds. The sample was then heated on a heatblock at 95°C for 45 minutes, mixed in a vortex and centrifuged for another 20 seconds.

Polymerase chain reaction

Samples were amplified at the d-loop locus (control region) using the PCR (Polymerase Chain Reaction)

method. Primers used in PCR are H16498 (5'-CCTGAAGTAAGAACCAGATG-3') as primary front and L15812 (5'-CCTCCCTAAGACTCAAGGAAG-3') as reverse primer [8]. The following is the mixture of reagents used in the PCR process for each sample. Master Mix: ddH2O: 10 ul, Primary 1: 1.25 ul, Primary 2: 1.25 ul, Bioline Ready Mix: 12.5 ul, Extraction template/DNA product: 1 ul.

All reagents are inserted and mixed into the PCR tube and run on a thermalcycler machine with the following temperature optimization. Pre-denaturation: 94°C 15 seconds, next denaturation 94°C 30 seconds, annealing 50°C 30 seconds, extension: 72°C 45 seconds, and continued to post-extension 72°C 5 minutes. The PCR process was carried out as many as 38 cycles.

Electrophoresis

One percent agarose gel was used for the electrophosesis. This gel is made from a mixture of 80 ml SB buffer (sodium & boric acid) and 0.8 mg agarose. Both ingredients are mixed in a glass beaker or erlenmeyer flask and heated until completely dissolved. Next, the liquid is poured into the mold, then cooled for ±45 minutes until the gel thickens. Samples were prepared by mixing 1µl loading dye in 3 µl PCR product. Mixed samples were inserted in to the gel well. Lader marker was inserted on one end of the gel.

Sequencing

The amplified PCR product was sent for sequensing to the UC Berkeley sequencing facility following the method ofSanger. The sequences of DNA was analyzed in MEGA 6 and BLAST by comparing with Gene Bank to identify the fish species.

III. RESULT AND DISCUSSION

The total lengths of the partial loop mitochondrial amplification was 419 bp. The alignment of the mitochondrial DNA to the Bass *et al*. (2005) with the accession of AY940824found 95% similarity with 1% nucleotide gaps (Table 1). Therefore, it was believe that using partial sequence mtDNA, the stranded sunfish found in Singaraja coastal region was similar to that of *Mola ramsayi* identified by Bass *et al* (2005) [3].

Mitochondrial DNA alignment in this study found that molecular characteristics of the stranded sunfish was very identical to the molecular data of the reference species from GenBank, that was *Mola ramsayi*. However, 5% differences was still recorded of all sequence analyzed. This is probably due to the difference of the amplification primer used, namely H16498 (5'-CCTGAAGTAAGAACCAGATG-3 ') as primary front (forward) and L15812 (5'-CCTCCCTAAGACTCAAGGAAG-3') as reverse primer [8]. Whereas the reference sequence uses primers A (5'-TTCCACCTCTAACTCCCAAAGCTAG-3'), E (5'-CCTGAAGTAGGAACCAGATG-3') and M (5'-

TABLE I
BLAST RESULT STRANDED OCEAN SUNFISH (*Mola ramsayi*) WITH *Mola ramsayi* ACCESSION NUMBER AY940824 (Bass *et al*., 2005)

Score	Expect	Identities	Gaps	Strand
540 bits (292)	3e-158	329/346 (95%)	5/346 (1%)	Plus/Plus

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Query  51   ATGGTGGGtatatacatatatgtattatcaccatatatatatatataCCATTAATCAATA  110
            ||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  1    ATGGTGGGTATATACATATATGTATTATCACCATATATATATATGTACCATTAATCAATA  60

Query  111  ATATCTTGCGAGCAATAAATTATATATGGGATAAAATGATCCAGAACATTGCAAGAAAAC  170
             |||||||||||||||||||||||||| ||||||||||||||||||  |||||||||
Sbjct  61   TTATCTTGCGAGCAATAAATTATATATGGAATAAAATGATCCAGAACATCACAAGAAAAC  120

Query  171  ACGAAATCTGAATGTATAAAAGACATAACACTAGAC-AGGGCATCCTGACTAAAAAGTTA  229
             | |||||  |||||||||||||||||||||||||| || ||| ||||||||||||||| |||
Sbjct  121  ATGAAATTTGAATGTATAAAAGACATAACACTA-ACTAGGACATCCTGACTAAAAAATTA  179

Query  230  AGCCCTAACACTTCAAATAATTTAAACAGATATACTTTGACTCAACATTCCTTCAAGGCA  289
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  180  AGCCCTAACACTTCAAATAATTTAAACAGATATACTTTGACTCAACATTCCTTCAAGACA  239

Query  290  AATGCTTAATGTAGTAAGAACCGACCATCAGTTGATTTCTTAATGCATACTCTTATTGAT  349
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  240  AATACTTAATGTAGTAAGAACCGACCATCAGTTGATTTCTTAATGCATACTCTTATTGAT  299

Query  350  GGTGAGGGACAATTATTCGTG--GGGGTCACACTTAGTGAATTATT  393
             ||||||||||||||| ||||  ||||||||| ||||||||||||
Sbjct  300  GGTGAGGGACAATTAT-CGTGGTGGGGTCACATTTAGTGAATTATT  344
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TATGCTTTAGTTAAGGCTACG-3') [3][9]. The primary difference allows the sequence variation amplified by the same in the same control loop region. To support the results of this alignment, morphology was also identified. The morphological characters refer to Fraser-Brunner [1].

