Analysis of the mtDNA D-loop Region Casts New Light on Philippine Red Junglefowl Phylogeny and Relationships to Other Junglefowl Species in Asia

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Red junglefowl (RJF) is considered the ancestor of domestic chickens. However, the possible maternal origin, genetic diversity, and subspecies classification of the Philippine (PH) RJF remains uncertain. In this study, the complete mitochondrial DNA (mtDNA) D-loop sequence of 55 PH RJFs collected from the mountainous areas of Occidental Mindoro, Palawan, Agusan del Norte, Capiz, Leyte, Iloilo, and Guimaras were analyzed and compared with chicken reference sequences. Phylogenetic analysis revealed multiple maternal origins of the PH RJFs based on haplogroups D, E, and Y classification. This was supported by PH RJFs and RJFs from other Asian countries sharing a clade. A median-joining network also revealed the haplotype sharing of the PH RJFs and Indonesian RJF, demonstrating common maternal ancestry. High haplotype and nucleotide diversity were also observed at all sampling sites. Analysis of molecular variance indicated that the principal molecular variance existed within populations (81.23%) rather than among populations (18.77%). A population neutrality test and Bayesian skyline plot (BSP) analysis elucidated the RJF maternal effective population size expansion in the Philippines that possibly started approximately 2,800–3,000 years ago. The co-existence of Gallus gallus bankiva and Gallus gallus gallus in the Philippines was also verified. The haplotype sharing of the current RJF samples with commercial chickens suggested the need to formulate conservation programs that would protect the RJFs in the Philippines.

Key words: Gallus gallus gallus, haplotype, mitochondrial D-loop, Philippines, phylogenetics, red junglefowl

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Introduction

Red junglefowl (RJF) is the primary wild ancestor of modern domestic chickens, whose domestication occurred less than 8,000 years ago (West and Zhou, 1989). RJFs inhabit areas with tropical climates and vegetation and are usually exposed to more stable daily and seasonal temperatures (Beebe, 1926; West and Zhou, 1989). They occur over a wide geographical range, throughout Southeast Asia, specifically the eastern and southernmost parts including the islands of Sumatra and Java to Bali, Sulawesi, and the Philippines. RJFs also inhabit the Malay Archipelago, northern and eastern India, and the Himalayan foothills of northern Pakistan (Nishibori et al., 2005; Bondoc, 2013).

Scientists have considered the molecular and evolutionary advantages of using mitochondrial DNA (mtDNA) for investigating the domestication origin of RJF (Hayashi et al., 1985; Birky, 2001; Shoubridge and Wai, 2007; Muchadeyi et al., 2008). The D-loop region is a distinct region of mtDNA that is noncoding and rapidly evolving compared to other mtDNA genome regions. Supporting the hypothesis of the monophyletic origin of domestication, the first molecular study of mtDNA in chickens suggested that a single domestication event occurred in Thailand and adjacent regions (Fumihito et al., 1994, 1996).

RJFs, both free-roaming and captive, can still be found in the Philippines (PH). However, sightings are becoming rare due to habitat destruction and human settlements in the forests (Masangkay et al., 2010). PH RJFs were categorized by Hachisuka (1939) and Bondoc (2013) under the G. g. philippensis while several studies identified Philippine RJFs...
under *G. g. gallus* (Nishida and Masangkay, 1978; Nishida et al., 1985, 2000; Nishibori et al., 2005; Godinez et al., 2019), thus creating confusion as to its true classification.

Due to insufficient studies on the genealogy of the PH RJF, its origin and domestication remain uncertain. Therefore, an in-depth molecular study and analysis could help pinpoint its genetic origin and present diversity. To date, no research has been published on the analysis of the D-loop region of the mtDNA of the PH RJF, representing the three main island regions (Luzon, Visayas, and Mindanao) of the Philippines. Therefore, this study was conducted to provide information on the origin, genetic status, and diversity of PH RJFs and their genetic relationships with previously identified jungle fowl in Asia. Furthermore, the results of this study could serve as a basis for conservation programs and policies that would be beneficial for the protection of RJFs.

