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GENETIC CHARACTERIZATION OF DWARF SNAKEHEAD, *Channa gachua* (Hamilton, 1822), FROM TWO POPULATIONS BASED ON *16S rRNA* GENE

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ABSTRACT

Indonesia is one of the countries with high biodiversity of freshwater fishes. One of the freshwater fish that has widely distribution in Asia with high economic potency for ornamental fish is dwarf snakehead. However, research on genetic characterization of dwarf snakehead from Indonesia is very limited. Therefore, this research aimed to compare genetic characterization of the dwarf snakehead from rice field irrigation at Donomulyo Village (Malang, East Java) and from Keji River (Magelang, Central Java). This study used a PCR method with universal primers: 16Sar and 16Sbr. The data obtained in this study were then analyzed using DNASTAR, BLAST, Mesquite, MEGA, DnaSP, and NETWORK. The results revealed the genetic distance between dwarf snakehead from rice field irrigation at Donomulyo Village and from Keji River was 1.36%. The divergence of GC content, haplotype number, variables sites, haplotype diversity, and nucleotide diversity in both populations exist. The results of this study are expected to arrange 16S mitochondrial DNA Library of dwarf snakehead from Indonesia which is useful for a reference in the conservation and utilization and management of dwarf snakehead in their habitat.

Keyword: Dwarf snakehead; genetic characterization; 16S rRNA gene

INTRODUCTION

Dwarf snakehead, *Channa gachua* (Hamilton, 1822), is a native fish from Asia and widely distributed from West Afghanistan to Indonesia through South and Central Asia (Kottelat, 2013). The fish is usually found in shallow waters, rivers, reservoirs, swamps, rice fields and irrigation at pH 6-7 and temperatures 10-28! (Ilmi & Arisuryanti, 2018). The fish has an elongated body reaching a size of 18-36 cm accompanied by an elongated dorsal fin, the mouth filled with teeth and has the superior type of mouth, and around caudal type of tail. The fish size from genus *Channa* is generally small, but *C. gachua* is the smallest. In addition, the fish has many color variations on their bodies, so they are suitable for use as ornamental fish (Gustiano *et al*., 2021). Furthermore, the albumin content in the fish can be used for therapy to healing wounds and reducing post-surgery pain (Ab Wahab *et al*., 2015; Hariati *et al*., 2019)

In order to increase the production of the fish in Indonesia through a breeding program, genetic characterization of the fish species has to be done using molecular approaches. The mitochondrial genome has been used as an effective tool for phylogenetic and population genetic analyses in vertebrates. One of the mitochondrial genes that is commonly used to explore the phylogenetic relationships of fishes at various taxonomic levels is *16S rRNA.* The *16S rRNA* gene is one of the genetic markers for investigating animal genetic variety and phylogeny. This is due to *the 16S* mitochondrial gene is conserved, and a change of a few nucleotides within or between populations could indicate a significant degree of variation (Cawthorn *et al.,* 2012; Yang *et al.,* 2014). The majority of *16S rRNA* studies were conducted on aquatic organisms, such as red sea parrotfish (Saad *et al*., 2019) and striped snakehead (Arisuryanti *et al*., 2020). This is due to the gene being considered as maternal inheritance, lack of introns, presence of single-copy orthologous genes, lack of recombination events, and high mutation rate. Mitochondrial *16S* rRNA gene also contains sufficient polymorphisms to unambiguously discriminate most species due to a high number of insertions and deletions in non-peptide coding DNA. In addition, the *16S* rRNA gene is ubiquitous (Faddagh *et al*., 2012; Srinivasan *et al*., 2015; Satoh *et al*., 2016; Hossain *et al*., 2019).

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Research on the genetic diversity of *C. gachua* has been carried out in several countries, including China (Zhou *et al*., 2019; He *et al*., 2019), Thailand (Tanomtong *et al*., 2013), Bangladesh (Ahmed *et al*., 2018), Malaysia, Borneo, Myanmar, Vietnam (Conte-Grand *et al*., 2017). In Indonesia, research on the genetic characterization of *C. gachua* is very limited. The previous study carried out by Ilmi and Arisuryanti (2018) revealed the nucleotide composition of the *16S* mitochondrial DNA *Channa gachua* from Keji River, Magelang, Central Java. Therefore, this study aimed to compare the genetic character of *C. gachua* from Keji River, Magelang, Central Java and *C. gachua* from rice field irrigation at Donomulyo Village, Malang, East Java.

