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A Study of the NGF Gene Intron in the Philippine Scrubfowl (*Megapodius cumingii*) from Kabetan Island, Central Sulawesi

Wayan Windyawati, I Made Budiarsa*, Zulaikhah Dwi Jayanti, Manap Trianto, Abdul Ashari, I Nengah Kundera
Departement of Biology Education, Faculty of Teacher Training and Education, Tadulako University.
Jl. Soekarno Hatta KM 9, 94148. Central Sulawesi, Tel./Fax. (0451) 422611, Indonesia.

Corresponding author*
budiarsa_imade@yahoo.com

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Abstract

The Philippine scrubfowl (*Megapodius cumingii*) is a bird species that utilizes environmental heat to incubate its eggs and has a wide geographic distribution across various habitats, including coastal forests, coastal areas, and small islands. This study aimed to describe and analyze the genetic diversity and phylogenetic relationships of *Megapodius cumingii* based on the Nerve Growth Factor (NGF) gene. Genomic DNA was isolated from blood samples of *M. cumingii* using the gSYNC™ DNA Extraction Kit (Geneaid) protocol and subsequently amplified using the Polymerase Chain Reaction (PCR) method with the forward primer AllNGF5′ and reverse primer AllNGF3′. Sequence data were analyzed using BLAST to determine their similarity to reference sequences available in GenBank. Genetic diversity was assessed using DnaSP v6.12.03, while phylogenetic reconstruction was performed using MEGA 11 based on the Neighbor-Joining (NJ) method with the Kimura 2-Parameter model. The results showed that the nucleotide composition consisted of Adenine (A) 29.3%, Thymine (T) 19.8%, Cytosine (C) 26.9%, and Guanine (G) 24.1%, with a G+C content (51.0%) slightly higher than the A+T content (49.0%). Genetic diversity analysis revealed a haplotype diversity value of 0.533 ± 0.172 and a nucleotide diversity value of 0.00145 ± 0.00047. Phylogenetic reconstruction produced a consistent tree topology across two outgroup models, with all *M. cumingii* samples forming a single clade supported by high bootstrap values (99–100%). The phylogenetic relationships among *M. cumingii* samples from Kabetan Island, Tolitoli, were very close, as indicated by genetic distance values ranging from 0.000 to 0.003.

Keywords*: Megapodius cumingii*; NGF gene; genetic diversity; phylogenetics; nuclear gene.

INTRODUCTION

The Philippine scrubfowl (*Megapodius cumingii*) is a bird species that utilizes environmental heat to incubate its eggs (Jones, 1999; Bashari et al., 2017). These heat sources include geothermal radiation, solar radiation, and heat generated by microbial decomposition (Birks & Edwards, 2002; Budiarsa et al., 2010; Harris et al., 2014). This unique reproductive strategy has enabled *M. cumingii* to achieve a broad geographic distribution across various habitats, including coastal forests, coastal areas, and small islands (Paguntalan et al., 2021). However, its wide distribution also makes the species highly vulnerable to environmental changes, particularly habitat degradation and disturbances at nesting sites. Human activities in coastal regions and habitat fragmentation may adversely affect the population stability of this species (Gorog et al., 2005; Radley et al., 2018). This condition is reflected in data from the International Union for Conservation of Nature (IUCN, 2021), which indicate that *M. cumingii* populations are declining, although the species is still classified as Least Concern.

Population decline may lead to a reduction in genetic diversity due to decreased gene flow among populations (Saputra & Yuda, 2020). Such conditions can negatively affect the adaptive capacity and long-term survival of the species in changing environments. Populations with low genetic diversity generally exhibit reduced resilience to environmental changes and selective pressures. According to Frankham et al. (2010), habitat fragmentation can result in population isolation, leading to a decline in genetic variation. Therefore, studies on genetic variation are essential for supporting effective population recovery and conservation strategies. Allendorf (2017) emphasized that genetic analysis is one of the most important approaches for biodiversity conservation. Consequently, molecular-based approaches have become among the most reliable methods for investigating genetic diversity and phylogenetic relationships among populations and species.

Molecular approaches employing DNA markers have been widely used in studies of avian phylogenetics and genetic diversity. Commonly used markers include mitochondrial DNA (mtDNA) and nuclear DNA. However, mitochondrial DNA has limitations because it only reflects maternal inheritance (Ladoukakis & Zouros, 2017). In contrast, nuclear DNA provides genetic information inherited from both parents and undergoes a higher level of recombination, making it particularly useful for evolutionary and population genetic studies (Zhang & Hewitt, 2003). One informative nuclear marker is the Nerve Growth Factor (NGF) gene, which plays an important role in the growth and development of the nervous system and contains nucleotide variations that are useful for phylogenetic analyses (Kimball et al., 2009; Jarvis et al., 2014; Prum et al., 2015). These characteristics suggest that the NGF gene has considerable potential for revealing genetic diversity and phylogenetic relationships at both population and species levels (Jarvis et al., 2014; Prum et al., 2015).

The use of the Nerve Growth Factor (NGF) gene in this study was motivated by previous research demonstrating its usefulness in phylogenetic reconstruction among species. Harris et al. (2014) and Novitasari et al. (2025) reported that several nuclear genes, such as *EEF2* and *RDP1*, have been successfully employed to investigate genetic relationships and evolutionary divergence within the family Megapodiidae. Furthermore, Budiarsa et al. (2010) demonstrated that nuclear DNA markers provide valuable phylogenetic information for the maleo (*Macrocephalon maleo*). Although nuclear DNA markers have been applied in studies of Megapodiidae, the use of the NGF gene to analyze phylogenetic relationships in *Megapodius cumingii*, particularly populations from Kabetan Island, Tolitoli Regency, Central Sulawesi, remains poorly documented. Therefore, this study aimed to describe and analyze the genetic diversity and phylogenetic relationships of the Philippine scrubfowl based on the Nerve Growth Factor (NGF) gene.

MATERIALS AND METHODS

Study Area

This study was conducted from September 2025 to January 2026. Samples of *Megapodius cumingii* were collected from Kabetan Island, Tolitoli District, Central Sulawesi, Indonesia. This location was selected because it represents one of the natural habitats where *M. cumingii* is still found in coastal areas. Kabetan Island is part of the species' distribution range in Sulawesi and is characterized by coastal ecosystems that provide important habitats for the daily activities and nesting behavior of *M. cumingii*. Furthermore, the occurrence of *M. cumingii* in the Tolitoli region has been documented in several previous studies, making this location a suitable representative of the local population for analyses of genetic diversity and phylogenetic relationships.

