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# Genetic Variation and Phylogenetic Analysis of Philippine Scrubfowl (*Megapodius cumingii*) Based on Mitochondrial NADH Dehydrogenase Subunit 2 (ND2) Gene

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**Abstract**

Central Sulawesi is one of the provinces in Indonesia with high avian diversity, including the Philippine scrubfowl (*Megapodius cumingii*). This species belongs to the family Megapodiidae and is distributed in the Philippines, parts of eastern Borneo, and several small islands in Indonesia. This study aimed to analyze the genetic variation and phylogenetic relationships of *Megapodius cumingii* from Poat Island based on sequences of the mitochondrial NADH dehydrogenase subunit 2 (ND2) gene. Genomic DNA extracted from blood samples of the Philippine scrubfowl was isolated using the gSYNC™ DNA Extraction Kit (Geneaid), amplified using the Polymerase Chain Reaction (PCR) method with primers L5145 (forward) and H6394 (reverse), and visualized by 1% agarose gel electrophoresis. The PCR products were purified using the QIAquick PCR Purification Kit. Sequence data were analyzed using BLAST for species confirmation, DnaSP v6.12.03 for genetic variation analysis, and MEGA 11 for phylogenetic tree reconstruction using the Maximum Likelihood and Neighbor-Joining methods. The results showed that the 1.072 base pair ND2 gene sequences generated three haplotypes, with a haplotype diversity value of 0.667 and a nucleotide diversity of 0.00249. The nucleotide base composition of the ND2 gene was dominated by A+T (52.03%) compared to G+C (47.95%). Phylogenetic tree reconstruction using both methods produced consistent topologies, in which all *Megapodius cumingii* samples from Poat Island formed a monophyletic clade with high bootstrap support, indicating strong and stable genetic relationships.

**Keywords:** Genetic variation; phylogenetics; *Megapodius cumingii*; NADH Dehydrogenase Subunit 2 (ND2); mitochondria.

## INTRODUCTION

Central Sulawesi is a province rich in avian diversity, encompassing numerous endemic species, range-restricted species, and protected taxa. This diversity holds high ecological value, as birds play crucial roles in pollination, seed dispersal, population control of other organisms, and the maintenance of balance and stability in natural ecosystems (Vikar et al., 2024). However, increasing anthropogenic pressures, such as coastal land conversion, industrial expansion, and hunting activities, have posed serious threats to the persistence of local bird populations. These pressures not only affect threatened species but are also beginning to impact species categorized as Least Concern, including the Philippine scrubfowl (*Megapodius cumingii*) (Supriatna, 2018; BirdLife International, 2021).

*Megapodius cumingii* belongs to the family Megapodiidae, distributed in the Philippines, parts of eastern Borneo, and several small islands in Indonesia, including Central Sulawesi, particularly the Banggai Islands (Harris et al., 2014; Bashari et al., 2017; Elliott & Kirwan, 2020). This species exhibits a unique reproductive strategy by utilizing environmental heat sources for egg incubation (Birks & Edwards, 2002; Radley et al., 2021). Its high dependence on environmental conditions makes *M. cumingii* particularly sensitive to habitat alteration, hunting pressure, and disturbances at nesting and natural incubation sites (BirdLife International, 2021). Although its global conservation status is categorized as Least Concern, local populations in Central Sulawesi are suspected to be under pressure, potentially leading to reduced genetic variation due to habitat fragmentation and decreased connectivity among populations (Sinclair et al., 1999; Paguntalan et al., 2021). A decline in genetic variation may directly reduce the species' adaptive capacity to environmental changes, increase the risk of inbreeding, and compromise long-term population resilience. Therefore, genetic analysis at the local population level is essential to understand the current genetic condition of this species. Such information will provide a strong scientific foundation for the formulation of effective, targeted, and molecular data–based conservation strategies (Tavakoli et al., 2017).

Molecular approaches, particularly genetic identification, form a critical basis for the development of modern conservation strategies grounded in scientific data (Budiarsa et al., 2010). In genetic studies, mitochondrial genes are frequently used because they are maternally inherited and exhibit relatively high mutation rates. One of the most informative genes is NADH dehydrogenase subunit 2 (ND2), which functions in the mitochondrial electron transport chain. The high nucleotide variability of the ND2 gene makes it an effective molecular marker for genetic analyses (Yue et al., 2021; Mailloux, 2022; Yin & Guo, 2025; Zink & Barrowclough, 2008; Quek et al., 2018). Such variation reflects differences in DNA base composition that form the basis for determining levels of genetic diversity and phylogenetic relationships among organisms (Elvyra, 2023; Wang et al., 2023; Hosseini et al., 2025).

The use of the mitochondrial ND2 gene in this study is supported by previous research demonstrating its effectiveness in resolving phylogenetic relationships within Megapodiidae. Birks and Edwards (2002) showed that the ND2 gene successfully reconstructed intergeneric relationships within the family, where *Megapodius* formed a clade with *Eulipoa wallacei* and was clearly separated from *Macrocephalon maleo*. These findings confirm that the mitochondrial ND2 gene provides strong phylogenetic resolution at interspecific and intergeneric levels. Compared with nuclear genes such as IRF2 intron 2 (Tala'a, 2021) and EEF2 (Novitasari et al., 2025), which are more conserved, biparentally inherited, and generally used to assess species stability and long-term evolutionary divergence patterns, the maternally inherited ND2 gene with its higher mutation rate is more sensitive in detecting intraspecific genetic variation, particularly at the scale of local and island populations. The application of ND2 to *Megapodius cumingii* populations from Poat Island is therefore relevant and complements previous studies by providing mitochondrial genetic variation data that have not previously been available, while also strengthening the understanding of phylogenetic relationships and the scientific basis for local conservation planning.

