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Phylogenetic Analysis of Endemic Fish from the Maros Karst Region, South Sulawesi, Indonesia

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Abstract

The Maros Karst is known for having a unique geomorphological structure with a diversity of endemic flora and fauna. The hydrology of this karst area is generally dominated by underground rivers with various freshwater ecosystems, which are generally dominated by endemic fish typical to Sulawesi. This study aims at identifying endemic fish of Maros Karst rivers using morphological and phylogenetic analysis as the baseline for further endemic fish conservation. Morphological analysis was done by comparing fish samples' morphological characteristics with those from fish taxonomy references. Phylogenetic analysis was carried out using mitochondrial DNA (mtDNA) analysis with the target gene Cytochrome C oxidase 1 (CO1). The results showed there are five endemic fish species from four families and three orders from the Maros Karst area with a genetic distance value between 0.0 to 0.275. These species make particular adaptations both morphologically and genetically to the karst ecosystem. However, it is necessary to understand the evolution of these fishes to establish effective conservation measures. Therefore, it is necessary to have a management strategy to ensure the sustainability of endemic fish populations and as well as the sustainability of the karst ecosystem as a whole. © 2021 Friends Science Publishers

Keywords: Maros Karst area; Endemic fish; Morphology; Phylogenetic; CO1

Introduction

The Maros Karst, known also as The Maros-Pangkep Karst, is an area that develops explicitly from the process of dissolving carbonate rocks (limestone and dolomite) within a specific time (Achmad and Hamzah 2016). According to Taslim (2014), Maros Karst area is unique, consisting of hills, valleys, dolina, uvala, polje, river systems and underground river networks and forests with different soil surface textures and compositions at each altitude, resulting uniqueness of the biota living in that karst area (Suhendar *et al*. 2018). According to Achmad and Hamzah (2016), this karst is one of the largest and second most beautiful karst in the world after the karst in China.

18 The Maros-Pangkep Karst is located in the Bantimurung-Bulusaraung National Park, which is known as one of the best conical karst areas in the world. This karst has a unique geomorphological form with a diversity of endemic flora and fauna (Putri *et al*. 2020). Endemic species is an organism whose distribution is limited in scope a21is only found in one particular area (Omar 2012; Dekić *et al*. 2015). The limited distribution and abundance of endemic species in a region allow knowledge of the rate of evolution such as area, age, isolation and environment (Hanly *et al*. 2017). Karst areas are highly fragmented habitats and could become a primary candidate for conservation due to their diversity and high levels of endemism (Çiçek *et al*. 2018), limited food sources, living under intense selective pressure and in natural isolation (Bichuette and Trajano 2015). The hydrology of the Maros Karst area is generally dominated by underground rivers (subsurface drainage). This condition is caused by the entry of rainwater through fractures which then concentrates and forms subsurface channels (Taslim 2014) forming Bantimurung River Watershed (Arsyad *et al*. 2014). Like other karst rivers, freshwater ecosystems within the karst are threatened with extinction because the permeability of the rocks could not filter well enough the contaminants from human, animal and industrial waste (Kolda *et al*. 2020). According to Omar *et al*. (2020) there are seven species of endemic fish in this karst need to be managed strategically to ensure the sustainability of those fish population as well as the watershed ecosystem as a whole.

The accuracy of species identification is necessary for fisheries management and conservation (Abdulmalik-Labe and Quilang 2019). Morphological and phylogenetic analysis is adequate tools for verifying species identity (Bayot *et al*. 2014). According to Serdiati *et al*. (2020), fish

morphological characters such as body shape, colour pattern, and the number of scales is used as an initial method to distinguish species. In accordance with phylogenetic ichthyofauna in the Maros Karst rivers, based on literature search, the information on this particular topic is still lacking. Out of seven endemic species of this area, our preliminary observation found only three species: *Marosatherina ladigesi*, *Lagusia micracanthus* and *Oryzias celebensis*. Several ichthyofauna studies that have been done were on *M. ladigesi,* related to its morphology such as taxonomic status (Hadiaty 2007); sexual dimorphism, colour patterns, habitat and distribution (Hadiaty 2017); reproductive biology (Omar *et al*. 2014; Kariyanti *et al*. 2019; Nasyrah *et al*. 2020); eco-biology (Nasyrah *et al*. 2019); genetics (Jayadi *et al*. 2015) and the length-weight relationship (Omar *et al*. 2020). More research was also done on *L. micracanthus* on the fish description and historical tracing (Hadiaty 2012; V and Hadiaty 2012), reproductive biology (Andy Omar *et al*. 2015; Nur *et al*. 2016), morphometrics (Nur *et al*. 2020a) and length-weight relationship (Nur *et al*. 2020b). As for *O. celebensis*, the research covered its gonad development (Hamaguchi 1983) and reproductive biology (Hasanah *et al*. 2019).