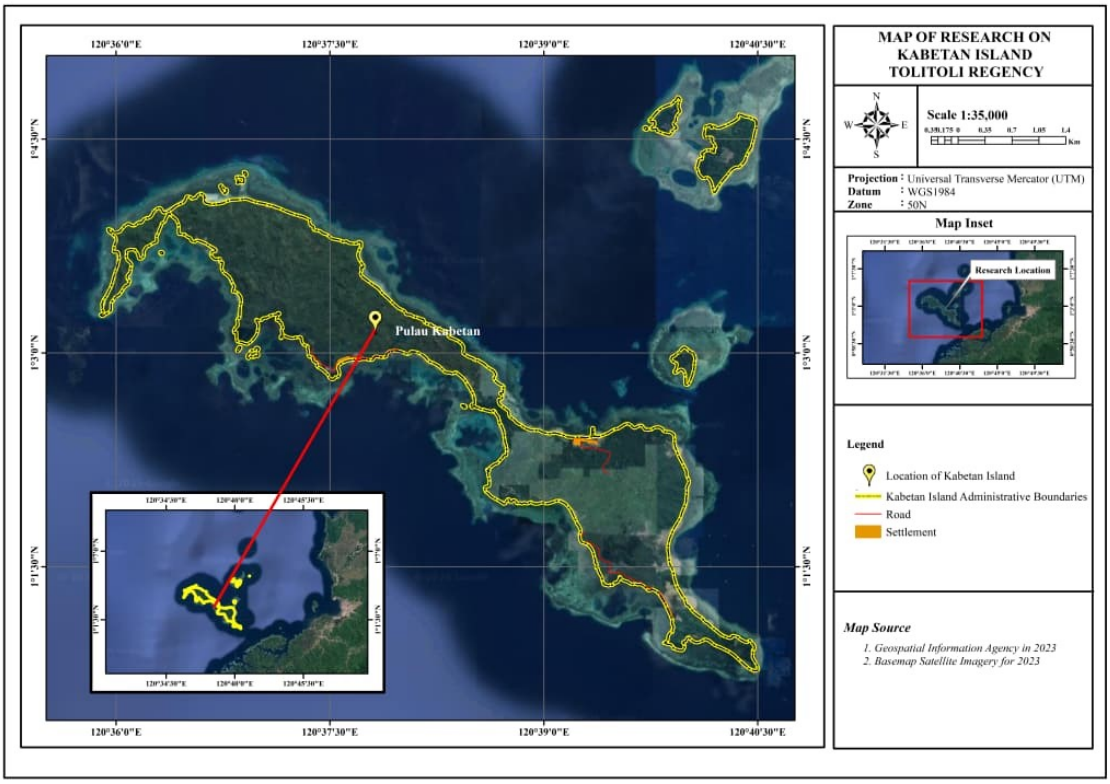

Figure 1. Study area map (Generated using ArcGIS 10.4).

Figure 2. Philippine scrubfowl (*Megapodius cumingii*) (Elliott & Kirwan, 2020).

Procedures

Sample Collection

Blood samples of the Philippine scrubfowl (*Megapodius cumingii*) were collected from Kabetan Island, Tolitoli Regency, Central Sulawesi, Indonesia. This location represents one of the natural habitats of the species. Approximately 0.1–0.2 mL of blood was collected from the brachial wing vein using a sterile syringe. During field sampling, blood samples were stored in an ice box to preserve sample quality and prevent DNA degradation.

DNA Extraction

Genomic DNA was extracted from blood samples at the Genetics Laboratory, Faculty of Biology, Universitas Gadjah Mada (UGM), using the gSYNC™ DNA Extraction Kit (Geneaid) following the manufacturer's protocol. Briefly, 100 µL of blood sample was mixed with Buffer BL and Proteinase K and incubated at 60°C. The mixture was then centrifuged to separate the DNA, followed by a series of washing steps using Wash Buffer to remove residual contaminants. DNA was subsequently eluted using Elution Buffer and stored at −20°C until further analysis.

DNA Amplification

DNA amplification was performed using the Takara Ex Taq™ PCR Kit. PCR reactions were carried out in a total volume of 50 µL using a Perkin-Elmer Thermal Cycler 9600. The thermal cycling conditions consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 35 s, annealing at 50°C for 30 s, and extension at 72°C. A final extension step was conducted at 72°C for 7 min. Amplification was performed to replicate the target gene sequence, producing amplicons as the final PCR products. The primers used for amplification were forward AllNGF5′ (GGTGCATAGCGTAATGTCCATG) and reverse AllNGF3′ (ATAATTTACAGGCTGAGGTAG) (Kimball et al., 2009). PCR products were subsequently sent to Genetika Science, Jakarta, Indonesia, for DNA sequencing.

Data Analysis

The NGF gene sequencing data were obtained in .ab1 file format and edited using GeneStudio version 2.2.0.0 to generate consensus sequences. The resulting sequences were compared with reference sequences available in the GenBank database through the National Center for Biotechnology Information (NCBI) website using the Basic Local Alignment Search Tool (BLAST) to confirm species identity based on sequence similarity. Consensus sequences were aligned and converted into FASTA format using MESQUITE. Genetic diversity analyses were performed using DnaSP version 6.12.03, while phylogenetic analyses were conducted using MEGA 11. Genetic distances were calculated using the Kimura 2-Parameter (K2P) model. Phylogenetic reconstruction was performed using the Neighbor-Joining (NJ) method under the Kimura 2-Parameter model with 1,000 bootstrap replicates to assess branch support.

RESULTS AND DISCUSSION

Amplification

The DNA sequences of *Megapodius cumingii* samples were successfully amplified using the forward primer AllNGF5′ and the reverse primer AllNGF3′. PCR visualization revealed a single, distinct DNA band with an approximate size of 737 bp, indicating that the amplification process was both efficient and specific to the target sequence. The absence of additional bands or smearing suggested that no non-specific amplification or DNA degradation occurred during the PCR process. Therefore, the PCR products were considered suitable for subsequent analyses, including DNA sequencing. The electrophoresis results are presented in Figure 3.