Figure 1. Map sampling collection of dwarf snakehead. (1) rice field irrigation at Donomulyo Village and (2) Keji River.

MATERIALS AND METHODS

Sampling for Collection for *16S* Mitochondrial Sequencing

Four samples of dwarf snakehead (sample code: KTM-01, KTM-02, KTM-03, dan KTM-04) were collected from rice field irrigation at Donomulyo Village, Malang, East Java (8°17'0"S 112°26'0"E) and two samples (sample code: KTS-01 and KTS-02) obtained from Keji River, Magelang, Central Java (7°35'34.85"S 110°16'19.48"E) (Figure 1). The 50-100 mg muscle tissue of each sample was dissected with sterilized scissors and put into a 1.5 ml sterile tube containing 99% ethanol. The muscle tissue samples were then transported to the Laboratory of Genetics and Breeding, Faculty of Biology, Gadjah Mada University (Yogyakarta, Indonesia) and stored in -20°C until further investigation.

DNA Extraction, Amplification, and Sequencing of *16S* Mitochondrial Gene: Procedure and Analysis

Total genomic DNA was extracted using Qiagen DNEasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocols. The *16S* mitochondrial gene from the six dwarf snakehead samples was amplified using MyTaq HS Red Mix PCR Kit for the polymerase chain reaction (PCR), and the primers used in this study was 16Sar (5'-CGCCTGTTTATCAAAAACAT-3') and 16Sbr (5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi, 1996). The PCR reaction mixture for each sample consisted of 12.5 µl PCR mix; 0.6 ìM primer forward; 0.6 µM primer reverse; 1 mM MgCl2; 5.5 µl ddH2O; and 3 µl DNA samples of the fish. The total volume of each PCR reaction was 25 µL, then amplified under the

thermal conditions following Arisuryanti *et al*. (2020). All of the PCR products were visualized using electrophoresis on a 1% agarose gel buffered with Tris-Acetate-EDTA (TAE), stained with FloroSafe (1st Base) nucleic acid stain, and visualized under UV light. All of the samples were then sent to First Base Services (Malaysia) through P.T. Genetics Science (Jakarta) for DNA purification and sequencing. The PCR products were sequenced bi-directionally by using both primers (16Sar and 16Sbr) and the Big Dye Terminator Ver. 3.1. sequencing kit (Applied Biosystems). The sequencing reactions were loaded into ABI 3730xl Genetic Analyzer (Applied Biosystems).

Chromatograms of the dwarf snakeheads investigated in this study were checked and assembled using SeqMan and edited manually using EditSeq Pro Program Lasergene DNASTAR software package. The sequence of each sample was verified using the BLAST program of NCBI. Multiple sequence alignments were done with Opal in MESQUITE v.3.61 (Maddison & Maddison, 2019) and ClustalW in MEGAX (Kumar *et al*., 2018). The genetic distance of the samples was analyzed using the MEGAX program with the Kimura 2 parameter model. The phylogeny relationship was reconstructed using the Neighbor-Joining (NJ) method in the MEGAX program using the Kimura 2 parameter model and 1,000 bootstraps. Genetic diversity indices such as the GC content, number of haplotypes, polymorphic sites, haplotype diversity and nucleotide diversity were analyzed using DnaSP v6.12.01 (Rozas *et al*., 2017). A haplotype network was analyzed using NETWORK 10.2.0.0 program with the Median Joining method (https://www.fluxus-engineering.com). Five dwarf snakeheads (*Channa gachua*) recorded at GenBank (accession number: KU986900, MH699846, KU238074, NC036948, MF924390) were used for comparison purposes. In addition, two species *C. striata* (KU986899) and *C. punctata* (EU342183) were used as outgroups.

