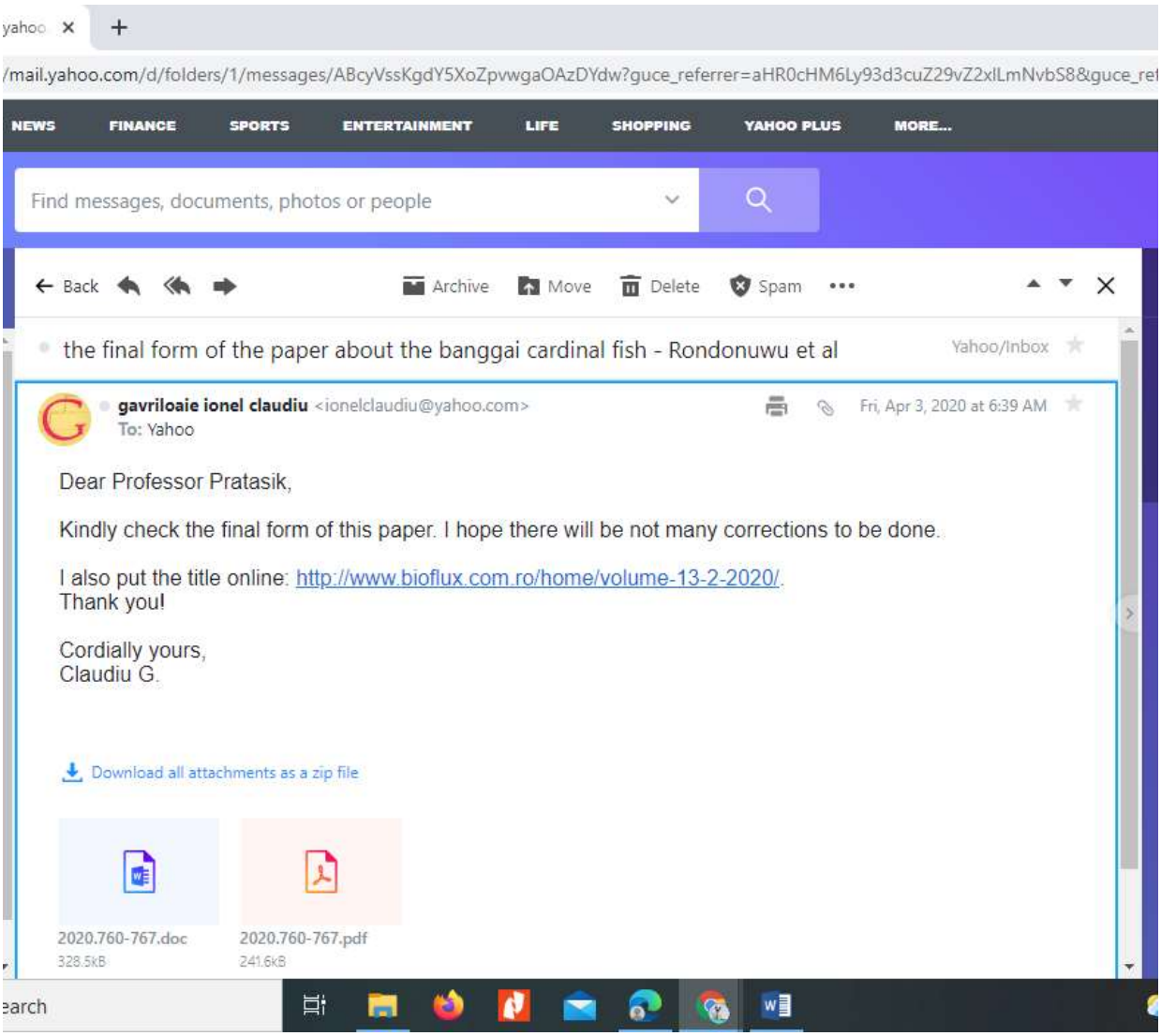
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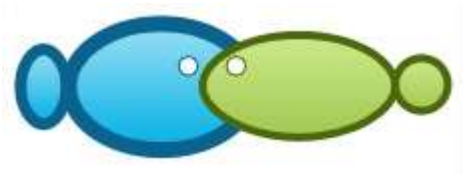