Materials and Methods

**Sample Collection and DNA Extraction**

A total of fifty-five (*n*=55) PH RJFs from the mountainous areas of the Philippines were classified according to their island region classification (Table 1). Proper handling and blood collection in RJFs were in accordance to the guidelines stipulated by the Guide for the Care and Use of Laboratory Animals. DNA extraction from blood was conducted using the phenol-chloroform method following the protocols of Nishibori et al. (2003) and Osman and Nishibori (2014).

The use of animals in this study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Matias H. Aznar Memorial College of Medicine, Philippines, with reference code MHAM-060919-01. Furthermore, the collection of wild chickens was also allowed by the Matias H. Aznar Memorial College of Medicine, Philippines under Gratuitous permit numbers 309 and R6-2019-007.

**DNA Amplification, Sequencing, and Analysis**

DNA concentration and purity were measured using a NanoDrop Lite Spectrophotometer (Thermo Scientific). A reading of ≥50 ng/µL for DNA concentration and ≥1.80 (A260/A280 nm) for DNA purity was considered ideal. Using the adjusted extracted DNA, 5kbp fragments of mtDNA were amplified using KOD-FX Neo DNA polymerase (KFX-201, Toyobo Co., Ltd., Osaka, Japan). Amplification of the 5 kbp fragment used the following primers: CybF: 5’TACACGAAATCGCTCAAAACACATGGCTTAG GCATC-3’, 16SR: 5’TGCACCATAGGTGTCTGTACCT GCATCAGGT-3’ (Nishibori et al., 2001). PCR amplification was performed in a 20 µL mixture containing 1.0 µL genomic DNA, 3.6 µL ddH2O, 10.0 µL 2xPCR buffer, 4.0 µL 2mM dNTPs, 0.6 µL Primer F (10 pmol/µL), 0.6 µL Primer R (10 pmol/µL), and 0.2 µL KOD-FX Neo DNA polymerase. The reaction began with a preliminary denaturation at 94°C for 2 min, followed by 30 cycles of DNA denaturation at 98°C for 10 s, annealing of primers at 57°C for 30 s, and primer extension at 68°C for 2 min and 30 s. The final step was an 8 min final extension of the primers at 15°C. PCR amplification was performed using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA).

Moreover, amplification of the 1.5 kbp mtDNA fragment targeting the D-loop region was carried out using the following primers: GalF1: 5’- AGGACTACGGCTTAGGCTGGGGAACAGG-3’, GalR1: 5’- GTCTGATACCGGTGGGTCAGG-3’ (Nishibori et al., 2001). The PCR amplification of the D-loop fragment PCR amplification was performed in a 20 µL mixture containing 1.0 µL template DNA (5.0 kbp), 4.5 µL ddH2O, 10.0 µL 2xPCR buffer, 4.0 µL 2mM dNTPs, 0.3 µL PrimerF (10 pmol/µL), 0.3 µL Primer R (10 pmol/µL), and 0.4 µL KOD-FX Neo DNA polymerase. Amplification was conducted using the GeneAmp PCR System 9700. The PCR cycle profile was as follows: preliminary denaturation at

| Island region | Province         | Sex | N   | Accession number          |
|---------------|------------------|-----|-----|----------------------------|
| Luzon         | Occidental Mindoro | ♂   | 18  | OL589006-OL589022, OL589024 |
|               |                  | ♀   | 1   | OL589023                   |
| Palawan       |                  | ♂   | 10  | OL589051-OL589060           |
| Visayas       | Capiz            | ♂   | 2   | OL589037-OL589038           |
|               |                  | ♂   | 4   | OL589029, OL589033, OL589035, OL589036 |
|               |                  | ♀   | 4   | OL589030-OL589032, OL589034 |
|               | Guimaras         | ♂   | 4   | OL589039-OL589042           |
|               | Leyte            | ♂   | 8   | OL589043-OL589050           |
| Mindanao      | Agusan del Norte | ♂   | 3   | OL589025-OL589027           |
|               | Agusan del Norte - Hybrid | ♂ | 1 | OL589028 |

♂=male; ♀=female; N=number of samples
94°C for 2 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 59°C for 30 s, and extension at 68°C for 30 s. The final step was the final extension of the primers for 5 min at 68°C and 15°C PCR mixture incubation.