RESULTS AND DISCUSSION

Results

All of the dwarf snakehead samples from rice field irrigation at Donomulyo Village (sample code: KTM-01, KTM-02, KTM-03 and KTM-04) and from Keji River (sample code: KTS-01 and KTS-02) were successfully amplified and resulted 539 bp in fragment length (Figure 2). The nucleotide BLAST analysis from NCBI (https://blast.ncbi.nlm.nih.gov) revealed that all of the dwarf snakehead samples examined in this study have 99% similarity with *Channa gachua* with accession number KU986900 recorded at GenBank. All of the *C. gachua* samples from rice field irrigation at Donomulyo Village and Keji River has been registered at GenBank

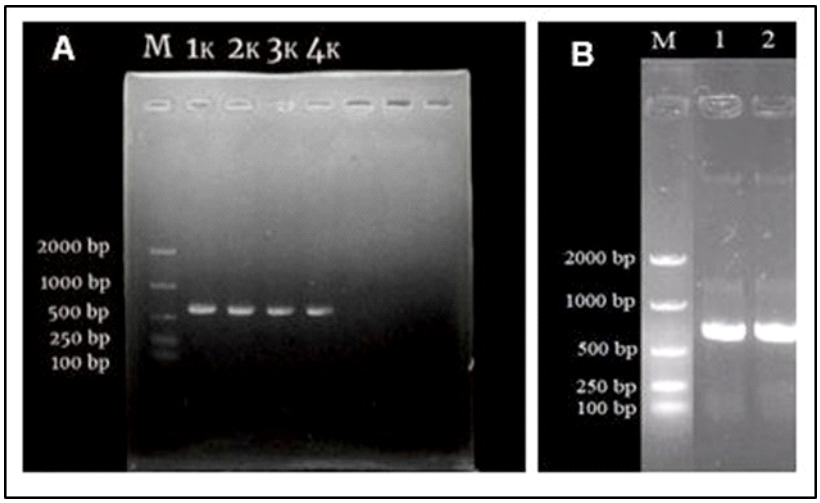

Figure 2. PCR amplification of the dwarf snakehead specimens from Rice Field Irrigation at Donomulyo Village, Malang, East Java (A) and from Keji River, Magelang, Central Java (B) using 16Sar and 16Sbr primers and were separated by electrophoresis in 1% gel electrophoresis (M= size marker 2000 bp, 1K=KTM-01, 2K=KTM-02, 3K=KTM-03, 4K=KTM-04, 1=KTS-01, 2=KTS-02).

with accession number MZ781215- MZ781220.

Based on the 539 bp fragment length, the estimate of genetic variation of *C. gachua* from rice field irrigation at Donomulyo Village and Keji River can be seen in Table 1. The result exhibited the GC content of *C. gachua* from Keji River was slightly higher than that of *C gachua* from rice field irrigation at Donomulyo Village. The difference of GC content of *16S* mitochondrial gene between *C. gachua* from rice field irrigation at Donomulyo Village and *C.*

Table 1. Estimates of genetic variation of *C. gachua* from two populations based on 16S mitochondrial gene. KTM is sample code of *C. gachua* from rice field irrigation at Donomulyo Village and KTS is sample code of *C. gachua* from Keji River

Sample code	GC content	Haplotype number	Variable sites	Parsimony informative sites	Haplotype diversity	Nucleotide diversity
KTM	46.71%	2	1	0	0,500±0,265	0,00083±0,00091
KTS	47.9%	2	5	0	1,00±0.250	0.00873±0.0000190

gachua from Keji River was 1.19%.

In addition, the results revealed that the number of variable sites, haplotype diversity and nucleotide diversity of *16S* mitochondrial sequences of *C. gachua* from Keji River was higher than that of *C. gachua* from rice field irrigation at Donomulyo Village. Both populations have similar haplotype numbers but different variable sites and no parsimony-informative site. In addition, there were 9 transitions on their pattern of nucleotide substitution (Table 2). If the *16S* sequences of both populations were analyzed together with *C. gachua* from GenBank database, 7 haplotypes with 22 variable sites and 16 parsimony-informative were detected (Table 2 and 3). Haplotype diversity and nucleotide diversity was 0.909 ± 0.066 and 0.01376 ± 0.00184, respectively. The *16S* sequence data also exhibited the variable sites contained 17 transitions, 3 transversions, and 2

Table 2. Summary of nucleotide variations in the partial *16S* mitochondrial gene of *C. gachua*. Only variable sites are shown. Dots indicate identity with the *C. gachua* sequence taken from GenBank with accession number KU986900 as a reference. Number above corresponds to the nucleotide base pair position.