Mitochondrial CO1 sequences of Banggai cardinal fish (BCF) from Lembeh Strait, North Sulawesi, Indonesia

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Abstract. This study determines the genetic structure of Banggai cardinal fish (BCF) from Lembeh Strait, North Sulawesi, based on the mitochondrial CO1 genetic marker. Sample collection used SCUBA gear and "chang net" fishing gear. One BCF specimen was collected in this study. Polymerase chain reaction (PCR) was performed using 5X Firepol PCR Master Mix. Bi-directional sequencing was done by First Base CO (Malaysia) using Big Dye© terminator chemistry (Perkin Elmer). PCR outcomes were separated using 0.8% agarose gel electrophoresis and shown by amplification of 718 bp band. The fish population found in the Lembeh Strait was *Pterapogon kauderni* Koumans, 1933 based on BLAST method. The lowest pairwise distance (d) was 0.000 (0%) and the highest was 0.324 (32.413%) with an average distance of 0.087±0.117 (8.692±11.746%). Genetic distance (d) of the ingroup was shorter than that of the outgroup. Based on the phylogenetic tree, the population of *P. cauderni* (ingroup) forms one cluster and is separated from other fish groups (outgroup). The closest population to *P. kauderni* is *Sphaeramia nematoptera*.
Key Words: SCUBA, chang net, *P. kauderni*, *S. nematoptera*, genetic marker.

Introduction. One of the recently endangered living resources in Indonesian waters is coral reef fish of Apogonidae, *Pterapogon kauderni* Koumans, 1933 well-known as Banggai cardinal fish (BCF) and locally called *capungan* Banggai. As one of the seawater ornamental fish of high commercial value, the demand for this species is very high, despite the fact it is overfished in nature, particularly in its original habitat, Banggai islands covering Banggai regency and Banggai Laut regency. Recently, the species is in the status of endangered species of Red List of IUCN (International Union for the Conservation of Nature) due to exploitation and habitat degradation (Allen & Donaldson 2007). Since 2018, under the decree of Indonesian Marine Affairs and Fisheries Minister Number 49/KEPMEN-KP/2018, the status of limited protection of BCF has been established.

The occurrence of BCF outside the natural habitat is interesting to be studied because it was recently classified as an endemic fish. A species is called endemic if it is a native species found in certain place and not found in other area (Hugget 2004; Levin 2009). The area can be an island, country, or certain zone. Norman Myers of Oxford University used the number of endemic species as a criterion for identifying hotspots because it tends to have highly specific habitat or dietary requirements, low dispersal ability, and restricted geographical distributions (Russell et al 2017). Meanwhile, the distribution of this species has been detected in extensive regions outside Banggai group of island, such as Luwuk, Bali, and Lembeh Strait, North Sulawesi Province, in sufficiently population (Erdmann & Vagelli 2001; Moore & Ndobe 2017; Vagelli 2008, 2011; Vagelli et al 2009).

Most biological experts think that major factor affecting speciation is geographic isolation, since as far as the population of the same species is still directly connecting or not, the gene flow still may occur (Sobel et al 2009). Nevertheless, species distribution can be controlled by geographic factors so that the exchange of gene structure in the population system and evolution will occur separately, and the evolution can make two populations be more different with time (Russell et al 2017).

Moreover, species identification of living creatures has developed, from morphological approach to molecular method. The latter used a short DNA part called "barcode DNA" that has applicative functions, such as ecological survey (Dick & Kress 2009), identification of cryptic taxa (Lahaye et al 2008), and confirmation of samples (Xue & Li 2011). As general understanding of barcode that can distinguish products, the DNA standard can ease the researcher to distinguish accurately and quickly the living species. Therefore, this study observes the biomolecular aspects of BCF in Lembeh Strait with the emphasis on the genetic characteristic and diversity (DNA barcode). This study was aimed at discerning the BCF from Lembeh Strait waters, North Sulawesi, using CO1 gene marker.

Material and Method

Sample collection. BCF samples were collected on July 5th, 2019 in Lembeh Strait waters, North Sulawesi, using SCUBA gear and "chang net" fishing gear, a modified beach seine facilitated with purse on the central part (Figure 1). The samples were preserved in 95% ethanol and stored at room temperature before DNA extraction.

