DOCTORAL THESIS

Morphological and Phylogenetic Studies on the Genetic Diversity and Origin of Philippine Red Junglefowl

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CHAPTER I

General Research Overview

1.1 Introduction

The genus *Gallus* is composed of four junglefowl species, the *Gallus gallus* (red junglefowl), *Gallus sonneratii* (gray junglefowl), *Gallus lafayettii* (Ceylon fowl) and *Gallus varius* (green junglefowl) (Nishida *et al.,* 1985), scattered around Asia (Lawal *et al.,* 2020). Among these species, the red junglefowl (RJF) subspecies was considered the ancestor of today's domestic chicken as proposed by Darwin (1986). Moreover, the recognized RJF subspecies based on their morphology and geographical distribution are *Gallus gallus gallus, Gallus gallus spadiceus*, *Gallus gallus jabouillei*, *Gallus gallus bankiva*, and *Gallus gallus murgi* (Johnsgard, 1999).

Among the five RJF subspecies, the continental *G.g.gallus* was the matriarchic origin of all the domestic chickens based on the monophyletic origin hypothesis (Fumihito *et al.,* 1994). In contrast, the polyphyletic origin hypothesis suggested multiple lineage origins of the domestic chickens (Nishibori *et al.,* 2005; Erikson *et al.,* 2008). However, current findings suggested that instead of the *G.g.gallus*, the *G.g. spadiceus* appeared to be the maternal origin of the domestic chickens (Wang *et al.,* 2020).

RJF is an important animal resource domesticated long ago for the use of man in different aspects of life. This domestication was established during the long and close interrelationship between the junglefowl and human beings, as humans started to provide the RJFs with ample supply of feeds under favorable conditions. RJFs could be easily identified through their morphology as they possessed certain phenotypic characteristics such as the boat-shaped body appearance, white or red earlobe, and slender greyish blue leg. On the other hand, sexual dimorphism between male and female RJF is easy to distinguish as male RJFs exhibit seasonal eclipse plumage absent

in females. In addition, male RJFs have prominent bright red combs while the females have very small single serrated combs (Syahar *et al.,* 2014).

The Philippines with more than 7,000 islands is part of Southeast Asia, located on the Western portion of the Pacific Ocean. Topographically, the Philippines is one of the countries with mega biodiversity and is home to many endemic species. However, despite its mega flora and fauna biodiversity, the Philippines also suffers from high-level deforestation, habitat destruction, and wildlife exploitation (Kittelberger *et al.,* 2020).

RJFs in the Philippines are locally called "Labuyo" or "manok y halas" due to their forest wandering nature (Bondoc, 2008). However, due to habit loss, and human migration into the forests, its sightings are becoming rare (Masangkay *et al.,* 2010). Furthermore, the first taxonomic classification of RJF in the Philippines was conducted by Hachisuka (1939), identifying the Philippine RJF under *Gallus gallus philippensis*. This subspecies classification was supported by the molecular DNA barcoding identification method by Bondoc (2013). However, PH RJF classification under *G.g.gallus* was reported by Nishida and Masangkay (1978) in their intensive investigation covering different islands in the Philippines. Their classification was based on the morphological characteristics inherent to the *G.g.gallus* subspecies, with high reference on earlobe color as the main taxonomic identification index.

Moreover, subspecies classification of the Philippine RJFs is still in question, given that the published results are often inconclusive and at times contradicting. Also, due to insufficient studies providing evidence on the true genealogy of the Philippine RJFs, their true subspecific classification, maternal origin, and domestication remain unclear to this day. To address this problem, an in-depth molecular study and analysis

encompassing the whole Philippine archipelago could help us understand its genetic identity, maternal origin, diversity and ecology. Furthermore, due to identification limitations, the need to use unconventional methods and DNA sources must be explored as well. Linking taxonomic classification, phylogenomics, and conservation of species will help us address the Sustainable Development Goals of the United Nations.

1.2 General Objectives

This research aimed to address RJF identification limitations using different conventional and unconventional genotypic and molecular methods. These methods include the molecular identification of species through morphological genotyping and qualitative analysis of a larger Philippine RJF data set that covers the three main island regions in the Philippines.

Furthermore, this study also aimed in the determination of the genetic diversity, population structure and demographic history, and possible maternal origin of Philippine RJF through mtDNA D-loop analysis. Evolutionary relationship, and divergence time estimation of the RJFs the from Philippines and Indonesia was also the goal of this study.

1.2.1 Specific objectives

1. Know the ecology and status of the Philippine RJFs under different settings.
2. Qualitative genotyping and quantitative morphological evaluation of the Philippine RJF in comparison to the RJFs in Asia.
3. Determine the possible maternal origin, genetic diversity, population structure, and population demographic history of the Philippine RJFs.

4. Elucidate the phylogenetic relationship of the Philippine RJFs with the RJFs in Asia.
5. Determine the evolutionary relationship of the Philippine and Indonesian RJFs.
6. Provide useful information that could contribute to the genetic resource's conservation programs of the RJFs in the Philippines.

1.3 Ethics on Animal Use

PH RJFs used in this study were approved by the Institutional Animal Care and use Committee (IACUC) of Matias H. Aznar Memorial College of Medicine, Inc. Cebu City, Philippines with reference code MHAM-060919-01.

Pursuant to the Executive Order No. 192, use of PH RJFs as research animals were approved by the Department of Environment and Natural Resources under the Republic Act No. 9147 otherwise known as the Wildlife Resources and Conservation and Protection Act, and Republic Act No. 7586 (National Integrated Protected Areas System) with Gratuitous permit no. 309.

1.4 Review of Related Literatures

1.4.1 Philippine geography

Philippines is an island nation composed of more than 7,000 islands. Luzon, Visayas, and Mindanao are the three major island regions, which constitute about 7 percent, 19 percent, and 34 percent of the total land area, respectively (Forest and Management Bureau, 2004). The country is divided into 17 administrative regions covering 81 provinces, 118 cities, 1,510 municipalities, and 41,995 barangays (NSCB, 2007). Philippines is strategically located on the Southeastern side of Asia, and western

part of the Pacific Ocean, which is also composed of islands. To the east, the South China Sea links the Philippines to continental Southeast Asian countries, among them are Vietnam, Malaysia, and Thailand. Due to the strategic geographical location of the Philippines, migration and exchange of trades between the Philippines and other Asian countries near it also occurred (Francia, 2013).

In a report made by the Forest Management Bureau (2004), 49.2% of the Philippines land area, or 14.76 million ha., have been officially classified as "forestland". In the context of the Philippines, "forestland" refers to all property owned by the national government that is still in the public domain based on the official system of classification. Topographically, most of the forestlands are hilly and mountainous with slopes ≥ 18% and hence are not suitable for agricultural purposes. Furthermore, the topographical description of the Philippines suites to the habitat of RJFs.

1.4.2 Ecology and habitat preference of red junglefowls

RJF utilize a variety of habitats, but are thought to prefer extensive, undisturbed mixed forests for foraging as well as breeding (Ali and Ripley, 1989). This subspecies occupies most tropical and sub-tropical habitats throughout its extensive range including mangroves, scrubland and plantations. RJFs seem to prefer flat or gently sloping terrain, forest edges and secondary forest. It is found from sea level up to around 2,500 meters (del Hoyo *et al.,* 2001) from rain forests to dry lands in Southeast Asia. The availability of RJF in wide specie range is maybe associated with availability of resources and physiological demands (Desta, 2019).

RJFs are generally considered common and widespread despite habitat loss and poaching within its range. The bird is affected relatively little by habitat loss because it can occupy a variety of habitats, including secondary vegetation and man-made habitats

such as rubber and oil-palm plantations and planted fields on forest edges (del Hoyo *et al.,* 2001).

RJF live in small flocks during the non-breeding season which extends from the summer through the autumn and winter. They have a hierarchical social system with a pecking order for both males and females. At the onset of breeding season in the spring, each of the stronger cock maintains a territory with three to five hens (Delacour, 1951). During the breeding season, the male birds announce their presence with the well known "cock-a-doodle-doo" call that is similar to that of the domestic cousin, but somewhat shriller and with a more abrupt ending. This call is uttered principally at dawn before sunrise and at dusk before sunset (Ali and Ripley, 1989). Like most pheasants, RJFs roost in trees singly or in pairs.

RJFs are threatened by habitat destruction, poaching, egg collection, predation, and genetic hybridization (Ali and Ripley, 1989; Peterson and Brisbin, 1999). Various species of animals serves as their predator which sometimes consumes either the egg, the live jungle fowl or its chicks (Johnsgard, 1986). However, a wide variety of ecological adaptation may have made junglefowl resilient to adverse effect of habitat loss (Callaway, 2016).

1.4.3 Philippine red junglefowl

As defined by Bondoc (1998), the name given to the Philippine RJFs is "Labuyo". Labuyo is the smallest and lightest type of chicken among the native strains that are indigenous to the Philippines. The adult male weighs 1 to 1.3kg. while the female is about 0.8 to 1.0kg only. It has a single comb with serrations and whitish tinge earlobes. As cited by Yebron *et al.* (2016) from Lambio and Gay (1993), the Philippines

native chicken was also believed to have descended from the wild red junglefowl domesticated by Filipino ancestors who arrived in the Philippines.

On a research conducted using ancient DNA to study the origin and dispersal of ancestral Polynesian chickens across the Pacific, they were able to find two modern specimens from the Philippines that carry haplotypes similar to the ancient Pacific samples, providing clues about a potential homeland of the Polynesian chicken (Thomson *et al.,* 2014). The findings of Peterson and Brisbin (1998) also suggested that the Philippines and other islands may even have been "seeded" with junglefowl previously modified by early human colonist, and hence hold junglefowl populations that are genetically contaminated from the outset.

Bondoc (2008) was able to successfully identified 25 red junglefowls found in the mountainous areas in the Philippines using DNA barcodes and the results of the analysis indicated existence of two main evolutionary clades based on the sample collected. Another significant study on the Philippine chickens is the study conducted by Yebron *et al.* (2016), wherein they reported on the genetic variation and relationship among Visayan native chicken genetic groups: Boholano and Darag. They used thirteen microsatellites or Simple Sequences Repeats (SSR) markers. However, this study was only on native chickens common in the Visayas. However, we still cannot trace and agree on the likelihood of the Philippine native chickens to the wild junglefowls because of insufficient molecular evidences regarding the wild junglefowls itself. To help in unlocking if not totally the origin and genetic diversity of RJFs, analysis on the complete mtDNA D-loop analysis on the chickens found in the forested area in the Philippines today must be conducted. RJF of economic and cultural importance to

humans, is apparently in danger of genetic extinction, so measures should be taken to assure its long-term survival (Peterson and Brisbin, 1998).

1.4.4 Morphological and genetic identification and breeding studies of the Philippine RJF

1.4.4.1 Morphological identification and classification. Hachisuka (1939) first classified the Philippine RJFs under a separate *Gallus gallus* subspecies which he called *G. g. philippensis*, denoting that this RJF was an endemic species widespread in the Philippines. Under a separate subspecies, he described the Philippine RJF to have small lappets, and golden hackle end more intensely colored than in *G. g. bankiva* and in Micronesian RJF subspecies. Parkes (1962) scrutinized the work of Hachisuka (1939) stating that the Philippine RJF was considered to be the same with *G. g. gallus,* adding that Hachisuka (1939) was faced with various limitations in conducting his work. There were however some inconsistent differences, as some avian characteristics were best developed in populations from the southern parts of the Philippines such as in Mindanao and Basilan, and lesser towards the northern part of the country. However, Parkes (1962) weighed on the evidences and concluded that the RJFs found in the Philippines were indigenous and were not introduced by man. In addition, Parkes (1962) conclusion was also based on an earlier study conducted by Rand and Rabor (1960) whose main work was on the general avifauna of the Philippine Islands.

The reports of Hachisuka (1939) and the generalization of Parkes (1962) based on collected evidence from the literature were all in disagreement with the findings of Nishida *et al.* (1985), and Nishida and Masangkay (1978). Through morphological identification of species, Nishida *et al.* (1985; 2000) reported that the RJF species found in the Philippines belongs to the *G. g. gallus* subspecies of the *Gallus gallus* species.

To support this claim, previous reports (Nishida *et al.* 1978; 1985; Nishida and Masangkay, 1978) documented nine Philippine RJF samples collected from different parts of Luzon, Mindoro, Negros, and Palawan during the first (1971) and second (1975) field investigations in the Philippines. These collected samples were all classified under the genus *Gallus* Linn. based on their white earlobes. With reference to the description suggested by Madoc (1956), and Wells (1999) on the RJFs in Malaysia as well as those described by Gilliard (1950), and Rand and Rabor (1958; 1960) on the morphological features of the Philippine RJFs, the nine collected samples were all categorized under the *G. g. gallus* subspecies. Nishida *et al.* (1985; 2000), on the other hand, did not mentioned about the existence of the *G. g. philippensis* subspecies, suggesting that this was really not considered as a subspecies of the *Gallus gallus* species even before. In addition, Johnsgard (1999) did not include *G. g. philippensis* as a recognized *Gallus gallus* subspecies. Figure 1.1 shows the distribution map of the different *Gallus gallus* subspecies in Southeast Asia. It was also noted that in this map the RJF subspecies represented in the Philippines was the *G. g. gallus* subspecies (Nishida *et al.,* 1985).

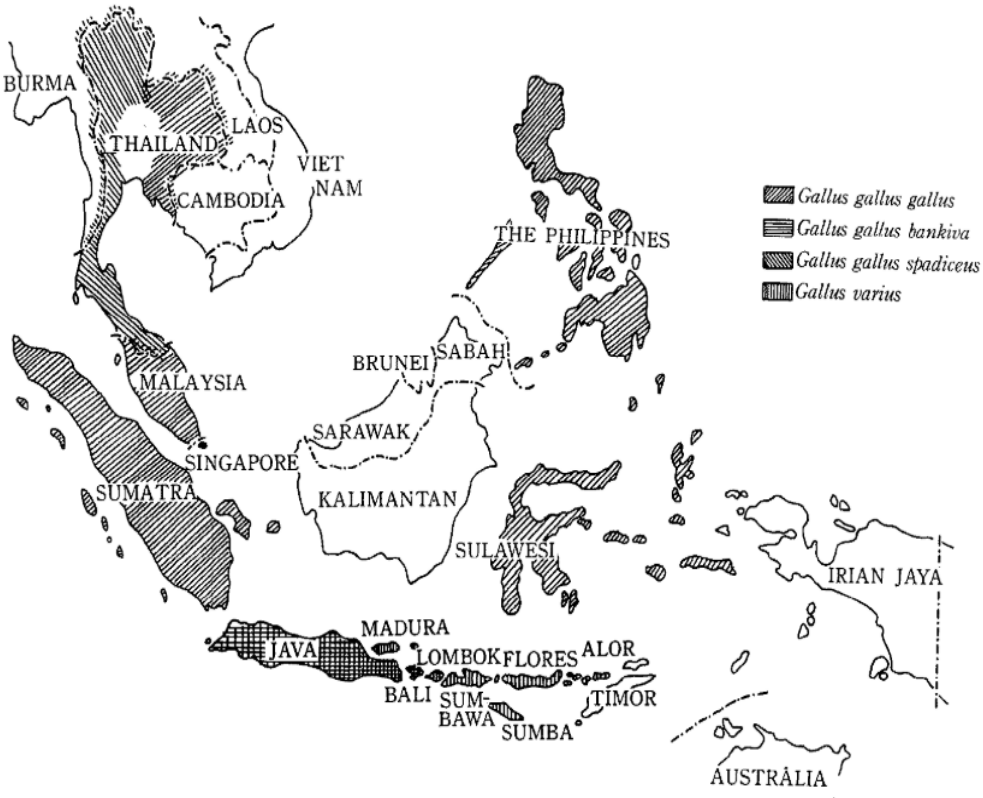


Figure 1.1. Map showing the distribution of the three subspecies of red junglefowl (*Gallus gallus*) and green junglefowl (*Gallus varius*) in Thailand, Indonesia, and the Philippines (Nishida *et al.,* 1985).

1.4.4.2 Genetic identification and studies of the Philippine RJF. To date, no finite molecular conclusion on the genetic identity of the Philippine RJF was published yet. Though there were few literatures available, these literatures however contradict with each other. Some literature classified the Philippine RJF under *G.g.philippensis* (Bondoc, 2013) while others recorded it under the *G.g.gallus* classification (Nishibori *et al.,* 2005). On a comprehensive research of Bondoc (2013) subjecting 25 Philippine RJF samples coming from different mountainous areas in the Philippines using DNA barcoding technique, he suggested that these RJFs were classified under *G.g.philippensis*. In this research Bondoc (2013) used the cytochrome c oxidase subunit

1 (*CO1*) of the mitochondrial genome for DNA barcoding to assess the diversity and genetic distances of RJFs obtained from the different mountains in the country and clustered these Philippine samples with the different RJF subspecies available in GenBank. His results had shown monophyletic branching of the Philippine RJF samples forming two separate clades different from the reference sequences. The monophyletic branching in his results suggests classification of Philippine RJF to a different subspecies. However, upon checking on the Barcode of Life Data System (BOLD), no DNA sequence data for the 25 RJFs analyzed by Bondoc (2013) was found and retrieved. The availability of these sequences could somehow serve as basis for future studies and proper identification of wild caught and introgresssed Philippine RJFs. The unavailability of these sequences made it difficult to justify the true existence of this subspecies. It was also noted that the subspecies *G.g. philippensis* was not included as a category of RJF classification in almost all genetic studies regarding RJFs (Nishibori *et al.,* 2005; Miao *et al.,* 2013; Godinez *et al.,* 2019; Wang *et al.,* 2020). In fact, the research of Bondoc (2013) was the only literature that genetically categorized the Philippine RJF under the *G.g. philippensis* subspecies. Figure 1.2 shows the phylogenetic tree of the Philippine RJF samples studied by Bondoc (2013).

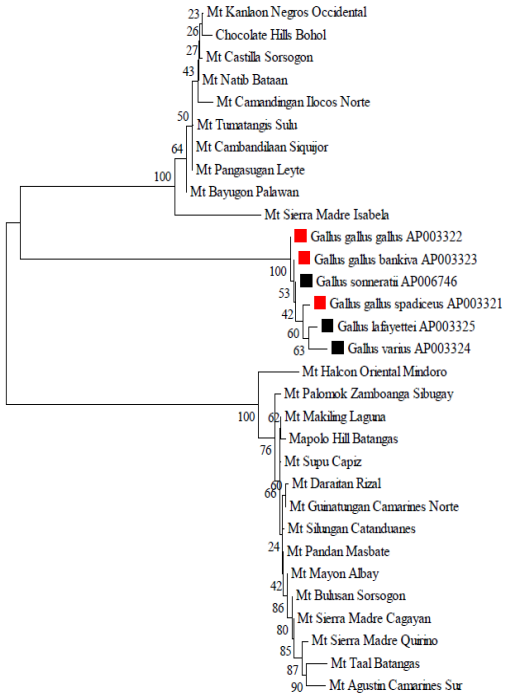


Figure 1.2. Neighbor-joining tree of the 25 Philippine RJF together with the different RJF subspecies and junglefowl species using *COI* sequences (Bondoc, 2013).

On the other hand, the DNA barcoding technique used by Bondoc (2013) is useful in assigning unknown individuals to species and enhances the discovery of new species (Hebert *et al.,* 2003; Stoeckle, 2003). However, using DNA barcodes in identifying species have some drawbacks, and one of which is limited phylogenetic resolution which arises from confusion about the scope of inference (Craig and Cicero, 2004). Also, the *COI* sequence is shorter as compared to the complete mtDNA sequence, making *COI* less informative as compared to the mtDNA D-loop. In addition, *COI* has the least variable mitochondrial gene, suggesting that it has slow evolutionary rate (Kerr, 2011) and has lower non-synonymous substitution rate among the protein-coding mitochondrial loci (Eo and DeWoody, 2010; Kerr, 2011). In contrast, mtDNA

is highly polymorphic with fast evolutionary rate and has high nucleotide substitution rate (Brown *et al.,* 1982).