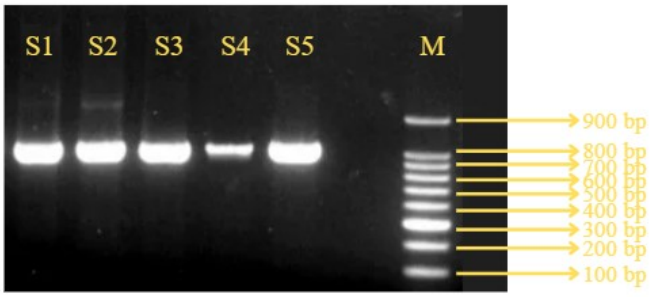

Figure 3. Agarose gel electrophoresis showing PCR amplification products of the NGF gene in the Philippine scrubfowl (*Megapodius cumingii*). The amplified fragment was approximately 737 bp in length.

Sequence analysis was performed by comparing the obtained sequences with reference data available in the GenBank database through the NCBI website using the Basic Local Alignment Search Tool (BLAST). This analysis aimed to determine the level of similarity between the sample sequences and reference sequences.

The results showed a query cover value of 100% and percent identity values ranging from 99.73% to 100.00%, indicating a very high degree of sequence similarity. These findings demonstrate that the obtained sequences closely match the reference sequences deposited in GenBank, thereby supporting the validity of species identification. Detailed results of the BLAST analysis are presented in Table 1.

Table 1. BLAST-based identification results of *Megapodius cumingii* sequences.

Code	BLAST		Verification	Location
	Query Cover (%)	Percent Identity (%)		
Sample 1	100	100.00	*Megapodius cumingii*	Tolitoli
Sample 2	100	100.00	*Megapodius cumingii*	Tolitoli
Sample 3	100	99.73	*Megapodius cumingii*	Tolitoli
Sample 4	100	99.73	*Megapodius cumingii*	Tolitoli
Sample 5	100	99.73	*Megapodius cumingii*	Tolitoli

Genetic Diversity

Analysis of the sample sequences together with *Megapodius cumingii* sequences from Sulawesi revealed a sequence length of 737 bp. The analysis identified two variable sites (S = 2), both of which were parsimony-informative sites (PI = 2). The haplotype diversity was estimated at Hd = 0.533 ± 0.172, while nucleotide diversity was $\pi = 0.00145 \pm 0.00047$. The low nucleotide diversity value indicates that genetic differences among individuals within the population are relatively small. However, the presence of more than one haplotype suggests that genetic variation is still maintained within the population (Frankham et al., 2010; Leffler et al., 2012). Detailed results of the genetic diversity analysis are presented in Table 2.

Table 2. Genetic diversity analysis of *Megapodius cumingii*.

Code	bp	Number of Individual	Number of Haplotype	Variable Site	Parsimony Site	Haplotype Diversity (Hd)	Nucleotide Diversity (π)
Sample 1 Sample 2 Sample 3 Sample 4 Sample 5 *Megapodius cumingii*	737	6	2	2	2	0,533±0,172	0,00145±0,00047

Nucleotide Composition

Based on the analysis, the nucleotide composition of the NGF gene in *Megapodius cumingii* consisted of Adenine (A) 29.3%, Thymine (T) 19.8%, Cytosine (C) 26.9%, and Guanine (G) 24.1%. These results indicate that the A+T content was 49.0%, whereas the G+C content was 51.0%, showing that the G+C composition was slightly higher than the A+T composition. The differences in nucleotide frequencies among samples were very small, indicating that nucleotide base composition was relatively uniform across individuals. Furthermore, the similarity in nucleotide composition between the study samples and the reference sequence *M. cumingii* suggests a close genetic relationship within the genus *Megapodius* (Table 3).

Table 3. Nucleotide composition analysis of *Megapodius cumingii.*

Code	T(U)	C	A	G	A+T	G+C	Location	References
Sample 1	19.8	27.0	29.2	24.0	49.0	51.0	Tolitoli	Research Data
Sample 2	19.7	26.8	29.3	24.2	49.0	51.0	Tolitoli	Research Data
Sample 3	19.7	26.8	29.3	24.2	49.0	51.0	Tolitoli	Research Data
Sample 4	19.8	27.0	29.2	24.0	49.0	51.0	Tolitoli	Research Data
Sample 5	19.8	27.0	29.2	24.0	49.0	51.0	Tolitoli	Research Data
Megapodius cumingii	19.7	26.8	29.3	24.2	49.0	51.0	Sulawesi	Harris et al. (2014)
Average	19.8%	26.9%	29.3%	24.1%	49.0%	51.0%		

Table 4. Nucleotide Composition Analysis of the Genus *Megapodius.*

Code	T(U)	C	A	G	A+T	G+C	Location	References
Megapodius decollatus	19.7	27.0	29.2	24.2	48.9	51.2	Papua New Guinea	Harris et al. (2014)
Megapodius eremita	19.7	26.8	29.3	24.2	49.0	51.0	Solomon	Harris et al. (2014)
Megapodius eremita	19.7	26.8	29.3	24.2	49.0	51.0	Papua New Guinea	Harris et al. (2014)
Megapodius forstenii	19.7	27.0	29.2	24.2	48.9	51.2	Maluku	Harris et al. (2014)
Megapodius forstenii	19.7	27.0	29.2	24.2	48.9	51.2	Maluku	Harris et al. (2014)
Megapodius freycinet	19.7	27.0	29.2	24.2	48.9	51.2	Irian Jaya	Harris et al. (2014)
Megapodius laperouse	19.7	26.8	29.5	24.0	49.2	50.8	USA	Harris et al. (2014)
Megapodius laperouse	19.7	26.8	29.5	24.0	49.2	50.8	USA	Harris et al. (2014)
Megapodius laperouse	19.7	26.8	29.5	24.0	49.2	50.8	Palau	Harris et al. (2014)
Megapodius layardi	19.7	26.9	29.2	24.2	48.9	51.1	Vanuatu	Harris et al. (2014)
Megapodius layardi	19.7	26.8	29.2	24.3	48.9	51.1	Vanuatu	Harris et al. (2014)
Megapodius pritchardii	19.8	26.7	29.3	24.2	49.1	50.9	Tonga	Harris et al. (2014)
Megapodius reinwardt	19.7	26.8	29.3	24.2	49.0	51.0	Papua New Guinea	Harris et al. (2014)
Megapodius reinwardt	19.7	26.8	29.3	24.2	49.0	51.0	Papua New Guinea	Harris et al. (2014)
Megapodius reinwardt	19.7	26.8	29.3	24.2	49.0	51.0	Australia	Harris et al. (2014)
Megapodius reinwardt	19.7	26.8	29.3	24.2	49.0	51.0	NTT	Harris et al. (2014)
Megapodius reinwardt	19.7	26.8	29.3	24.2	49.0	51.0	Aru	Harris et al. (2014)
Megapodius tenimberensis	20.0	26.7	29.2	24.2	49.2	50.9	Tanimbar	Harris et al. (2014)
Average	19.7	26.8	29.3	24.2	49.0	51.0		