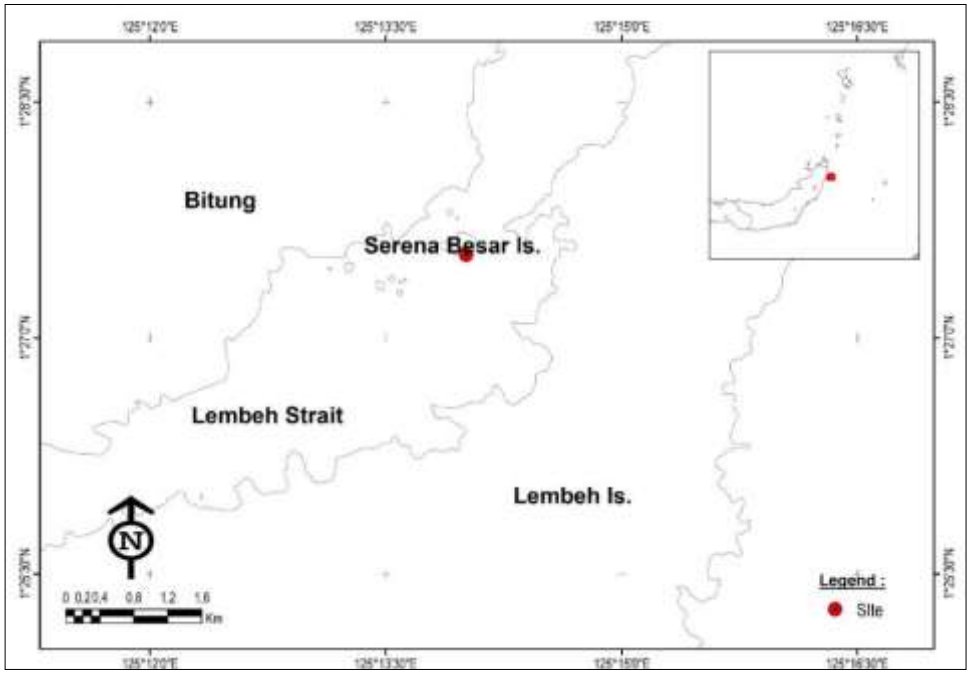

Figure 1. Study site.

Sampling and preparation. About 3 g of BCF flesh was taken from the abdominal part. The flesh was preserved in 95% technical ethanol for at least 1 day before DNA extraction. The purpose of this preservation was to wash the sample from salt water and draw water from the cell so as to facilitate the DNA extraction process.

Extraction, PCR, and sequencing. Total DNA extraction (nucleus and mitochondria) of BCF employed innuPrep DNA Micro Kit (Analytical Jena, Germany). Polymerase chain

reaction (PCR) was performed using 5X Firepol PCR Master Mix. The CO1 gene (primary types) used are FF2d: 5'-TTC TCC ACC AAC CAC AAR GAY ATY GG-3' and FR1d 5'CAC CTC AGG GTG TCC GAA RAA YCA RAA-3' (Ivanova et al 2007). PCR was carried out in 35 cycles at 95°C (50 sec). The PCR product was visualized in 1% (b/v) agarose gel electrophoresis. Bi-directional sequencing was done by First Base CO (Malaysia) using Big Dye© terminator chemistry (Perkin Elmer).

Data analysis. The chromatogram obtained was edited using Mega X v10.1 software (Kumar et al 2018). Sequencing outcomes were compared with 8 other sequences of the same species, 4 sequences of different species of the same family Apogonidae, *Cheilodipterus quinquelineatus, Apogon doederleini, Pristicon rhodopterus,* and *Sphaeramia nematoptera*, and one butterflyfish *Chaetodon kleinii* (Chaetodontidae) from the Gen Bank using BLAST (Basic Local Alignment Search Tools) and BOLD systems (National Center for Biotechnology Information, U. S. National Library of Medicine, https://www.ncbi.nlm.nih.gov/genbank/). The sequence analysis of the BCF DNA was done by using p-distance model *maximum composite likelihood* (Tamura et al 2004), and phylogenetic analysis using Neighbour-Joining method (Saitou & Nei 1987).

