

Detection of 16S mitochondrial sequence polymorphism and haplotype network of *Ophiocara porocephala* (Valenciennes, 1837) from Tekolok Estuary (NTB, Indonesia) using DnaSP and NETWORK

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Abstract. The two genetic software, DnaSP and NETWORK, is commonly used to analyse gene polymorphism and haplotype network which is useful for the understanding pattern of genetic variation and genetic relationship. In this study, the two software were applied to detect polymorphism of 16S sequence gene of *Ophiocara paracephala* from Tekolok Estuary (NTB, Indonesia) which has not been investigated. This study used a PCR method with 16Sar and 16Sbr primers. The results revealed two haplotypes and two variable sites with one parsimony informative within *O.paracephala* from Tekolok Estuary population. Next, if the 16S sequence data of *O.paracephala* from Tekolok Estuary was combined with the 16S gene data of *O.paracephala* from GenBank, three haplotypes were detected with five variables sites and one parsimony informative. This finding showed intra-population and intraspecific genetic variation of *O.paracephala*. In addition, haplotype network using NETWORK and a phylogenetic tree using Neighbor Joining and Maximum Likelihood methods exhibited that *O.paracephala* from Tekolok Estuary has a close genetic relationship with *O.paracephala* from Japan.

Keywords: 16S gene, *O.paracephala*, polymorphism, haplotype network

1 Introduction

The use of genetic software to analyse DNA polymorphism is now considered essential [1, 2]. DNA polymorphism can be detected from a single base pair change or many base pair changes and repeated sequences. DNA Sequence Polymorphism (DnaSP), a genetic software package, is commonly used to analyse DNA polymorphism of a single locus or several loci [3]. This software can measure DNA sequence variation at the intra-population level or intraspecific level in noncoding, synonymous or nonsynonymous sites, or in various sorts of codon positions. Another software, NETWORK (<https://www.fluxus-engineering.com>), is usually used to examine haplotype network among samples within and between populations. Both software are currently used to detect DNA polymorphism and haplotype network in freshwater fish species such as *Urechis unicinctus* [4], *Steindachneridion scriptum* [5], and *Percocypris pingi* [6].

This study used direct 16S mtDNA sequencing to determine intra-population and intraspecific genetic variation of *O.paracephala* and their haplotype network. The 16 mtDNA is a conserved gene which means the change of few nucleotides within or among the population can be used to indicate intra-population or intraspecific genetic variation [7]. No information

related to genetic information of *O.paracephala* from Tekolok Estuary located at East Lombok (West Nusa Tenggara, Indonesia). Therefore, the objective of this study was to detect 16S mtDNA polymorphism within the population of *O.paracephala* from Tekolok Estuary and also examine intraspecific genetic variation and haplotype network of *O.paracephala* combined with *O.paracephala* from GenBank database.

2 Materials and methods

2.1 Sampling collection for 16S mitochondrial sequencing

Seven samples of *Ophicara porocephala* (code LTK01-LTK07) were collected from Tekolok Estuary (East Lombok, West Nusa Tenggara, Indonesia). The sampling location can be seen in Figure 1 (8°20'30.0"S 116°42'31.0"E). The fish samples were placed on ice in the field for short term preservation before photographing. The representative of fish sample was then documented (Figure 2). Next, small pieces of muscle tissue of each sample (50 mg) was put into 1.5 mL tube and preserved with 99 % ethanol for long time preservation and kept in -20 °C until further investigation

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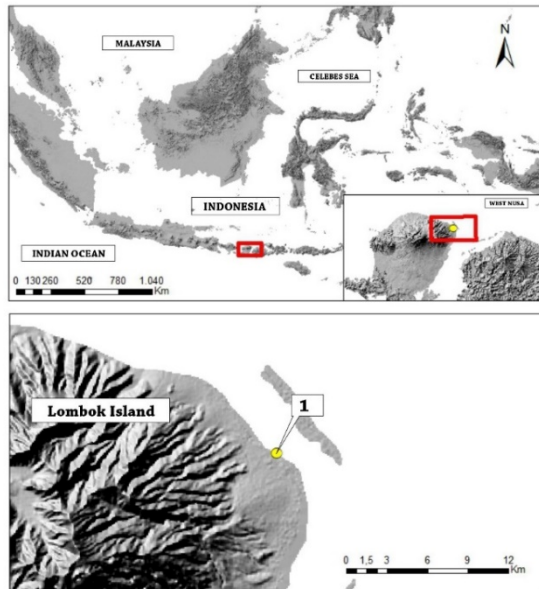