Moreover, molecular genetics literatures categorizing Philippine RJF under *G.g.gallus* was very limited. One research using this subspecies classification was by Nishibori *et al.* (2005), wherein they used a male RJF from Manila, Philippines collected on the year 1998. For accessibility and future reference, the sequence of this sample was made public in DDBJ/EMBL/GenBank (AP003322/ NC_007236). Their results presented phylogenetic trees based on amino acid sequence and based on the sequences of the first and second nucleotide/codon of mtDNA and these results have shown the genetic relatedness of the Philippine RJF and the *G.g.bankiva.* Using BLAST homology this Philippine RJF sequence (AP003322/ NC_007236) by Nishibori *et al.* (2005) was inferred with selected *Gallus gallus* subspecies reference sequence (Table 1.1) together with Philippine RJF sequences published in Genbank. The results showed that it shared 99.84% similarity with the *G.g.bankiva* (AP003323) which has an accession length of 1232 bp. It also shared 99.59%-99.84% similarity with the RJFs collected by Godinez *et al.* (2019) with an accession length of 1231 bp, lower than *G.g.bankiva* (AP003323). Given the limited sample number, the similarity test result only showed close genetic relatedness and did denote final classification of AP003322/ NC_007236 (Nishibori *et al.,* 2005) to *G.g. bankiva.* In addition, AP003322/ NC_007236 was collected only in one area in the Philippines.

Recently, on a research by Godinez *et al.* (2019), the Philippine RJF they collected from Samar Philippines was classified under the species *Gallus gallus* in their research paper, and was furtherly categorized under *G.g.gallus* in GenBank database. On the other hand, their sample was limited to only one island and did not significantly

represent the 3 major island regions in the Philippines. Moreover, their result was in agreement with the findings of Miao *et al.* (2013) wherein, the Philippine RJF (AP003322/ NC_007236) showed close similarity with the SNP sites with the haplotypes of Samar RJFs except at bases 199, 293, 309, 417. In addition, the Philippine RJF (AP003322/ NC_007236) (Nishibori *et al.,* 2005) sample from Miao *et al.* (2013) was classified as under the *G.g. gallus* subspecies of the genus *Gallus.* Furthermore, their results also implied that the Samar RJFs is not a unique local group and might have been derived from neighboring countries. Also, their results agreed with Osman and Nishibori (2014) affirming close genetic relationship in terms of mtDNA D-loop haplogroup classification among Southeast Asian neighboring countries including Philippines. This result further added to the evidence that the Philippine RJF did not belong to a separate and unique taxon. This also further suggested that the Philippine RJFs does not belong to a separate RJF subspecies.

On the ancient DNA research on the dispersal of ancestral Polynesian chickens across the Pacific, Thomson *et al.* (2014) found modern specimens from the Philippines carrying similar haplotypes with the ancient Pacific samples providing clues about a potential homeland of the Polynesian chicken. In addition, the DNA sequence which provided clue for the relationship of the Polynesian chickens and the chicken from the Philippines was also the sequence sample (AP003322) published by Nishibori *et al.* (2005). On the other hand, Peterson and Brisbin, Jr. (1998) suggested that Philippines together with other countries might have been introduced with junglefowl brought by human settlements from different countries, and hence hold junglefowls populations that were genetically contaminated from the outset, thus contradicting the conclusion of Parkes (1962).

Table 1.1. List of reference sequence used for homology testing.

Accession number	Subspecies	Reference
MK085033	*Gallus gallus gallus*	Godinez *et al.,* 2019
MK085037	*Gallus gallus gallus*	Godinez *et al.,* 2019
MK085036	*Gallus gallus gallus*	Godinez *et al.,* 2019
MK085035	*Gallus gallus gallus*	Godinez *et al.,* 2019
AB007725	*Gallus gallus gallus*	Miyake, 1997
AB007723	*Gallus gallus domesticus*:White Leghorn	Miyake, 1997
AP003318	*Gallus gallus:* White Plymouth Rock	Nishibori *et al.,* 2003
NC_007239	*Gallus lafayetii*	Nishibori *et al.,* 2005
AP003324	*Gallus varius*	Nishibori *et al.,* 2005
NC_007240	*Gallus sonneratii*	Nishibori *et al.,* 2005
AP003323	*Gallus gallus bankiva*	Nishibori *et al.,* 2005
GU261690	*Gallus gallus spadiceus*	Miao *et al.,* 2013
GU261709	*Gallus gallus murghi*	Miao *et al.,* 2013
GU261696	*Gallus gallus jabouillei*	Miao *et al.,* 2013

1.4.4.3 Breeding studies on the Philippine RJF. In a study conducted by Buctot and Espina (2015) utilizing Philippine RJFs coming from selected areas in Leyte, they assessed the breeding performance of the RJF (♂) x native chicken (♀) and the quality of the egg produced under confinement system and under natural mating method. The result of their study had shown that the egg fertility and hatchability produced by the RJF (♂) x native chicken (♀) crossing is comparable with the native chicken (♂) x native chicken (♀) mating. In addition, though all of the RJFs used were collected from different areas in Leyte, Philippines, the results had shown no significant difference with each other with regards to breeding performance and egg production indices. On the other hand, Buctot (2016) also reported comparable production potential and egg

quality traits between two RJFs sourced from the mountainous area in Southern Leyte, Philippines mated with the native chicken hens under confinement and under natural mating method and compared it with the performance of native roosters mated with native chicken hens. These were assessed based on their breeding and production performance under confinement system. Their results have shown that the performance of RJFs were comparable with the performance shown by the native chicken, though difference was observed only on the egg weight due to genetic and non-genetic factors.

1.4.5 Supplementary information from different DNA data banks and ornithology websites

1.4.5.1 DNA data banks sources. With regards to the DNA sequence availability of *G. g. philippensis* there are no available sequences in GenBank. Another website which stores important DNA sequences is the Barcode of Data Life System (BOLD) which was designed to support the generation and application of DNA barcode data of different species including those of chickens. However, in this website, no *COI* sequence or DNA barcode of *G. g. philippensis* or any Philippine RJF samples can be found. With the unavailability of the *G. g. philippensis* DNA sequence in any DNA public domain websites, we could not use this subspecies as basis for the molecular classification of the newly collected Philippine RJF samples, thus using this classification for future studies would be unlikely. On the other hand, Philippine RJFs classified under *G. g. gallus* (Nishibori *et al.,* 2005; Godinez *et al.,* 2019) are available online.

1.4.5.2 Other Sources of information on the classification of the Philippine RJF. There are several ornithology websites that did not catalogue *G. g. philippensis* as subspecies of *Gallus gallus*. These websites include "The Cornell Lab of Ornithology", which contains comprehensive life histories of all bird families. Unfortunately, the subspecies *G. g. philippensis* is not among their list. Another reliable website is the Avibase which is an online database that organizes bird taxonomic and distribution data globally. Though *G. g. philippensis* is available in Avibase (https://avibase.ca/CF6BDC57), it was however, indicated with an invalid subspecies status. This could probably be attributed to the lack of morphology and molecular classification literatures that would support its existence.

On another account, one sample of Philippine RJF was catalogued in the Smithsonian Institution (SI) (https://www.si.edu/object/nmnhvz_4016513) which is a male RJF published as *G. g. philippensis* and recorded as nmnhvz_4016513. The specimen which was a whole skin preparation was collected in Palawan on July 19, 1888. This record is perhaps the oldest known existing sample of this subspecies. However, despite its existence, no other description regarding its morphology and molecular identity were stated. Understandably only morphological information is available for this record.

1.4.6 Mitochondrial DNA: genomics and evolution

Advancements in molecular genetics aimed to trace the genealogy of different animal species including humans. These advancements helps not just in the tracking down our history but also the revelation of genetic variations distribution of a population. The eagerness of the scientific community to know about origin and

genetic diversity among species resulted to the realization of the scientific advantage of mitochondrial DNA (mtDNA).

mtDNA is present in most cell cytoplasm in high copy number and is easy to be amplified, rapid, and inexpensive to sequence (Zink and Barrowclough, 2008; Galtier *et al.,* 2009). It has shaken the field of molecular evolutionary biology by storm, as it can be obtained easily from animals, it rapidly evolves, and holds potential information at a variety of taxonomic levels (Zink and Barrowclough, 2008). mtDNA is highly polymorphic with an evolutionary rate of more than 5 times faster as compared to nuclear DNA (Brown *et al.,* 1982). One distinct region is the D-loop region which is non-conding and evolves much faster than other regions of the mtDNA genome, making it a marker of choice in genetic diversity within and between species. In addition, the mitochondrial gene content is strongly conserved, has little duplication, has no intron, and has very short intergenic regions (Gissi *et al.,* 2008).

Mitochondrial DNA's inheritance is maternally transmitted (Birky, 2001; Muchadeyi *et al.,* 2008), co-occur within a zygote (Avise *et al.,* 1987), does not undergo recombination (Hayasi *et al.,* 1985), thus all sites shares common maternal genealogy. This transmission of mtDNA in the female germ line reduces the within individual diversity (Shoubridge and Wai, 2007). The hypervariable D-loop region of mtDNA sequence can be used to detect ancient population structures (Muchadeyi *et al.,* 2008). The lack of genetic exchange has been considered as a useful feature, as it implies that the within-species history of mtDNA can be appropriately represented by a unique tree, which traces back the origins and geographic movements of maternal lineages (Avise *et al.,* 1987). The field of phylogeography heavily relies on the clonal inheritance of mtDNA (Galtier *et al.,* 2009). In addition, mitochondrial encoded genes supposed to

evolved in neutral way and have been considered as less likely to be involved in adaptive process. The evolutionary rate of mtDNA has been frequently assumed to be clock-like which means, it helps in determining divergence times. Clonal, neutral and clock-like mtDNA apparently stands as the ideal witness of population and species history (Galtier *et al.,* 2009). These practical issues presumably clearly explain the popularity of mtDNA in molecular ecology. The reasons most often invoked to justify this choice, however, are more fundamental (Ballard and Whitlock, 2004; Ballard and Rand, 2005).

1.4.7 Mitochondrial DNA studies in chickens

Over the years, numerous studies have taken the advantage, usefulness and reliability of mtDNA in the reconstruction of the maternal lineage history and domestication in domestic chickens. In fact, the complete sequence of mtDNA D-loop region was successfully used in genetic study especially to determine phylogenetic relationship, including genetic distance and genetic variability within and among populations (Oka *et al.,* 2007; Miao *et al.,* 2013; Osman and Nishibori, 2014).

The first mtDNA molecular study on chickens was on the study conducted by Fumihito *et al.* (1994) which suggested that the primary maternal ancestor of the domestic chicken (*Gallus gallus domesticus*) was the *Gallus gallus gallus* subspecies which is an Indochinese RJF. They also suggested that domestic chickens have monophyletic origin and that the continental population of the RJFs subspecies (*Gallus gallus gallus*) found in Southeast Asia (SEA) sufficed as the sole ancestor of all domestic chicken. They also suggested that a single domestication event occurred in Thailand and adjacent regions (Fumihito *et al.,* 1994, 1996). They examined 400 bp of the mitochondrial D-loop region and provided support for Darwin's conclusion that the

chicken was established through domestication of RJF (Fumihito *et al.,* 1996). However, because only 400 bp of mtDNA sequence was analyzed, the statistical validity of this conclusion remains a concern (Hasegawa and Adachi, 1996). Furthermore, the conclusion they presented is supported by other studies using microsatellite DNA (Hillel *et al.,* 2003). In addition, a study conducted by Nui *et al.* (2002) on the mtDNA of Chinese native chicken also suggested the same area of domestication site reported by Fumihito *et al.* (1994, 1996). In contrast, multiple-origins hypothesis of Nishibori *et al.* (2005) showed molecular evidences of hybridization between species in the genus *Gallus*. The phylogenetic analysis of the entire mtDNA genome and nuclear DNA (nucDNA) regions for four CR1 (chicken repeat 1) regions and *OTC* (ornithine carbamoyltransferase) revealed evidence of hybridization with other jungle fowl species (Nishibori *et al.,* 2005). Multiple rather than single origins of domestication were now accepted (Liu *et al.,* 2006; Kanginakudru *et al.,* 2008; Xiang *et al.,* 2014), with mtDNA providing support for localized domestication events in South Asia (Oka *et al.,* 2007; Kanginakudru *et al.,* 2008), Northeast India, Southwest China, and a further event in Southwest China and Southeast Asia (Miao *et al.,* 2013). These were also supported by the archeological findings on the domestication of chickens by the people living in the Indus Valley (Zeuner, 1963) and in the Huber and Henna Provinces of China date to 6000 B.C. (West and Zhou, 1989).

Numerous studies about the genealogy of chicken were also conducted in different parts of the world. Muchadeyi *et al.,* (2008) observed two distinct haplogroups in Zimbabwe native chickens, which may be originated from Southeast Asia and the Indian subcontinent. Analysis on the partial mtDNA D-loop sequences in

East African native chickens was conducted also. The analysis revealed the existence of at least five genetically distinct mtDNA D-loop haplogroups, which originated from South and Southwest China and/or surrounding regions as well in Southeast Asian countries such as Myanmar and Thailand (Razafindraibe *et al.,* 2008; Mwacharo *et al.,* 2011) observed two haplotypes in Madagascar native chicken originated from Indonesia and African continent or an introgression from commercial lines. Adebambo *et al.* (2010), in their study in Nigerian native chickens reported that a single haplogroup seemed to be of Indian origin. There were also comparison of the phylogenetic differentiation between two Egyptian chicken breeds with mtDNA D-loop region conducted by Ramadan *et al.* (2011). Indian chickens were found out to have originated from *G.g. spadiceus, G.g. gallus* and *G.g. murghi (*Kanginakudru *et al.,* 2008). Native chickens in Bangladesh as reported by Islam and Nishibori (2012) were strongly influenced by the *G.g. murghi*. Based on the analysis conducted by Zhang *et al.* (2017), Tibetan chickens were dominated by seven major haplogroups, but were not distinguishable from the indigenous chickens in its surrounding areas, thus some clads may have originated from gamefowls. Sulandari *et al.* (2008) reported that Indonesian indigenous chickens have been associated to reference sequences from India, China and Indonesia. Laotian indigenous chickens have also been believed to have originated in Southeast Asian continent and China based on the clade where it belongs (Kawabe *et al.,* 2014). Diversity of the Vietnamese local chickens was found out to be related to the chickens of Indian, Chinese and Southeast Asian origins (Cuc *et al.,* 2011). Studies mentioned only showed that mtDNA has been a useful marker to trace back the origin of livestock species thus, it has been widely used to reconstruct domestication patterns (Groeneveld *et al.,* 2010).

CHAPTER II

Morphology and Genetics of the Philippine Red Junglefowl

2.1 Abstract

Red junglefowls (RJF) are distributed widely in the different areas in Asia, including the Philippines. However, no morphology study representing the three main island regions in the Philippines was conducted. Thus, in this study, a total of 34 Philippine (PH) RJFs (29 males; 5 females) were sourced from Luzon, Visayas, and Mindanao for morphological identification and profiling using different qualitative and quantitative parameters. Taxonomic classification based on its morphology is validated through mitochondrial DNA (mtDNA) D-loop region analysis. This study revealed that black/slate gray shank and white/whitish red earlobe color appeared to be the most reliable RJF indicator in addition to the eclipse plumage in males and rust red to yellow mantle color in female RJFs as expressed by the e^{+} allele. Spur length (2.23 $\pm$ 0.87 cm) and body length (34.31$\pm$3.47 cm) in male RJFs were also identified as good RJF morphometric index. The strongest indicator of introgression observed in this study was the expression of yellow shank color in the hybrid PH RJF. The morphological and genetic assessment in this study revealed the coexistence of *G.g.gallus* and *G.g.bankiva* subspecies classification in the Philippines by phylogenetic analysis. The sole use of earlobe color as RJF subspecies identification index is also invalidated in this study. Furthermore, this study highlighted the importance of primary identification of RJFs using qualitative and quantitative morphology parameters as supported by the mtDNA D-loop region.

Keywords: morphology, mtDNA, Philippines, red junglefowls

2.2 Introduction

Red junglefowl (RJF) is considered to be the ancestor of domestic chickens present today as supported by the monophyletic (Fumihito *et al.,*1994; 1996) and polyphyletic (Nishibori *et al.,* 2005) maternal origin of chickens. RJFs are further classified into different subspecies: *Gallus gallus gallus, Gallus gallus spadiceus, Gallus gallus jabouillei, Gallus gallus bankiva,* and *Gallus gallus murghi* (Johnsgard, 1999). These RJFs were exclusively found and widely distributed in the different areas in South and Southeast Asia (Hanh *et al.,* 2015) and utilized variety of habitats for foraging as well as breeding (Ali and Ripley, 1989). However, the existence of RJFs today is threatened by habitat destruction, poaching, egg collection, and genetic hybridization (Ali and Ripley, 1989; Peterson and Brisbin, 1998). Various species of animals also served as their predator, consuming either the egg, the live junglefowl, or its chicks (Johnsgard, 1986).

The Philippine (PH) RJF are forest dwelling chickens (Masangkay *et al.,* 2010) that were characterized by their small body size (Bondoc, 1998), and varying shade of white earlobe color (Nishida and Masangkay, 1978; Nishida *et al.,* 1985; 2000). Its initial morphology classification suggested that the PH RJFs belonged to a separate RJF subspecies called *Gallus gallus philippinesis* (Hachisuka, 1939). However, other studies classified PH RJFs under *G.g.gallus* based on its earlobe and hackle color (Nishida and Masangkay, 1978; Nishida *et al.,* 1985; 2000) and genetic analysis (Nishibori *et al.,* 2005). Though identifications on PH RJFs were already made, intensive profiling and classification as supported by genetic analysis that represents Luzon, Visayas and Mindanao has not been conducted to date. Thus, the purpose of this study was to provide baseline data on the morphological characteristics of the PH

RJFs found in the different areas in the Philippines through genotyping, and morphometric and genetic analysis. In addition, proper identification and status survey are important in formulating conservation strategies that will help curb the erosion of RJF genetic resources (Liyanage *et al.,* 2015), thus this study.

2.3 Materials and Methods

2.3.1 Experimental site and animals

The RJFs used in this study were sampled from the selected provinces of Luzon, Visayas and Mindanao (Table 2.1; Fig. 2.1). Collection of samples were conducted during the breeding (January) and non-breeding (August) season of the RJFs.

In addition, 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, Inc. Cebu City, Philippines with reference code: MHAM-060919-01. RJF sampling in the different areas in the Philippines was also permitted by the Department of Environment and Natural Resources of the Philippines under Gratuitous Permit number 309 and R6-2019-007.

Table 2.1. PH RJF information and distribution per region.