Results and Discussion. The PCR outcomes were separated using 0.8% agarose gel electrophoresis and shown by amplification of 718 bp band (Figure 2). The DNA sequence of BCF from Lembeh Strait had genetic similarity to that of BCF (GenBank), especially sequences AB890097.1 and AP005997.1 with a total score of 1230 and query cover of 97%.

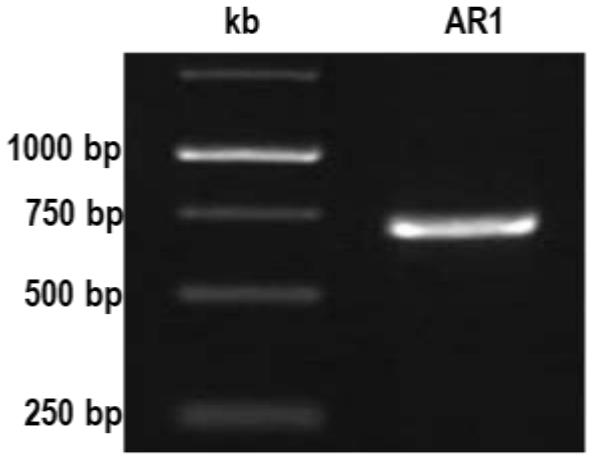

Figure 2. CO1 gene amplification of BCF samples from Lembeh Strait on 0.8% agarose gel (1kb: GeneRuler, Thermo Scientific as marker; AR1: sample number).

Based on species identity, all sequences of BCF had high similarity, 99.33-99.57%. The lowest similarity to BCF was found in that of the outgroup JF434784.1, the comparison with *Chaetodon kleinii,* with total cover of 606, 88% query, and 80.19% identity (Table 1). Thus, the fish sample collected from Lembeh Strait was *Pterapogon kauderni* Koumans, 1933 (Steinke et al 2009; Hubert et al 2012; Mabuchi et al 2014; Matias & Hereward 2018).

Table 1

Identification of banggai cardinal fish in Lembeh Strait using BLAST method (comparison with several sequences)

Sequence ID	*Sequence*	*Total score*	*Query cover*	*Identity (%)*
AB890097.1	*Pterapogon kauderni* mitochondrial COI gene for cytochrome c oxidase subunit I	1230	97	98.43
AP005997.1	*Pterapogon kauderni* mitochondrial DNA, complete genome	1230	97	98.43
FJ583995.1	*Pterapogon kauderni* voucher BIOUG:HLC-10728 cytochrome oxidase subunit 1 (COI) gene	1179	90	99.54
FJ583998.1	*Pterapogon kauderni* voucher BIOUG:HLC-10730 cytochrome oxidase subunit 1 (COI) gene	1173	90	99.38
MH049167.1	*Pterapogon kauderni* isolate dki102 cytochrome c oxidase subunit 1 (COI) gene	1099	86	98.33
MH049167.1	*Pterapogon kauderni* isolate dki102 cytochrome c oxidase subunit 1 (COI) gene	1079	83	99.33
FJ346810.1	*Pterapogon kauderni* isolate A12 cytochrome oxidase subunit I (COI) gene	856	65	99.57
FJ346809.1	*Pterapogon kauderni* isolate A11 cytochrome oxidase subunit I (COI) gene	684	96	84.63
NC_040863.1	*Cheilodipterus quinquelineatus* mitochondrion	682	98	84.18
AB890062.1	*Apogon doederleini* mitochondrial COI gene for cytochrome c oxidase subunit I	676	98	84.02
AB890095.1	*Pristicon rhodopterus* mitochondrial COI gene for cytochrome c oxidase subunit I	667	98	83.85
AB890106.1	*Sphaeramia nematoptera* mitochondrial COI gene for cytochrome c oxidase subunit I	797	98	83.76
JF434784.1	*Chaetodon kleinii* voucher REU0758 cytochrome oxidase subunit 1 (COI) gene	606	88	80.19

The sequence analysis of the 13 nucleotide sequences of 718 bp using the *maximum composite likelihood* model showed the lowest genetic distance (d) of 0.000 (0%) and the highest of 0.324 (32.413%) with mean population distance of 0.087±0.117 (8.692±11.746%). The genetic distance (d) of the ingroup was shorter than that of the outgroup (Table 2 and Appendix 1).