Samples	Polymorphic sites 11122222222233333444 17366222333445033497 89 55612568257796269326 52
KU986900**	CATACAGTATCATTTAATCATA
KU238074**	.GC...A.GGT...CG.....
NC036948**	..C.TCT.G......G..A.AG
MF924390**	..C.TCT.G......G..A.AG
MH699846**	.....
KTS-01	...G...CGC.GC..G.C....
KTS-02	.......CGC..C..G.C.G..
KTM-01	T......CG....C.G.....
KTM-02	T......CG....C.GG.....
KTM-03	T......CG....C.GG.....
KTM-04	T......CG....C.GG.....

** samples were taken from GenBank (KU986900=Malaysia; MH699846=Vietnam; KU238074, NC036948, MF924390=China)

Table 3. Haplotype data of *C. gachua* from rice field irrigation at Donomulyo Village and from GenBank database based on *16S* mitochondrial gene.

Haplotype	Sample	Accession Number	Location
Hap_1	2	KU986900 MH699846	Malaysia Vietnam
Hap_2	1	KU238074	China
Hap_3	2	NC036948 MF924390	China China
Hap_4	1	KTS-01	Keji River, Magelang, Indonesia
Hap_5	1	KTS-02	Keji River, Magelang, Indonesia
Hap_6	1	KTM-01	Rice field irrigation at Donomulyo village, Malang, Indonesia
Hap_7	3	KTM-02 KTM-03 KTM-04	Rice field irrigation at Donomulyo village, Malang, Indonesia

multiple substitutions.

The haplotype networking among the *C. gachua* from rice field irrigation at Donomulyo Village (East Java, Indonesia), *C. gachua* from Keji River (Central Java, Indonesia), and *C. gachua* from other countries can be seen in Figure 3. One and another haplotype was separated with 5-22 mutation points. The *C. gachua* from rice field irrigation at Donomulyo village and *C. gachua* from Keji River was separated with 9 mutation points, whereas *C. gachua* from rice field irrigation at Donomulyo village and *C. gachua* from Malaysia and Vietnam was separated only with 6 mutation point. This indicates the *C. gachua* from the three populations were genetically close compared with *C. gachua* from Keji River. Either *C. gachua* samples from rice field irrigation at Donomulyo Village or *C. gachua* from Keji River formed into two different haplotypes in the same haplogroup. This finding can be used as a molecular