Island region	Collection period	Sex	N	Accession number
Luzon	Breeding season	♂	19	OL589006-OL58902, OL589024
		♀	1	OL589023
Visayas	Non-breeding season	♂	6	OL589029, OL589033, OL589035-OL589038
		♀	4	OL589030, OL589031, OL589032, OL589034
Mindanao	Breeding season	♂	4	OL589025-OL589028

♂ = male; ♀ = female; N= number of samples

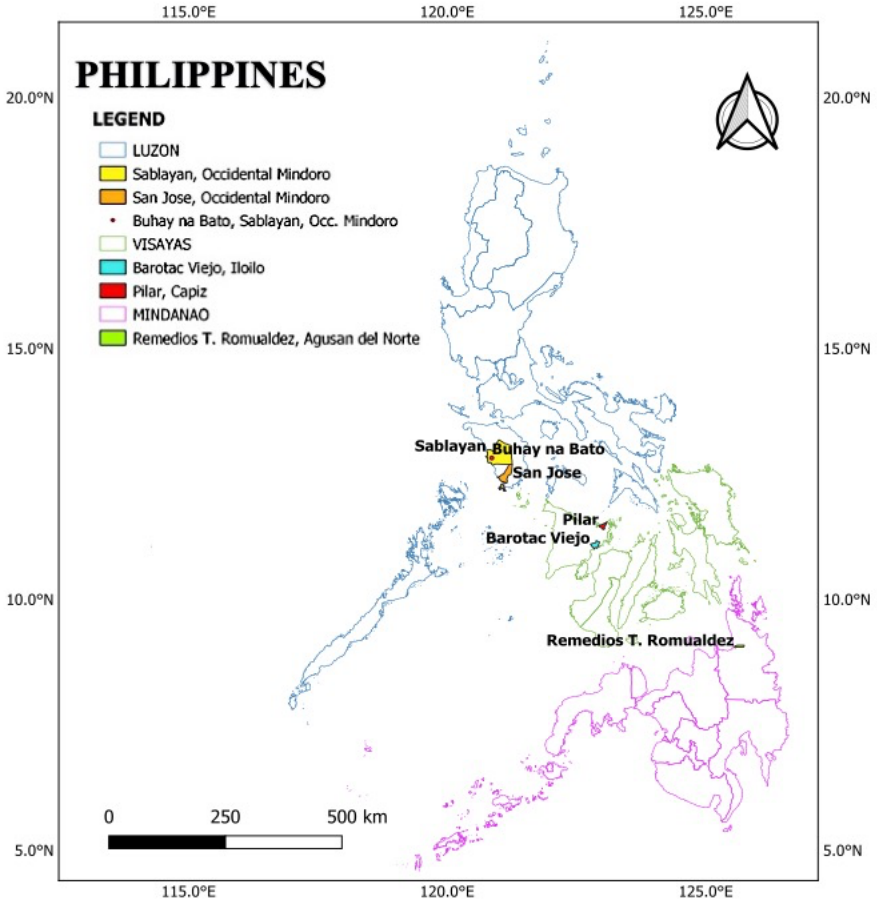


Figure 2.1. Red junglefowl collection sites in the Philippines.

2.3.2 Qualitative and quantitative data analysis

Qualitative other data parameters and methods followed were based on different RJF and chicken studies (Beebe, 1926; Baker, 1928; Delacour, 1947; Kimball, 1958; Morejohn, 1968; Delacour, 1977; Nishida *et al.*, 1985; Crawford, 1990; Nishida *et al.,* 2000; Condon, 2012; Syahar *et al.,* 2014; Desta, 2019). These qualitative data include plumage color, hackle and mantle characteristics, comb type, earlobe and tarsus color, beak and skin color. The morphometric data collected were based on the guidelines set by FAO (2012).

Moreover, quantitative parameters measured and evaluated were the following:

1. *Shank length*. Measured from the hock joint to the spur of either leg.
2. *Spur length.* Measured from its base to the spur tip.
3. *Body length*. Measured when the bird's body is completely drawn throughout its length from the tip of the beak to that of the base of the tail
4. *Wingspan*. Measured between the tips of the right and left wings after both are stretched out in full
5. *Body circumference*. Taken at the tip of the pectus (hind breast)
6. *Body weight*. Measured using a standardized weighing scale

2.3.3 Genetic analysis and statistical computation

Descriptive frequencies were analyzed using Microsoft Excel ver. 16. 56 and SPSS ver. 28. Qualitative data were analyzed using frequency analysis, while the difference between population was analyzed using the least significant differences (LSD) of the General linear model (GLM) using SPSS ver. 28. Regions represented by less than 3 RJFs were excluded in the statistical analysis.

The DNA sequences of the PH RJFs used in this study were previously indexed and stored in GenBank. Sequence alignments of the 1232 bp long mtDNA D-loop sequenced data were conducted using Molecular Evolutionary Genetic Analysis (MEGA X) (Kumar *et al.,* 2018) to improve and refine difficult alignment and trap errors in input sequences. Sequences were aligned with the reference sequences (Miao *et al.,* 2013) for haplogroup classification. Haplogroup group classification was

conducted using Neighbor-joining (NJ) phylogenetic tree, using the Kimura 2-parameter model. Sequence similarity analysis with the RJF subspecies reference sequences was conducted using BlastN. The Philippine map with sampling sites was created using QGIS 3.16.8.

2.4 Results and Discussions

2.4.1 Qualitative traits

2.4.1.1 Plumage color expression and characteristics. All male and female PH RJFs identified and evaluated expressed the wild type e^+ allele (Appendix table 2.6.1; Fig. 2.2 A,B,D). The male PH RJFs has black breast, ventral plumage (Crawford, 1990), upper major and median secondary coverts, reddish-brown edge of the primary flights (Nishida, *et al.,* 2000). However, the wild plumage patterns were also observed in the hybrid RJF in this study (Fig. 2.2C), thus suggesting that e^+ allele expression in male RJFs should not be used as a gauge in the genetic purity assessment of wild chickens. Moreover, eclipse plumage was observed only in the Visayas (66.67%) (Fig. 2.2D), however, there were two juvenile RJFs sampled in the same area which have not expressed the eclipse plumage yet. Expressed also by the e^+ allele, the eclipse plumage was only present in male RJFs (Syahar *et al.,* 2014), during the non-breeding season (Baker, 1928; Delacour, 1947; Kimball, 1958; Morejohn, 1968; Delacour, 1977; Crawford, 1990). Furthermore, female PH RJFs were phenotypically observed to express a mixture of brown pigment in a stippled pattern, while the breast has a salmon-brown color devoid of stippling.

Moreover, majority of the male PH RJFs from Luzon (94.74%) and Mindanao (75.00%) has golden yellow hackle. The juvenile RJFs from Visayas also exhibited the

same hackle color (33.33%). In addition, the hackle color of the 66.67% RJFs from Visayas can't be determined since these RJFs were molting (Appendix table 2.6.1). During the RJF nonbreeding season (June-September), the bright golden yellow hackles of the male RJFs will molt and will be replaced immediately with short, black, spatulate-shaped feathers (Baker, 1928; Delacour, 1947; Kimball, 1958; Morejohn, 1968; Delacour 1977; Crawford, 1990). On the other hand, the dark hackle color (orange) observed in the male RJFs from Luzon and Mindanao was a characteristic of a *G.g.bankiva* (Nishida *et al.,* 2000). Hachisuka (1939) added that the hackle of *G.g.philippensis* appeared to be more golden than the hackle ends of *G.g.bankiva.* Furthermore, the only RJF that exhibited the red hackle color in this study was the hybrid RJF from Mindanao. On the other hand, all female PH RJFs collected has rust red colored head to yellow and yellowish-orange colored mantle end (Appendix table 2.6.2).

Figure 2.2. (A) female, (B) male, (C) hybrid PH RJF and (D) PH RJF expressing the eclipse plumage.

2.4.1.2 Comb type. Male PH RJFs used in this study has bright fleshy prominent single red comb except for the hybrid RJF from Mindanao. On the other hand, all female PH RJFs has a small single serrated comb on top of its head which was very evident upon closer examination. These comb type observations were in agreement with the previous studies on RJF comb characteristics (Beebe, 1926; Delacour, 1977; Brisbin and Peterson, 2007; Syahar *et al.,* 2014; Kaila *et al.,* 2015). Furthermore, single comb was also known to exist in traditional chickens in the tropics (Duguma, 2006), and among Philippine native chickens (Cabarles *et al.,* 2012; Salces *et al.,* 2015). This result suggested that this trait should not be used in differentiating wild RJFs from domestic chickens.

2.4.1.3 Earlobe color. The most common earlobe color observed in this study was white and whitish red/reddish white color (Fig. 2.3B). This earlobe colors were expressed by 55.17% (n=16) of the total male PH RJFs. Red earlobe color was also more prominent (24.14%) (Fig. 2.3C) over white earlobe color (17.24%) (Fig. 2.3A) among the male RJFs analyzed (Appendix table 2.6.1). A removed earlobe was also observed in Mindanao (3.45%). Furthermore, all female RJFs in this study has pale white earlobe color (Appendix table 2.6.2). Earlobe color was recognized as the most important RJF subspecies taxonomic identification index (Nishida *et al.*, 2000). The same authors also mentioned that the RJF subspecies distributed in the Philippines was the *G.g. gallus* with white earlobes that varied in shades from white to whitish pink in color. This characterization was based on the earlobe color and characteristic description provided by Gillard (1950); Rand and Rabor (1960). Furthermore, the predominance of white and whitish red earlobe color among the analyzed PH RJFs suggested that the RJFs used in this study were under *G.g.gallus,* confirming the

previous RJF studies (Nishida and Masangkay, 1978; Nishida *et al.,* 1985; 2000). Moreover, the PH RJFs exhibiting the red earlobe color expression in this study could be classified under *G.g.bankiva* (Nishida *et al.,* 2000), thus, suggesting the possible coexistence of *G.g.bankiva* and *G.g.gallus* in the Philippines. Furthermore, earlobe color expression in chickens was suggested to be due to a single SNP in the *TP63* gene (Luo *et al.,* 2018).

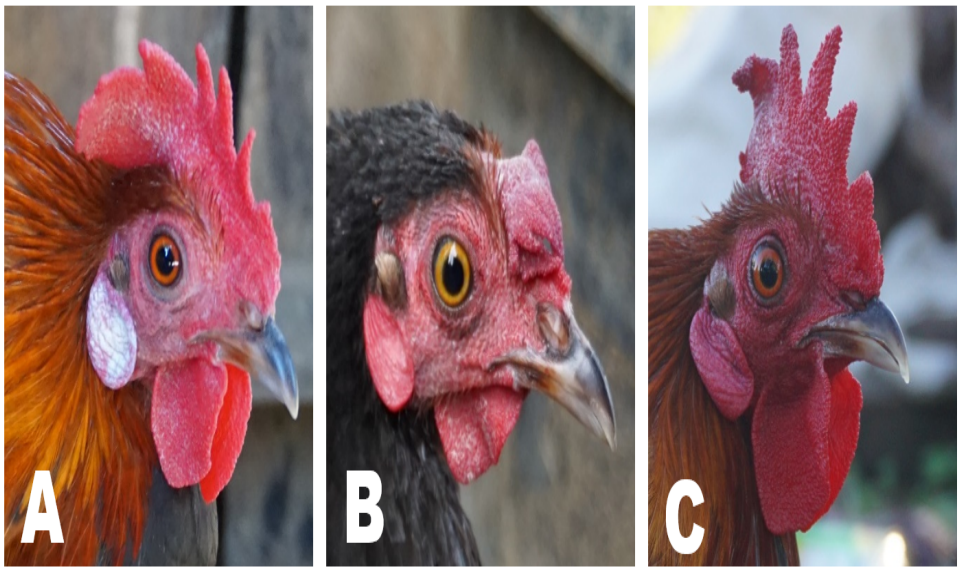


Figure 2.3. White (A), whitish red/reddish white (B) and red (C) earlobe color in PH RJFs.

2.4.1.4 Beak color. The results have shown that all male and female RJF samples in this study were observed to have black to slate gray upper beak with a slight tint of yellowish color at the end, and a yellowish-gray lower mandible (Appendix table 2.6.1 and 2.6.2). The same beak color was also observed in the hybrid PH RJF in this study. Furthermore, the upper beak which appeared to have a tint of yellow could be due to the continuous direct sunlight exposure of the upper part of the beak in addition to the differential act of location-specific biochemical processes (Desta *et al.,* 2013).

2.4.1.5 Shank and skin color. The most common shank color in the male PH RJFs sampled from the different Philippine regions was black/slate gray (Fig. 2.4A), followed by white (Fig. 2.4B), and then yellow shank color (Fig. 2.4C) which was only observed on the hybrid PH RJF (Appendix table 2.6.1). On the other hand, all female Philippine RJFs sampled have slender slate gray shank and none of them showed other shank color (Appendix table 2.6.2). The result of this study supported the findings of Baker (1928), who mentioned that RJFs possessed dark slate to dark gray tarsi. Moreover, the black shank exhibited by the RJFs could be due to the presence of dermal melanin expressed by the *ididWW* genotype (Hutt, 1949). Bump (1961) also suggested that any yellow color in the shanks of an RJF was a result of hybridization with domestic chickens. This was supported by the identification of beta-carotene deoxygenase 2 (*BCDO2*) gene which was responsible for the expression of yellow colored shank on hybrids and domestic chickens (Eriksson *et al.,* 2008). The non-pigmented skin characteristic of an RJF (Nishida *et al.*, 2000), was observed in all sampled male and female PH RJFs across population. The same skin color was also observed on the hybrid PH RJF, thus, suggesting that it should not be used as an RJF identification index.

Figure 2.4. Shank color differences in PH RJFs: (A) black/slate-gray, (B) white, and (C) yellow.

2.4.2 Morphometric traits

Table 2.2 shows the morphometric traits variability between regions. The results showed that only spur length (SL) and body length (BL) were not significantly different ($p>0.01$) between RJF populations. This suggested that SL and BL were good PH RJF identification indices since the difference between regions for these traits doesn't not vary significantly. The body length (BL), wingspan (WS), body circumference (BC), and body weight (BW) were not good PH RJF identification index since these traits vary significantly ($p<0.01$) between regions. Furthermore, the morphometric measures in this study were lower than the Boholano native chickens in the Philippines reported by Salces *et al.* (2015). The average BW (950g) reported in this study was almost the same with the male Thai RJFs (938.2g), and higher than the male Indonesian RJFs (863.3g) (Nishida *et al.,*1985). Moreover, the 12 male Philippine RJFs collected by Nishida *et al*. (1985) has lower average BW (853.8g) than the mean BW sampled in this study (950.0g). Furthermore, the Malaysian RJFs (Syahar

et al., 2014) has longer SL (8.70cm) and BL (39.40cm), and has shorter SpL (1.90cm), WS (24.70cm) and lower BW (901.60g) than the PH RJFs in this study (Table 2.3).

Table 2.2. Quantitative difference of the male PH RJF.

Morphometric traits	Luzon (n=19)	Visayas (n=6)	Mindanao (n=4)	Mean (n=29)
SL (cm)	5.13±0.50[b]	6.67±0.52[a]	6.00±0.82	5.57±0.82
SpL(cm)	2.10±1.00	2.67±0.29	2.50±0.41	2.23±0.87
BL(cm)	34.26±3.54	34.50±1.87	34.25±5.91	34.31±3.47
WS(cm)	29.84±2.27[c]	31.17±3.19	33.75±1.26	30.66±2.63
BC(cm)	32.76±1.92[bc]	24.75±2.09[ac]	36.88±2.72[ab]	31.67±4.27
BW(grams)	957.89±289.28	725.00±112.92[c]	1250.00±251.66[b]	950.00±289.17

SL= Shank length; SpL= Spur length; BL=Body length; WS=Wingspan; BC=Body circumference; BW= body weight
No mean superscript = not significantly different. Means with superscript were highly significantly (p>0.01) from the region that superscript represents: a=Luzon, b=Visayas, c=Mindanao.

Table 2.3. Mean body measurement comparison between Malaysian and Philippines RJFs.

Parameter	Malaysia*		Philippines**	
	Male	Female	Male	Female
Shank length(cm)	8.70	7.20	5.57	6.00
Spur length(cm)	1.90	-	2.20	-
Body length(cm)	39.40	30.10	34.31	31.00
Wing span(cm)	24.70	18.80	30.66	28.60
Body weight (g)	901.60	498.00	950.00	640.00

** Syahar et al. 2014*
***in this study*

Moreover, the female PH RJFs in this study (Table 2.4) were heavier (640.00g) as compared to the Indonesian *G.g.gallus* (675g) (Nishida *et al.,* 1985), and Malaysian RJF (498.00g) (Syahar *et al.,* 2014). Furthermore, female PH RJFs also has shorter SL (6.00cm), and longer BL (31cm) and WS (28cm) than the female Malaysian RJF (Syahar *et al.,* 2014). Comparing the female PH RJFs and Boholano native chicken in the Philippines, the Boholano native chicken has higher BW (1.15kg), chest

circumference/BC (24.90cm), SL (8.60cm) and WS (37.82cm) (Salces *et al.,* 2014). This result provided further evidence that RJFs are smaller as compared to native chickens.

Table 2.4. Quantitative linear body measurements of female PH RJFs.

Morphometric traits	Luzon (n=1)	Visayas (n=4)	Mean (n=5)
SL(cm)	6	6.00	6.00
BL(cm)	34	30.25	31.00
WS(cm)	26	29.25	28.60
BC(cm)	3	22.63	24.50
BW (g)	900	575.00	640.00

SL= Shank length; SpL= Spur length; BL=Body length; WS=Wingspan; BC=Body circumference; BW= body weight

2.4.3 Genetic classification and morphology difference between haplogroups

Genetically, not all PH RJFs morphologically classified under *G.g.gallus* and *G.g.bankiva* based on the color of their earlobe (Nishida *et al.,* 1985; 2000) coincides with the genetic similarity analysis. Only 42.86% of the morphologically classified RJFs under *G.g.bankiva* was also genetically classified under the same classification. On the other hand, 69.23% of the PH RJFs classified under *G.g.gallus* based on their earlobe color was in congruent with the subspecies classification through qualitative morphology assessment (Appendix table 2.6.3). Although this result suggested that earlobe color should not be used as an absolute measure of RJF subspecies classification, the genetic analysis in this study supported the co-existence of *G.g.bankiva* and *G.g.gallus* as initially revealed through qualitative morphology assessment.

Moreover, the PH RJFs in this study were classified under haplogroup D (n=30), haplogroup E (n=2) and haplogroup E (n=1) (Appendix fig. 2.6.1; Appendix table

2.6.3). The PH RJFs classified under haplogroup D shared the same cluster with the *G.g.gallus* and *G.g.bankiva* from Philippines and Indonesia, respectively. It also shared the same haplogroup with the domestic chickens from China, Laos and India. Moreover, the PH RJFs classified under haplogroup E shared the same clade with the commercial and domestic chickens of China and India, while haplogroup Y was represented by a wild RJF reference sequence from China (Miao *et al.,* 2013).

Furthermore, all PH RJFs classified under haplogroup D, E and Y exhibited the wild-type plumage allele (e^{+}), white skin color, slate gray beak color, and rust red to yellow colored mantle in female (Appendix table 2.6.4). Moreover, a sample which was morphologically classified as a wild chicken, was phylogenetically clustered with the commercial chickens in haplogroup E, thus revealing its domestic chicken matrilineal origin. In addition, only the yellow shank color trait in haplogroup E was not evident among PH RJFs classified under haplogroup D and Y. As mentioned, yellow shank color was a characteristic of a hybrid chicken (Ericsson *et al.,* 2008), which further verified the hybrid classification of PH.M4.

The morphometric difference between haplogroups showed that the male hybrid RJF classified under haplogroup E has higher morphometric traits as compared to the RJFs under haplogroup D (Table 2.5). On the other hand, the female PH RJF under haplogroup E has higher BL (31cm), WS (30cm) and BW (600g) than the RJFs in haplogroup E. Haplogroup Y has the highest BW (900g) and BL (34cm) as compared with the other haplogroups (Table 2.5). Table 2.5 also shows the average morphometric profile of the RJFs in the Philippines as represented by haplogroup D and Y. Samples classified under haplogroup E were excluded since these were classified together with the commercial chickens.

Table 2.5. Haplogroup difference based on quantitative parameters of male and female PH RJFs.