Phylogenetic Reconstruction and Genetic Distance

Phylogenetic analysis was conducted to investigate the evolutionary relationships and degree of relatedness among species based on genetic variation. In this study, the analysis included five *Megapodius cumingii* sequences, 18 reference sequences representing the genus *Megapodius*, and three outgroup sequences: *Callipepla californica*, *Phasianus colchicus*, and *Penelope purpurascens*. Phylogenetic reconstruction was performed using two different outgroup datasets to evaluate the stability of the resulting tree topology. The first phylogenetic tree (Figure 4) was reconstructed using three outgroups (*Callipepla californica*, *Phasianus colchicus*, and *Penelope purpurascens*), whereas the second tree (Figure 5) was reconstructed using two outgroups (*Phasianus colchicus* and *Penelope purpurascens*). Despite differences in the number of outgroups included, the major clustering pattern within the genus *Megapodius* remained consistent. All *Megapodius cumingii* samples formed a single clade supported by high bootstrap values (99–100%). These results indicate that the phylogenetic relationships among *Megapodius cumingii* and other members of the genus *Megapodius* are stable, and that variations in the number of outgroups used did not affect the overall topology of the reconstructed phylogenetic trees.

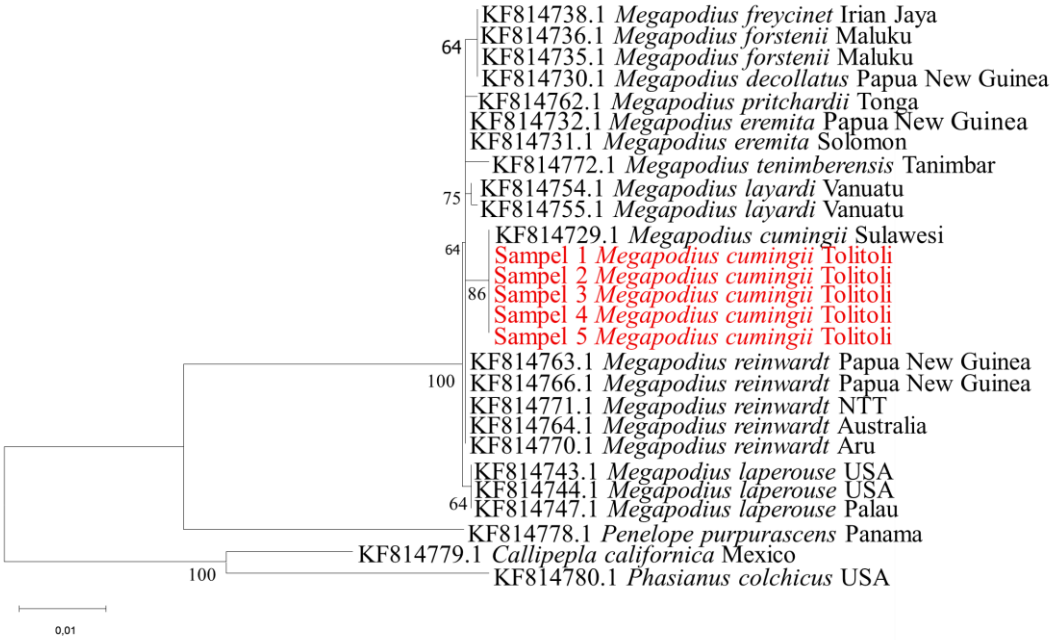

Figure 4. Neighbor-Joining (NJ) phylogenetic tree of *Megapodius cumingii* reconstructed from NGF gene sequences with 1,000 bootstrap replicates using three outgroup taxa.

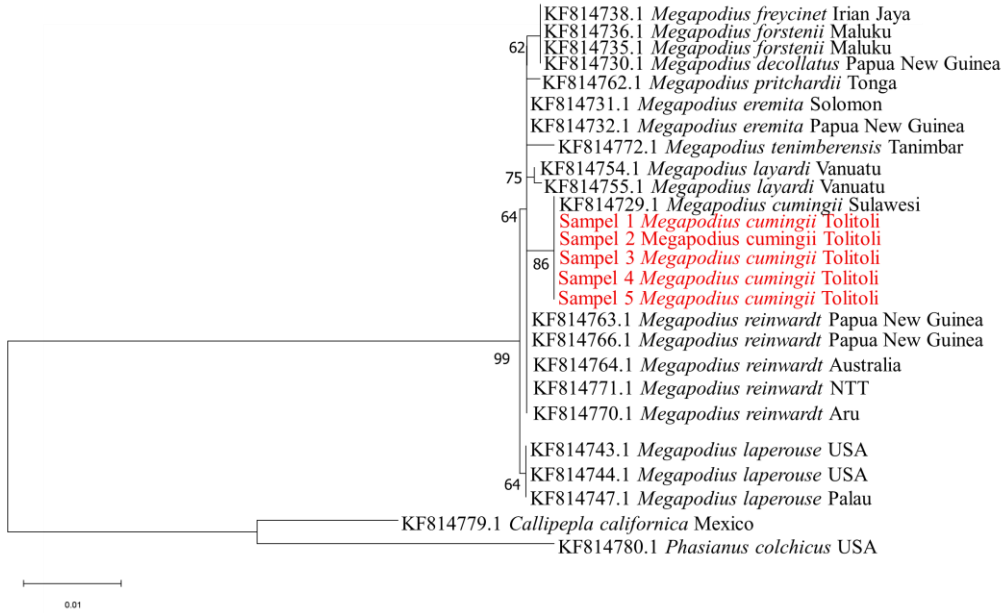

Figure 5. Neighbor-Joining (NJ) phylogenetic tree of *Megapodius cumingii* reconstructed from NGF gene sequences with 1,000 bootstrap replicates using two outgroup taxa.