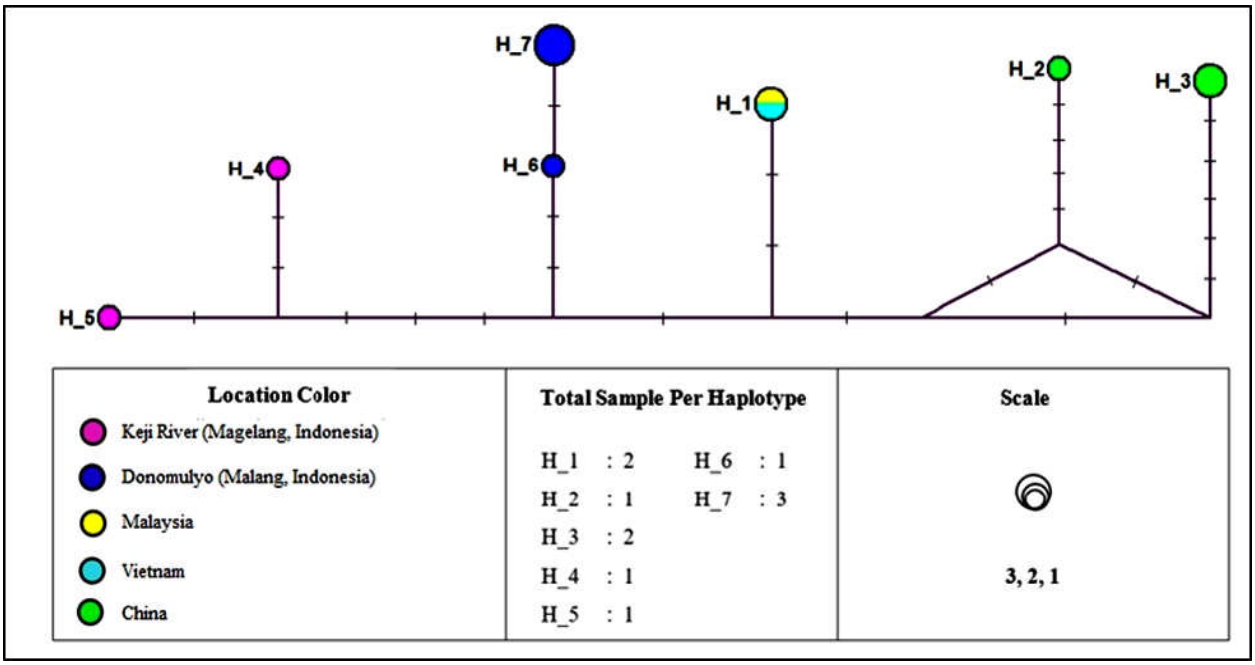

Figure 3. Construction of Haplotype Networking from 539 bp of *16S* sequence data.

marker for both populations.

The genetic distance between *C. gachua* from rice field irrigation at Donomulyo village and *C. gachua* from Keji River was 1.36 %, whereas the genetic distance between *C. gachua* from rice field irrigation at Donomulyo village and *C. gachua* from other countries was range 1.08 – 2.02% (Table 4). The genetic distance between *C. gachua* from rice field irrigation at Donomulyo Village and *C. gachua* from Malaysia, Vietnam, and China was 1.08%, 1.08%, and 1.96% respectively. The genetic distance between *C. gachua* samples from rice field irrigation at Donomulyo village, Malang and *C. gachua* from Keji River, Magelang was higher than *C. gachua* from Malaysia and Vietnam. The same value of genetic distance between *C. gachua* samples from rice field irrigation at Donomulyo village (Malang, Indonesia) and *C. gachua* from Malaysia and Vietnam which was 1.08% revealed that the *C. gachua* from the three populations were close genetically compared

Table 4. Percentage of genetic distance between *C. gachua* from rice field irrigation at Donomulyo Village (KTM) and Keji River (KTS), and GenBank database

	1	2	3	4	5	6	7
1.KU238074**	-						
2.NC036948**	1.88	-					
3.MF924390**	1.88	0.00	-				
4.KU986900**	1.50	1.69	1.69	-			
5.MH699846**	1.50	1.69	1.69	0.00	-		
6.KTS	1.98	2.36	2.36	1.41	1.41	-	
7.KTM	1.84	2.02	2.02	1.08	1.08	1.36	-