Morphometric traits	Male		Female		
	Hap D (n=28)	Hap E (n=1)	Hap D (n=3)	Hap E (n=1)	Hap Y (n=1)
SL(cm)	5.56	7.00	6.00	6.00	6
SpL(cm)	2.21	2.50	-	-	-
BL(cm)	34.31	40.00	30.00	31.00	34
WS(cm)	30.65	35.00	28.67	31.00	26
BC(cm)	31.13	40.50	23.00	21.50	32
BW (g)	932.69	1600.00	566.67	600.00	900

SL= Shank length; SpL= Spur length; BL=Body length; WS=Wingspan; BC=Body circumference; BW= body weight; Hap=haplogroup

2.5 Conclusion and Recommendations

The morphological and genetic assessment revealed the *G.g.gallus* and *G.g.bankiva* subspecies classification of the PH RJFs in this study. Shank color, earlobe color and eclipse plumage appeared to be the most important parameter in determining the wild junglefowl ancestry of an RJF. Spur and body length were also good RJF indicators since these were the only morphometric trait that did not differ between regions. Marked qualitative and quantitative difference between haplogroups were also observed in this study, particularly the expression of yellow shank color of the hybrid PH RJF in haplogroup E. Moreover, this study also highlighted the importance of primary identification of RJFs using qualitative and quantitative morphology parameters. This research also emphasized that validating the gathered morphology data through genetic analysis is important in the proper identification of RJFs.

2.6 Appendices

Appendix table 2.6. 1. Qualitative traits of the male PH RJFs.

Qualitative trait	Luzon		Visayas		Mindanao	
	n=19	%	n=6	%	n=4	%
Plumage color						
Wild-type	19	100	6	100	4	100
Others	0	0	0	0	0	0
Eclipse plumage						
Eclipse plumage	0	0	4	66.67	0	0
Non-Eclipse plumage	19	100	2	33.33	4	100
Hackle						
Golden yellow	12	63.16	2	33.33	2	50
Orange	7	36.84	0	0	1	25
Red	0	0	0	0	1	25
Others	0	0	4	66.67	0	0
Comb type						
Single	19	100	6	100	4	100
Others	0	0	0	0	0	0
Earlobe color						
White	2	10.53	3	50.00	0	0
Red	6	31.58	1	16.67	0	0
Whitish red/reddish white	11	57.89	2	33.33	3	75
Removed	0	0	0	0	1	25
Beak color						
Slate gray	19	100	6	100	4	100
Yellow	0	0	0	0	0	0
Other	0	0	0	0	0	0
Shank color						
Black/slate gray	18	94.74	4	66.67	3	75
White	1	5.26	2	33.33	0	0
Yellow	0	0	0	0	1	25
Skin color						
White/non-pigmented	19	100	6	100	4	100
Yellow	0	0	0	0	0	0
Others	0	0	0	0	0	0

Appendix table 2.6.2. Qualitative traits of the female PH RJFs.

Qualitative trait	Luzon		Visayas	
	n=1	%	n=4	%
Plumage color				
Wild-type	1	100	4	100
Others	0	0	0	0
Mantle				
Rust red to yellow	1	100	4	100
Others	0	0	0	0
Comb type				
Single	1	100	4	100
Others	0	0	0	0
Earlobe color				
White	1	100	4	100
Red	0	0	0	0
Whitish	0	0	0	0
Beak color				
Slate gray	1	100	4	80
Yellow	0	0	0	0
Other	0	0	0	0
Shank color				
Black/slate gray	1	100	4	100
White	0	0	0	0
Yellow	0	0	0	0
Skin color				
White/non-pigmented	1	20	4	100
Yellow	0	0	0	0
Others	0	0	0	0

Appendix table 2.6.3. Subspecies classification based on morphology and mtDNA of the PH RJFs.

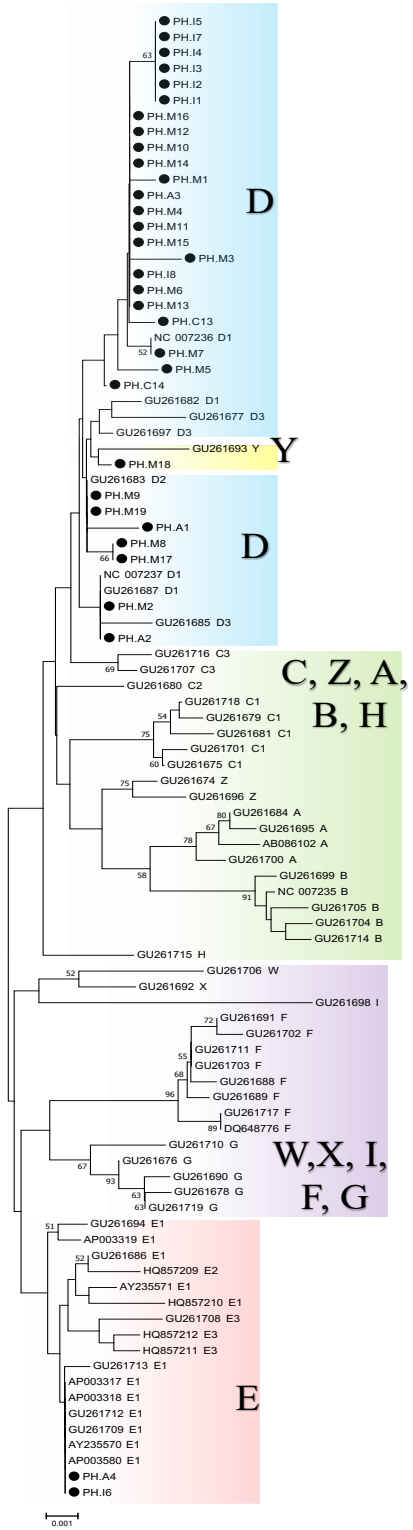
Island Region	PH RJF	Morphology		Genetics	Haplogroup	Accession number
		Earlobe color	Classification	Classification		
Luzon	L1	WR/RW	*G.g.gallus*	*G.g.gallus*	D	OL589006
Luzon	L2	WR/RW	*G.g.gallus*	*G.g.bankiva*	D	OL589007
Luzon	L3	White	*G.g.gallus*	*G.g.gallus*	D	OL589008
Luzon	L4	R	*G.g.bankiva*	*G.g.gallus*	D	OL589009
Luzon	L5	WR/RW	*G.g.gallus*	*G.g.bankiva*	D	OL589010
Luzon	L6	R	*G.g.bankiva*	*G.g.gallus*	D	OL589011
Luzon	L7	WR/RW	*G.g.gallus*	*G.g.gallus*	D	OL589012
Luzon	L8	W	*G.g.gallus*	*G.g.bankiva*	D	OL589013
Luzon	L9	R	*G.g.bankiva*	*G.g.bankiva*	D	OL589014
Luzon	L10	WR/RW	*G.g.gallus*	*G.g.gallus*	D	OL589015
Luzon	L11	R	*G.g.bankiva*	*G.g.gallus*	D	OL589016
Luzon	L12	WR/RW	*G.g.gallus*	*G.g.gallus*	D	OL589017
Luzon	L13	WR/RW	*G.g.gallus*	*G.g.gallus*	D	OL589018
Luzon	L14	WR/RW	*G.g.gallus*	*G.g.gallus*	D	OL589019
Luzon	L15	WR/RW	*G.g.gallus*	*G.g.gallus*	D	OL589020
Luzon	L16	R	*G.g.bankiva*	*G.g.gallus*	D	OL589021
Luzon	L17	R	*G.g.bankiva*	*G.g.bankiva*	D	OL589022
Luzon	L18	W	*G.g.gallus*	*G.g.gallus*	Y	OL589023
Luzon	L19	WR/RW	*G.g.gallus*	*G.g.bankiva*	D	OL589024
Luzon	L20*	WR/RW	*G.g.gallus*	-	-	-
Visayas	V1	WR/RW	*G.g.gallus*	*G.g.gallus*	D	OL589029
Visayas	V2	W	*G.g.gallus*	*G.g.gallus*	D	OL589030
Visayas	V3	W	*G.g.gallus*	*G.g.gallus*	D	OL589031
Visayas	V4	W	*G.g.gallus*	*G.g.gallus*	D	OL589032
Visayas	V5	W	*G.g.gallus*	*G.g.gallus*	D	OL589033
Visayas	V6	W	*G.g.gallus*	*G.g.murghi*	E	OL589034
Visayas	V7	W	*G.g.gallus*	*G.g.gallus*	D	OL589035
Visayas	V8	W	*G.g.gallus*	*G.g.gallus*	D	OL589036
Visayas	V13	R	*G.g.bankiva*	*G.g.bankiva*	D	OL589037
Visayas	V14	WR/RW	*G.g.gallus*	*G.g.bankiva*	D	OL589038
Mindanao	M1	WR/RW	*G.g.gallus*	*G.g.bankiva*	D	OL589025
Mindanao	M2	WR/RW	*G.g.gallus*	*G.g.bankiva*	D	OL589026
Mindanao	M3	WR/RW	*G.g.gallus*	*G.g.gallus*	D	OL589027
Mindanao	M4**	Rvd	*G.g.gallus*	*G.g.murghi*	E	OL589028

** Not successfully sequenced; **hybrid;* W= *white;* R= *red;* WR= *whitish red;* RW= *reddish white;* Rvd=*removed*

Appendix table 2.6.4. Qualitative traits of PH RJFs under different haplogroups.

Parameter	Haplogroup D		Haplogroup E		Haplogroup Y	
	n=30	%	n=2	%	n=1	%
Plumage color						
Wild-type	30	100.00	2	100	1	100
Others	0	0	0	0	-	-
*Eclipse plumage**						
Evident	4	14.81	0	0	-	-
Not evident	19	70.37	1	50	1	100
Hackle						
Golden yellow	19	70.37	0	0	-	-
Orange					1	100
Red	0	0	1	50	-	-
Others	4	14.81	0	0	-	-
*Mantle***						
Rust red to yellow	3	100	1	100	1	100
Others	0	0	0	0	-	-
Comb type						
Single	30	100.00	1	50	1	100
Others	0	0	1	50	-	-
Earlobe color						
White	8	25.81	1	50	1	100
Red	7	22.58	0	0	-	-
Whitish red/reddish white	16	51.61	0	0	-	-
Removed	0	0	1	50	-	-
Beak color						
Slate gray	27	100.00	2	100	1	100
Yellow	0	0	0	0	-	-
Other	0	0	0	0	-	-
Shank color						
Black/slate gray	24	88.89	1	50	1	100
White	3	11.11	0	0	-	-
Yellow	0	0	1	50	-	-
Skin color						
White/non-pigmented	27	100.00	2	100	1	100
Yellow	0	0	0	0	-	-
Others	0	0	0	0	-	-

*male only; **female only



Appendix figure 2.6.1. Neighbor-joining network showing the haplogroup classification of the PH RJFs together with the reference sequences (Miao *et al.,* 2013). *Haplogroup A, B, C, D, E, F, G, H, I, Y, Z . Black dots denote the novel PH RJF samples.*

CHAPTER III

Phylogenetic Studies on Philippine Red Junglefowls and its Relationship with the Junglefowls in Asia based on mtDNA D-loop Region

3.1 Abstract

Red junglefowl (RJF) is considered the ancestor of today's 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 the chicken reference sequences. Phylogenetic analysis revealed multiple maternal origin of the PH RJFs based on its haplogroup D, E, and Y classification. This was supported by the clade sharing of the PH RJFs and the RJFs from other Asian countries. Median-joining network also revealed the haplotype sharing of the PH RJFs and Indonesian RJF demonstrating common maternal ancestry. High haplotype and nucleotide diversity was also observed in all sampling sites. Analysis of molecular variation indicated that the principal molecular variance existed within populations (81.23%) rather than among population (18.77%). Neutrality test and Bayesian Skyline Plot (BSP) analysis elucidated RJF maternal effective population size expansion in the Philippines that possibly started between 2,800-3,000 years before present. Through mtDNA, the co-existence of *the Gallus gallus bankiva* and *Gallus gallus gallus* in the Philippines was also verified. The haplotype sharing of the current RJF samples with the commercial chickens suggested the need to formulate conservation programs that would protect the present and future RJFs in the Philippines.

Keywords: *Gallus gallus gallus*, haplotype, mitochondrial D-loop, Philippines, phylogenetics, red junglefowl

3.2 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 climate and vegetation and are usually exposed to a more stable daily and seasonal temperatures (Beebe, 1926; West and Zhou, 1989). They occur over a wide geographical range just like in Southeast Asia, specifically the eastern and southern most parts of Southeast Asia in the islands of Sumatra and Java to Bali, Sulawesi and the Philippines. RJFs also inhabits the Malay Archipelago, the northern and eastern India, and the Himalayan foothills of northern Pakistan (Nishibori *et al.,* 2005; Bondoc, 2013).

To trace the origin of species domestication, scientists considered the molecular and evolutionary advantages of mitochondrial DNA (mtDNA) (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 as compared to the other mtDNA genome regions. Supporting the hypothesis of 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 or captive, can still be found in the Philippines (PH) Unfortunately, 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 subspecies classification.

Due to insufficient studies and evidence on the genealogy of the PH RJF, its origin and domestication remain uncertain to this day. Therefore, an in-depth molecular study and analysis could help pinpoint its genetic origin and diversity to address this problem. Unfortunately, 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) in the Philippines. Therefore, this study was conducted to provide information on the origin, genetic status, and diversity of PH RJFs and their genetic relationship with the previously identified jungle fowls in Asia. Furthermore, the result of this study could serve as a basis for conservation programs and policies that would be beneficial for the protection of RJFs.

3.3 Materials and Methods

3.3.1 Blood sample collection and DNA extraction

A total of fifty-five (n=55) extracted PH RJF DNA sequences from mountainous areas of the Philippines were classified according to their island region classification (Table 3.1). 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, Inc. Cebu City, Philippines, with reference code: MHAM-060919-01. Furthermore, the collection of wild chickens was also allowed by the Department of Environment and Natural Resources of the Philippines under a Gratuitous permit number 309 and R6-2019-007. DNA extraction was conducted using

the phenol-chloroform method following the protocols of Nishibori *et al*. (2003) and Osman and Nishibori (2014).

Table 3.1. Number and collection sites of Philippine red junglefowls used in this study.

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
	Iloilo	♂	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

Figure 3.1. PH RJFs collected from different areas in the Philippines.

3.3.2 DNA amplification, sequencing, and analysis

DNA concentration and purity were measured using the Thermo Scientific NanoDrop Lite Spectrophotometer. A reading of ≥ 50 ng/µl for DNA concentration and ≥1.80 (A260/A280nm) for DNA purity was considered ideal. Using the adjusted extracted DNA, 5k bp fragments of the mtDNA were amplified using KOD-FX Neo DNA polymerase (KFX-201, TOYOBO CO., LTD., Osaka, Japan). Amplification of the 5k bp fragment used the following primers:

CytbF: 5'TACACGAATCAGGCTCAAACAACCCCCTAGGCATC-3', 16SR: 5'TGCACCATTAGGTTGTCCTGATCCAACATCGAGGT-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 last step was an 8

min final extension of primers at 15°C. The PCR amplification was conducted using the GeneAmp PCR System 9700 (Applied Biosystems, Foster, CA, USA).

Moreover, amplification of the 1.5k bp mtDNA fragment targeting the D-loop region was carried out using the following primers: GalF1: 5'-AGGACTACGGCTTGAAAAGCCATTG – 3', GalR1: 5'-GCTGAGTACCCGTGGGGGTGTGGCT -3' (Nishibori *et al.,* 2001). The PCR amplification of the D-loop fragment PCR amplification was performed in a 20 µl mixture with 0.5 µl template DNA (5.0 k bp), 4.5 µl ddH2O, 10.0 µl 2xPCR buffer, 4.0 µl 2mM dNTPs, 0.3 µl Primer F (10 pmol/ µl), 0.3 µl Primer R (10 pmol/ µl), and 0.4 µl KOD-FX Neo DNA polymerase. The amplification was conducted using GeneAmp PCR System 9700. The PCR cycle profile began with a preliminary denaturation at 94°C for 2 min, followed by 30 cycles of DNA denaturation at 98°C for 10 s, primer annealing at 59°C for 30 s, and extension at 68°C for 30 s. The last step was the final extension of 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 1232 bp-long mtDNA D-loop sequenced data were performed through the window interface progressive multiple sequence alignment program of the molecular evolution genetic analysis (MEGA 7) (Kumar *et al.,* 2016) to improve and refine difficult alignment and trap errors in 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 3.5.2.2 (Excoffier and Lischer, 2010). Furthermore, percent similarity of the PH RJFs with the reference sequences from the RJF subspecies was conducted using BlastN.

Past population dynamics of the Philippine RJFs was 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.

3.4 Results and Discussions

3.4.1 Haplogroup classifications of PH RJFs

Determining the evolutionary relationship of the PH RJFs in this study, the maximum-likelihood phylogenetic tree (Fig. 3.2) showed the classification of PH RJF haplotypes into haplogroups D, E, and Y. The result revealed that the PH RJF haplotypes showed 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 current PH RJF samples under *G.g.gallus* (Nishibori *et al.,* 2005; Godinez *et al.,* 2019), which was also previously 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 result of this study also agreed with the previous findings of Godinez *et al.* (2019) on the haplogroup classification of the RJFs from Samar, Philippines. Five PH RJF haplotypes were also classified under the 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.

On the other hand, the PH RJF11 haplotype of 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 of PH RJF11 haplotype with subhaplogroup E1 suggests its close genetic relationship with the domestic and commercial chickens from India and China. This result also provided evidence on 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 (Appendix table 3.6.1).

Although the RJF is the main ancestral contributor to chicken genetic diversity, post-domestication events involving crosses with other junglefowl species also took place (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 result of this study proved otherwise. On the other hand, haplogroup H was a rare haplogroup that was notably present in fighting cocks (Hata *et al.,* 2021).

A median-joining network was constructed to support the haplogroup classification of the PH RJFs (Fig. 3.3). In this figure, 21.82% of the total RJF samples in this study shared the same haplotype as the 61 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. Though the PH haplotypes were categorized under haplogroup D, only 1.82% and 3.64% shared the same haplotype with the wild junglefowl from the Philippines and Indonesia, respectively. The remaining 78.18% of the total PH RJFs formed a unique haplotype not similar with the reference sequences. On the basis of this haplotype sharing, the result suggested that the PH RJFs could have come from Indonesia. However, a larger data set is needed to confirm this claim. Although the result in Fig. 3.2 showed 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. 3.3).

Furthermore, the result of this study agreed with 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.