Mola ramsayi and *M. mola* almost similar morphological character, described by Fraser-Brunner [1]. They share several unique anatomical features, of which the most striking is the lack of almost all osteological elements of the caudal fin structures, which are replaced by a pseudocaudal fin [4][10]. *M. mola* have rougher skin and reduced band of denticles running between their dorsal and anal fins along the pseudotail known as a clavus. In *M. ramsayi*, the clavus region is supported by 14-24 fin rays and 12 ossicle [5][11]. This character is in accordance with stranded sunfish in this study, after surgery to ensure ray structure found 16 fin rays in clavus and ends at the ossicle at the end of clavus. Whereas when on *M. mola* the clavus has 12 fin rays, eight of which bear ossicles [12]. Beside that, *M. ramsayi* does not have the smooth band at the base of the clavus [13-14], it similar to the specimen in this research. The stranded ocean sunfish were we found in this research has 173cm total length, and 130 cm width (Table 2).

The phylogenetic tree (Figure 1) shown the relationship between species of *Mola mola* and *Mola ramseyi*, presented in three branches. The evolutionary history was inferred using the UPGMA method [15].

TABLE II
MORPHOMETRIC OF STRANDED OCEAN SUNFISH IN PRESENT STUDY (*Mola ramsayi*)

Body Length (cm)		Fins Length (cm)	
Total length	173	Length of ventral fin	78
body width	130	Width of ventral fin	30
Length of clavus	36	Length of dorsal fin	83
Width of clavus	82	Width dorsal fin	31

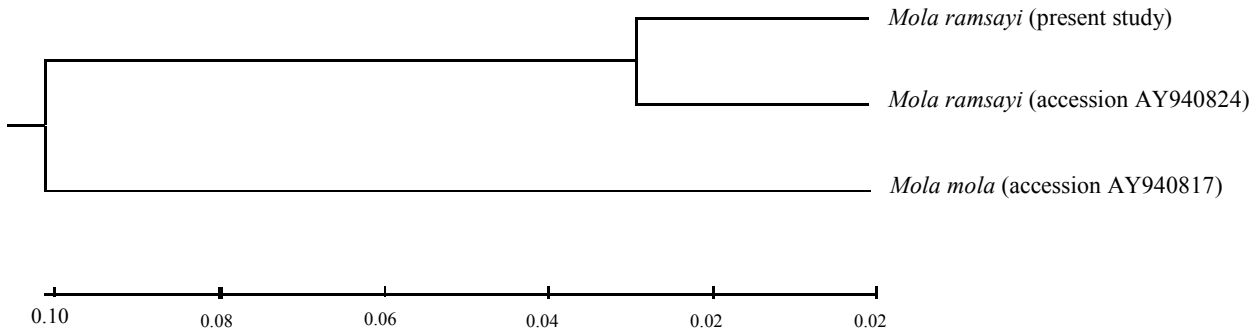

Fig. 1. Relationships of Mola ramsayi from present study and Mola from previous study by Bass et al., (2005)

The optimal tree with the sum of branch length= 0.23223534 was presented. The same lengh of tree units was used for the evolutionary distances. The evolutionary distances were calculted employing the Maximum Composite Likelihood method [16] and were in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 339 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [17]. It was found that the specific cluster of *Mola ramsayi* species has different molecular character to the *Mola mola.*

The morphological similarity between the specimens *M. ramsayi* (present study), and *M. ramsayi* were presented in Table 3. Both have similar morphological characters, those were by the present of smooth band, number of clavus fin rays, the ratios of ossicle size versus space between ossicle. [12]. The morphological characters of *Mola* seem to be well consistent with the key character of *Mola ramsayi* proposed by Fraser-Brunner [1], accept for the presence or absence of the smooth band. It may caused by age of the specimens, but further study is necessary [13][18].

In comparison *Mola mola*, the only other ocean sunfish species, they show similar dive behavior. *Mola mola* has been recorded in temperatures as low as 1.8^{o} C off Japan [6][18] and high as 30^{0}C off the southeast coast of the US and Bahamas [19-20]. The stranded *M. ramsayi* found in this study may have originated from the population of *M. ramsayi* in Lembongan Bali, the closest habitat of *M. ramsayi* found around Bali island. Research that has been carried out, *M. ramsayi* has random migration pattern They spent around 89 days for travelling in the distance of 747 km. Migration of ocean sunfish was also found depended on abundance of prey, temperature and other factors [19][21-22]. Further study is still required to investigated the cause of the stranded *M. Ramsayi*. Water pollution or other factor may contribute to this accorence.

TABLE III
COMPARISON OF MORPHOLOGICAL CHARACTERS OF MOLA IN PRESENT STUDY SPECIMEN WITH OTHERS MOLA SPECIMEN FROM PREVIOUS STUDIES

Species specimen	Present study	Sawai *et al.,* 2017a		Jawad, 2012	Jawad *et al.,* 2013
	M. ramsayi	*M. ramsayi*	*M. mola*	*M. ramsayi*	*M. mola*
Total length (cm)	173.0	91.6	91.6	135.0	135.0
Ossicles	12	12-13	11-13	12	8
Clavus fin rays	14	19	No data	16	12
Smooth band	Present	Present	Present	absent	Present
Ossicle size (OS) versus width of spaces between ossicles (SS)	OS>SS	OS>SS	OS>SS	OS>SS	OS<SS
Head bump	Absent	Absent	Absent	No data	No data
Shape of clavus	Round	Round	Round	No data	No data

IV. CONCLUSION

The sunfih which stranded on Singaraja coast was identified as *Mola ramsayi* which has been proven by 95% similarity from molecular identification of d-loop control region, partial sequence of mtDNA and morphological characters that correspond to the key characters of *M. ramsayi*.

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