** samples were taken from GenBank (KU986900=Malaysia; MH699846=Vietnam; KU238074, NC036948, MF924390=China)

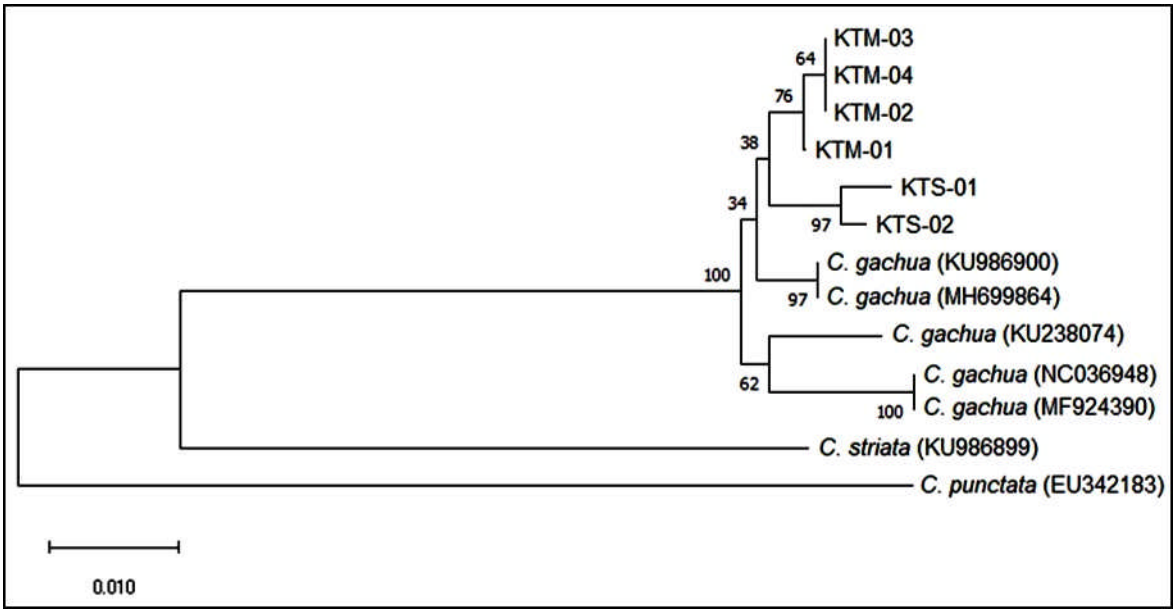

Figure 4. Neighbor-Joining phylogenetic tree of *C. gachua* inferred from DNA sequences of *16S* mitochondrial gene. KTM is the sample code of *C. gachua* from rice field irrigation at Donomulyo village, KTS is the sample code of *C. gachua* from Keji River, and other *C. gachua* was taken from GenBank.

with *C. gachua* from Keji River.

The *C. gachua* from rice field irrigation at Donomulyo village (East Java, Indonesia) was clade together with *C. gachua* from Keji River (Central Java, Indonesia), Malaysia, and Vietnam, whereas the other clade was *C. gachua* from China. The separation of the two clades was supported by genetic distance 1.86% and haplotype networking with 22 mutation points. Further study is needed to confirm whether *C. gachua* is cryptic species or is still conspecific with high genetic variation.

Discussion

The analysis of Pairwise Distance Calculation with Kimura 2 parameter model was used to analyze the genetic distance or the genetic relatedness of *C. gachua* from two populations investigated in this study and from GenBank. The genetic distance of *C. gachua* from the two populations was 1.36%. This result had a higher value than that of Lakra *et al*. (2010) which found the genetic distance of *C. gachua* in India was about 0.41%. However, the phylogenetic analysis revealed that *C. gachua* from the two populations were still grouped in one lineage together with *C. gachua* from Malaysia and Vietnam and genetically separated from China. The close genetic relationship between *C. gachua* from the two populations and *C. gachua* from Malaysia and Vietnam compared to China might be due to migration which would have been possible during periods of lower sea levels (Hall, 2002), when these land masses and island were connected and creating the ancient landmass of what is referred to as Sundaland (Voris, 2000). The Sundaland landmass would have appeared several times during the last ice age when sea levels were lower and would have also been present during earlier periods extending in the Miocene (Hall, 2012). The presence of Sundaland has been used to explain the otherwise unusual and disjunct distribution of freshwater fish species in Southeast Asia including dwarf snakehead.

The detected 9 variable sites, which all are transitions, indicated genetic divergence of *C. gachua* in both populations investigated in this study, rice field irrigation at Donomulyo Village (Malang, East Java) and Keji River (Magelang, Central Java). The high transitions obtained in this study agree with the regularity of mitochondrial DNA evolution in animals (Yang *et al*., 2014). In addition, the sequence data of *16S rRNA* region revealed that *C. gachua* from rice field irrigation at Donomulyo Village had lower average haplotype diversity and nucleotide diversity compared to *C. gachua* from Keji River, which indicated the abundant diversities of the fish species in Keji River, even though the sample number of *C. gachua* collected from Keji River is lower than that of *C. gachua* from rice field irrigation at Donomulyo Village. This is due to the *16S* mitochondrial gene is a conserved gene, and nucleotide divergences among samples within a group may reflect genetic heterogeneity within a population (Cawthorn *et al.,* 2012; Yang *et al.,* 2014). In addition, different levels of genetic variation between dwarf snakehead wild populations can be related to population sizes and different pressures of exploitation. This is due to

overfishing being a main factor causing the decrease in genetic variation in wild fish populations (Pinsky & Palumbi, 2014). Furthermore, the finding, which revealed no share haplotype network between the two populations, indicated *C. gachua* from each population were specific and it can be used for molecular marker.

CONCLUSION

The *16S* mitochondrial sequence data show genetic divergences and intraspecies genetic variation between *C. gachua* collected from two populations (rice field irrigation at Donomulyo Village, Malang, East Java and Keji River, Magelang, Central Java). Those populations form specific haplotypes without sharing with other populations. This finding can be used as a molecular marker for the two populations of *C. gachua* to assembly *16S* mitochondrial DNA library of *C. gachua* from Indonesia.

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