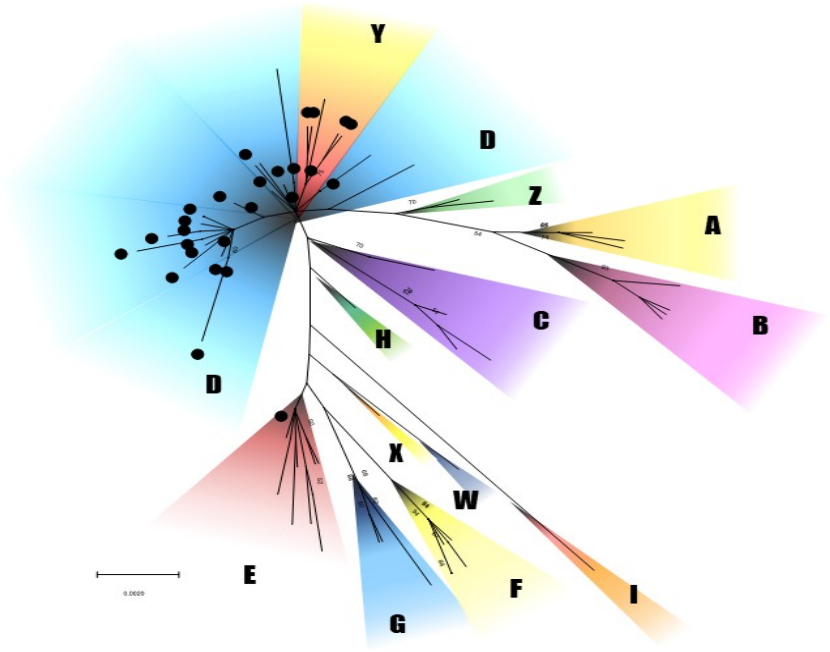


Figure 3.2. Phylogenetic tree of PH RJF haplotypes together with the 61 reference sequences (Miao *et al.,* 2013) based on the ML method. *Black dots denote PHRJF haplotypes. Haplogroups A, B, C, D, E, F, G, H, I, X,Y, Z are denoted with different colors for differentiation.*

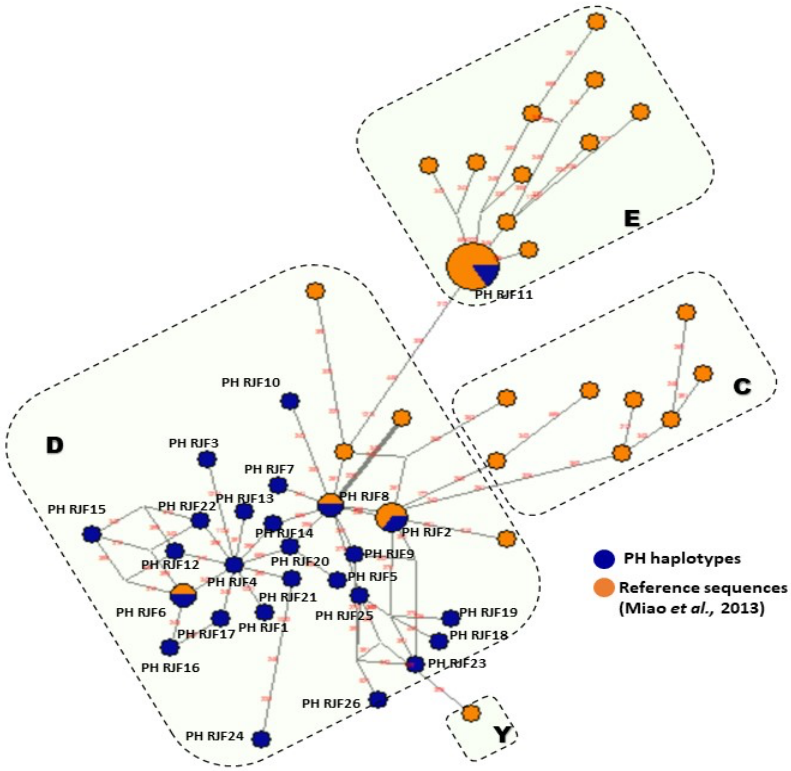


Figure 3.3. Median-joining network of PH RJF haplotypes with haplogroup D, E and Y reference sequences from Miao *et al.,* (2013)

3.4.2 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 3.2 showed a total of 30 variable sites and 314 total polymorphisms detected in the RJF sequence samples in this study as inferred with the reference sequence (NC_007235). The result revealed 0.64% and 99.36%, transversion and transition mutation, respectively. The highest number of substitutions was observed in PH RJF3, a unique haplotype that did not share the same haplotype with the reference sequences from Miao *et al.* (2013). High nucleotide substitution rate is common in mtDNA (Brown *et al.,* 1982), thus supporting the result 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 on all PH RJF sampling sites.

Table 3.2. Observed sequence variation among PH RJF haplotypes mitochondrial D-loop sequences.

Haplotype	N	Luzon	Visayas	Mindanao	21	21	22	22	24	44	44	55	61	70	81	96	06	01	15	24	34	55	51	69	99	44	47	51	68	102	115	131	141	152	No of polymorphism
					27	12	25	26	41	43	66	66	10	07	18	69	60	10	55	21	44	51	95	16	99	46	71	86	53	24	31	41	51	62	
NC 007235	-	-	-	-	A	T	C	A	A	T	T	T	C	T	A	C	T	C	T	A	T	T	C	T	G	C	T	A	G	T	T	C	A	T	30
PH RJF1	1	1			G	.	.	.	.	C	C	C	T	.	G	T	C	T	C	.	.	.	.	.	.	.	.	.	A	.	.	.	G	A	13
PH RJF2	2	1		1	G	.	.	.	.	C	C	C	T	.	G	.	C	T	C	G	.	.	.	.	.	.	.	.	.	.	.	.	G	.	11
PH RJF3	1	1			G	.	.	.	.	C	C	C	T	.	G	T	C	T	C	.	.	.	.	.	.	.	.	.	A	.	G	G	G	.	15
PH RJF4	12	9	2	1	G	.	.	.	.	C	C	C	T	.	G	T	C	T	C	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	12
PH RJF5	1	1			G	.	.	.	.	C	C	C	T	.	G	T	C	T	C	.	.	C	.	.	.	.	.	.	.	.	.	.	G	.	12
PH RJF6	1	1			G	.	.	.	.	C	C	C	T	.	G	T	C	T	C	G	.	.	.	.	.	.	.	.	A	.	.	.	G	.	13
PH RJF7	2	2			G	.	.	.	G	C	C	C	T	.	G	.	C	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	11
PH RJF8	7	5	2		G	.	.	.	.	C	C	C	T	.	G	.	C	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	10
PH RJF9	1	1			G	.	.	.	.	C	C	C	T	.	G	.	C	T	C	.	.	.	T	.	.	.	.	.	.	.	.	.	G	.	11
PH RJF10	1			1	G	.	.	.	.	.	C	C	T	.	G	.	C	T	C	.	C	.	.	.	.	.	.	.	.	.	.	.	G	.	10
PH RJF11	2		1	1*	G	C	.	.	.	C	C	C	T	.	.	.	.	T	C	.	.	.	.	.	.	T	.	.	.	.	.	T	G	.	11
PH RJF12	7		7		G	.	.	.	.	C	C	C	T	.	G	T	C	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	11
PH RJF13	1		1		G	.	.	.	.	C	C	C	T	.	G	T	C	T	C	.	.	.	T	.	.	.	.	.	A	.	.	.	G	.	13
PH RJF14	1		1		G	.	.	.	.	C	C	C	T	.	G	.	C	T	C	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	11
PH RJF15	2		2		G	.	.	.	.	C	C	C	T	.	G	T	C	.	C	G	.	.	.	.	A	.	.	.	A	.	.	.	G	.	13
PH RJF16	1		1		G	.	.	.	.	.	C	C	T	.	G	T	C	T	C	G	.	.	.	.	.	.	.	.	A	.	.	.	G	.	12
PH RJF17	1		1		G	.	.	.	.	.	C	C	T	.	G	T	C	T	C	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	11
PH RJF18	1		1		G	.	.	.	.	C	C	C	T	C	G	.	C	T	C	.	.	.	.	C	.	.	C	.	.	.	.	.	G	.	13
PH RJF19	1		1		G	.	.	G	.	C	C	C	T	C	G	.	C	T	C	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	13
PH RJF20	1		1		G	.	.	.	.	C	C	C	T	.	G	T	C	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	11
PH RJF21	2		2		G	.	.	.	.	C	C	C	T	.	G	T	C	T	C	.	.	.	.	C	.	.	.	.	A	.	.	.	G	.	13
PH RJF22	1	1			G	.	.	.	.	C	C	C	T	.	G	T	C	T	C	.	.	.	.	.	A	.	.	.	A	.	.	.	G	.	13
PH RJF23	2	2			G	.	.	.	.	C	C	C	T	C	G	.	C	T	C	G	.	.	T	.	.	.	.	.	.	.	.	.	G	.	13
PH RJF24	1	1			G	.	T	.	.	C	.	C	T	.	G	T	C	T	C	.	.	.	.	C	.	.	.	.	A	C	.	.	G	.	14
PH RJF25	1	1			G	.	.	.	.	C	C	C	T	C	G	.	C	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	11
PH RJF26	1	1			G	.	.	.	.	C	C	C	T	C	G	.	C	T	C	.	.	.	T	.	.	.	.	G	.	.	.	.	G	.	13
TOTAL	55	28	23	4																															

**=hybrid RJF. N= number of individuals sharing the same haplotype. Dots (.) = denotes identity with the reference sequence.*

3.4.3 Genetic diversity of the PH RJFs

The genetic diversity of this study revealed that the Agusan del Norte and Capiz RJF population have the highest haplotype diversity among the PH RJF populations studied (Table 3.3). This is probably due to the wide haplotype distribution of the RJFs in these two populations brought about by the small sample number that were sampled from a broader geographical location. In addition, Agusan del Norte also has the highest nucleotide diversity (0.1333±0.1159). The high genetic diversity in this study was supported by 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.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 in the conceptualization and management of species conservation (Milligan *et al.,* 1994). Thus, the result of this study suggested that the genetic diversity of the PH RJFs is not at risk.

This study also conducted a pairwise distance test analysis of the different RJF populations to determine which population appeared to be the closest (Table 3.4). High genetic differentiation was observed between the Guimaras and Leyte RJF populations. This is supported by the distant geographical distance of these two locations, leading to population differentiation. The negative and zero *Fst* values in this study suggested high genetic sharing among the population. On the other hand, Capiz and Iloilo RJF populations were expected to have the lowest *Fst* value due to their site proximity.

However, the result of this analysis showed otherwise. Furthermore, *Fst* analysis between collection sites is supported by the ML tree based on the maximum composite likelihood pairwise distance estimation (Figure 3.4). In this figure, low *Fst* distance difference between Occidental Mindoro, Capiz, and Iloilo is supported by the clad sharing of these collection sites.

Table 3.3. Haplotype and nucleotide diversity of PH RJFs.

Population	N	PH RJF haplotypes	Hd	π	Mean pairwise difference
Occidental Mindoro	19	9	0.7778 ±0.0956	0.0021±0.0013	2.6082±1.4591
Agusan del Norte	3	3	1.0000 ± 0.2722	0.1333±0.1159	3.3333±2.3231
Iloilo	8	3	0.4643 ± 0.2000	0.0020±0.0014	2.4286±1.4679
Capiz	2	2	1.0000 ± 0.5000	0.0024±0.0028	3.0000±2.4495
Guimaras	4	3	0.8333 ± 0.2224	0.0020±0.0016	2.5000±1.6855
Leyte	8	6	0.9286 ± 0.0844	0.0031±0.0020	3.8214±2.1474
Palawan	10	7	0.9111 ± 0.0773	0.0036±0.0022	4.4222±2.3822

N= number of samples; Hd = haplotype diversity; π= nucleotide diversity.

Table 3.4. Pairwise distance (*Fst*) difference between RJF populations in the Philippines.

RJF Population	Palawan	Leyte	Guimaras	Capiz	Iloilo	Agusan del Norte
Occidental Mindoro	0.1144	0.10159	0.32749	-0.0817	0.22274	-0.0373
Agusan del Norte	-0.0550	-0.0039	0.2222	0.0000	0.2397	-
Iloilo	0.32126	0.26081	0.25143	0.16429	-	-
Capiz	0.05364	0.11688	0.30769	-	-	-
Guimaras	0.36959	0.38776	-	-	-	-
Leyte	0.03114	-	-	-	-	-

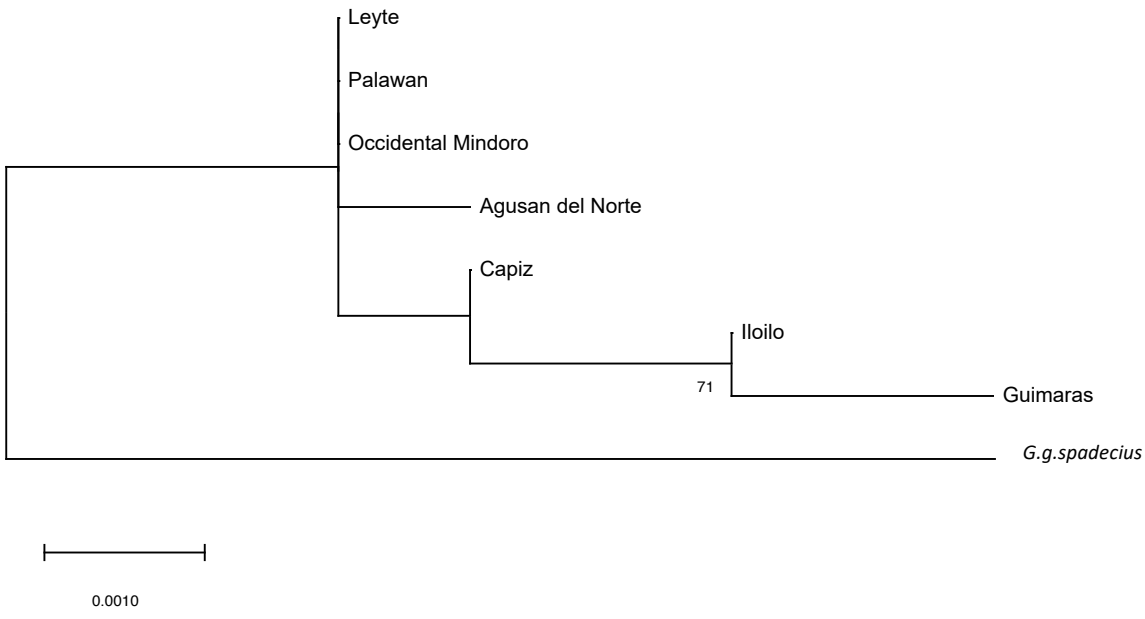


Figure 3.4. Maximum likelihood tree of the PH RJFs based on collection site.
Bootstrap value lower than 50% was removed.

3.4.4 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 AMOVA analysis result of this study revealed 18.77% and 81.23% between and within the variation of the PH RJF population, respectively (Table 3.5). The low genetic differentiation between populations suggested that the RJF population under study had not been subdivided between regions. On the other hand, the high percentage of variation among populations could also be due to the high genetic recombination within the PH RJF populations and a relatively high degree of gene flow between them, thus, preventing genetic differentiation (Bekerie *et al.,* 2015).

To infer and analyze signatures of historical demographic events of the PH RJFs, the population neutrality test (Sharma *et al.,* 2013) was conducted. The negative Tajima's *D* test (Tajima, 1993) for the Occidental Mindoro (-0.9534), Palawan (-0.46000), 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 suggested that this RJF population is evolving according to mutation-drift equilibrium (Joshi *et al.,* 2013), probably due to the small sample size (n=2) analyzed in this area.

On the other hand, Fu's *Fs* test is a better fit for a larger sample size since 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 on its population expansion (Lopez and Lama, 2007) thus, dismissing the possibility of genetic hitchhiking, background selection, and evolutionary forces that produced 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 *Fs* (-19.3980) (p=0.0000) suggested a population expansion of Philippines RJFs analyzed in this study (Table 6).

To support the result of the neutrality analysis of this study, a Bayesian skyline plot (BSP) analysis was carried out to further assess the population expansion of the PH RJFs. Utilizing the total 55 PH RJFs, including the hybrid PH RJF, with 95% high posterior density interval. The overall BSP result pointed out an increase in maternal effective population size between 2,800-3,000 years before present (Fig. 3.5).

Table 3.5. Analysis of molecular variation within and between PH RJF populations.

Source of the sum of variance percentage	Sum of squares	Variance components	Percentage of variation
Among populations	19.62	0.289	18.77
Within populations	58.25	1.24	81.23
Total	77.87	1.53	-

Table 3.6. Test of neutrality within Philippine RJF populations.

Population	Tajima's *D*	Tajima's *D* p-value	Fu's *Fs*	Fu's *Fs* p-value
Occidental Mindoro	-0.9534	0.1660	-3.7839	0.0060
Palawan	-0.4600	0.3170	-1.6400	0.1200
Iloilo	-1.4213	0.0850	1.7637	0.8280
Capiz	0.0000	1.0000	0.6932	0.3460
Leyte	0.2775	0.66130	-1.7245	0.0860
Guimaras	1.3652	0.8550	0.4611	0.5310
Agusan del Norte	-0.8338	1.0000	-0.0770	0.2160
Philippines (overall)	-1.5013	0.0400	-19.3980	0.0000

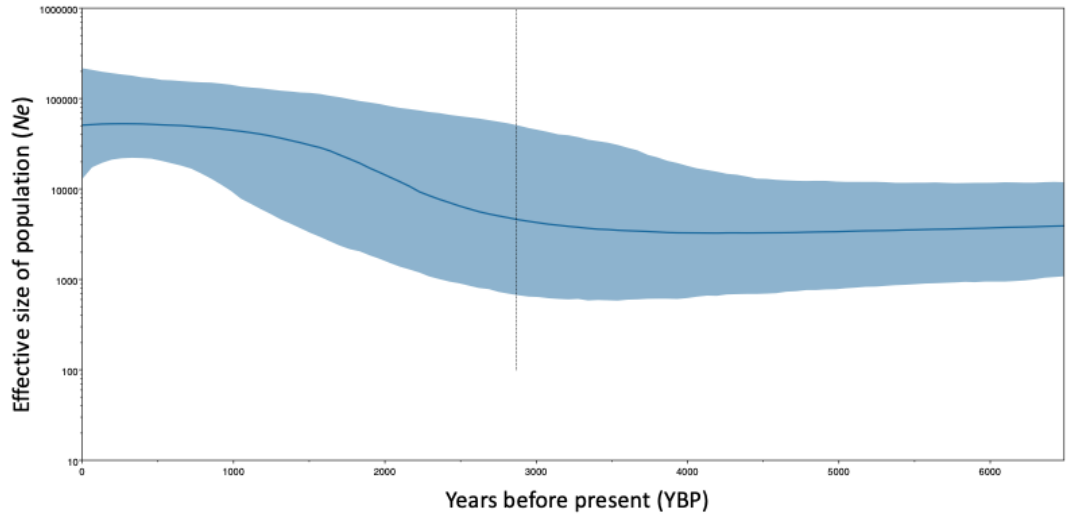


Figure 3.5. Bayesian Skyline plot showing the population expansion of RJFs in the Philippines. *The light blue line represents the median estimate effective population size, while the light blue shade represents the 95% high posterior density interval.*

3.4.6 Relationship of PH RJF with the different *G.gallus* species and RJF subspecies from different Asian countries

To elucidate the genetic relationship of the PH RJFs with the RJFs from different Asian countries, an ML tree (Fig. 3.6A) was constructed based on Tamura-Nei model (Tamura and Nei, 1993). Citing the relationship of the current PH RJF samples with the different RJF sequences in Asia, the results have shown that majority of the PH RJF sequences in this study shared the same clade with NC_007236 (Nishibori *et al.,* 2005), an RJF from the Philippines. To support the phylogenetic tree of this study, 54.55% of the total PH RJF sequences were 99.81% by average identical with NC_007236.

Furthermore, the clade sharing of PH RJFs with KY039428, an Indonesian RJF also supported the result shown in Fig. 3.2. The close percent homology of the minority of the PH RJFs in this study with the RJF from Indonesia could be due to the gene flow of RJF genes from the Philippines to Indonesia or vice versa brought by geographical proximity of the two countries and human migration. According to Tan-Cullamar (1993), one of the factors supporting the Indonesian migration to southern Mindanao in the 1900s is the geographical proximity, similar climate environment, and resources, thus providing clues on the genetic proximity and sharing of the RJF gene pool of the Philippines and Indonesia.

Tracing the relationship of the PH RJFs with the different *G.gallus* species and subspecies, the results have shown (Fig. 3.6B) close genetic relationship of the PH RJF haplotypes with *G.g.gallus* (AP003322) and *G.g.bankiva* (AP003323) with the exception of the PH RJF11 haplotype which formed a close genetic relationship with *G.g.murghi* (GU261709). Determining the similarity of the current PH RJFs with the

different *G.gallus* species and RJF subspecies using BlastN, the results had shown that majority (50%) of the detected PH RJF haplotypes shared 99.76% average similarity with *G.g.gallus* (AP003322). On the other hand, 46.15% of the total PH haplotypes shared 99.77% similarity with *G.g.bankiva* (AP003323). Compendio and Nishibori (2021) previously reported 99.84% similarity of a Philippine RJF (NC_007236) sequence with a *G.g.bankiva* (AP003323) sequence. This result also suggested possible gene flow and admixture between these RJF subspecies in the Philippines, where they coexist.