Fig.1. Sample collection sites (1) of *Ophiocara porocephala* at Tekolok Estuary, West Nusa Tenggara



Fig. 2. *Ophiocara porocephala* collected from Tekolok Estuary (Documented by Lukman Hakim)

2.2 DNA extraction, amplification, and sequencing of 16S mitochondrial gene: procedure and analysis

Total genomic DNA was extracted from muscle tissue using DNeasy tissue kit (QIAGEN, Valencia, USA) following the manufacturer's standard protocol. The mtDNA 16S rRNA gene was amplified using forward 16Sar (5'-CGCCTGTTTA TCAAAAACAT-3') and reverse 16Sbr (5'-CGGTCTGAACTCAGATCACGT-3') primers [8]. The PCR was performed in a total volume of 50 μ l, containing approximately 10-100 ng of genomic DNA, 25 μ l MyTaq HS Red Mix PCR Kit (Bioline), 2 mM MgCl₂, 0.6 μ M of each primer, and 11 μ l ddH₂O. The PCR cycling was carried out in a thermocycler (Biorad) following cycle procedures conducted by Arisuryanti et al. [9]. Next, 1 % agarose gel was used to check the PCR products through electrophoresis and they were visualized under UV light after staining with GelRed (Bioline). The PCR products were then purified and sequenced in both direction at a

commercial laboratory (First Base, Malaysia) in an ABI 3730xl DNA sequencer (Applied Biosystem)

SeqMan and EditSeq program (Lasergene, DNASTAR) were used to check and edit manually by eyes to detect ambiguous bases. The program Opal in MESQUITE v.3.51 [10] and ClustalW in MEGAX [11] was used for multiple sequence alignments. The nucleotide compositions (A, T, G and C) for each of the 16S mitochondrial gene fragments were calculated. Numbers of haplotypes, numbers of nucleotide polymorphic sites, haplotype diversity (h), and nucleotide diversity (π) were analysed with DnaSP ver 6.0 [3]. Haplotype diversity (h) is the average number of differences between random pairs of homologous haplotype sequences in a sample and nucleotide diversity (π) is the average number of differences between random pairs of homologous nucleotide sites in a sample [12]. Next, haplotype joining network was constructed using Median Joining Network in NETWORK v 10.1 (<https://www.fluxus-engineering.com>). Kimura-2-parameter model using MEGAX [11] was used to estimate intraspecific genetic distance and was summarised in Neighbor-Joining (NJ) and Maximum Likelihood (ML) trees. The NJ and ML trees with bootstrap values (1,000 replication) were constructed using MEGAX [11]. The 1,000 repeated sampling tests were performed to obtain the support values of the clade nodes. In this study, two 16S sequence data of *O.paracephala* from Genbank with accession number AB079256 and KF415426 were included in analyses for a comparison purpose and two 16S sequence data of *Oxyeleotris lineolate* (KP684140) and *Bostrychus sinensis* (JQ665462) were used for the outgroup.

3 Results and discussion

The partial sequences of the mtDNA 16S genes of the seven *O.porocephala* (with code LTK-01, LTK-02, LTK-03, LTK-04, LTK-05, LTK-06, and LTK-07) examined in this study can be amplified (Figure 3). All of the sequences were 599 bp long, and neither insertions nor deletions were found.