The successfully sequenced DNA samples were cleaned and translated using GENESTUDIO™ Professional (Sequence Analysis Software). Profile alignments of 1232bp-long mtDNA D-loop sequenced data were performed through the window interface progressive multiple sequence alignment program of the molecular evolutionary genetic analysis (MEGA 7) (Kumar et al., 2016) to improve and refine difficult alignment and trap errors in the input sequences. The genetic distance matrix analysis between PH RJF populations and some ancestral and outgroup sequences was carried out using the maximum likelihood (ML) method using the same software.

Pairwise distance and haplotype analysis were performed using DnaSP v6.12.03. Analysis of molecular variance within and between populations, as well as the genetic and nucleotide diversity of the RJF samples, was performed using Arlequin ver 3.52.2 (Excoffier and Lischer, 2010). Furthermore, the percent similarity of the PH RJFs with the reference sequences from the RJF subspecies was conducted using BlastN.

Past population dynamics of Philippine RJFs were demonstrated through Bayesian Skyline Plot (BSP) (Drummond et al., 2005) using BEAST v. 2.6. 6 (Bouckaert et al., 2019), following the method described by Godinez et al., 2021. The generation time (8.09 years) used was the accumulated divergence time between domestic chickens and RJF (Lawal et al., 2020). Tracer v.1.7.2 (Rambaut et al., 2018) was used to visualize the generated Markov chain Monte Carlo (MCMC) trace files.

**Results and Discussions**

**Haplogroup Classifications of PH RJFs**

Determining the evolutionary relationship of the PH RJFs in this study, the maximum-likelihood phylogenetic tree (Fig. 1) showed the classification of PH RJF haplotypes into...
haplogroups D, E, and Y. The results revealed that the PH RJF haplotypes share a close genetic relationship with NC_007236 (Nishibori et al., 2005), a wild chicken from the Philippines classified under subhaplogroup D1. This result also provided further evidence on the molecular classification of some PH RJF samples under G. gallus (Nishibori et al., 2005; Godinez et al., 2019), which has also been morphologically classified under the same RJF subspecies (Nishida and Masangkay 1978; Nishida et al., 1985, 2000). In addition, the classification of PH RJF haplotypes in haplogroup D was in accordance with the distribution of chickens under haplogroup D in African, South, and East Asian and Southeast Asian countries, as stated by Miao et al. (2013). Furthermore, the results of this study also agreed with the previous findings of Godinez et al. (2019) on the haplogroup classification of RJFs from Samar, Philippines. Five PH RJF haplotypes were also classified under haplogroup Y, which was represented by a wild chicken (GU261693) from Yunnan, China (Miao et al., 2013). This clustering further suggests the wild chicken origin of the PH RJFs generated in this study.

In contrast, the PH RJF11 haplotype in this study was classified under subhaplogroup E1. Miao et al. (2013) mentioned that chickens classified under subhaplogroup E1 were globally distributed and present in all geographically defined populations. The close genetic relationship between the PH RJF11 haplotype and subhaplogroup E1 suggests a close genetic relationship with domestic and commercial chickens from India and China. This result also provided evidence of the co-existence of domestic chicken and wildfowl (Miao et al., 2013) in the sampling area where the RJFs under PH RJF11 haplotype were collected. Moreover, the PH RJF11 haplotype was composed of two PH RJF sequence samples (PH.I6 and PH.A4), of which only PH.A4 was morphologically classified as a hybrid RJF (Table S1).