This study aims to examine the level of genetic variation and the phylogenetic relationships of *Megapodius cumingii* from Poat Island based on mitochondrial NADH dehydrogenase subunit 2 (ND2) gene sequences. The analysis is expected to provide insights into genetic relationship patterns at the local population scale, identify potential genetic differentiation, and serve as a scientific foundation for the development of sustainable, molecular data–based genetic conservation strategies for this species.

## MATERIALS AND METHODS

This study was conducted from October 2025 until the completion of all research stages. Sample collection was carried out on Poat Island Beach, Pagimana District, Banggai Regency, Central Sulawesi. DNA isolation and amplification were performed at the Genetics Laboratory, Universitas Gadjah Mada, while DNA sequencing was conducted at Genetika Science, Jakarta, Indonesia. Bioinformatics analyses were carried out in Palu City.

### Prosedures

*Sample Collection*

Blood samples of the Philippine scrubfowl (*Megapodius cumingii*) were collected from Poat Island, Pagimana District, Banggai Regency, Central Sulawesi Province. Blood was drawn from the pectoral vein beneath the wing using a sterile syringe, with a volume of approximately 0.1–0.2 mL per individual. The collected blood was transferred into collection tubes containing EDTA (Ethylene Diamine Tetraacetic Acid) as an anticoagulant and gently homogenized to ensure proper mixing. During fieldwork, all blood samples were stored in a cooling box containing ice to preserve DNA quality prior to further laboratory analyses.

*DNA Isolation*

DNA isolation from *Megapodius cumingii* blood samples was performed using the gSYNC™ DNA Extraction Kit (Geneaid). A total of 100 µL of blood sample was mixed with Buffer BL and Proteinase K, then incubated at 60°C until complete lysis was achieved. The homogenized solution was subsequently transferred to a spin column and centrifuged. The column was washed sequentially using Wash Buffer to remove contaminants. DNA was eluted using Elution Buffer and stored at −20°C for further amplification.

*DNA Amplification*

DNA amplification was carried out using a commercial Taq DNA polymerase kit in a total reaction volume of 25 µL, consisting of 25 ng DNA template, 2.5 µL 10× buffer, 1 µL dNTPs (2.5 mM), 1 µL of each primer (L5145 forward and H6394 reverse), and 0.5 units of Taq polymerase (Thermo Scientific). The PCR program consisted of pre-denaturation at 95°C for 5 minutes; followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 52°C for 30 seconds, and elongation at 72°C for 1 minute; with a final extension at 72°C for 10 minutes. PCR products were verified by electrophoresis on a 1% agarose gel using a DNA marker, run at 90–100 V for 30–40 minutes. A DNA band of approximately 1,000 bp observed under a UV transilluminator indicated successful amplification of the ND2 gene.

*Electrophoresis and Visualization*

DNA electrophoresis was performed using a 1% agarose gel prepared by dissolving 0.2 g of agarose in 20 mL of 1× TAE buffer. The gel was stained with FloroSafe, poured into a casting tray, and allowed to solidify.

Amplified DNA samples along with a DNA ladder were loaded into the wells and electrophoresed at 50 V for 17–20 minutes. DNA bands were visualized using a UV transilluminator connected to a gel documentation system.

*DNA Sequencing*

Purification was performed to obtain high-quality amplicons using the QIAquick PCR Purification Kit protocol. Sequencing was subsequently conducted to determine the nucleotide sequence for further analysis. The sequencing process utilized the BigDye® Terminator v3.1 Cycle Sequencing Kit and was run on an ABI PRISM 3100 Avant Genetic Analyzer.

**Data analysis**

ND2 gene sequencing results (forward and reverse.ab1 files) were edited using GeneStudio 2.2.0.0 to trim low-quality regions and generate consensus sequences. Sequence identity was confirmed using BLAST (NCBI). Consensus sequences were aligned using MESQUITE and converted into FASTA format for analysis in MEGA 11 (Tamura et al., 2021). The multiple alignment results were exported to DnaSP v6.12.03 to calculate genetic variation parameters, including number of individuals, number of haplotypes (h), number of polymorphic sites, parsimony-informative sites, haplotype diversity (Hd), and nucleotide diversity ($\pi$) (Rozas et al., 2017). Phylogenetic reconstruction was performed in MEGA 11 using the Maximum Likelihood (ML) method with the Tamura–Nei model and the Neighbor-Joining (NJ) method with the Kimura 2-Parameter model, with 1,000 bootstrap replicates.

## RESULTS AND DISCUSSION

**DNA Amplification, Visualization, and Sequencing of Samples**

The results of 1% agarose gel electrophoresis showed that the mitochondrial ND2 gene PCR products from five *Megapodius cumingii* samples (S1–S5) were successfully amplified. Amplification was performed using primers L5145 (5′ forward) and H6394 (5′ reverse). Visualization under a UV transilluminator revealed a single DNA band of approximately ±1,000 bp in all samples, consistent with the expected fragment length of the targeted ND2 gene. The absence of non-specific bands or smearing indicated good DNA quality and successful amplification, thereby supporting the acquisition of high-quality sequences in the subsequent sequencing stage. Differences in band intensity among samples were likely associated with variations in DNA concentration or PCR amplification efficiency; however, these differences did not affect the consistency of the fragment length produced (Figure 1).