Although *G.g.gallus* and *G.g.spadiceus* have little genetic difference with each other (Lawal *et al.,* 2020), the results of this study have shown that none of the PH RJFs forms close genetic relationship with *G.g.spadiceus*, suggesting no relationship between the PH RJFs and this particular subspecies. Furthermore, Wang *et al.* (2020) suggested that domestic chickens were initially derived from the RJF subspecies *G.g. spadiceus*, distributed in southwestern China, northern Thailand, and Myanmar. However, the domestic chickens detected in this study do not share the same clade with *G.g. spadiceus*, suggesting that the former might have been derived from a different domestication center as well as species of origin. Furthermore, the result of this analysis further suggested that the wild chickens in the Philippines are not classified under *G.g. philippensis* as reported previously by Hachisuka (1939) and Bondoc (2013).

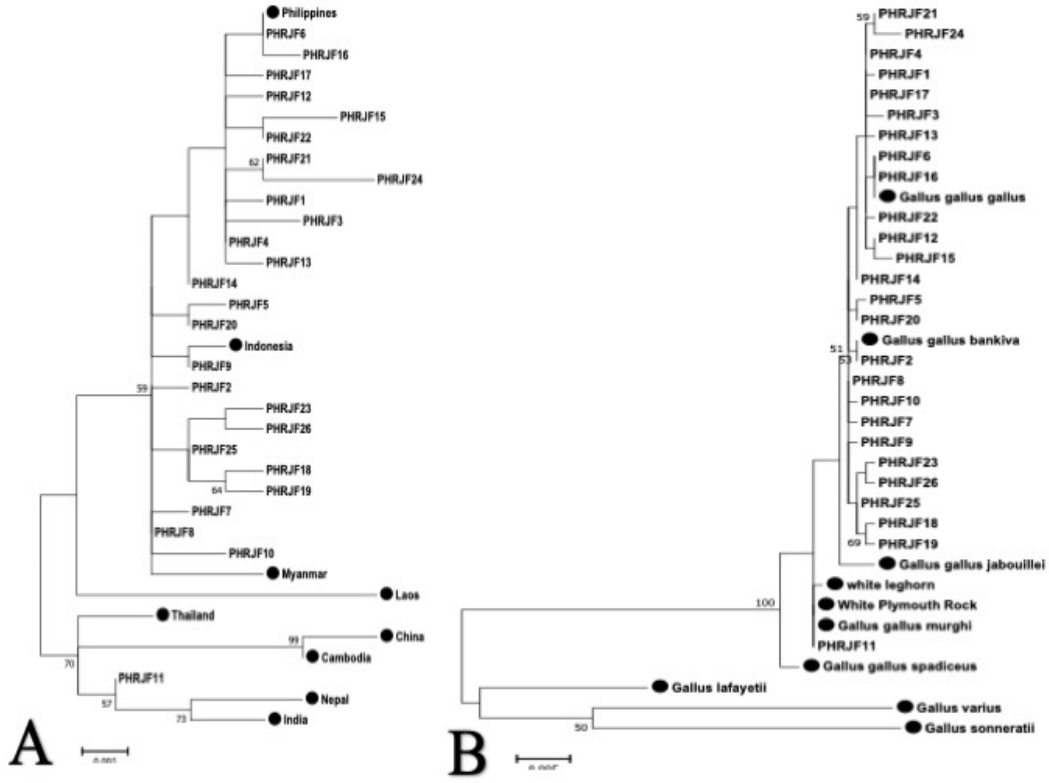


Figure 3.6. Phylogenetic tree between the (A) PH RJFs in relation to RJFs from different Southeast Asian countries, and (B) between the 26 PH RJF haplotypes and different *G.g.* species and subspecies. *Black dot denotes reference sequences.*

3.5 Conclusion and Recommendations

The result of this study elucidates the subspecies classification of the PH RJFs under *G.g.gallus* and *G.g.bankiva* suggesting that the Philippines has no separate RJF subspecies. This study also suggests the Southeast Asian origin of the PH RJFs. Given the high genetic and nucleotide diversity as well as population expansion of the PH RJFs, this study suggests that the RJFs in the Philippines are not at risk of endangerment. However, haplogroup sharing with the commercial chickens is of biodiversity concern. The need for RJF conservation programs that would prevent gene flow of genetic material from domestic chickens to RJFs is suggested. Moreover, the important role of mtDNA D-loop control region in tracing the possible maternal origin of the PH RJFs, as well as their phylogenetic interrelationships with the different RJF populations in Asia is also emphasized in this study. The need for a wider sampling site coverage is recommended to further verify the claims made in this study.

3.6 Appendices

Appendix table 3.6.1. Philippine RJF distribution based on their haplotype classification and its polymorphic sites in relation to the NC 007235 reference sequence.

Haplotype	Luzon	Visayas	Mindanao	Variable sites
PHRJF1	PH.M1	-	-	212, 243, 246, 256, 261, 281, 296,306, 310, 315, 686, 1215, 1221
PHRJF2	PH.M2	-	PH.A2	212, 243, 246, 256, 261, 281, 306, 310, 315, 342, 1215
PHRJF3	PH.M3	-	-	212, 243, 246, 256, 261, 281, 296,306, 310, 315, 686, 1134, 1214, 1215,
PHRJF4	PH.M4, PH.M6, PH.M10, PH.M11, PH.M12, PH.M13, PH.M14, PH.M15, PH.M16,	PH.I8, PH.P17	PH.A3	212, 243, 246, 256, 261, 281, 296,306, 310, 315, 686, 1215
PHRJF5	PH.M5	-	-	212, 243, 246, 256, 261, 281, 296, 306, 310, 315, 355, 1215
PHRJF6	PH.M7	-	-	212, 243, 246, 256, 261, 281, 296,306, 310, 315, 342, 686, 1215
PHRJF7	PH.M8, PH.M17	-	-	212, 241, 243, 246, 256, 261, 281, 306, 310, 315, 1215

PHRJF8	PH.M9, PH.M19, PH.P13, PH.P14, PH.P15	PH.L8, PH.L9,	-	212, 243, 246, 256, 261, 281, 306, 310, 315,1215
PHRJF9	PH.M18	-	-	212, 243, 246, 256, 261, 281, 306, 310, 315, 319,1215
PHRJF10	-	-	PH.A1	212, 246, 256, 261, 281, 306, 310, 315,354, 1215
PHRJF11	-	PH.I6	PH.A4	212, 217, 243, 246, 256, 261, 310, 315, 446,1214, 1215
PHRJF12	PH.I1, PH.I2, PH.I3, PH.I4, PH.I5, PH.I7,	PH.L3	-	212, 243, 246, 256, 261, 281, 296,306, 315, 686, 1215
PHRJF13	-	PH.C13	-	212, 243, 246, 256, 261, 281, 296,306, 310, 315, 391, 686, 1215
PHRJF14	-	PH.C14	-	212, 243, 246, 256, 261, 281, 306, 310, 315, 686, 1215
PHRJF15	-	PH.G73, PH.G74	-	212, 243, 246, 256, 261, 281, 296,306, 315, 342, 399, 686, 1215
PHRJF16	-	PH.G75	-	212, 246, 256, 261, 281, 296,306, 310, 315,342, 686, 1215

Continuation…..

Haplotype	Luzon	Visayas	Mindanao	Variable sites
PHRJF17	-	PH.G76	-	212, 246, 256, 261, 281, 296,306, 310, 315, 686, 1215
PHRJF18	-	PH.L1	-	212, 243, 246, 256, 261, 270, 281, 306, 310, 315, 396, 447, 1215
PHRJF19	-	PH.L2	-	212, 226, 243, 246, 256, 261, 270, 281, 306, 310, 315, 391, 1215
PHRJF20	-	PH.L4	-	212, 243, 246, 256, 261, 281, 296,306, 310, 315, 1215
PHRJF21	-	PH.L5, PH.L6	-	212, 243, 246, 256, 261, 281, 296,306, 310, 315, 396, 686, 1215
PHRJF22	PH.P10	-	-	212, 243, 246, 256, 261, 281, 296,306, 310, 315, 399, 686, 1215
PHRJF23	PH.P11, PH.P12	-	-	212, 243, 246, 256, 261, 270, 281,306, 310, 315, 342, 391, 1215
PHRJF24	PH.P18,	-	-	212, 225, 243, 256, 261, 281, 296,306, 310, 315, 396, 686, 1052, 1215
PHRJF25	PH.P19	-	-	212, 243, 246, 256, 261, 270, 281, 306, 310, 315, 686, 1215
PHRJF26	PH.P20	-	-	212, 243, 246, 256, 261, 270, 281, 306, 310, 315, 391, 521, 1215

CHAPTER IV

Evolutionary Relationship of the Philippine and Indonesian Red Junglefowls

4.1 Abstract

Red junglefowls (RJF) inhabits the forested areas in Southeast Asian countries including the Philippines (PH) and Indonesia (IND). Given the geographical proximity of the Philippines and Indonesia, the evolutionary relationship of the RJFs in these two countries and its relationship with the RJFs from other Asian countries is still unresolved. Utilizing the phylogenomic advantage of the mitochondrial DNA (mtDNA), a phylogenetic analysis was conducted on the RJFs (N=80) from Indonesia and the Philippines in this study. The results revealed the classification of IND RJFs in haplogroup D in contrast to the haplogroup D, Y and E classification of the PH RJFs. The haplotype sharing of the IND RJFs with a *G.g.bankiva* from Indonesia aside from the domestic chickens from Laos and China suggested multiple maternal origin of the IND RJFs. The possible maternal origin sharing of the PH and IND RJF is supported by its 0.27 *Fst* pairwise distance difference. The high haplotype and nucleotide diversity which is essential for biodiversity was also observed in PH and IND RJFs. The negative neutrality test result of this study elucidated population expansion of the two RJF populations. Coalescent time estimates revealed earlier divergence of PH RJFs (~7.14kya) than IND RJFs (~5.1kya). This study also revealed separate migration of RJFs from the Philippines to Indonesia, and Philippines to Polynesia. The importance of mtDNA in determining the population demography and evolutionary relationship of the Philippine and Indonesian RJFs was also highlighted in this study.

Keywords: *G.g.bankiva, G.g.gallus, Indonesia, mtDNA, Philippines, RJF*

4.2 Introduction

The forest dwelling junglefowls in Asia are mainly composed of four different species, the *Gallus gallus*, *Gallus lafayettei*, *Gallus varius*, and *Gallus sonneratii*. Presently, there are five subspecies of red junglefowls (RJFs), the *Gallus gallus gallus* (*G.g. gallus*), and *Gallus gallus spadiceus* (*G.g.spadiceus*), *Gallus gallus bankiva* (*G.g. bankiva*), *Gallus gallus murghi (G.g.murghi*) and *Gallus gallus jabouillei* (*G.g. jabouillei*) (Niu *et al.,* 2002; Sawai *et al.,* 2010). Among these RJF subspecies, Fumihito *et al.* (1994;1996) suggests that the *G.g. gallus* subspecies, an Indochinese red Junglefowl, is the primary maternal ancestor of the domestic chicken. However, current findings suggested that *G.g.spadiceus* is the maternal origin of the domestic chickens (Wang *et al.,* 2020).

RJF utilized a variety of habitats, but are thought to prefer extensive, undisturbed mixed forests for foraging and breeding (Ali and Ripley, 1989). They are also found in areas with flat or gently sloping terrain, forest edges and secondary forest (del Hoyo *et al.,* 2001). Sighted in the mountainous areas in South and Southeast Asia, these RJFs were also documented and reported in the Philippines and Indonesia (Nishibori *et al.,* 2005; Bondoc, 2013; Ulfah *et al.,* 2016).

Although these RJFs still inhabits the forests in the Philippines, its sightings are now becoming uncommon due to natural and anthropogenic habit destruction, and poaching (Masangkay *et al.,* 2010). In Indonesia, wild RJFs were reported to inhabit Sumatra, Java, and Madura islands, alongside the green junglefowls (Ulfah *et al.,* 2016). Moreover, *G.g.bankiva* was reported to be an endemic RJF in Indonesia which was described by Nishida *et al.* (1958) as an RJF with red earlobes and round hackles.

Mitochondrial DNA (mtDNA) is widely used in phylogenetic studies due to its high copy number, rapid and clockwise evolutionary rate for divergence time estimation (Brown *et al.,* 1979). This genetic marker was also used to study the phylogeographic structures and diversity in avian species (Desjardins and Morais, 1990; Oka *et al.,* 2007; Miao *et al.,* 2013), as well as in the phylogenetic analysis of Southeast Asian RJFs (Osman and Nishibori, 2014).

Genetically, molecular studies using mtDNA have already been conducted in RJFs in the Philippines (Bondoc, 2013; Godinez *et al.,* 2019). Although the results reported ambiguous classifications, these studies verified the existence of wild chickens in the country. Furthermore, studies have already been conducted on the genetic characteristics of red and green junglefowl (Ulfah *et al.,* 2016) and Indonesian indigenous chickens using mtDNA (Sulandari *et al.,* 2008). However, no phylogenetic studies that focuses solely on Indonesian RJFs have been reported to date.

Due to the geographical proximity of the Philippines and Indonesia, this study aimed to determine the evolutionary relationship of RJFs in these two countries while utilizing the phylogenomic advantage of mtDNA. Knowledge about their diversity and relationship could help us understand the complex genetic relationship of RJFs in Asia.

4.3 Materials and Methods

4.3.1 RJF sample collection and DNA extraction

A total of 25 IND (Appendix table 4.6.1) and 55 PH RJF sequences (Appendix table 4.6.2) were used in this study. All IND RJF samples were collected from the different areas in Sulawesi Indonesia, while all PH RJF samples were collected from the three island regions in the Philippines (Luzon, Visayas and Mindanao). All DNA samples were extracted using the phenol-chloroform method following the protocol described by Green and Sambrook (2002) and Nishibori *et al.* (2002; 2003).

4.3.2 DNA extraction from feather samples

A total of 22 individual feathers from 18 fowls kept in different storage periods and conditions were used in this study (Appendix figure 4.6.1). The samples were geographically collected from: Nepal (n=2), Malaysia (n=3), Indonesia (n=4), Chiba zoological park (n=1), and Philippines (n=8). To prevent deterioration, the majority of these feather samples were obtained from various countries and zoos and preserved in a -210C non-frozen freezer, while others were simply stored at room temperature. The oldest sample analyzed in this study was a Malaysian fowl feather (Figure 4.1).

The feathers used in this study were divided mainly into two main regions, the calamus and the barbs. Prior to DNA extraction, the feather parts of interest were individually prepared, cut and washed with 70% ethanol for 15-20 min and washed with distilled water for 3-5min thereafter. Pre-extraction cleaning of samples was conducted to avoid possible contamination brought by the adhering exogenous materials. The calamus of the feather samples was cut off longitudinally

and furtherly diced with a sterile surgical scissor and forceps to help facilitate enzymatic digestion. Moreover, 3-5 barbs of the feathers were also used and reduced to 2-3mm for the same purpose.

DNA from feather samples were extracted using the ISOHAIR: Hair and nail (DNA extraction kit protocol) of the Nippon Gene Co., LTD with the addition of phenol-chloroform/isoamyl alcohol. Since the calamus and the barbs were heavily keratinized old samples, the initial incubation period was changed to 60^0C for 30 min. The second incubation was also modified to 60^0C for 24 hrs. instead of 10 min under 55^0C incubation temperature.

Figure 4.1. Malaysian fowl feather (MY5) from an unidentified fowl preserved for 47 years.

4.3.3 DNA amplification, sequencing and analysis

mtDNA amplification was conducted using KOD-FX Neo DNA polymerase (KFX-201, TOYOBO CO., LTD., Osaka, Japan) following the procedure described by Nishibori *et al.* (2001), and Osman and Nishibori (2014).

Amplification of the 5k bp mtDNA fragment was conducted using the following primers:

CytbF: 5'TACACGAATCAGGCTCAAACAACCCCCTAGGCATC-3', 16SR: 5'TGCACCATTAGGTTGTCCTGATCCAACATCGAGGT-3' (Nishibori *et al.,* 2001). The PCR amplification was conducted in a 20 µl volume of mix containing 1.0 µl genomic DNA, 3.6 µl ddH_2O, 10.0 µl 2xPCR buffer, 4.0 µl 2µM dNTPs, 0.6 µl F primer (10 pmol/ µl), 0.6 µl R primer (10 pmol/ µl), and 0.2 µl KOD-FX Neo polymerase. The reaction begun with a preliminary denaturation at 94°C for 2 min, followed by 30 cycles of DNA denaturation at 98°C for 10 sec, annealing of primers at 57°C for 30 sec, and primer extension at 68°C for 2 min and 30 sec. The last step was an 8 min final extension of primers at 15°C. The PCR amplification was conducted using the GeneAmp PCR System 9700 (Applied Biosystems, Foster, USA).

Amplification of the complete mtDNA D-loop region with using Gal1F: 5'AGGACTACGCTTGAAAAGCCATTG-3' and Gal1R 5'GCTGAGTACCCGTGGGGGTGTGGCT-3' (Nishibori *et al.,* 2001) was also conducted using the amplified DNA product from 5k bp amplification. The D-loop fragment PCR amplification was performed in a 20 µl volume of mix with 0.5 µl template DNA (5.0k bp), 4.5 µl ddH_2O, 10.0 µl 2xPCR buffer, 4.0 µl 2µM dNTPs, 0.3 µl F primer (10 pmol/ µl), 0.3 µl R primer (10 pmol/ µl and 0.4 µl KOD-FX Neo polymerase. The amplification was conducted using GeneAmp PCR System 9700 (Applied Biosystems, Foster, USA). The PCR cycle profile begun with a preliminary denaturation at 94°C for 2 min, followed by 30 cycles of DNA denaturation at 98°C for 10 sec, annealing of primers at 59°C for 30 sec, and primer

extension at 68°C for 30 sec. The last step was the 5 min final extension of primers at 68°C and 15°C PCR mixture incubation. The following primers: GalF1-25′ - TCCACCTCACGAGAGATCAGCAACCC-3′ and GalR1-2 (Gal1R) 5′ - TTTGGTAGTGGAGTTTCTC-TAATAA-3′ (Nishibori *et al.,* 2001) were used in sequencing reactions.

The successfully sequenced IND RJFs (n=25), PH RJFS (n=55) and feather samples (n=11) were cleaned and translated using GENESTUDIO^{TM}. Molecular evolutionary Genetic Analysis of the molecular evolution (MEGA X) (Kumar *et al.,* 2018) was used to align all DNA sequences in this study. The genetic distance matrix analysis between RJFs and reference sequences (Appendix table 4.6.3; 4.6.4) was carried out using Maximum likelihood (ML) method, following the General Time Reversible model. The bootstrap consensus tree inferred from 3000 replicates is taken to represent the evolutionary history of taxa analyzed. Branches with bootstrap value <50% was removed. Genetic and nucleotide diversity as well as haplotyping was conducted using DnaSP v6.12.03. The analysis of molecular variance (AMOVA) within and between RJF populations, and test for neutrality was conducted using the Arlequin ver 3.5.2.2 (Excoffier and Lischer, 2010). Network 10.1.0.0 was used to create the median-joining network. Divergence time analysis was conducted using BEAST v. 2.6. 6 (Bouckaert *et al.,* 2019), following the method described by Godinez *et al*. (2021). Tracer v.1.7.2 (Rambaut *et al.,* 2018) was used in the analysis of the generated Markov chain Monte Carlo (MCMC) trace files. FigTree v1.4.4 was used in the visualization of the annotated phylogenetic tree produced from BEAST v. 2.6. 6.