However, species identification through morphological analysis is complicated and considered subjective in deciding a species within the same genus; therefore, the current molecular approach using DNA barcode is preferably done to strengthen the morphological approach. The DNA barcoding is carried out by utilizing mitochondrial DNA as a basis for observing variations, kinship in species and studies related to genetics (Bucklin *et al*. 2010). Mitochondrial DNA (mtDNA) has become one of the most common molecular markers and used for phylogenetic and taxonomic studies in animals because of maternal inheritance. Although mtDNA sequencing data successfully determine the phylogenetic relationships, there are considerable differences in the characteristics of the various gene types and of great importance. Cytochrome c oxidase subunit I (COI) is the largest of the mtDNA cytochrome oxidase subunits (Clark *et al*. 2010). It is one of the largest protein-coding genes in the metazoan mitochondrial genome, which has been used as a target gene for molecular phylogenetic and identification studies (Naim *et al*. 2012).

According to Hebert *et al*. (2003), analysis of variations in CO1 sequences is an analytical method that has a high diversity and is very useful in determining the relatedness of species. Subunit 1 cytochrome oxidation was chosen because it is the most stable gene and its relatively fast evolution rate when compared to other genes in mtDNA (Bucklin *et al*. 2010). Besides, CO1 is also considered capable of differentiating between individuals at the species level (Lefébure *et al*. 2006). Cytochrome c oxidase subunit 1 PCR has been used by several researchers to identify freshwater fish in Lake Towuti, South Sulawesi (Larson *et al*. 2014) and managed to identify a new species of goby fish, while Jayadi *et al*. (2019) successfully identified endemic fish of the Termatherinidae family using PCR Cytochrome c Oxidase 1. Inadequate information on fish species identification, morphologically and phylogenetically, in Maros Karst became our research rationale to identify endemic species using CO1 target gene with the hope for baseline data in fish endemic conservation.

Materials and Methods

Sampling site and collection

A total of 15 fish samples were collected from three rivers (Batubassi, Bantimurung, and Pattunuang) of the Maros Karst, South Sulawesi, Indonesia (Fig. 1) from July to December 2020. Morphological identification was conducted according to the guideline from Kottelat *et al*. (1993) and Hadiaty (2012), prior to DNA analysis. Each individual fish sample was photographed using a digital camera. Species then confirmed through molecular identification using the CO1 gene region. In addition, 50 mg of anal fin tissues from each sample was preserved with 70% alcohol solution. Before the DNA extraction process, the collected samples were stored at room temperature.

DNA extraction, PCR and data analysis

DNA extraction was done using genomic DNA mini kit (Geneaid). Genetic analysis was performed using Cytochrome c Oxidase Subunit 1 (CO1) with FISH-BCL (5'-TCA ACY AAT CAY AAA GAT ATY GGC AC) and FISH-BCH (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA) primers referred to Baldwin *et al*. (2009) and Andriyono *et al*. (2020). CO1 gene amplification was conducted using PCR with initial denaturation program at a temperature of 95ºC for 5 min following 40 cycles consisting of denaturation at 95ºC for 30 s, annealing at 50ºC for 30 s, extension at 72ºC for 45 s and a final extension at 72ºC for 5 min. The PCR amplification product was then separated using electrophoresis of 1% agarose gel. A DNA fragment from agarose gel was documented using the Gel Documentation System (Biometra). Finally, the size of the DNA fragment was then measured using a 100 bp Plus DNA Ladder. DNA sequencing was performed at PT Genetics Science in Jakarta as a private agent. Gene purification and sequencing were carried out at 1st Base in Malaysia. The PCR product was 20 μL and the total primer was 150 μL.

DNA sequence data were used for forward and reverse primers which were put together or aligned using BioEdit7 software. The BLAST (Basic Local Alignment Search Tool) analysis at http://www.ncbi.nih.nlm.gov/BLAST (Madden 2013) was carried out on DNA sequences from each fish sample to determine its similarity to DNA COI sequences in the GenBank database. Accessions were of high similarity, and sequences were downloaded to make phylogenetic trees using the MEGA (Molecular Evolutionary Genetics Analysis) version 7.0 program (Kumar *et al*. 2016).