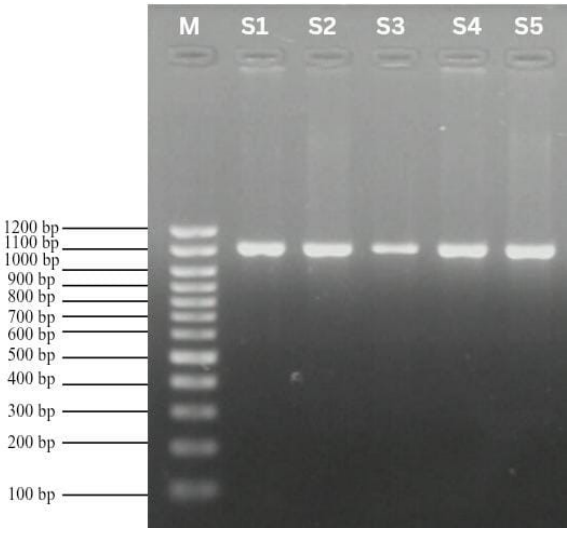


**Figure 1.** DNA amplification visualization of the mitochondrial ND2 gene showed that Samples 1–5 represent *Megapodius cumingii* collected from Poat Island Beach, Pagimana District, Banggai Regency, Central Sulawesi, while M indicates the DNA marker (ladder).

**BLAST Sequence Results**

Based on the BLAST analysis of the Philippine scrubfowl (*Megapodius cumingii*) DNA sequences, all tested samples (Sample 1–Sample 5) exhibited a very high level of similarity to the reference sequence available in GenBank under accession number KF854310.1, as indicated by a query cover value of 100% for all samples. The percentage identity values ranged from 99.44% to 99.91%, demonstrating very strong nucleotide similarity and molecularly confirming the species identity (Table 1).

**Table 1.** Sequence identification results of the Philippine scrubfowl (*Megapodius cumingii*) using the BLAST method from GenBank

| Code | *Query cover* (%) | *Percent Identity* (%) | Verification | Location |
|---|---|---|---|---|
| 2851141 Sample 1 | 100 | 99,91 | *Megapodius cumingii* | Banggai |
| 2851142 Sample 2 | 100 | 99,44 | *Megapodius cumingii* | Banggai |
| 2851143 Sample 3 | 100 | 99,91 | *Megapodius cumingii* | Banggai |
| 2851149 Sample 4 | 100 | 99,44 | *Megapodius cumingii* | Banggai |
| 2851148 Sample 5 | 100 | 99,91 | *Megapodius cumingii* | Banggai |

### Genetic Variation of *Megapodius cumingii*

Based on the analysis using DnaSP, the ND2 gene (1,072 bp) of *Megapodius cumingii* from seven individuals (n = 7) yielded three haplotypes (h = 3), with six polymorphic sites (S = 6) and five parsimony-informative sites (PI = 5). The haplotype diversity was Hd = 0.667, and the nucleotide diversity was $\pi$ = 0.00249. These results indicate moderate haplotype diversity but low nucleotide diversity within the analyzed population. This pattern suggests that nucleotide differences among individuals are relatively small, reflecting a high level of genetic relatedness within the population studied (Fan et al., 2024) (Table 2).

**Table 2.** Results of genetic variation analysis of *Megapodius cumingii* sample sequences and comparative samples from NCBI

| Code | bp | Individual | Haplotipe | Polimorfik Site | Parsimory Site | Haplotype Diversity (Hd) | Nucleotide Diversity ($\pi$) |
|---|---|---|---|---|---|---|---|
| 2851141 Sampel 1<br>2851142 Sampel 2<br>2851143 Sampel 3<br>2851149 Sampel 4<br>2851148 Sampel 5<br>KF854310.1<br>AF394624.1 | 1072 | 7 | 3 | 6 | 5 | 0,667 ± 0,160 | 0,00249± 0,00078 |

The grouping of *Megapodius cumingii* samples was conducted based on haplotype analysis using DnaSP software. This classification provides an overview of the variation patterns in the mitochondrial ND2 gene sequences and the distribution of individuals according to haplotype similarity and sampling location. Therefore, it can serve as a basis for understanding the genetic structure of the analyzed population (Table).

**Table 3.** Sample groups based on haplotype analysis using DnaSP

| Haplotype | Code | Location |
|---|---|---|
| H1 | AF394624.1 *Megapodius cumingii* | Sulawesi |
| | Sample 1 *Megapodius cumingii* | Banggai |
| | Sample 3 *Megapodius cumingii* | Banggai |
| | Sample 5 *Megapodius cumingii* | Banggai |
| H2 | KF854310.1 *Megapodius cumingii* | Sulawesi |
| H3 | Sample 2 *Megapodius cumingii* | Banggai |
| | Sample 4 *Megapodius cumingii* | Banggai |

### Nucleotide Composition of *Megapodius cumingii*

The nucleotide composition of the ND2 gene in *Megapodius cumingii* showed a relatively uniform base composition across samples, with an average content of T(U) at 23.33%, C at 37.42%, A at 28.70%, and G at 10.54%. Overall, the total A+T content reached approximately 52.03%, whereas G+C accounted for 47.95%, indicating a slight predominance of A+T bases, which is a common characteristic of avian mitochondrial DNA. The uniformity of base composition suggests that the detected genetic variation in the ND2 gene is primarily due to differences in nucleotide arrangement at specific sites rather than overall differences in base composition. Consequently, this pattern is unlikely to introduce bias in genetic distance calculations or in the reconstruction of phylogenetic relationships (Table 4).