Although the RJF is the main ancestral contributor to chicken genetic diversity, post-domestication events involving crosses with other junglefowl species also occurred (Eriksson et al., 2008; Lawal et al., 2020). Thus, since PH.A4 was an RJF x fighting cock hybrid, it was expected to be classified under haplogroup H, providing historical links between the Philippines, Thailand, and Japan through cock-fighting activities. However, the results of this study proved otherwise. In contrast, haplogroup H is a rare haplogroup that is notably present in fighting cocks (Hata et al., 2021).

A median-joining network was constructed to support the haplogroup classification of PH RJFs (Fig. 2). Of the total RJF samples in this study, 21.82% shared the same haplotype with some of the reference sequences. Most of these (12.73%) shared the same haplotype with domestic chickens from China (GU2161683), and 3.64% of the PH RJF sequences shared the same haplotype with the same subhaplogroup E1. Although the PH haplotypes were categorized under haplogroup D, only 1.82% and 3.64% shared the same haplotype as the wild junglefowl from the Philippines and Indonesia, respectively. The remaining 78.18% of the total PH RJFs formed a unique haplotype that was not similar to the reference sequences. Based on this haplotype sharing, the results suggested that the PH RJFs could have come from Indonesia. However, a larger dataset is needed to confirm this claim. Although the results in Fig. 1 show the clustering of PH RJF haplotypes with the wild chicken from China in haplogroup Y, no haplotype sharing was observed between the two countries (Fig. 2).

Furthermore, the results of this study agree with those of Osman and Nishibori (2014) on the close genetic relationship of Southeast Asian RJFs in terms of their D-loop nucleotide position. The genetic relationship of the PH RJF with the wild and domestic chickens in Asia observed in this study agreed with Peterson and Brisbin (1998), who suggested that the Philippines, together with other countries, might have accepted introduced junglefowl brought by human settlements.

Patterns of mtDNA Variability

In this study, the detected PH RJF haplotypes were aligned with the Burmese RJF reference sequence (NC 007235) (Nishibori et al., 2005) from GenBank. Table 2 shows a total of 30 variable sites and 314 total polymorphisms detected in the RJF sequence samples in this study, as inferred from the reference sequence (NC_007235). The results revealed 0.64% and 99.36% transversion and transition mutations, respectively. The highest number of substitutions was observed in PH RJF3, a unique haplotype that did not share the same haplotype as the reference sequences from Miao et al. (2013). A high nucleotide substitution rate is common in mtDNA (Brown et al., 1982), thus supporting the results of this study.

Among the haplotypes detected, the PH RJF4 haplotype was found to be the most common haplotype in this study, where the three Philippine regions (Luzon, Visayas, and Mindanao) shared the same haplotype. Although all island regions shared the same haplotype, no haplotype sharing was observed at any of the PH RJF sampling sites.

Genetic Diversity of the Philippine RJFs

The genetic diversity observed in this study revealed that the Agusan del Norte and Capiz RJF populations had the highest haplotype diversity among the PH RJF populations studied (Table 3). This is probably due to the wide haplotype distribution of the RJFs in these two populations due to the small sample number that was sampled from a broader geographical location. In addition, Agusan del Norte also had the highest nucleotide diversity (0.1333±0.1159). The high genetic diversity observed in this study corresponds with the high haplotype diversity (1.00±0.20) in RJFs from Samar, Philippines, previously reported by Godinez et al. (2019). On the other hand, the low haplotype (0.4643±0.2000) and nucleotide diversity (0.0020±0.0014) of the Iloilo RJF population was probably due to the close genetic relatedness of the RJFs collected in this area. Generally, the nucleotide diversity of the Philippine RJF populations in this study was higher than that of the G. gallus subspecies (0.01080±0.00059) in Indonesia, India, and China (Liu et al., 2006). Knowledge of genetic variation within and between populations is essential for the conceptualization and management of species conservation (Milligan et al., 1994). Thus, the
results of this study suggest that the genetic diversity of PH RJFs is not at risk.