The genetic distance analysis revealed that the differences among *Megapodius cumingii* samples from Tolitoli were very small, ranging from 0.000 to 0.003. These values indicate a very close phylogenetic relationship and suggest that all individuals likely originated from the same population. The genetic distances between *M. cumingii* and other species within the genus *Megapodius* were slightly higher, ranging from approximately 0.003 to 0.006, indicating the presence of genetic differentiation among species despite their close evolutionary relationships. In contrast, comparisons with species outside the family Megapodiidae, such as *Callipepla californica*, *Phasianus colchicus*, and *Penelope purpurascens*, yielded substantially higher genetic distance values, ranging from approximately 0.045 to 0.113, reflecting more distant evolutionary relationships. In general, low genetic distance values indicate close relatedness among taxa, whereas higher values reflect greater evolutionary divergence over time (Kumar & Nei, 2008).

	Sampel 1 *Megapodius cumingii* Tolitoli	Sampel 2 *Megapodius cumingii* Tolitoli	Sampel 3 *Megapodius cumingii* Tolitoli	Sampel 4 *Megapodius cumingii* Tolitoli	Sampel 5 *Megapodius cumingii* Tolitoli	KF814729.1 *Megapodius cumingii* Sulawesi	KF814730.1 *Megapodius decollatus Papua* New Guinea	KF814731.1 *Megapodius eremita* Solomon	KF814732.1 *Megapodius eremita* Papua New Guinea	KF814735.1 *Megapodius forstenii* Maluku	KF814736.1 *Megapodius forstenii* Maluku	KF814738.1 *Megapodius freycinet* Irian Jaya	KF814743.1 *Megapodius laperouse* USA	KF814744.1 *Megapodius laperouse* USA	KF814747.1 *Megapodius laperouse* Palau	KF814754.1 *Megapodius layardi* Vanuatu	KF814755.1 *Megapodius layardi* Vanuatu	KF814762.1 *Megapodius pritchardii* Tonga	KF814763.1 *Megapodius reinwardt* Papua New Guinea	KF814766.1 *Megapodius reinwardt* Papua New Guinea	KF814764.1 *Megapodius reinwardt* Australia	KF814771.1 *Megapodius reinwardt* NTT	KF814770.1 *Megapodius reinwardt* Aru	KF814772.1 *Megapodius tenimberensis* Tanimbar	KF814779.1 *Callipepla californica* Mexico	KF814780.1 *Phasianus colchicus* USA
Sampel 1 *Megapodius cumingii* Tolitoli																										
Sampel 2 *Megapodius cumingii* Tolitoli	0,000																									
Sampel 3 *Megapodius cumingii* Tolitoli	0,003	0,003																								
Sampel 4 *Megapodius cumingii* Tolitoli	0,000	0,000	0,003																							
Sampel 5 *Megapodius cumingii* Tolitoli	0,000	0,000	0,003	0,000																						
KF814729.1 *Megapodius cumingii* Sulawesi	0,003	0,003	0,000	0,003	0,003																					
KF814730.1 *Megapodius decollatus* Papua New Guinea	0,004	0,004	0,001	0,004	0,004	0,001																				
KF814731.1 *Megapodius eremita* Solomon	0,003	0,003	0,000	0,003	0,003	0,000	0,001																			
KF814732.1 *Megapodius eremita* Papua New Guinea	0,003	0,003	0,000	0,003	0,003	0,000	0,001	0,000																		
KF814735.1 *Megapodius forstenii* Maluku	0,004	0,004	0,001	0,004	0,004	0,001	0,000	0,001	0,001																	
KF814736.1 *Megapodius forstenii* Maluku	0,004	0,004	0,001	0,004	0,004	0,001	0,000	0,001	0,001	0,000																
KF814738.1 *Megapodius freycinet* Irian Jaya	0,004	0,004	0,001	0,004	0,004	0,001	0,000	0,001	0,001	0,000	0,000															
KF814743.1 *Megapodius laperouse* USA	0,004	0,004	0,001	0,004	0,004	0,001	0,003	0,001	0,001	0,003	0,003	0,003														
KF814744.1 *Megapodius laperouse* USA	0,004	0,004	0,001	0,004	0,004	0,001	0,003	0,001	0,001	0,003	0,003	0,003	0,000													
KF814747.1 *Megapodius laperouse* Palau	0,004	0,004	0,001	0,004	0,004	0,001	0,003	0,001	0,001	0,003	0,003	0,003	0,000	0,000												
KF814754.1 *Megapodius layardi* Vanuatu	0,003	0,003	0,000	0,003	0,003	0,000	0,001	0,000	0,000	0,001	0,001	0,001	0,001	0,001	0,001											
KF814755.1 *Megapodius layardi* Vanuatu	0,004	0,004	0,001	0,004	0,004	0,001	0,003	0,001	0,001	0,003	0,003	0,003	0,003	0,003	0,003	0,000										
KF814762.1 *Megapodius pritchardii* Tonga	0,004	0,004	0,001	0,004	0,004	0,001	0,003	0,001	0,001	0,003	0,003	0,003	0,003	0,003	0,003	0,001	0,003									
KF814763.1 *Megapodius reinwardt* Papua New Guinea	0,003	0,003	0,000	0,003	0,003	0,000	0,001	0,000	0,000	0,001	0,001	0,001	0,001	0,001	0,001	0,000	0,001	0,001								
KF814766.1 *Megapodius reinwardt* Papua New Guinea	0,003	0,003	0,000	0,003	0,003	0,000	0,001	0,000	0,000	0,001	0,001	0,001	0,001	0,001	0,001	0,000	0,001	0,001	0,000							
KF814764.1 *Megapodius reinwardt* Australia	0,003	0,003	0,000	0,003	0,003	0,000	0,001	0,000	0,000	0,001	0,001	0,001	0,001	0,001	0,001	0,000	0,001	0,001	0,000	0,000						
KF814771.1 *Megapodius reinwardt* NTT	0,003	0,003	0,000	0,003	0,003	0,000	0,001	0,000	0,000	0,001	0,001	0,001	0,001	0,001	0,001	0,000	0,001	0,001	0,000	0,000	0,000					
KF814770.1 *Megapodius reinwardt* Aru	0,003	0,003	0,000	0,003	0,003	0,000	0,001	0,000	0,000	0,001	0,001	0,001	0,001	0,001	0,001	0,000	0,001	0,001	0,000	0,000	0,000	0,000				
KF814772.1 *Megapodius tenimberensis* Tanimbar	0,006	0,006	0,003	0,006	0,006	0,003	0,004	0,003	0,003	0,004	0,004	0,004	0,004	0,004	0,004	0,003	0,004	0,004	0,003	0,003	0,003	0,003	0,003			
KF814779.1 *Callipepla californica* Mexico	0,096	0,096	0,093	0,096	0,096	0,093	0,095	0,093	0,093	0,095	0,095	0,095	0,093	0,093	0,093	0,093	0,095	0,095	0,093	0,093	0,093	0,093	0,093	0,096		
KF814780.1 *Phasianus colchicus* USA	0,113	0,113	0,109	0,113	0,113	0,109	0,111	0,109	0,109	0,111	0,111	0,111	0,109	0,109	0,109	0,110	0,111	0,111	0,109	0,109	0,109	0,109	0,109	0,113	0,045	