**Table 4.** Average Nucleotide Composition of *Megapodius cumingii*

| Code | T(U) | C | A | G | A+T | G+C | Location | References |
|---|---|---|---|---|---|---|---|---|
| 2851141 Sampel 1 | 23,32 | 37,40 | 28,73 | 10,54 | 52,05 | 47,94 | Banggai | Research Data |
| 2851142 Sampel 2 | 23,41 | 37,40 | 28,63 | 10,54 | 52,02 | 47,94 | Banggai | Research Data |
| 2851143 Sampel 3 | 23,32 | 37,40 | 28,73 | 10,54 | 52,05 | 47,94 | Banggai | Research Data |
| 2851149 Sampel 4 | 23,41 | 37,40 | 28,63 | 10,54 | 52,02 | 47,94 | Banggai | Research Data |
| 2851148 Sampel 5 | 23,32 | 37,40 | 28,73 | 10,54 | 52,05 | 47,94 | Banggai | Research Data |
| AF394624.1 | 23,32 | 37,40 | 28,73 | 10,54 | 52,05 | 47,94 | Sulawesi | Birks & Edwards, 2002 |
| KF854310.1 | 23,22 | 37,50 | 28,73 | 10,54 | 51,95 | 48,04 | Sulawesi | Birks & Edwards, 2002 |
| Average | 23,33% | 37,42% | 28,70% | 10,54% | 52,03% | 47,95% | | |

The nucleotide composition of the ND2 gene within the genus *Megapodius* shows a higher A+T content (52.80%) compared to G+C (47.21%), with G being the least abundant base. Differences in base composition among geographic locations were relatively small, indicating that the observed genetic variation is primarily driven by differences in nucleotide sequences rather than overall base proportions. This pattern reflects the stability of the ND2 gene while maintaining its informativeness for phylogenetic analysis (Table 5).

**Table 5.** Average Nucleotide Composition of the Genus *Megapodius*.

| Code | T(U) | C | A | G | A+T | G+C | Location | References |
|---|---|---|---|---|---|---|---|---|
| AF394627.1 | 23,88 | 37,12 | 28,63 | 10,35 | 52,51 | 47,47 | Papua Nugini | Birks & Edwards, 2002 |
| AF394626.1 | 23,97 | 36,94 | 28,73 | 10,35 | 52,70 | 47,29 | Kepulauan Solomon | Birks & Edwards, 2002 |
| AF394633.1 | 23,78 | 37,07 | 29,29 | 9,88 | 53,07 | 46,95 | Australia | Birks & Edwards, 2002 |
| AF394635.1 | 23,69 | 37,5 | 28,63 | 10,16 | 52,32 | 47,66 | Vanuatu | Birks & Edwards, 2002 |
| AF394634.1 | 23,78 | 37,31 | 28,63 | 10,26 | 52,41 | 47,57 | Vanuatu | Birks & Edwards, 2002 |
| AF394625.1 | 23,97 | 36,94 | 28,73 | 10,35 | 52,70 | 47,29 | Papua Nugini | Birks & Edwards, 2002 |
| AF394632.1 | 24,06 | 36,75 | 29,01 | 10,16 | 53,07 | 46,91 | Papua | Birks & Edwards, 2002 |
| AF394631.1 | 24,06 | 36,75 | 28,91 | 10,62 | 52,97 | 47,37 | Papua | Birks & Edwards, 2002 |
| AF394630.1 | 24,34 | 36,47 | 29,01 | 10,16 | 53,35 | 46,63 | Maluku | Birks & Edwards, 2002 |
| AF394629.1 | 24,44 | 36,38 | 29,10 | 10,07 | 53,54 | 46,45 | Maluku | Birks & Edwards, 2002 |
| AF394637.1 | 23,50 | 37,5 | 28,63 | 10,35 | 52,13 | 47,85 | Tanimbar Maluku | Birks & Edwards, 2002 |
| AF394628.1 | 23,97 | 36,94 | 29,19 | 9,88 | 53,16 | 46,82 | Halmahera Maluku | Birks & Edwards, 2002 |
| AF394636.1 | 23,50 | 37,59 | 28,91 | 9,98 | 52,41 | 47,57 | Tonga | Birks & Edwards, 2002 |
| KF854315.1 | 23,97 | 37,03 | 28,91 | 10,07 | 52,88 | 47,10 | Palau | Harris et al., 2014 |
| Rata-rata | 23,92 % | 37,02 % | 28,88 % | 10,18 % | 52,80 % | 47,21 % | | |

## Phylogenetic Tree and Genetic Distance

Phylogenetic trees were constructed to determine the evolutionary relationships of *Megapodius cumingii* with other species within the family Megapodiidae as the ingroup, and *Numida meleagris* and *Dendragapus obscurus* as the outgroup, based on the mitochondrial ND2 gene using the Maximum Likelihood (ML) method. The Tamura–Nei (TN93) nucleotide substitution model was applied because it accounts for differences in base frequencies and variation in substitution rates between transitions and transversions. A bootstrap test with 1,000 replicates was performed to assess the stability and reliability of the branching patterns. The analysis showed that the family Megapodiidae formed a stable clade, with the genus *Megapodius* exhibiting monophyly. The *M. cumingii* samples from Poat Island consistently clustered with *M. cumingii* from Sulawesi. A similar clustering pattern was also obtained in the phylogenetic tree reconstructed using the Neighbor-Joining method with the Kimura 2-Parameter model and 1,000 bootstrap replicates. Meanwhile, *Numida meleagris* and *Dendragapus obscurus* were clearly separated from the Megapodiidae clade and functioned as outgroups to determine the direction of branching and to clarify phylogenetic boundaries within the analysis (Figures 2 and 3).