A pairwise distance test analysis was also conducted to determine which population appeared to be the most closely related (Table 4). High genetic differentiation was observed between the Guimaras and Leyte RJF populations. In addition, the negative and zero $F_{st}$ values in this study suggest high genetic similarity among the populations. Capiz and Iloilo RJF populations were expected to have the lowest $F_{st}$ values because of their site proximity. However, the results of this analysis shows otherwise.

**Population Structure and Demographic History**

To elucidate the genetic variations between and within PH RJF populations (Excoffier et al., 1992; Excoffier and Lischer, 2010), the analysis of molecular variance (AMOVA) results of this study showed 18.77% between and 81.23% within the PH RJF (Table 5). The low genetic differentiation between populations suggested that the RJF population had not been subdivided between regions. However, the high percentage of variation among populations could also be due to the high
Table 2.  *Observed sequence variation among mitochondrial D-loop sequences of Philippine red junglefowl haplotypes of populations present in the Philippines*

| Haplotype     | N Luzon | Visayas | Mindanao | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
|---------------|---------|---------|----------|---|---|---|---|---|---|---|---|---|---|
| PH RJF1       | 1 1     | 1       |          | A | T | C | A | T | T | C | T | A | T |
| PH RJF2       | 2 1     | 1       |          | A | T | C | A | T | T | C | T | A | T |
| PH RJF3       | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF4       | 12 9 2  | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF5       | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF6       | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF7       | 2 2     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF8       | 7 5 2   | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF9       | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF10      | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF11      | 2 1     | 1*      |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF12      | 7 7     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF13      | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF14      | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF15      | 2 2     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF16      | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF17      | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF18      | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF19      | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF20      | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF21      | 1 1     | 2       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF22      | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF23      | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF24      | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF25      | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |
| PH RJF26      | 1 1     | 1       |          | A | G | G | G | G | G | G | G | G | G |

* = hybrid RJF. N= number of individuals sharing the same haplotype. Dots (.)= denotes identity with the reference sequence.
The population neutrality test (Sharma et al., 2013) was conducted to infer and analyze the signatures of historical demographic events of the PH RJFs. The negative Tajima’s D test (Tajima, 1993) for the Occidental Mindoro (−0.9534), Palawan (−0.4600), Iloilo (−1.4213), and Agusan del Norte (−0.8338) RJF population suggested recent expansion of the population size. The positive Tajima’s D test observed in Leyte (0.2775) and Guimaras (1.3652) suggested a decline in population size in these areas. The zero (0) Tajima’s D value for the Capiz RJF population suggests that this RJF population is evolving according to mutation-drift equilibrium (Joshi et al., 2013), probably because of the small sample size (n=2) analyzed in this area.

However, Fu’s Fs test is a better fit for a larger sample size because this test is sensitive to population growth (Rozas et al., 2003). Thus, this study’s non-significant Fu’s Fs p-value could be attributed to the low sample size in most RJF sampling areas except for Occidental Mindoro (n=19) (p =0.0060). Furthermore, the negative Fu’s Fs value (−3.7839) in the Occidental Mindoro RJF population also provided strong evidence of its population expansion (Lopez and Lama, 2007). This, rules out possibilities such as genetic hitchhiking, background selection, and evolutionary forces resulting in a pattern similar to population expansion (Fu and Li, 1993; Fu, 1997; Okello et al., 2005).

The consistent negative Tajima’s D and Fu’s Fs results of the RJF populations in Luzon suggested a rapid demographic expansion from a small effective population size (Avise, 2000). This result is also supported by the high haplotype and nucleotide diversity of the Palawan and Occidental Mindoro RJF populations. Furthermore, the overall negative Tajima’s D (−1.5013) (p=0.0400) and Fu’s Fs (−19.3980) (p=0.0000) suggested a recent population expansion of PH RJFs analyzed.
In this study (Table 6).

To bolster the results of the population neutrality analysis, a Bayesian skyline plot (BSP) analysis was performed to further assess the population expansion of PH RJFs. Utilizing 55 PH RJFs, including the hybrid PH RJF, with a 95% high posterior density interval. The overall BSP result indicated an increase in the maternal effective population size approximately 2,800-3,000 years ago (Fig. 3).