Fig. 1: Map showing study sites from where fishes were collected

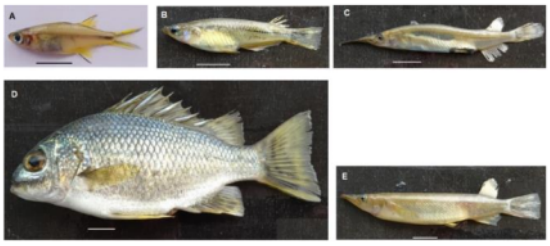

Fig. 2: Endemic fish found in the Maros Karst Region. (**A**) *Marosatherina ladigesi* (Ahl 1936); (**B**) *Oryzias celebensis* (Weber 1894); (**C**) *Dermogenys orientalis* (Weber 1894); (**D**) *Lagusia micracanthus* (Bleeker 1860) and (**E**) *Nomorhamphus liemi* (Vogt 1978); scale bar 1 cm

Results

A total of 15 CO1 samples were obtained from 5 endemic fish species originating from the karst region representing 5 genera, 4 families and 3 orders (Table 1 and Fig. 2). Of the five genera of these endemic fish, the CO1 sequence that represents these fish in the gene bank is only three genera, namely *M. ladigesi* (1 sequence), *N. liemi* (1 sequence) and *O. celebensis* (2 sequences). Two other fish species have not been registered in the genbank sequence, namely *D. orientalis* and *L. micracanthus*.

Furthermore, gene sequencing of these endemic fish samples, especially *M. ladigesi* and *O. celebensis*, showed similarities with similar fish sequences registered in the gene bank (Fig. 3), namely *M. ladigesi* (Accession No.: AY 290808.1) with a 97.09% similarity with the sample sequence we obtained, as well as *O. celebensis* (Accession No.: LC153105.1) with a 95–96% similarity to our sample sequence. For *N. liemi* fish, it has a similarity with the sample sequence *N. liemi* (Accession No.: LC1533118.1) of 85.78% and *Nomorhampus spp*. (Accession No.: JQ430374) of 95%.

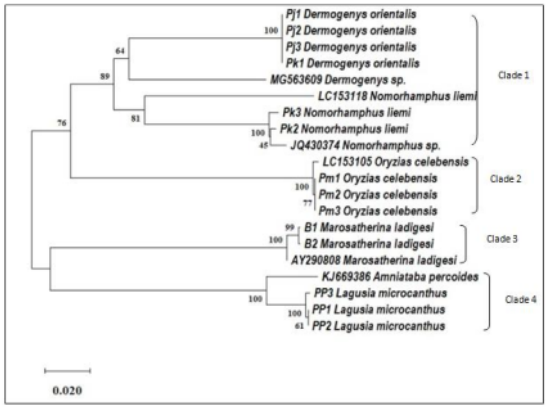

Fig. 3: Phylogenetic tree of Maros Karst's endemic fish by Maximum Likelihood method

Discussion

Phylogenetic trees using the Maximum Likelihood method produced four clades based on family groups. Clade II to clade IV showed each species genetics, except for clade I, which grouped two species, namely *N. liemi* and *D. orientalis*. Phylogenetic analysis shows that these two species are genetically closely related, although they have differences in morphology. They are still in one family, namely Zenarchopteridae. Apart from these two species, species *O. celebensis* in clade III also shows a kinship relationship with *N. liemi* and *D. orientalis*, because *O. celebensis* is still in the same order as the two species, namely Beloniformes.

According to Kobayashi *et al.* (2020), the order Beloniformes is divided into five families, namely Belonidae (having an elongated upper and lower jaw), Hemiramphidae (fish that have beaks), Zenarchopteridae (having beaks with viviparous reproductive characteristics), Exocoetidae (flying fish) and Adrinichthyidae (rice fish). Hemirhamphidae and Zenarchopteridae only have elongated mandibles, while Exocoetidae and Adrinichthyidae have non-elongated upper and lower jaws. However, in this study, we only found endemic fish that belong to two families, namely Zenachopteridae and Adrinichthyidae. In addition to the relationship between clade I and clade II, clade IV (*M. ladigesi*) shows almost the same reproductive

Table 1: Types of endemic fish caught in the Maros Karst Area

Order	Family	Species	Common name	Indonesian name	Local name
Atheriniformes (1.6%)	Telmatherinidae	*Telmatherina ladigesi* (Ahl 1936)	Celebes rainbowfish	Ikan pelangi Maros	Beseng-beseng
Beloniformes (0.3%)	Adrianichthyidae	*Oryzias celebensis* (Weber 1894)	Celebes medaka		Binishi
	Zenarchopteridae	*Dermogenys orientalis* (Weber 1894)		Ikan julung-julung	Anculung
		Nomorhamphus liemi (Vogt 1978)			Anculung
Perciformes (0.5%)	Terapontidae	*Lagusia micracanthus* (Bleeker 1860)			Piri'

characteristics as clade I and clade II, namely having unique types of testes and ovaries, which in turn affect reproductive modifications such as spermatogenesis, internal fertilization, hermaphrodites, and viviparity (Malabarba and Malabarba 2020). The *L. micracanthus* (Clade III) is the only distinct endemic species in South Sulawesi (Vari and Hadiati 2012). The only fish from the Terapontidae family that lives in freshwaters with distribution is limited to certain rivers in South Sulawesi (Nur *et al*. 2020a). This finding suggests that phylogenetic trees can describe the relationship between clades and taxonomic groups (Alotaibi *et al*. 2020).