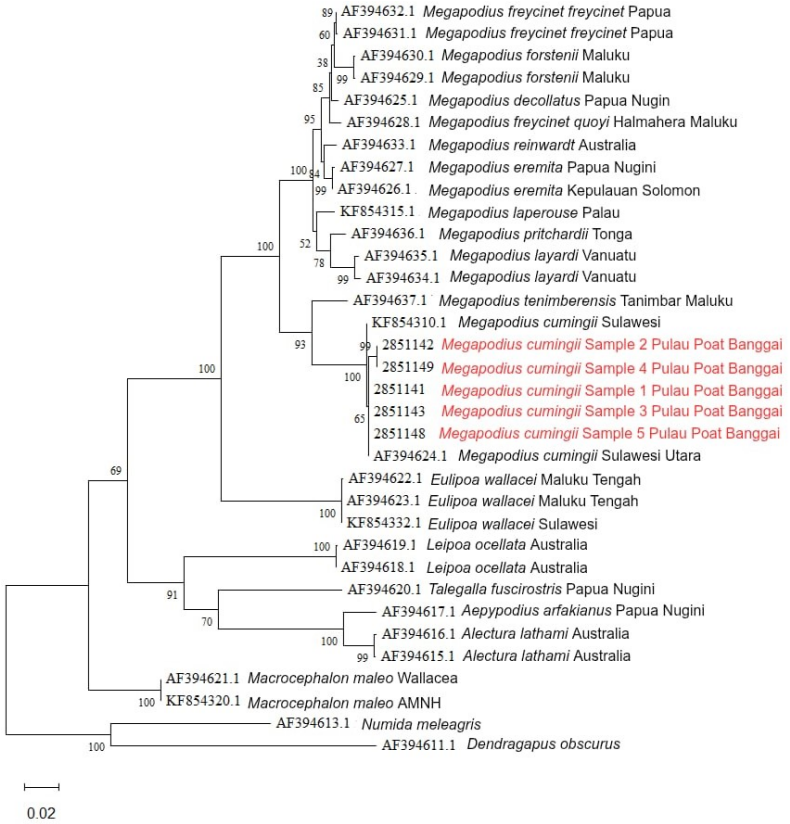


**Figure 2.** The phylogenetic tree was constructed using MEGA 11, incorporating *Megapodius cumingii* samples and other Megapodiidae species retrieved from the GenBank database, along with two outgroup samples, using the Maximum Likelihood (ML) method under the Tamura–Nei model with 1,000 bootstrap replicates.

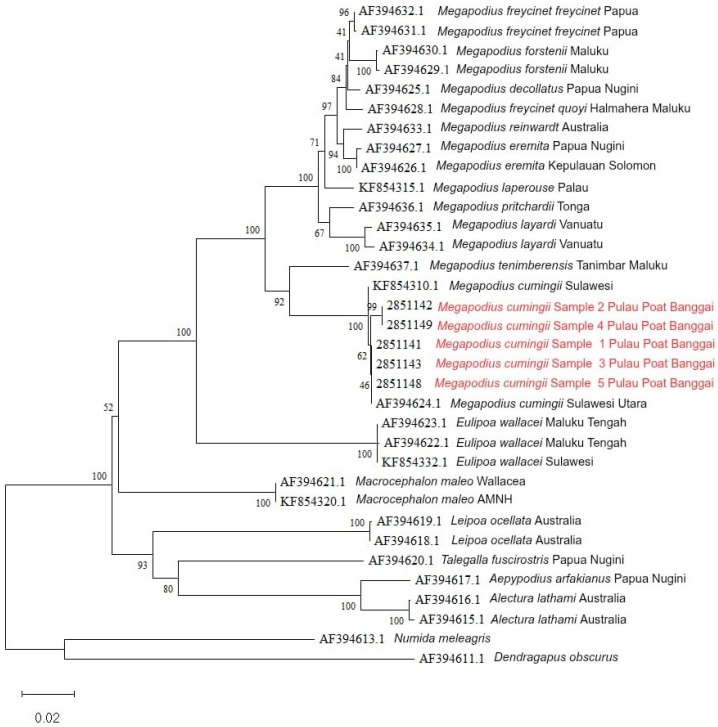


**Figure 3.** The phylogenetic tree of *Megapodius cumingii* samples and other Megapodiidae species retrieved from the GenBank database, including two outgroup samples, was reconstructed using the Neighbor-Joining method under the Kimura 2-Parameter model with 1,000 bootstrap replicates.

Genetic distance analysis based on the mitochondrial ND2 gene shows that the genetic distances among Megapodius cumingii samples from Poat Island and comparative sequences from NCBI range between 0.00–

0.01, equivalent to 0–1% nucleotide differences. This very low level of divergence indicates an extremely close genetic relationship and high genetic homogeneity within the local population, reflecting minimal sequence differentiation among individuals.