Figure 6. Genetic Distances between *Megapodius cumingii* and other *Megapodius* species.

Discussion

DNA amplification is the process of generating multiple copies of a target DNA fragment in vitro using the Polymerase Chain Reaction (PCR) technique, enabling the production of millions to billions of DNA copies within a relatively short period (Antara et al., 2025). This process utilizes DNA polymerase enzymes, specific primers, nucleotides, and thermal cycling consisting of denaturation, annealing, and extension steps to exponentially amplify DNA fragments (Nabholz et al., 2008; Barnes et al., 2021). The DNA amplification results obtained in this study showed that all samples (S1–S5) produced a single DNA band of approximately 737 bp. This finding indicates that the PCR process was successfully optimized and that the primers specifically amplified the target DNA fragment. The absence of additional bands or smearing suggests that neither non-specific amplification nor DNA degradation occurred during the amplification process. Visualization of DNA bands on agarose gel electrophoresis was used to verify amplification success based on the expected fragment size and the clarity of the resulting bands (Pamulang & Haryanto, 2021). The presence of distinct DNA bands also indicates that the concentration and quality of the amplified DNA were sufficient for downstream applications such as DNA sequencing. These results are consistent with those reported by Savitri et al. (2021), who obtained clear and specific PCR products of the CHD1 gene in *Cacatua galerita*. Similarly, Speller et al. (2011) stated that distinct PCR bands without smearing indicate high DNA quality and successful amplification suitable for subsequent sequencing analyses.

Genetic diversity analysis using DnaSP version 6 identified two haplotypes and two variable sites (S = 2), both of which were parsimony-informative sites (PI = 2). The haplotype diversity value (Hd = 0.533 ± 0.172) indicates the presence of haplotype variation among individuals, whereas the nucleotide diversity value (π = 0.00145 ± 0.00047) suggests that genetic differences among sequences are relatively low. The combination of moderate haplotype diversity and low nucleotide diversity generally reflects the presence of recent genetic variation with limited differentiation, which may be associated with demographic processes such as recent population expansion or ongoing gene flow (Yang et al., 2025). The low nucleotide diversity observed in this study indicates that most sequences retain highly similar nucleotide arrangements, resulting in close genetic relationships among individuals. Nevertheless, the presence of more than one haplotype demonstrates that the *Megapodius cumingii* population still harbors genetic variation that may serve as an important foundation for phylogenetic studies, population structure analyses, and species conservation efforts.

Analysis of nucleotide base composition in *Megapodius cumingii* revealed relatively balanced proportions of Thymine (19.8%), Cytosine (26.9%), Adenine (29.3%), and Guanine (24.1%), resulting in an A+T content of 49.0% and a G+C content of 51.0%. This pattern indicates a nearly balanced nucleotide composition with a slight predominance of G+C content. Similar nucleotide composition patterns were observed across all samples and reference sequences. Moreover, this pattern is consistent with NGF gene sequences from other *Megapodius* species distributed across Papua New Guinea, the Maluku Islands, Australia, and the Pacific region, which exhibit nearly identical nucleotide compositions, suggesting a highly conserved genetic structure within the genus (Harris et al., 2014). In general, nucleotide composition in avian nuclear DNA tends to be relatively stable and exhibits limited variation because it is subject to evolutionary constraints. Variations in GC content are known to contribute to genome evolution and influence phylogenetic inference, although they often remain relatively conserved within a genus (Zhou et al., 2024).

Phylogenetic reconstruction using the Neighbor-Joining (NJ) method under the Kimura 2-Parameter model revealed a consistent pattern of evolutionary relationships, particularly among *Megapodius cumingii* samples from Kabetan Island. All *M. cumingii* sequences from Kabetan Island, Tolitoli, formed a single clade supported by high bootstrap values, reaching 100% in Figure 4 and 99% in Figure 5. These values indicate strong support for the inferred evolutionary relationships and the resulting tree topology. High bootstrap support reflects the stability of the phylogenetic tree and demonstrates that the relationships among samples and other *Megapodius* species are robust and consistently recovered. The primary difference between the two phylogenetic trees lies in the number of outgroup taxa included. The first tree (Figure 4) incorporated three outgroups, namely *Penelope purpurascens*, *Callipepla californica*, and *Phasianus colchicus*, whereas the second tree (Figure 5) included only two outgroups, *Callipepla californica* and *Phasianus colchicus*. Despite these differences, the major clustering pattern within the genus *Megapodius* remained unchanged, indicating that the phylogenetic relationships among samples were stable regardless of the outgroup configuration employed (Jarvis et al., 2014; Prum et al., 2015).