Table 2

Average of pairwise distance of maximum composite likelihood model-based sequences

Sequences	*P-distance (d)*	*%*	*SD*
Total	0.087	8.692	0.117
Ingroup			
Pterapogon kauderni (7 populations)	0.001	0.140	0.002
Outgroup			
Apogonidae (4 populations)	0.199	19.901	0.001
Chaetodontidae (1 population)	0.324	32.413	

Appendix 1

Pair distances of banggai cardinal fish from Lembeh Strait with several populations - estimates of evolutionary divergence between sequences

No	*Sequence*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
1	*Pterapogon kauderni*_Lembeh_COI_AR1	0.000											
2	AB890097_*Pterapogon kauderni*_(Japan)	0.002	0.000										
3	AP005997_*Pterapogon kauderni*_(Japan)	0.000	0.002	0.000									
4	FJ583995_*Pterapogon kauderni*_(Canada)	0.000	0.002	0.000	0.000								
5	FJ583998_Pterapogon_kauderni_(Canada)	0.002	0.005	0.002	0.002	0.000							
6	MH049167_*Pterapogon kauderni*_(Indonesia)	0.005	0.007	0.005	0.005	0.007	0.000						
7	FJ346810_*Pterapogon kauderni*_(USA)	0.000	0.002	0.000	0.000	0.002	0.005	0.000					
8	FJ346809_*Pterapogon kauderni*_(USA)	0.000	0.002	0.000	0.000	0.002	0.005	0.000	0.000				
9	AB890106_*Sphaeramia nematoptera*_(Japan)	0.196	0.201	0.196	0.196	0.193	0.204	0.196	0.196	0.000			
10	NC_040863_*Cheilodipterus quinquelineatus*_(USA)	0.194	0.198	0.194	0.194	0.198	0.195	0.194	0.194	0.252	0.000		
11	AB890062_*Apogon doederleini*_(Japan)	0.192	0.188	0.192	0.192	0.196	0.192	0.192	0.192	0.169	0.180	0.000	
12	AB890095_*Pristicon rhodopterus*_(Japan)	0.213	0.218	0.213	0.213	0.217	0.214	0.213	0.213	0.194	0.195	0.180	0.000
13	JF434784_*Chaetodon kleinii*_(France)	0.324	0.330	0.324	0.324	0.324	0.313	0.324	0.324	0.300	0.252	0.241	0.272

The pairwise distance method is the basis of the phylogenetic tree reconstruction. The value of p-distance index gives information on kinship among tree-like compared organisms. The more the p-distance value is, the farther the kinship between the organism, and vice versa. The genetic distance could reflect the genetic kinship between species, individuals or population. The present study indicated that the BCF population in Lembeh Strait had very close kinship to 7 other populations, although there is no information on the locality of population collected as ornamental fish.

Based on phylogenetic analysis using Neighbor-Joining method (Saitou & Nei 1987), the eight populations of BCF (ingroup) formed one cluster separated from other fish populations compared (outgroup) (Figure 2). According to Bernardi & Vagelli (2004), genetic isolation could occur in very short distance. However, the phylogenetic analysis of BCF in several natural habitats or the introduced habitat showed that BCF in Lembeh Strait belonged to the same clade as that in several localities of Banggai islands. Hoffman et al (2005) who studied the same species in Banggai islands and Luwuk waters using microsatelite method found no significant relationship between genetic and geographic distance despite increased genetic distance when removing Luwuk population from the analysis. Thus, to more accurately answer the genetic variations of BCF, more comprehensive phylogenetic studies need to be done with more populations from different locations.