4.4 Results and Discussion

4.4.1 Evolutionary relationship of PH and IND RJFs

All detected IND RJF haplotypes in this study were classified under haplogroup D, forming the same cluster with a wild chicken from Indonesia (NC_007237), and domestic chickens from Laos, China, and Northeast India (Miao *et al.,* 2013). This clustering revealed multiple maternal origin of the IND RJFs (Fig. 4.2). Moreover, none of the IND RJFs were clustered with the reference sequences under subhaplotype E1. This study also revealed that 76.92% of the total PH RJF haplotypes shared the same clade with the wild chicken reference sequences from the Philippines and Indonesia, as well as with the domestic chickens from Laos, India and China in haplogroup D. On the other hand, 19.23% of the PH RJF haplotypes were classified in haplogroup Y, together with a wild chicken from China, and only 3.85 % formed the same cluster with the commercial and domestic chickens from India and China in haplogroup E. The classification of the PH and IND RJFs with haplogroup D agreed with Miao *et al.* (2013) on the distribution of chickens in Asia.

The ML tree (Fig. 4.2) of this study is supported by the median-joining network (Fig. 4.3) which revealed the haplotype sharing of the PH and IND RJF haplotypes with an RJF from Indonesia (NC_007237) and a domestic chicken from Laos (GU261687). Although some PH RJF haplotypes shared the same haplogroup with the wild chickens of China, the median-network joining results showed no haplotype sharing between the PH RJFs and the haplogroup Y reference sequence. The result also showed that none of the IND RJFs shared the same haplotype with other RJF subspecies reference sequences except with the *G.g. bankiva* from

Indonesia. In addition, 92.31% of the IND RJFs in this study were classified as *G.g. bankiva* in reference to the RJF subspecies reference sequences used. Furthermore, the median-joining result suggested possible origin of the PH RJFs in China, Laos, India and Indonesia. This result also suggested that the RJFs in the Philippines were not endemic, thus, supporting the results of Peterson and Brisbin (1998). The haplotype sharing of the PH RJF haplotypes with the commercial and domestic chickens in subhaplogroup E1 also provided evidence on the introgressed RJF population present in the Philippines.

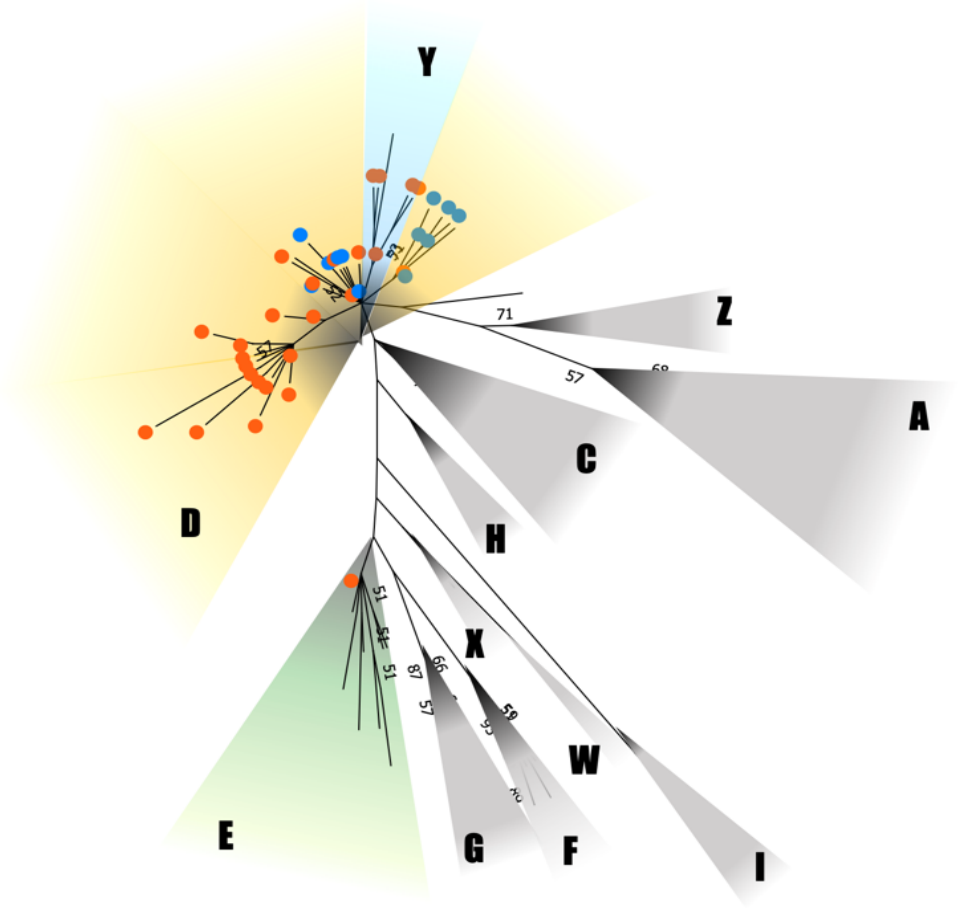


Figure 4.2. Maximum likelihood tree of PH and IND RJF haplotypes together with 61 reference sequences (Miao *et al.,* 2013). Haplogroup: A, B, C, D, E, F, G, H, I, X,Y, Z
Orange dots: PHRJF haplotypes. Blue dots: INDRJF haplotypes.

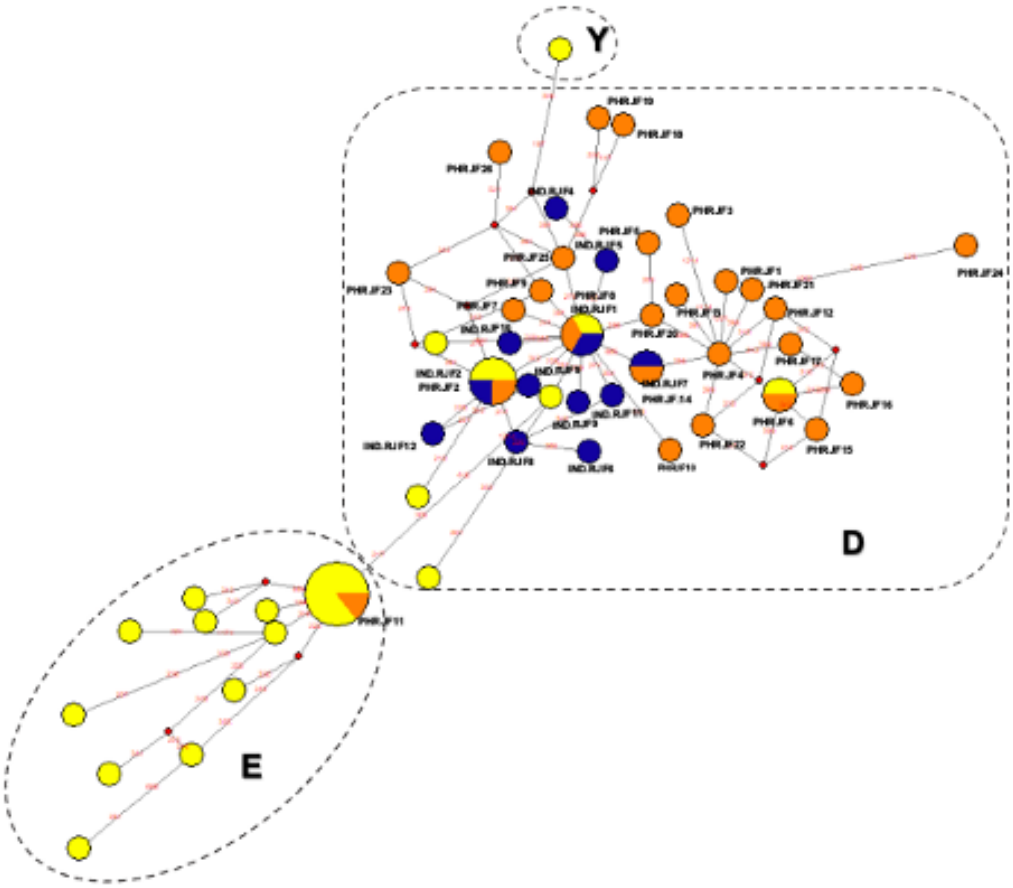


Figure 4.3. Median-joining network of PH and IND RJFs with reference sequences from Miao *et al.* (2013). *Blue and orange dots denote IND and PH RJF haplotypes, respectively. Yellow dots denote the reference sequence (Miao et al., 2013).*

4.4.2 Nucleotide polymorphism of PH and IND RJFs inferred with the Burmese RJF reference sequence

The PH and IND RJF haplotypes were aligned and inferred with a Burmese RJF reference sequence (NC_007235) to determine polymorphisms and mutation differences between the two RJF populations. Table 4.1 revealed 35 variable sites for the two RJF populations. PH and IND RJF haplotypes recorded 136 and 314 total polymorphisms, respectively. Sites 228, 244, 269, and 280 were unique IND RJF polymorphism, while PH RJF haplotypes were detected to have 16 unique polymorphisms different from polymorphic sites identified in IND RJFs. Among

the variable sites identified, the polymorphisms in sites 217, 306, and 446 were inherently unique to the PHRJF11 haplotype. Furthermore, among the 35 variable sites detected, 14 sites with transition mutation were shared by both RJF populations. Among all the mutations identified, only PH RJF3 exhibited transversion mutations (1134, 1214). The high transition mutation identified in this study is expected considering that this type of mutation is common in molecular evolution over the transversion mutation (Stoltzfus and Norris, 2015).

Table 4.1. Nucleotide polymorphism of Philippine (PH) and Indonesian (IND) RJF haplotypes inferred with NC 007235 reference

	2 1 2	2 1 7	2 2 5	2 2 6	2 2 8	2 4 1	2 4 3	2 4 4	2 4 6	2 5 6	2 6 1	2 6 9	2 7 0	2 8 0	2 8 1	2 9 6	3 0 6	3 1 0	3 1 5	3 4 2	3 5 3	3 5 4	3 5 5	3 9 1	3 9 6	3 9 9	4 4 6	4 4 7	5 2 1	6 8 6	1 0 5 2	1 1 3 4	1 2 4	1 2 5	1 2 1
NC 007235	A	T	C	A	C	A	T	T	T	T	C	C	T	T	A	C	T	C	T	A	A	T	T	C	T	G	C	T	A	G	T	T	C	A	T
IND.RJF1	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	.	C	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
IND.RJF2	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	.	C	T	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
IND.RJF3	G	.	.	.	T	.	C	.	C	C	T	.	.	.	G	.	C	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
IND.RJF4	G	.	.	.	.	.	C	.	C	C	T	.	.	C	G	.	C	T	C	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	G	.
IND.RJF5	G	.	.	.	.	.	C	.	C	C	T	.	.	C	G	.	C	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
IND.RJF6	G	.	.	.	.	.	C	C	C	C	T	.	.	.	G	.	C	T	C	G	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.
IND.RJF7	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	.	C	T	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.
IND.RJF8	G	.	.	.	.	.	C	C	C	C	T	.	.	.	G	.	C	T	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
IND.RJF9	G	.	.	.	.	.	C	.	C	C	T	T	.	.	G	.	C	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
IND.RJF10	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	.	C	T	C	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
IND.RJF11	G	.	.	.	.	.	C	C	C	C	T	.	.	.	G	.	C	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
IND.RJF12	G	.	.	.	T	.	C	.	C	C	T	.	.	.	G	.	C	T	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
PHRJF1	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	T	C	T	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	A	
PHRJF2	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	.	C	T	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	
PHRJF3	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	T	C	T	C	.	.	.	.	.	.	.	.	.	.	A	.	G	G	G	.	
PHRJF4	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	T	C	T	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	
PHRJF5	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	T	C	T	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.	G	.	
PHRJF6	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	T	C	T	C	G	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	
PHRJF7	G	.	.	.	.	G	C	.	C	C	T	.	.	.	G	.	C	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	
PHRJF8	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	.	C	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	
PHRJF9	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	.	C	T	C	.	.	.	.	T	.	.	.	.	.	.	.	.	.	G	.	
PHRJF10	G	.	.	.	.	.	.	.	C	C	T	.	.	.	G	.	C	T	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	G	.	
PHRJF11	G	C	.	.	.	.	C	.	C	C	T	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	T	.	.	.	.	.	T	G	.	
PHRJF12	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	T	C	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	
PHRJF13	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	T	C	T	C	.	.	.	.	T	.	.	.	.	.	A	.	.	.	G	.	
PHRJF14	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	.	C	T	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	
PHRJF15	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	T	C	.	C	G	.	.	.	.	.	A	.	.	.	A	.	.	.	G	.	
PHRJF16	G	.	.	.	.	.	.	.	C	C	T	.	.	.	G	T	C	T	C	G	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	
PHRJF17	G	.	.	.	.	.	.	.	C	C	T	.	.	.	G	T	C	T	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	
PHRJF18	G	.	.	.	.	.	C	.	C	C	T	.	C	.	G	.	C	T	C	.	.	.	.	.	C	.	.	C	.	.	.	.	.	G	.	
PHRJF19	G	.	.	G	.	.	C	.	C	C	T	.	C	.	G	.	C	T	C	.	.	.	.	.	C	.	.	.	.	.	.	.	.	G	.	
PHRJF20	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	T	C	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	
PHRJF21	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	T	C	T	C	.	.	.	.	.	C	.	.	.	.	A	.	.	.	G	.	
PHRJF22	G	.	.	.	.	.	C	.	C	C	T	.	.	.	G	T	C	T	C	.	.	.	.	.	.	A	.	.	.	A	.	.	.	G	.	
PHRJF23	G	.	.	.	.	.	C	.	C	C	T	.	C	.	G	.	C	T	C	G	.	.	.	T	.	.	.	.	.	.	.	.	.	G	.	
PHRJF24	G	.	T	.	.	.	C	.	.	C	T	.	.	.	G	T	C	T	C	.	.	.	.	.	C	.	.	.	.	A	C	.	.	G	.	
PHRJF25	G	.	.	.	.	.	C	.	C	C	T	.	C	.	G	.	C	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	
PHRJF26	G	.	.	.	.	.	C	.	C	C	T	.	C	.	G	.	C	T	C	.	.	.	.	T	.	.	.	.	G	.	.	.	.	G	.	

PHRJF11=hybrid RJF. Dots (.) = denotes identity with the reference sequence.

4.4.3 Genetic relationship of IND RJFs and PH RJFs with the RJFs in Asia

The ML tree (Fig. 4.4) revealed 91.67% of the total INDRJF haplotypes were closely related to *G.g. bankiva* (AP003323). This was supported by 99.84%-100% similarity (ave. 99.91%) with the *G.g. bankiva* (AP003323), as compared to 99.59%-99.84% similarity (ave. 99.76%) with the *G.g.gallus* (AP003322). The clade sharing of INDRJF7 with *G.g.gallus* reference sequence is supported by the 99.84% (ave.) similarity of the INDRJF7 with the said RJF subspecies (Appendix table 4.6.5). These results also supported the haplotype sharing of IND RJFs with an Indonesian RJF (*G.g.bankiva*) (NC 007237) (Nishibori *et al.,* 2005) (Figure 4.3). This genetic analysis result of this study added information on the presence also of *G.g.bankiva* in Sulawesi Indonesia, in addition to the distribution of morphologically classified *G.g.gallus* as initially reported by Nishida *et al.* (1985).

Moreover, PH RJFs was observed to form clusters with the *G.g.gallus* (AP003322) and *G.g.bankiva* (AP003323) with an exemption of the PH RJF11 haplotype which formed close genetic relationship with *G.g.murghi* (GU261709) (Figure 4.4). This result was supported by 99.76% average similarity of the 50% of the total PH RJF haplotypes with the *G.g.gallus (*AP003322*)*, while 46.15% of the total PH haplotypes shared 99.77% similarity with *G.g.bankiva* (AP003323) (Appendix table 4.6.5). This result also supported the haplotype sharing of the PH RJF haplotypes to both *G.g.bankiva* and *G.g.gallus.*

In addition, Philippines and Indonesia did not share the same haplotype with the other junglefowl reference sequence used in this study, suggesting that PH and IND RJFs were not a product of interspecies hybridization. Moreover, this result

was supported by the separate clade classification of the IND RJFs and *Gallus varius*, a green junglefowl inhabiting Indonesia together with the RJFs (Ulfah *et al.,* 2016).

None of the detected PH and IND haplotypes shared the same haplotypes with the *G.g. spadiceus* providing further evidence that the domestic chicken detected in the Philippines were derived from a separate domestication center, and not related to the chicken domestication event reported by Wang *et al.* (2020).

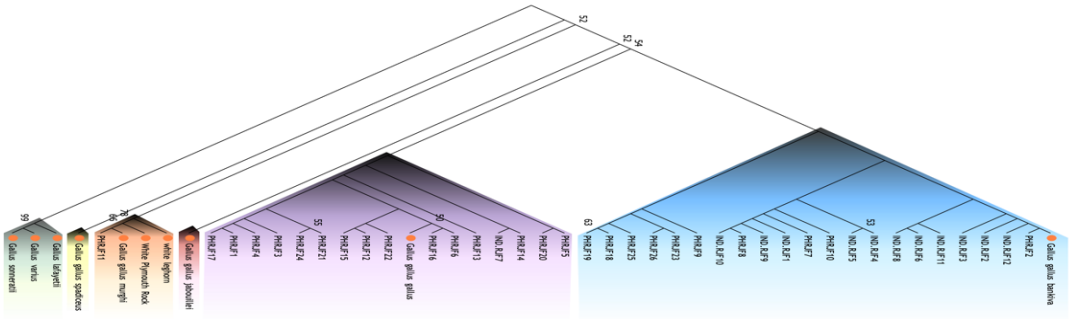


Figure 4.4. Maximum likelihood tree of PH and IND RJF haplotypes with the different RJF subspecies based on General time reversible model. *Orange dots denote reference sequences.*

Inferring with the RJFs from other Asian countries, the results revealed that all PH and IND RJF haplotypes shared the same clade with each other except for PH RJF 11 which was detected to be closely related to the RJFs of Nepal and India (Fig. 4.5). The clade sharing of the PH and IND haplotypes with the RJFs from the Philippines, Indonesia, and Myanmar in this study agreed with the findings of Osman and Nishibori (2014).

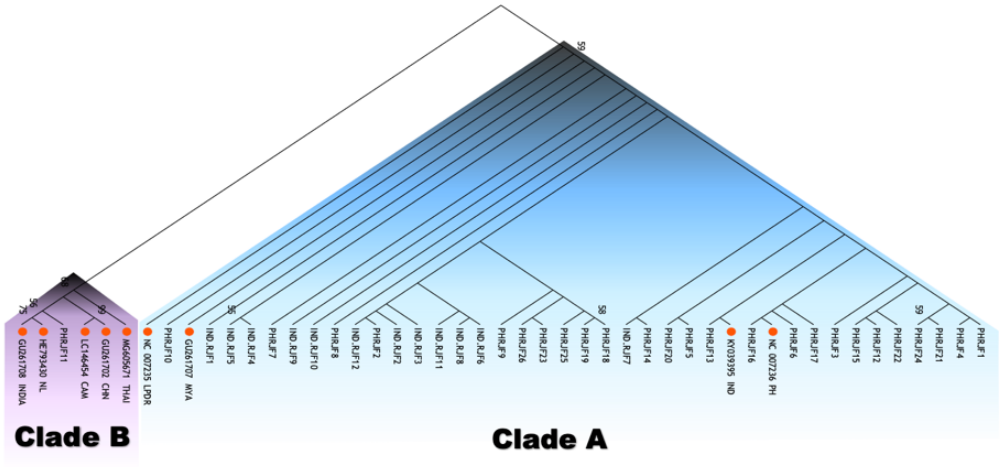


Figure 4.5. Phylogenetic tree of PH and IND RJF together with the different RJFs from different Asian countries based on maximum likelihood method. *Orange dots denote reference sequence. CAM=Cambodia; NL=Nepal; THAI=Thailand; India; MYA=Myanmar; IND=Indonesia; LPDR=Peoples Democratic Republic of Laos. Bootstrap lower than 50% was removed.*

Furthermore, Figure 4.6 shows the ML tree of the PH and IND RJF together with DNA sequences extracted from feathers stored under different condition and duration. This figure showed the unrelatedness of the Malaysian chicken (barbs and calamus) (~47 years old) with the PH and IND RJFs. The unrelatedness of the PH and IND RJFs with the Malaysian chickens could be attributed to its 100% similarity with a native chicken (*G.g. domesticus*) from the Philippines (MK085053). Although the Philippine RJF sequences extracted from feathers and blood shared the same clade, no branch sharing between these sequences was observed.