| | 2851141 | 2851142 | 2851143 | 2851149 | 2851148 | AF394624.1 | KF854310.1 | AF394616.1 | AF394615.1 | AF394619.1 | AF394618.1 | AF394627.1 | AF394626.1 | AF394633.1 | AF394620.1 | AF394623.1 | AF394622.1 | AF394621.1 | KF854332.1 | KF854320.1 | AF394635.1 | AF394634.1 | AF394625.1 | AF394632.1 | AF394631.1 | AF394630.1 | AF394629.1 | AF394637.1 | AF394628.1 | AF394636.1 | KF854315.1 | AF394617.1 | AF394613.1 | AF394611.1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2851141 *Megapodius cumingii* Sample 1 Pulau Poat Banggai | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2851142 *Megapodius cumingii* Sample 2 Pulau Poat Banggai | 0,00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2851143 *Megapodius cumingii* Sample 3 Pulau Poat Banggai | 0,00 | 0,00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2851149 *Megapodius cumingii Sample* 4 Pulau Poat Banggai | 0,00 | 0,00 | 0,00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2851148 *Megapodius cumingii Sample* 5 Pulau Poat Banggai | 0,00 | 0,00 | 0,00 | 0,00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| AF394624.1 *Megapodius cumingii* Sulawesi Utara | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| KF854310.1 *Megapodius cumingii* Sulawesi | 0,00 | 0,01 | 0,00 | 0,01 | 0,00 | 0,00 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| AF394616.1 *Alectura lathami* Australia | 0,22 | 0,22 | 0,22 | 0,22 | 0,22 | 0,22 | 0,22 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| AF394615.1 *Alectura lathami* Australia | 0,22 | 0,22 | 0,22 | 0,22 | 0,22 | 0,22 | 0,22 | 0,00 | | | | | | | | | | | | | | | | | | | | | | | | | |
| AF394619.1 *Leipoa ocellata* Australia | 0,20 | 0,20 | 0,20 | 0,20 | 0,20 | 0,20 | 0,20 | 0,18 | 0,19 | | | | | | | | | | | | | | | | | | | | | | | | |
| AF394618.1 *Leipoa ocellata* Australia | 0,20 | 0,20 | 0,20 | 0,20 | 0,20 | 0,20 | 0,20 | 0,18 | 0,18 | 0,00 | | | | | | | | | | | | | | | | | | | | | | | |
| AF394627.1 *Megapodius eremita* Papua Nugini | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,21 | 0,21 | 0,20 | 0,19 | | | | | | | | | | | | | | | | | | | | | | |
| AF394626.1 *Megapodius eremita* Kepulauan Solomon | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,07 | 0,21 | 0,21 | 0,19 | 0,19 | 0,00 | | | | | | | | | | | | | | | | | | | | | |
| AF394633.1 *Megapodius reinwardt* Australia | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,21 | 0,21 | 0,18 | 0,19 | 0,01 | 0,01 | | | | | | | | | | | | | | | | | | | | |
| AF394620.1 *Talegalla fuscirostris* Papua Nugini | 0,19 | 0,19 | 0,19 | 0,19 | 0,19 | 0,19 | 0,19 | 0,16 | 0,16 | 0,17 | 0,17 | 0,20 | 0,20 | 0,18 | | | | | | | | | | | | | | | | | | | |
| AF394623.1 *Eulipoa wallacei* Maluku Tengah | 0,13 | 0,14 | 0,13 | 0,14 | 0,13 | 0,13 | 0,13 | 0,22 | 0,22 | 0,20 | 0,20 | 0,13 | 0,13 | 0,12 | 0,19 | | | | | | | | | | | | | | | | | | |
| AF394622.1 *Eulipoa wallacei* Maluku Tengah | 0,13 | 0,14 | 0,13 | 0,14 | 0,13 | 0,13 | 0,13 | 0,22 | 0,22 | 0,20 | 0,20 | 0,13 | 0,13 | 0,12 | 0,19 | 0,00 | | | | | | | | | | | | | | | | | |
| AF394621.1 *Macrocephalon maleo* Wallacea | 0,15 | 0,16 | 0,15 | 0,16 | 0,15 | 0,15 | 0,15 | 0,17 | 0,17 | 0,17 | 0,16 | 0,15 | 0,15 | 0,15 | 0,15 | 0,16 | 0,16 | | | | | | | | | | | | | | | | |
| KF854332.1 *Eulipoa wallacei* Sulawesi | 0,13 | 0,14 | 0,13 | 0,14 | 0,13 | 0,13 | 0,13 | 0,22 | 0,22 | 0,20 | 0,20 | 0,13 | 0,13 | 0,12 | 0,19 | 0,00 | 0,00 | 0,16 | | | | | | | | | | | | | | | |
| KF854320.1 *Macrocephalon maleo* AMNH | 0,15 | 0,16 | 0,15 | 0,16 | 0,15 | 0,15 | 0,15 | 0,17 | 0,17 | 0,17 | 0,16 | 0,15 | 0,15 | 0,15 | 0,15 | 0,16 | 0,16 | 0,00 | 0,16 | | | | | | | | | | | | | | |