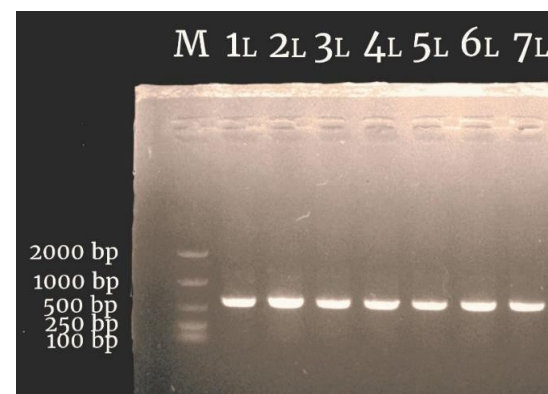


Fig. 3. PCR amplification of 16S mitochondrial gene fragments generated by primers (16Sar and 16Sbr) from *O.porocephala* collected from Tekolok Estuary and coded with LTK (M= size marker 2000 bp, 1L=LTK-01, 2L=LTK-02, 3L=LTK-03, 4L=LTK-04, 5L=LTK-05, 6L=LTK-06, and 7L=LTK-07)

The average rate of *16S* nucleotide composition of the *O.paracephala* from Tekolok River can be seen in Table 1. The difference in the nucleotide between *O.paracephala* from Tekolok River and *O.paracephala* from Japan and UK recorded at GenBank was C=0-0.13 %; G=0.05-0.18 %; and A=0.19-0.24 %. The *O.paracephala* from Tekolok River had the highest frequency of nucleotide A compared to *O.paracephala* from Japan and UK. Based on Table 1, it can be seen that the composition of nucleotide T of *O.paracephala* from Tekolok Estuary, Japan and UK had the same value.

Table 1. The average composition of 16S nucleotide of *O.porocephala*

Sample	T (%)	C (%)	A (%)	G (%)	A+T (%)	G+C (%)
LTK 01-07	20.33	28.49	27.64	23.54	47.96	52.04
AB079256*	20.33	28.49	27.59	23.59	47.91	52.09
KF415426*	20.33	28.86	27.40	23.41	47.73	52.27

* Samples were taken from *GenBank*

The analysis of *16S* nucleotide sequences of *O.paracephala* using DnaSP ver.6.0 was made after the *16S* sequences of *O.paracephala* from Tekolok Estuary was parallelized with the standard *16S* sequences of *O.paracephala* recorded at GenBank. Within Tekolok Estuary population with 599 bp fragment length, two haplotypes with two variable sites and one parsimony informative site were identified from seven samples of *O.paracephala* investigated in this study. Next, among three populations of *O.paracephala* investigated in this study and combined with *O.paracephala* from GenBank database with 551 bp fragment length, three haplotypes with five variable sites and one parsimony informative site were detected (Table 2 and Table 3). The level of divergence among haplotypes was low with haplotypes differing between 1 bp to 4 bp. The variable sites contained three transitions and two transversions. No insertion and deletion were found on *O.paracephala* from Tekolok Estuary, Japan and UK recorded at GenBank database. Next, haplotype diversity and nucleotide diversity were 0.556 and 0.002 respectively. In addition, five samples of *O.paracephala* from Tekolok Estuary (LTK 03-07) have identical *16S* sequences with *O.paracephala* from Japan (AB079256) which exhibited that both populations have similar haplotype.

Table 2. Summary of nucleotide variations in the partial *16S* mitochondrial gene of *O.paracephala*. Only variable sites are shown. Dots indicate identity with the *O.paracephala* sequence taken from GenBank with accession number AB079256 as a reference. The number above corresponds to nucleotide base pair position.

Samples	Polymorphic sites				
	1	1	2	3	5
	1	9	9	6	5
	7	4	9	5	8
AB079256*	A	G	A	G	T
KF415426*	C	.	T	A	C
LTK-01	.	A	.	.	.
LTK-02	.	A	.	.	.
LTK-03
LTK-04
LTK-05
LTK-06
LTK-07

*Samples were taken from GenBank

The analysis of mt-DNA *16S* sequence using Median Joining Network using NETWORK ver. 10 revealed the haplotype networking among the *O.paracephala* from (West Nusa Tenggara, Indonesia) and *O.paracephala* from other countries (Figure 4). One and other haplotypes were separated with 1 mutation point except for the *O.paracephala* from UK which was separated with 4 mutation points. This data supported the variable sites between one and another population of *O.paracephala* showed in Table 2.