**Relationship of PH RJF with the Different G. gallus Species and RJF Subspecies from Other Asian Countries**

An ML tree (Fig. 4A) was constructed based on the Tamura-Nei model (Tamura and Nei, 1993) to elucidate the relationships between PH RJFs and those from other Asian countries. Using the relationship of the current PH RJF samples with the different RJF sequences in Asia, the results showed that most of PH RJF sequences in this study shared the same clade with NC_007236 (Nishibori et al., 2005), an RJF from the Philippines. In fact, it can be shown that 54.55% of the total PH RJF sequences were 99.81%, identical to that of NC_007236.

Furthermore, the fact that PH RJFs share a clade with KY039428, an Indonesian RJF, also supports the results shown in Fig. 1. The high percentage of homology observed in a minority of the PH RJFs with the RJF from Indonesia could be due to the gene flow of RJF genes from the Philippines to Indonesia or vice versa. This is possible due to the geographical proximity of the two countries and human migration. According to Tan-Cullamar (1993), factors supporting the Indonesian migration to southern Mindanao in the 1900s was geographical proximity, as well as a similar
climate environment, and resources. This provides clues on the genetic proximity and sharing of the RJF gene pool between the Philippines and Indonesia.

Tracing the relationship of the PH RJFs with the different *G. gallus* species and RJF subspecies, the results showed a close genetic relationship between the PH RJF haplotypes and *G. g. gallus* (AP003322) and *G. g. bankiva* (AP003323), except for the PH RJF11 haplotype, which formed a close genetic relationship with *G. g. murghi* (GU261709; Fig.4B). The similarity of current PH RJFs with the different species of *G. gallus* and the subspecies of RJFs was determined using BlastN. The results revealed that 50% of the detected PH RJF haplotypes shared 99.76% average similarity with *G. gallus* (AP003322). However, 46.15% of the total PH haplotypes shared 99.77% similarity with *G. bankiva* (AP003323). This corresponds with the findings of Compendio and Nishibori (2021) who previously reported a 99.84% similarity between a Philippine RJF (NC_007236) sequence and a *G. g. bankiva* (AP003323) sequence. This also suggests gene flow between these coexisting RJF subspecies in the Philippines.

Although there is little genetic distance between *G. gallus* and *G. spadiceus* (Lawal et al., 2020), the results of this study showed that none of the PH RJFs formed close genetic relationships with the *G. g. spadiceus*. This indicates that there is no relationship between the PH RJFs and this particular subspecies. It was recently suggested by Wang et al. (2020) that domestic chickens were initially bred from the *G. g. spadiceus*, distributed in southwestern China, northern Thailand, and Myanmar. However, the domestic chickens present in this study do not share the same clade with *G. spadiceus*, suggesting that PH domestic chickens might have been derived from a different domestication center and RJF subspecies origin. This result also showed that the wild chickens in the Philippines were not classified under *G. philippensis*, as reported previously by Hachisuka (1939) and Bondoc (2013).

The results of this study elucidated the classification of the subspecies of the PH RJFs under *G. g. gallus* and *G. bankiva*, suggesting that the Philippines does not have a separate RJF subspecies; this study also suggests that the PH RJFs originate from Southeast Asia. Given the high genetic and nucleotide diversity and population expansion of PH RJFs, this study suggests that the RJFs in the Philippines are not at risk. However, haplogroup sharing with commercial chickens is a
concern for biodiversity. Therefore, the need for RJF conservation programs to prevent gene flow of genetic material from domestic chickens to RJFs is suggested.

Furthermore, in this study, the important role of the mtDNA D-loop control region in tracing the possible maternal origin of PH RJF and its phylogenetic interrelationships with different populations of RJF in Asia is also emphasized. A more comprehensive sampling site coverage is recommended to further verify the claims made in this study.

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Author Contributions

All authors contributed equally to this manuscript’s conceptualization, analysis, and finalization.

Conflict of Interest

The authors declare no conflict of interest in this study.

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