| AF394635.1 *Megapodius layardi* Vanuatu | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,21 | 0,21 | 0,19 | 0,19 | 0,04 | 0,04 | 0,04 | 0,19 | 0,13 | 0,13 | 0,14 | 0,13 | 0,14 | | | | | | | | | | | | | |
| AF394634.1 *Megapodius layardi* Vanuatu | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,21 | 0,21 | 0,19 | 0,19 | 0,04 | 0,04 | 0,04 | 0,19 | 0,13 | 0,13 | 0,15 | 0,13 | 0,15 | 0,01 | | | | | | | | | | | | |
| AF394625.1 *Megapodius decollatus* Papua Nugini | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,07 | 0,21 | 0,21 | 0,20 | 0,20 | 0,02 | 0,02 | 0,02 | 0,19 | 0,13 | 0,13 | 0,16 | 0,13 | 0,16 | 0,04 | 0,04 | | | | | | | | | | | |
| AF394632.1 *Megapodius freycinet freycinet* Papua | 0,07 | 0,08 | 0,07 | 0,08 | 0,07 | 0,07 | 0,07 | 0,20 | 0,21 | 0,20 | 0,20 | 0,02 | 0,01 | 0,02 | 0,20 | 0,13 | 0,14 | 0,16 | 0,13 | 0,16 | 0,04 | 0,04 | 0,01 | | | | | | | | | | |
| AF394631.1 *Megapodius freycinet freycinet* Papua | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,07 | 0,20 | 0,20 | 0,20 | 0,20 | 0,02 | 0,02 | 0,02 | 0,20 | 0,14 | 0,14 | 0,16 | 0,14 | 0,16 | 0,04 | 0,04 | 0,01 | 0,00 | | | | | | | | | |
| AF394630.1 *Megapodius forstenii* Maluku | 0,08 | 0,09 | 0,08 | 0,09 | 0,08 | 0,08 | 0,08 | 0,22 | 0,22 | 0,20 | 0,20 | 0,03 | 0,02 | 0,03 | 0,20 | 0,14 | 0,14 | 0,16 | 0,14 | 0,16 | 0,04 | 0,04 | 0,02 | 0,01 | 0,01 | | | | | | | | |
| AF394629.1 *Megapodius forstenii* Maluku | 0,08 | 0,09 | 0,08 | 0,09 | 0,08 | 0,08 | 0,08 | 0,21 | 0,22 | 0,20 | 0,20 | 0,03 | 0,02 | 0,03 | 0,20 | 0,14 | 0,14 | 0,16 | 0,14 | 0,16 | 0,04 | 0,04 | 0,02 | 0,01 | 0,01 | 0,00 | | | | | | | |
| AF394637.1 *Megapodius tenimberensis* Tanimbar Maluku | 0,05 | 0,06 | 0,05 | 0,06 | 0,05 | 0,05 | 0,05 | 0,20 | 0,20 | 0,18 | 0,18 | 0,07 | 0,07 | 0,07 | 0,19 | 0,14 | 0,14 | 0,14 | 0,14 | 0,14 | 0,07 | 0,07 | 0,06 | 0,07 | 0,07 | 0,07 | 0,07 | | | | | | |
| AF394628.1 *Megapodius freycinet quoyii* Halmahera Maluku | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,21 | 0,21 | 0,19 | 0,19 | 0,02 | 0,02 | 0,02 | 0,20 | 0,13 | 0,13 | 0,15 | 0,13 | 0,15 | 0,04 | 0,04 | 0,01 | 0,01 | 0,01 | 0,02 | 0,02 | 0,07 | | | | | |
| AF394636.1 *Megapodius pritchardii* Tonga | 0,08 | 0,09 | 0,08 | 0,09 | 0,08 | 0,08 | 0,08 | 0,21 | 0,21 | 0,19 | 0,19 | 0,03 | 0,03 | 0,03 | 0,20 | 0,13 | 0,13 | 0,15 | 0,13 | 0,15 | 0,03 | 0,03 | 0,03 | 0,03 | 0,03 | 0,03 | 0,03 | 0,07 | 0,03 | | | | |
| KF854315.1 *Megapodius laperouse* Palau | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,08 | 0,21 | 0,21 | 0,18 | 0,18 | 0,03 | 0,02 | 0,02 | 0,19 | 0,13 | 0,13 | 0,15 | 0,13 | 0,15 | 0,03 | 0,04 | 0,03 | 0,02 | 0,02 | 0,03 | 0,03 | 0,07 | 0,03 | 0,02 | | | |
| AF394617.1 *Aepypodius arfakianus* Papua Nugini | 0,22 | 0,22 | 0,22 | 0,22 | 0,22 | 0,22 | 0,22 | 0,04 | 0,04 | 0,18 | 0,17 | 0,21 | 0,21 | 0,20 | 0,16 | 0,22 | 0,22 | 0,18 | 0,22 | 0,18 | 0,21 | 0,20 | 0,21 | 0,20 | 0,20 | 0,21 | 0,21 | 0,20 | 0,20 | 0,21 | 0,21 | | |
| AF394613.1 *Numida meleagris* | 0,26 | 0,26 | 0,26 | 0,26 | 0,26 | 0,26 | 0,26 | 0,27 | 0,27 | 0,25 | 0,25 | 0,25 | 0,25 | 0,25 | 0,26 | 0,26 | 0,26 | 0,22 | 0,26 | 0,22 | 0,25 | 0,25 | 0,26 | 0,26 | 0,26 | 0,26 | 0,26 | 0,26 | 0,26 | 0,26 | 0,25 | 0,27 | |
| AF394611.1 *Dendragapus obscurus* | 0,30 | 0,30 | 0,30 | 0,30 | 0,30 | 0,30 | 0,30 | 0,29 | 0,29 | 0,32 | 0,31 | 0,29 | 0,29 | 0,30 | 0,29 | 0,29 | 0,29 | 0,26 | 0,29 | 0,26 | 0,29 | 0,29 | 0,29 | 0,29 | 0,29 | 0,30 | 0,29 | 0,29 | 0,29 | 0,29 | 0,29 | 0,30 | 0,23 |