Table 3. Haplotype data of *O.paracephala* from Tekolok Estuary and GenBank database based on *16S* mitochondrial gene

Haplotype	Sample Number	Accession Number	Location
Hap-1	2	LTK-01 LTK-02	Tekolok Estuary (NTB, Indonesia)
Hap-2	6	AB079256 LTK-03 LTK-04 LTK-05 LTK-06 LTK-07	Japan Tekolok Estuary (NTB, Indonesia)
Hap-3	1	KF415426	UK

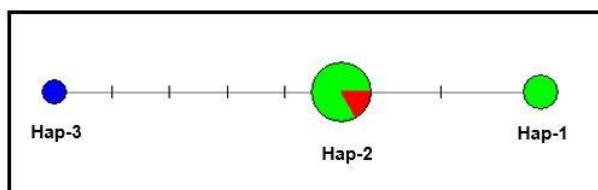


Fig. 4. Construction of Median Joining Network from 599 bp *16S* sequence of *O.paracephala*. Each haplotype is represented by a circle and sizes of circles are relative to the number of individuals sharing a specific haplotype. Haplotypes are labelled according to the population (Green=Tekolok Estuary, Red=Japan, Blue=UK).

The separation among haplotypes determined in Figure 4 was also supported by the phylogenetic tree using Neighbour-Joining (NJ) and Maximum Likelihood (ML) methods (Figure 5). The phylogenetic tree revealed the separation among haplotype-1 (LTK-01 and LTK-02), haplotype-2 (LTK 03-07 and AB079725), and haplotype-3 (KF415426). The separation among the haplotypes was supported by genetic distance between one and other haplotypes. The analysis of *Pairwise Distance Calculation* with the Kimura 2 parameter (K2P) model revealed that the genetic distance within *O.paracephala* from Tekolok Estuary was between 0-0.182 (average=0.087 %) whereas the intraspecific genetic distance of *O.paracephala* from Tekolok Estuary (West Nusa Tenggara, Indonesia) and *O.paracephala* from other countries (Japan and UK) was between 0-0.913 % (average=0.223 %). In addition, the genetic distance between *O.paracephala* from Tekolok Estuary and *O.paracephala* from UK was slightly higher than that from Japan. This finding revealed that *O.paracephala* from Tekolok Estuary (West Nusa Tenggara, Indonesia) and Japan was close genetically.

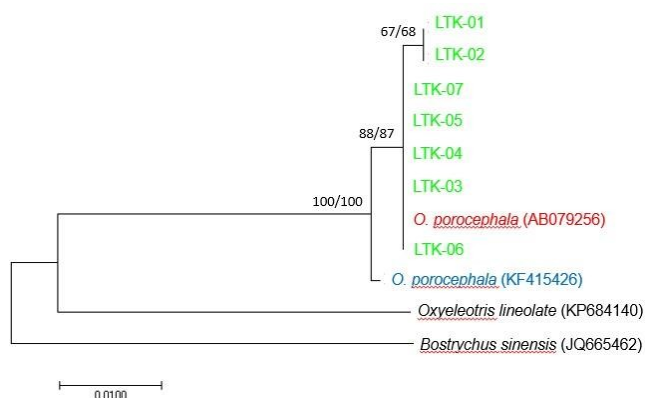


Fig. 5. Phylogenetic tree of *O.paracephala* inferred from DNA sequences of *16S* mitochondrial gene. The number of each node represent bootstrap NJ (left) and ML (right). Scale corresponds to substitution/site. LTK is sample code of *O.porocephala* from Tekolok Estuary and other *O.porocephala* was taken from GenBank.

4 Conclusion

The analysis using DnaSP and NETWORK revealed the intra-population and intraspecific polymorphism of *O.paracephala* based on *16S* mitochondrial gene. In addition, this finding identified the close genetic relationship between *O.paracephala* from Tekolok Estuary (NTB, Indonesia) and *O.paracephala* from Japan.

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