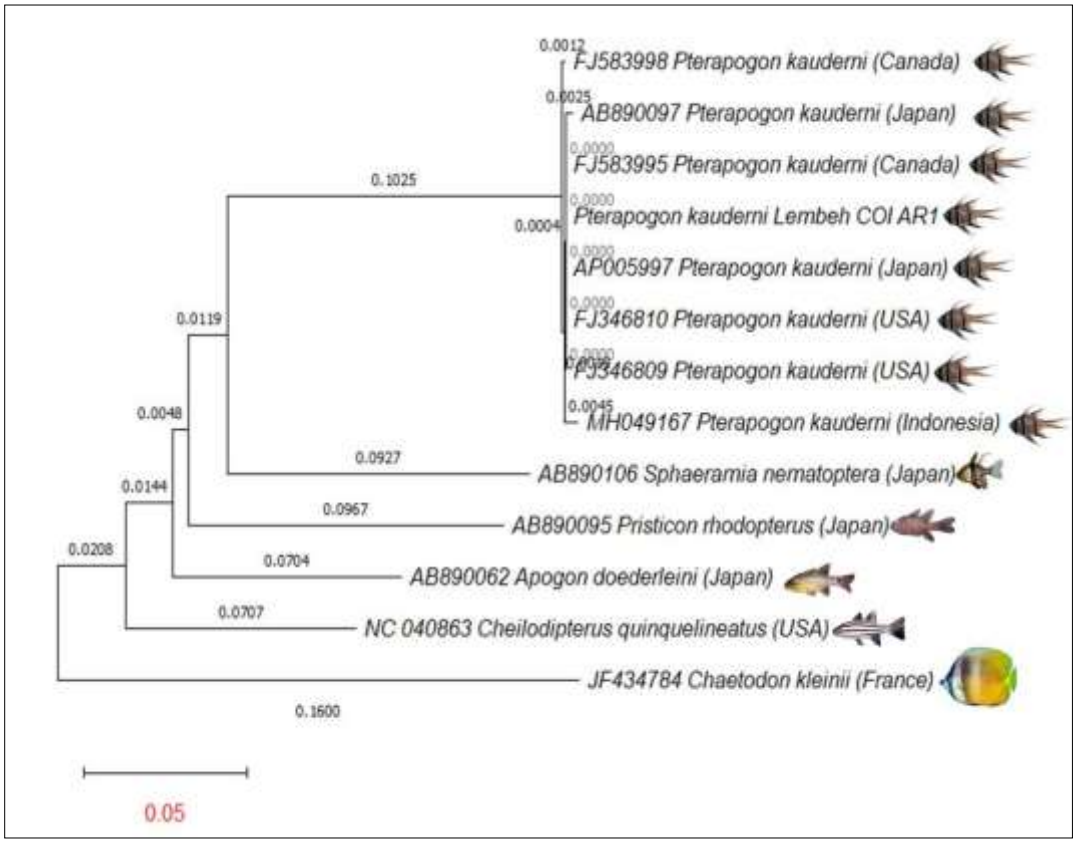

Figure 2. The phylogenetic tree of *Pterapogon kauderni*.

The genetic distance analysis showed that four species of family Apogonidae, *Sphaeramia nematoptera*, *Pristicon rhodopterus*, *Cheilodipterus quinguelineatus* and *Apogon doederleini* are separated from *P. kauderni* population (Figure 2), but these four species occur in separate cluster. Besides Apogonidae cluster, Chaetodontidae population makes different cluster as well. This finding reveals that the kinship of fish species and family has

distinguishing genetic marker. *Chaetodon kleinii* is ecologically a fish that possesses the same habitat as *P. kauderni,* coral reef habitat (Allen & Erdmann 2012a, b; Kuiter & Tonozuka 2001; Sale 1991, 2002). BCF occupies most physiographic zones including reef flats, reef crests, and even the upper section of some reef slopes, but is limited to a narrow depth range of < 3 m, so that both species are grouped as coral fish (Vagelli 2011). In spite of that, the biological characteristics of both species are not mixed, such as inbreeding that will yield homozigotic individuals. It means that the biological character is a major factor determining the intraspecific genetic variations so that both species are always separate. Figure 2 also reveals that the population with the closest kinship to *P. kauderni* is *S. nematoptera.* In the revision of the systematics of the cardinal fishes (Percomorpha: Apogonidae) based on molecular analyses and comparative reevaluation of morphological characters, both species are classified as new tribe, Sphaeramiini (Mabuchi et al 2014).

Conclusions. The fish sample collected in Lembeh Strait was Banggai cardinal fish (BCF) *Pterapogon kauderni* Koumans, 1933. The genetic distance (d) of the ingroup is shorter than that of the outgroup. The BCF population in Lembeh Strait and 7 other BCF populations had very close kinship. In the phylogenetic tree, the eight populations formed one cluster that was separated from the other species compared and outgroup. Other population having the closest kinship to *P. kauderni* was *S. nematoptera.*

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