Phylogenetic trees can also explain the evolutionary relationships between individuals or groups of organisms, which initially use morphological characteristics, based on similarities and are considered to have a close kinship. However, classical systematic analysis is sometimes confusing due to environmental variables, so that the use of proteins and DNA characters is developed to infer the phylogenetic relationship (Samsudin 2017).

Mitochondrial DNA analysis that is commonly used to classify and identify fish is the cytochrome 1 (CO1) subunit. The CO1 gene is a highly conserved region that has been used at all levels of organisms to identify species, differing only by a few sequences (variable locations) (Serdiati *et al*. 2020). According to In *et al*. (2013), universal primers for the CO1 gene are very stable and attach to the 5' end of the animal gene. The CO1 gene has advantages over other mitochondrial genes such as cytochrome b because changing the amino acid sequence is slower in evolution. The application of the CO1 gene in identifying species is called a DNA barcode (Samsudin 2017). DNA barcode is very relevant to use in habitats that have a diversity of species that are threatened with extinction due to anthropogenic activities (Hubert *et al*. 2016), such as in karst areas.

The karst area is one of the most fragmented habitats, and limited food sources (Bichuette and Trajano 2015), causing organisms in the ecosystem to adapt specifically and depend on the karst environment (Putri *et al*. 2020). These organisms have the potential to be specialized resources and could create ecological opportunities for exploration of tropical organisms and promote greater species diversity (Hanly *et al*. 2017).

The results of molecular analysis indicate that in general, the endemic fish groups based on species in this karst area are monophyletic (or having a common ancestor). Due to a geographic barrier, the distribution of these fish is limited, except for *D. orientalis*, which is paraphyletic (the ancestors are the same, but not all individuals belong to the same clade). This phenomenon is also examined by Samsudin (2017), that *D. orientalis* are paraphyletic based on histological analysis of the gonads and embryonic modifications and osteological characteristics. Unlike the case with *D. orientalis*, *N. liemi* is monophyletic. Although it can be seen in Fig. 3, there is one individual *N. liemi* in the subclade *D. orientalis*, similar to that found by Bruyn *et al*. (2013).

Another fish that is monophyletic is the *Oryzias* fish group, including *O. celebensis*. According to Mokodongan and Yamahira (2015), these fish are scattered in several areas of Sulawesi (South, West, Central) with various species. Their ancestors are thought to have originated from Asia (Borneo) and were isolated by the opening of the Makassar Strait during the Eocene period, which occurred about 45 million years ago, which resulted in them diverging within the island from a common ancestor (Mandagi *et al*. 2018).

Information on molecular studies of two other fish species, *M. ladigesi* and *L. micracanthus*, is still lacking. Even for L. *micracanthus* fish, there is no information related to the life history and habitat in which it lives, due to the lack of samples obtained during this research. The phylogenetic placement of these fish in the Terapontidae family is only based on morphological analysis (Vari and Hadiaty 2012). Similar to *L. micracanthus* fish, *M. ladigesi* fish also has no information related to DNA barcode analysis. However, DNA barcode analysis of various types of fish from the Telmatherinidae family has been carried out in previous studies (Jayadi *et al*. 2019). Previously, this fish was described by Ahl (1936) as a type of fish from the *Telmatherina* genus, namely *T. ladigesi*. Until Aarn *et al*. (1998) found a clear difference between *M. ladigesi* fish and other *Telmatherina* species; they determined it as a genus. Furthermore, this fish is named according to the habitat, namely Maros (Hadiaty 2007).

Many species are endemic to this land with various life challenges due to continuous environmental changes such as pollution, habitat loss, modification and development. Further studies are needed to conserve this native biodiversity and their habitats. Molecular studies have also been used to understand the evolutionary relationships of organisms which are very important in providing insights and establishing appropriate conservation steps (Samsudin 2017).

Conclusion

In this study, we obtained five endemic fish species from the

Maros Karst area. These five species represent three orders and four families. These species make particular adaptations both morphologically and genetically to the karst ecosystem. However, it is necessary to understand the evolution of these fishes to establish effective conservation measures.

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Author Contributions

SBAO and DY: proposed the research and finalizing the manuscript, MTU: data collection, AAH and SA: DNA analysis and drafted the manuscript. All authors provided critical feedback and helped to shape the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Ethics Approval

Not applicable in this paper

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