Genetic distance analysis showed that genetic divergence among *Megapodius cumingii* samples from Tolitoli was very low, ranging from 0.000 to 0.003. These low genetic distance values reflect limited genetic divergence among individuals and are consistent with the generally conserved nature of nuclear DNA within a species. In contrast, genetic distances between *M. cumingii* and other species within the genus *Megapodius* were slightly higher (approximately 0.003–0.006), indicating interspecific genetic differentiation while still reflecting close relationships within the same genus. Considerably higher genetic distances were observed when *M. cumingii* was compared with species outside the family Megapodiidae, such as *Callipepla californica*, *Phasianus colchicus*, and *Penelope purpurascens*

(approximately 0.045–0.113). These values indicate greater evolutionary divergence and more distant phylogenetic relationships. In general, low to moderate genetic distance values reflect close evolutionary relationships, whereas higher values indicate longer periods of evolutionary divergence (Kumar et al., 2008; Zhao et al., 2021).

The findings of this study are consistent with previous studies on birds, particularly members of the order Galliformes, which have demonstrated that nuclear genes are relatively conserved and effective for investigating phylogenetic relationships and evolutionary affinities among species (Shen et al., 2010; Jiang et al., 2019). Similar results were reported by Novitasari et al. (2025), who found that *M. cumingii* populations in Central Sulawesi exhibited haplotype variation among populations while all samples remained grouped within the same clade, reflecting close genetic relationships. These findings suggest that the presence of multiple haplotypes represents existing genetic variation within the population, which can be utilized to infer dispersal patterns, population structure, and the evolutionary history of *M. cumingii*.

CONCLUSIONS

Megapodius cumingii from Kabetan Island, Tolitoli Regency, Central Sulawesi, exhibited genetic diversity with a haplotype diversity value of 0.533 ± 0.172 and a nucleotide diversity value of 0.00145 ± 0.00047. Phylogenetic reconstruction showed that all *M. cumingii* samples clustered within the same clade, supported by high bootstrap values of 99% and 100%. The phylogenetic relationships among *M. cumingii* individuals from Kabetan Island, Tolitoli, were very close. This finding was further supported by the genetic distance analysis, which revealed values ranging from 0.000 to 0.003.

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REFERENCES

Allendorf, F. W. (2017). Genetics and the conservation of natural populations : allozymes to genomes. *Molecular Ecology*, *26*, 420–430. https://doi.org/10.1111/mec.13948

Antara, N. Y., Sari, B. A., Trianes, J., Rastuti, M. R., Bastian., Zamzam, M. K., Fajarido, A., Baihaki, I., Mukhlis, A. Z. R., Gunawan, A., Gustaman, F. A. I., Aryani, D., Mufidah, H., & Amin, A. (2025). Polymerase Chain Reaction (PCR). In F. H. M. Mawo & M. F. Nurmidin (Eds.), *Biologi Molekuler Untuk Teknologi Laboratorium Medis* (pp. 27–48). Yayasan Bina Lentera Insan.

Barnes, W. M., Zhang, Z., Kermekchiev, M. B., & Steiger, M. G. (2021). A Single Amino Acid Change to Taq DNA Polymerase Enables Faster PCR , Reverse Transcription and. *Frontiers in Bioengineering and Biotechnology*, *8*, 1–10. https://doi.org/10.3389/fbioe.2020.553474

Bashari, H., Mangangue, B., & Mangangue, A. (2017). Incubation strategy of Philippine Scrubfowl Megapodius cumingii on Manumpitaeng islet, North Sulawesi, Indonesia. *Birdingasia*, *27*, 82–85.

Birks, S. M., & Edwards, S. V. (2002). A Phylogeny of the Megapodes (Aves: Megapodiidae) Based on Nuclear and Mitochondrial DNA Sequences. *Molecular Phylogenetics and Evolution*, *23*(3), 408–421. https://doi.org/10.1016/S1055-7903(02)00002-7

Budiarsa, I., Artama, W., A., Sembiring, L., & Situmorang, J. (2010). Analisis Filogenetik Burung Maleo (Macrocephalon maleo) Berdasarkan Sekuen Intron Satu Gen Rhodopsin (RDP1) Nukleus Phylogenetic Analysis of the Maleo (Macrocephalon maleo) based on the First Intron of Rhodopsin (RDP1) Nuclear Gene Sequences. *Biota*, *15*(2), 160–166.

Elliott, A., & Kirwan, G. M. (2020). *Philippine Megapode (Megapodius cumingii)*. Birds of the World. https://doi.org/10.2173/BOW.TABSCR1.01

Frankham, R., Ballou, J. D., & Briscoe, D. A. (2010). *Introduction to Conservation Genetics,* (2Edition ed.). Cambridge University Press. https://doi.org/http://dx.doi.org/10.1017/CBO9780511809002

Gorog, A. J., Pamungkas, B., & Lee, R. J. (2005). Nesting ground abandonment by the maleo (Macrocephalon maleo) in North Sulawesi : Identifying conservation priorities for Indonesia ' s endemic megapode. *Biological Conservation*, *126*, 548–555. https://doi.org/10.1016/j.biocon.2005.07.006

Harris, R. B., Birks, S. M., & Leaché, A. D. (2014). Incubator birds: Biogeographical origins and evolution of underground nesting in megapodes (Galliformes: Megapodiidae). *Journal of Biogeography*, *41*(11), 2045–2056. https://doi.org/10.1111/jbi.12357

IUCN. (2021). Megapodius cumingii, Philippine Scrubfowl. *The IUCN Red List of Threatened Species 2021: T22678588A195820544*. https://dx.doi.org/10.2305/IUCN.UK.2021-3.RLTS.T22678588A195820544.en

Jarvis, E. D., Mirarab, S., Aberer, A. J., Li, B., Houde, P., Li, C., Ho, S. Y. W., Faircloth, B. C., Nabholz, B., Howard, J. J., Suh, A., Weber, C. C., Fonseca, R. R. da., Li, J., Zhang, F., Li, H., Zhou, L., Narula, N., Liu, L., … Zhang, G. (2014). Whole-genome analysesresolve early branches in the tree of life of modern birds. *Science*, *346*(6215), 1320–1331.

Jiang, K., Zhao, F., & Huang, Z. (2019). Inter-familial relationships of the Gamefowl (Aves : Galliformes) based on complete mitochondrial genome sequences complete

mitochondrial genome sequences. *Mitochondrial DNA Part B*, *4*(1), 723–724. https://doi.org/10.1080/23802359.2018.1553509

Jones, D., N. (1999). What we don't know about megapodes. *Proceedings Third International Megapode Symposium*, *327*, 160–168.