**Figure 4.** Genetic distance analysis based on the ND2 gene revealed that the genetic distances among *Megapodius cumingii* samples from Poat Island and comparative samples from NCBI ranged from 0.00 to 0.01, corresponding to 0–1% nucleotide differences. This indicates a very close evolutionary relationship and a high level of genetic homogeneity within the local population.

## Discussion

The successful amplification of the mitochondrial ND2 gene in *Megapodius cumingii* indicates high DNA quality and optimal PCR conditions, resulting in specific amplification that supports the reliability of subsequent molecular and phylogenetic analyses (Zink & Barrowclough, 2008). BLAST results confirmed that the ND2 sequences originated from *M. cumingii*, showing very high similarity to reference sequences in GenBank and reaffirming the effectiveness of the ND2 gene as a molecular marker. Minor sequence differences reflect natural intraspecific genetic variation (Khan et al., 2023).

Genetic variation analysis of the ND2 gene revealed that the *Megapodius cumingii* population from Poat Island exhibits relatively low genetic diversity, characterized by moderate haplotype diversity and low nucleotide diversity. This pattern suggests that genetic variation is mainly represented by haplotypes that differ minimally at the nucleotide level, indicating shallow genetic differentiation due to the occurrence of mutations without substantial accumulation of nucleotide substitutions (Grant & Bowen, 1998; Yue et al., 2021). Such conditions are commonly observed in isolated island bird populations with small effective population sizes. This pattern is further reinforced by the ecological characteristics of *M. cumingii*, which depends on specific natural incubation sites and has limited dispersal capacity on small islands, thereby restricting gene flow (Sinclair et al., 2002; Paguntalan et al., 2021). Haplotype analysis identified three ND2 haplotypes, indicating the presence of intraspecific genetic variation, although differences were relatively small, with several individuals sharing identical haplotypes and reflecting overall genetic homogeneity within the population (Birks & Edwards, 2002).

The nucleotide composition of the ND2 gene in *Megapodius cumingii* from Poat Island showed a relatively uniform pattern across samples, with a slight predominance of A+T (52.03%) over G+C (47.95%), a common feature of avian mitochondrial DNA. A similar pattern was observed at the genus level (*Megapodius*), with A+T content of 52.80% and G+C of 47.21%, and G as the least abundant base. This indicates compositional conservatism of the ND2 gene across species and geographic regions. The uniform nucleotide proportions suggest that the detected genetic variation is primarily attributable to differences in nucleotide sequences at specific sites rather than overall base composition. Consequently, the ND2 gene remains compositionally stable yet informative and reliable for genetic distance analysis and phylogenetic reconstruction within the genus *Megapodius*.

Phylogenetic reconstruction using the Maximum Likelihood (ML) method under the Tamura–Nei model and the Neighbor-Joining (NJ) method under the Kimura 2-Parameter model produced generally consistent relationship patterns, particularly in clustering members of the family Megapodiidae. All *Megapodius cumingii* samples from Poat Island, Banggai, formed a clear monophyletic clade with high bootstrap support (≥90–100) in both trees, reflecting strong genetic relationships and stable phylogenetic topology (Kumar et al., 2018;

Tamura et al., 2021). The clustering of *Megapodius* with related genera such as *Eulipoa* and *Macrocephalon* was also broadly similar across both methods. Meanwhile, the outgroup species *Numida meleagris* and *Dendragapus obscurus* were clearly separated from the ingroup, confirming the appropriateness of outgroup selection in avian phylogenetic analysis (Prum et al., 2015; Claramunt & Cracraft, 2015). The primary difference between methods was observed in bootstrap values at certain internal nodes, where Maximum Likelihood generally provided higher support and more stable topology compared to Neighbor-Joining, which is distance-based and computationally simpler and showed lower support at some internal branches (Yang, 2014).

Genetic distance analysis based on the mitochondrial ND2 gene showed that all *Megapodius cumingii* samples from Poat Island had a genetic distance of 0.00, indicating high genetic homogeneity likely resulting from geographic isolation and limited gene flow. Genetic distances between *M. cumingii* and other Megapodiidae species ranged from 0.07 to 0.22, with the lowest value observed with *M. freycinet*, reflecting closer phylogenetic affinity. Higher distances with other genera indicate deeper evolutionary divergence. The highest distances (>0.25) with the outgroup species further confirm substantial evolutionary separation and support the reliability of the ND2 gene as a phylogenetic marker in birds.

These findings have important conservation implications, as low genetic variation may limit the adaptive capacity of *Megapodius cumingii* populations under environmental pressures. The ecological vulnerability of Poat Island highlights the importance of protecting key habitats, particularly natural incubation sites. The observed genetic homogeneity suggests that this population may be treated as a single local management unit. The consistency of genetic patterns with other island megapodes emphasizes the need for habitat- and population-based conservation approaches to ensure the long-term persistence of *M. cumingii* in Wallacea (Shafer et al., 2015; Frankham et al., 2017; Hoban et al., 2020).

The congruence between the genetic variation patterns and phylogenetic structure of *Megapodius cumingii* from Poat Island and previous studies indicates that this population represents part of a relatively homogeneous evolutionary unit within Wallacea. Studies on other megapodes, such as *Macrocephalon maleo*, have also reported low ND2 nucleotide variation in geographically isolated populations, associated with small effective population sizes and dependence on specific reproductive habitats (Budiarsa et al., 2010). Similar patterns have been documented in island Galliformes in Southeast Asia and China, where shallow phylogenetic structure reflects relatively recent island colonization despite clear geographic separation (Wang et al., 2023). The consistency of ND2 results with previous studies employing nuclear and other mitochondrial markers in *M. cumingii* from Central Sulawesi further confirms that low genetic divergence is a general characteristic of this species, underscoring the importance of habitat management and protection of breeding sites in conservation efforts (Tala'a, 2021; Novitasari et al., 2025).

## CONCLUSIONS

Based on this study, it can be concluded that *Megapodius cumingii* from Poat Island exhibits a low level of genetic variation based on mitochondrial ND2 gene analysis. This is indicated by the presence of three haplotypes with moderate haplotype diversity (Hd = 0.667) and low nucleotide diversity ($\pi$ = 0.00249). The nucleotide composition of the ND2 gene showed relatively uniform base proportions across samples, with a slight predominance of A+T (52.03%) compared to G+C (47.95%). Genetic distance analysis revealed very high genetic homogeneity among Poat Island samples, with a genetic distance value of 0.00, and a very close relationship with *M. cumingii* from Sulawesi, which also showed a genetic distance of 0.00. In contrast, genetic distances increased with other species within the genus *Megapodius* (0.02–0.09), with species from different genera (0.13–0.23), and reached the highest values in the outgroup (approximately 0.29–0.30). Phylogenetic reconstruction placed all *M. cumingii* samples from Poat Island into a single, strongly supported monophyletic clade, confirming that this population represents a relatively homogeneous evolutionary unit with consistent phylogenetic topology.

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