Kimball, R. T., Braun, E. L., Barker, F. K., Bowie, R. C. K., Braun, M. J., Chojnowski, J. L., Hackett, S. J., Han, K., Harshman, J., Heimer-torres, V., Holznagel, W., Huddleston, C. J., Marks, B. D., Miglia, K. J., Moore, W. S., Reddy, S., Sheldon, F. H., Smith, J. V, Witt, C. C., & Yuri, T. (2009). Molecular Phylogenetics and Evolution Short Communication A well-tested set of primers to amplify regions spread across the avian genome. *Molecular Phylogenetics and Evolution*, *50*(3), 654–660. https://doi.org/10.1016/j.ympev.2008.11.018

Kumar, S., Nei, M., Dudley, J., & Tamura, K. (2008). MEGA : A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics*, *9*(4), 299–306. https://doi.org/10.1093/bib/bbn017

Ladoukakis, E. D., & Zouros, E. (2017). Evolution and inheritance of animal mitochondrial DNA : rules and exceptions. *Journal of Biological Research-Thessaloniki*, *24*(2), 1–7. https://doi.org/10.1186/s40709-017-0060-4

Leffler, E. M., Bullaughey, K., Matute, D. R., Meyer, W. K., Se, L., Venkat, A., Andolfatto, P., & Przeworski, M. (2012). Revisiting an Old Riddle : What Determines Genetic Diversity Levels within Species ? *PLoS Biology*, *10*(9), 1–9. https://doi.org/10.1371/journal.pbio.1001388

Nabholz, B., Gle, S., & Galtier, N. (2008). Strong Variations of Mitochondrial Mutation Rate across Mammals — the Longevity Hypothesis. *Molecular Biology and Evolution, 25*(1), 120–130. https://doi.org/10.1093/molbev/msm248

Novitasari, Budiarsa, I. M., Suleman, S. M., Jayanti, Z. D., & Trianto, M. (2025). Burung Gosong Filipina (Megapodius cumingii) di Sulawesi Tengah : Variasi Haplotipe dan Hubungan Filogenetik Berdasarkan Gen EEF2. *Bioscientist: Jurnal Ilmiah Biologi*, *13*(3), 2086–2095.

Paguntalan, L., J., Feliza, M., O., Bonares, B., A., & Villegas, G., M. (2021). Ecology of Philippine Scrubfowl Megapodius cumingii on Palawan With Notes on Other Islands. *Journal of ASIAN Ornithology*, *37*, 99–106.

Pamulang, Y. V., & Haryanto, A. (2021). Short Communication : Molecular bird sexing on kutilang (Pycnonotus sp .) based on amplification of CHD-Z and CHD-W genes by using polymerase chain reaction method. *Biodiversitas*, *22*(1), 449–452. https://doi.org/10.13057/biodiv/d220155

Prum, R. O., Berv, J. S., Dornburg, A., Field, D. J., Townsend, J. P., Lemmon, E. M., & Lemmon, A. R. (2015). A comprehensive phylogeny of birds (Aves) using targeted next-generation DNA sequencing. *Nature*. https://doi.org/10.1038/nature15697

Radley, P. M., Davis, R. A., Dekker, R. W. R. J., Molloy, S. W., Blake, D., & Heinshon, R. (2018). Vulnerability of megapodes (Megapodiidae , Aves) to climate change and. *Environmental Conservation*, 1–11. https://doi.org/10.1017/S0376892918000152

Saputra, A. W., & Yuda, P. (2020). Low genetic diversity and no genetic differentiation between maleo hatched at coastal and inland nesting grounds in North Sulawesi , Indonesia. *Biodiversitas*, *21*(10), 4772–4777. https://doi.org/10.13057/biodiv/d211042

Savitri, D., Putri, I., Nugrahani, W. P., Purwaningrum, M., & Haryanto, A. (2021). Molecular bird sexing of sulphur - crested cockatoo (Cacatua galerita) by polymerase chain reaction method. *Indonesian Journal of Biotechnology*, *26*(1), 1–6. https://doi.org/10.22146/ijbiotech.54611

Shen, Y.-Y., Liang, L., Sun, Y.-B., Yue, B.-S., Yang, X.-J., Murphy, R. W., & Zhang, Y.-P. (2010). A mitogenomic perspective on the ancient, rapid radiation in the Galliformes with an emphasis on the Phasianidae. *BMC Evolutionary Biology*, *10*(132), 1–10. https://doi.org/10.1186/1471-2148-10-132

Speller, C. F., Nicholas, G. P., & Yang, D. Y. (2011). Feather barbs as a good source of mtDNA for bird species identification in forensic wildlife investigations. *Investigative Genetics*, *2*(1), 16. https://doi.org/10.1186/2041-2223-2-16

Yang, T., Sun, Y., & Xiao, P. (2025). Genome-Wide Genetic Diversity and Population Structure of Sillago sinica (Perciformes , Sillaginidae) from the Coastal Waters of China : Implications for Phylogeographic Pattern and Fishery Management. *Biology*, *14*(1329), 1–15. https://doi.org/https://doi.org/10.3390/biology14101329

Zhang, D. X., & Hewitt, G. M. (2003). Nuclear DNA analyses in genetic studies of populations: Practice, problems and prospects. In *Molecular Ecology* (Vol. 12, Issue 3, pp. 563–584). https://doi.org/10.1046/j.1365-294X.2003.01773.x

Zhao, Y., Zhu, X., Jiang, Y., Li, Z., Li, X., Xu, W., Wei, H., & Li, Y. (2021). Genetic diversity and variation of seven Chinese grass shrimp (Palaemonetes sinensis) populations based on the mitochondrial COI. *BMC Ecology and Evolution*, *21*(167), 1–10. https://doi.org/10.1186/s12862-021-01893-8

Zhou, S., Wang, X., Wang, L., Gao, X., Lyu, T., & Xia, T. (2024). Different Evolutionary Trends of Galloanseres : Mitogenomics Analysis. *Animals*, *14*(1437), 2–13. https://doi.org/https://doi.org/10.3390/ani14101437

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