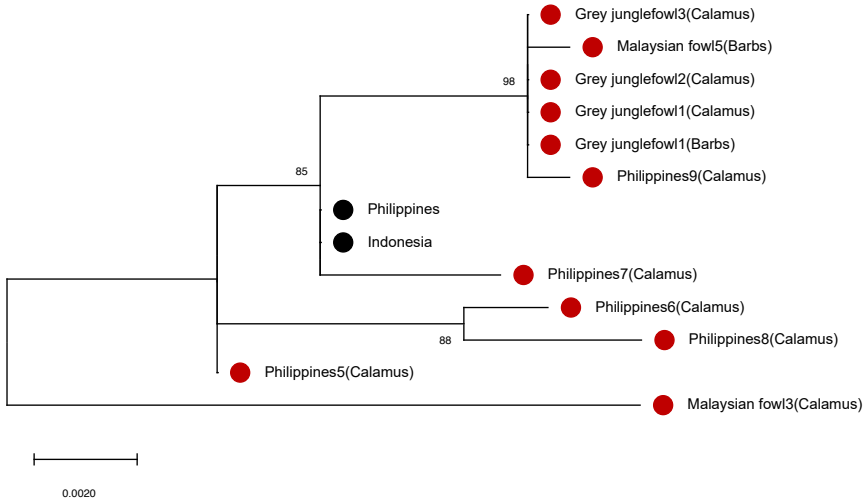


Figure 4.6. Maximum likelihood tree of PH and IND RJF together with sequences extracted from feathers. *Black dots denote DNA sequences from blood samples. Red dots denote DNA sequences from feather samples.*

4.4.4 Genetic diversity, population genetic differentiation and demographic history

Genetic diversity analysis revealed that the PH RJFs has higher haplotype (Hd) (0.92± 0.02) and nucleotide diversity (π) (0.11± 0.06) than IND RJFs (Table 4.2). This result suggested that the PH RJFs were genetically more diverse than IND RJFs. This result was supported by the classification of the PH RJFs under haplotypes Y, D and E, in comparison to the IND RJFs that were only classified under haplotype D (Fig. 4.1). The high Hd and π observed in this study suggested rapid population growth from a small population, assuming that there has been sufficient time for the recovery of haplotype variation via mutation but too short for the accumulation of large sequence differences (Lowe *et al.,* 2004).

In addition, the Hd of IND RJFs reported in this study was higher than the Hd of Indonesian indigenous chickens (0.88045) (Sulandari *et al.,* 2008). Also, the Hd of the *G.g.* subspecies (0.01080 ± 0.00059) in Indonesia, India, and China (Liu

et al., 2006) was lower than the Hd of the PH RJFs (0.92 ± 0.02), and IND RJFs (0.90 ± 0.04) in this study. The *Fst* difference (0.27) between the RJF populations in this study suggested a lack of significant genetic structuring or population subdivision. This was supported by the low genetic differentiation between the two RJF population. Thus, suggesting maternal origin sharing of the PH and IND RJF populations.

Table 4.2. Haplotype and nucleotide diversity of the PH and IND RJFs and their *Fst* distance difference

Population	Hd	π	*Fst*
			Indonesia
Philippines	0.92 ± 0.02	0.11± 0.06	0.27
Indonesia	0.90 ± 0.04	0.07± 0.04	-

Moreover, the neutrality test of this study revealed that Philippines has lower negative Tajima's *D* (-1.27) and Fu's *FS* (-18.71) as compared to the Indonesian RJF population (Tajima's *D*= -0.92; Fu's *FS* = -7.47). Although Tajima's *D p-value* of Philippines (*p=0.09*) and Indonesia (*p=0.21*) were not significant, these results suggested population expansion of the two RJF populations (Table 4.3). On the other hand, the observed negative Tajima's *D* indicated that the Philippine and Indonesian RJF population departed from equilibrium, possibly due to past or recent population expansion, bottleneck effect, or heterogeneity of mutation rates (Tajima, 1996). However, the observed negative Fu's *FS* value provided strong evidence of past population expansion of the PH and IND RJFs. This population expansion might be due to genetic hitchhiking, background selection, and evolutionary force producing the observed population

expansion pattern (Okello *et al.,* 2005; Joshi *et al.,* 2013). The combination of high haplotype diversity and low nucleotide diversity in this study also provided evidence of the past and rapid demographic expansion from a small effective population size (Avise, 2000).

Table 4.3. Test of neutrality of the PH and IND RJF population.

Population	N	No. of haplotypes	Tajima's *D*	Tajima's *D p-value*	Fu's *FS*	Fu's *FS* p-value
Philippines	53	25	-1.27	0.09	-18.71	0.00
Indonesia	25	12	-0.92	0.21	-7.47	0.00

N= number of RJF samples

The AMOVA analysis of this study revealed 25.21% variation between PH and IND RJFs and 74.79% within the two populations. The low variation percentage suggested that the Philippine and Indonesian RJF population were not genetically structured. This result was supported by the low *Fst* pairwise difference (0.27) between the two population. This result also suggested the possibility of breeding female exchange between the two RJF populations. Moreover, the high variation within the two RJF population could be due to their collection site difference and distance.

Table 4.4. AMOVA of the PH and IND RJF population.

Source of Variation	Sum of squares	Variance components	Variation (%)
Between populations	15.18	0.41	25.21
Within populations	92.63	1.22	74.79

4.4.5 Evolutionary relationship of PH and IND RJFs based on coalescent time estimate

Utilizing estimated divergence time (8.09kya) between RJFs and domestic chicken (Lawal *et al.,* 2020) as basis for the divergence time calibration of the PH and IND RJFs, the results showed that two PH RJFs (PH.L4 and PH.M5) diverge first (~7.14kya) from the least common RJF ancestor than the IND RJFs. These two PH RJFs equally shared 99.67% similarity with the *G.g.gallus* (AP003322) and *G.g.bankiva* (AP003323) reference sequences. The result also revealed that IND RJFs did not diverge separately from the least common RJF ancestor but has probably diverged from the PH RJFs at around ~5.1kya. Moreover, at around ~1.75kya, the IND RJFs showed clear separate divergence from the PH RJF population to form a separate cluster (Appendix fig. 4.6.2).

Although Capiz and Iloilo are geographically adjacent from each other, the results showed distant divergence period. In addition, though Guimaras is part of Panay group of islands together with Capiz and Iloilo, the results showed that the Guimaras RJF population was a younger RJF population that is closely related to the ancient Polynesian chicken samples (Appendix fig. 4.6.2). This is based on the divergence period (~2.7kya ago) between the Guimaras RJF and the ancient Pacific chickens. Thus, supporting the findings of Thomson *et al.* (2014) on the close genetic relationship of Philippines chickens and ancient Pacific chicken samples.

Moreover, the coalescent time estimate result of this study does not support the north to south (Luzon to Visayas to Mindanao) distribution of RJFs in the Philippines, considering that the samples that diverged first were from Luzon and Visayas, with the RJFs from Guimaras (Visayas) as the youngest RJFs sampled.

Moreover, the result of this study supported the proposed route of Austroasiatic and Austronesian migration into Indonesia, tracing the geographic distribution sites that have produced red-slipped and cord-marked pottery from South China to Philippines then to Indonesia at around 4000-4200 ybp (years before present) (Simanjuntak, 2017). The result of this study also supported the migration routes of early Austronesian groups (Hung, 2016), based on the Out-of-Taiwan migration hypothesis proposed by Bellwood (1984).

4.5. Conclusion and Recommendations

This study concluded the close evolutionary relationship of the Philippine and Indonesian RJFs based on its haplogroup and haplotype sharing, coalescent divergence time estimates, and low *Fst* distance difference. The possibility of maternal sharing and exchange of breeding female between the two countries was also elucidated in this study. Moreover, the high genetic and nucleotide diversity of this study suggested that the RJFs in Indonesia and Philippines were not considered as a threatened population. However, the haplotype sharing of some samples in this study with the domestic chickens is of biodiversity concern that needs immediate action.

Consequently, this study also concluded the *G.g.gallus* and *G.g.bankiva* classification of PH RJFs confirming further the coexistence of these two RJF subspecies in the Philippines. This study also concludes the *G.g.bankiva* classification of the IND RJFs.

Due to few sample number and limited RJF population used in this study, the need to analyze larger data set which includes other RJF populations could elucidate further the RJF migration in Asia and in the Pacific regions.

4.6. Appendices

Appendix table 4.6.1. IND RJF sample information and accession number.

IND RJF	Sex	Sampling site	Accession number
IndRJF 1	♂	Sulawesi, Indonesia	OM100841
IndRJF 2	♂	Sulawesi, Indonesia	OM100842
IndRJF 3	♂	Sulawesi, Indonesia	OM100843
IndRJF 5	♂	Sulawesi, Indonesia	OM100844
IndRJF 6	♂	Sulawesi, Indonesia	OM100845
IndRJF 7	♂	Sulawesi, Indonesia	OM100846
IndRJF 9	♂	Sulawesi, Indonesia	OM100847
IndRJF10	♂	Sulawesi, Indonesia	OM100848
IndRJF 12	♂	Sulawesi, Indonesia	OM100849
IndRJF 16	♂	Sulawesi, Indonesia	OM100850
IndRJF 19	♂	Sulawesi, Indonesia	OM100851
IndRJF 20	♂	Sulawesi, Indonesia	OM100852
IndRJF 21	♂	Sulawesi, Indonesia	OM100853
IndRJF 22	♂	Sulawesi, Indonesia	OM100854
IndRJF 23	♂	Sulawesi, Indonesia	OM100855
IndRJF 27	♂	Sulawesi, Indonesia	OM100856
IndRJF 29	♂	Sulawesi, Indonesia	OM100857
IndRJF 30	♂	Sulawesi, Indonesia	OM100858
IndRJF 31	♂	Sulawesi, Indonesia	OM100859
IndRJF 32	♂	Sulawesi, Indonesia	OM100860
IndRJF 33	♂	Sulawesi, Indonesia	OM100861
IndRJF 34	♂	Sulawesi, Indonesia	OM100862
IndRJF 35	♂	Sulawesi, Indonesia	OM100863
IndRJF 36	♂	Sulawesi, Indonesia	OM100864
IndRJF 38	♂	Sulawesi, Indonesia	OM100865

Appendix table 4.6.2. PH RJF sample information and accession number.

PH RJF	Sex	Sampling site	Island region	Accession number
PH.M1	♂	Occidental Mindoro	Luzon	OL589006
PH.M2	♂	Occidental Mindoro	Luzon	OL589007
PH.M3	♂	Occidental Mindoro	Luzon	OL589008
PH.M4	♂	Occidental Mindoro	Luzon	OL589009
PH.M5	♂	Occidental Mindoro	Luzon	OL589010
PH.M6	♂	Occidental Mindoro	Luzon	OL589011
PH.M7	♂	Occidental Mindoro	Luzon	OL589012
PH.M8	♂	Occidental Mindoro	Luzon	OL589013
PH.M9	♂	Occidental Mindoro	Luzon	OL589014
PH.M10	♂	Occidental Mindoro	Luzon	OL589015
PH.M11	♂	Occidental Mindoro	Luzon	OL589016
PH.M12	♂	Occidental Mindoro	Luzon	OL589017
PH.M13	♂	Occidental Mindoro	Luzon	OL589018
PH.M14	♂	Occidental Mindoro	Luzon	OL589019
PH.M15	♂	Occidental Mindoro	Luzon	OL589020
PH.M16	♂	Occidental Mindoro	Luzon	OL589021
PH.M17	♂	Occidental Mindoro	Luzon	OL589022
PH.M18	♀	Occidental Mindoro	Luzon	OL589023
PH.M19	♂	Occidental Mindoro	Luzon	OL589024
PH.P10	♂	Palawan	Luzon	OL589051
PH.P11	♂	Palawan	Luzon	OL589052
PH.P12	♂	Palawan	Luzon	OL589053
PH.P13	♂	Palawan	Luzon	OL589054
PH.P14	♂	Palawan	Luzon	OL589055
PH.P15	♂	Palawan	Luzon	OL589056
PH.P17	♂	Palawan	Luzon	OL589057
PH.P18	♂	Palawan	Luzon	OL589058
PH.P19	♂	Palawan	Luzon	OL589059

PH.P20	♂	Palawan	Luzon	OL589060
PH.I1	♂	Iloilo	Visayas	OL589029
PH.I2	♀	Iloilo	Visayas	OL589030
PH.I3	♀	Iloilo	Visayas	OL589031
PH.I4	♀	Iloilo	Visayas	OL589032
PH.I5	♂	Iloilo	Visayas	OL589033
PH.I6	♀	Iloilo	Visayas	OL589034
PH.I7	♂	Iloilo	Visayas	OL589035
PH.I8	♂	Iloilo	Visayas	OL589036
PH.C13	♂	Capiz	Visayas	OL589037
PH.C14	♂	Capiz	Visayas	OL589038
PH.L1	♂	Leyte	Visayas	OL589043
PH.L2	♂	Leyte	Visayas	OL589044
PH.L3	♂	Leyte	Visayas	OL589045
PH.L4	♂	Leyte	Visayas	OL589046
PH.L5	♂	Leyte	Visayas	OL589047
PH.L6	♂	Leyte	Visayas	OL589048
PH.L8	♂	Leyte	Visayas	OL589049
PH.L9	♂	Leyte	Visayas	OL589050
PH.673	♂	Guimaras	Visayas	OL589039
PH.674	♂	Guimaras	Visayas	OL589040
PH.675	♂	Guimaras	Visayas	OL589041
PH.676	♂	Guimaras	Visayas	OL589042
PH.A1	♂	Agusan del Norte	Mindanao	OL589025
PH.A2	♂	Agusan del Norte	Mindanao	OL589026
PH.A3	♂	Agusan del Norte	Mindanao	OL589027
PH.A4	♂	Agusan del Norte	Mindanao	OL589028

Appendix table 4.6.3. Reference sequence samples (Miao *et al.,* 2013).

Accession number	Haplogroup	Chicken Type	Location	Reference
AB086102	A	domestic chicken	Japan: Hiroshima	Wada *et al.* 2005
GU261684	A	domestic chicken	China: Yunnan	Miao *et al.,* 2013
GU261695	A	wild fowl	China: Yunnan	Miao *et al.,* 2013
GU261700	A	wild fowl	Myanmar	Miao *et al.,* 2013
NC_007235	B	wild fowl	Laos: Vientiane	Nishibori *et al.,* 2005
GU261704	B	wild fowl	China: Yunnan	Miao *et al.,* 2013
GU261705	B	domestic chicken	China: Yunnan	Miao *et al.,* 2013
GU261714	B	domestic chicken	China: Yunnan	Miao *et al.,* 2013
GU261699	B	domestic chicken	China: Yunnan	Miao *et al.,* 2013
GU261674	Z	wild fowl	China: Hainan	Miao *et al.,* 2013
GU261696	Z	wild fowl	China: Hainan	Miao *et al.,* 2013
GU261693	Y	wild fowl	China: Yunnan	Miao *et al.,* 2013
GU261701	C1	domestic chicken	China: Henan	Miao *et al.,* 2013
GU261675	C1	domestic chicken	China: Hunan	Miao *et al.,* 2013
GU261681	C1	domestic chicken	China: Hunan	Miao *et al.,* 2013
GU261718	C1	domestic chicken	China: Yunnan	Miao *et al.,* 2013
GU261679	C1	domestic chicken	China: Henan	Miao *et al.,* 2013
GU261680	C2	domestic chicken	Southern India	Miao *et al.,* 2013
GU261716	C3	wild fowl	Myanmar	Miao *et al.,* 2013
GU261707	C3	wild fowl	India	Miao *et al.,* 2013
NC_007236	D1	wild fowl	Philippine: Manila	Nishibori *et al.,* 2005
NC_007237	D1	wild fowl	Indonesia: Bali	Nishibori *et al.,* 2005
GU261687	D1	domestic chicken	Laos	Miao *et al.,* 2013
GU261682	D1	domestic chicken	Laos	Miao *et al.,* 2013
GU261683	D2	domestic chicken	China: Xinjiang	Miao *et al.,* 2013
GU261677	D3	domestic chicken	China: Zhejiang	Miao *et al.,* 2013
GU261697	D3	domestic chicken	Southern India	Miao *et al.,* 2013
GU261685	D3	domestic chicken	Northeast India	Miao *et al.,* 2013
GU261686	E1	domestic chicken	China: Henan	Miao *et al.,* 2013
GU261713	E1	domestic chicken	China: Yunnan	Miao *et al.,* 2013
AP003317	E1	domestic chicken	Commercial Line	Nishibori *et al.,* 2003
AY235571	E1	domestic chicken	Commercial Lines	Froman and Kirby, 2005
AP003318	E1	domestic chicken	Commercial Line	Nishibori *et al.,* 2003

GU261712	E1	domestic chicken	China: Yunnan	Miao *et al.,* 2013
GU261709	E1	domestic chicken	India	Miao *et al.,* 2013
AY235570	E1	domestic chicken	Commercial Line	Froman and Kirby, 2005
AP003580	E1	domestic chicken	Commercial Line	Nishibori *et al.,* 2003
GU261694	E1	domestic chicken	China: Hebei	Miao *et al.,* 2013
AP003319	E1	domestic chicken	Laos: Vientiane	Miao *et al.,* 2013
HQ857210	E1	domestic chicken	Northeast India	Miao *et al.,* 2013
HQ857209	E2	domestic chicken	Northeast India	Miao *et al.,* 2013
GU261708	E3	wild fowl	India	Miao *et al.,* 2013
HQ857212	E3	domestic chicken	Northeast India	Miao *et al.,* 2013
HQ857211	E3	domestic chicken	Northeast India	Miao *et al.,* 2013
GU261691	F	wild fowl	Myanmar	Miao *et al.,* 2013
GU261702	F	wild fowl	China: Yunnan	Miao *et al.,* 2013
GU261688	F	domestic chicken	China: Yunnan	Miao *et al.,* 2013
GU261711	F	domestic chicken	China: Yunnan	Miao *et al.,* 2013
GU261689	F	domestic chicken	China: Yunnan	Miao *et al.,* 2013
GU261703	F	wild fowl	Myanmar	Miao *et al.,* 2013
GU261717	F	domestic chicken	China: Yunnan	Miao *et al.,* 2013
DQ648776	F	domestic chicken	China: Yunnan	Tong *et al.,* 2006
GU261678	G	domestic chicken	China: Henan	Miao *et al.,* 2013
GU261710	G	domestic chicken	China: Yunnan	Miao *et al.,* 2013
GU261676	G	domestic chicken	China: Yunnan	Miao *et al.,* 2013
GU261719	G	domestic chicken	China: Yunnan	Miao *et al.,* 2013
GU261690	G	wild fowl	China: Yunnan	Miao *et al.,* 2013
GU261715	H	domestic chicken	China: Yunnan	Miao *et al.,* 2013
GU261706	W	wild fowl	China: Yunnan	Miao *et al.,* 2013
GU261692	X	wild fowl	China: Yunnan	Miao *et al.,* 2013
GU261698	I	domestic chicken	Northeast India	Miao *et al.,* 2013

Appendix table 4.6.4. *Gallus gallus* reference sequence samples.

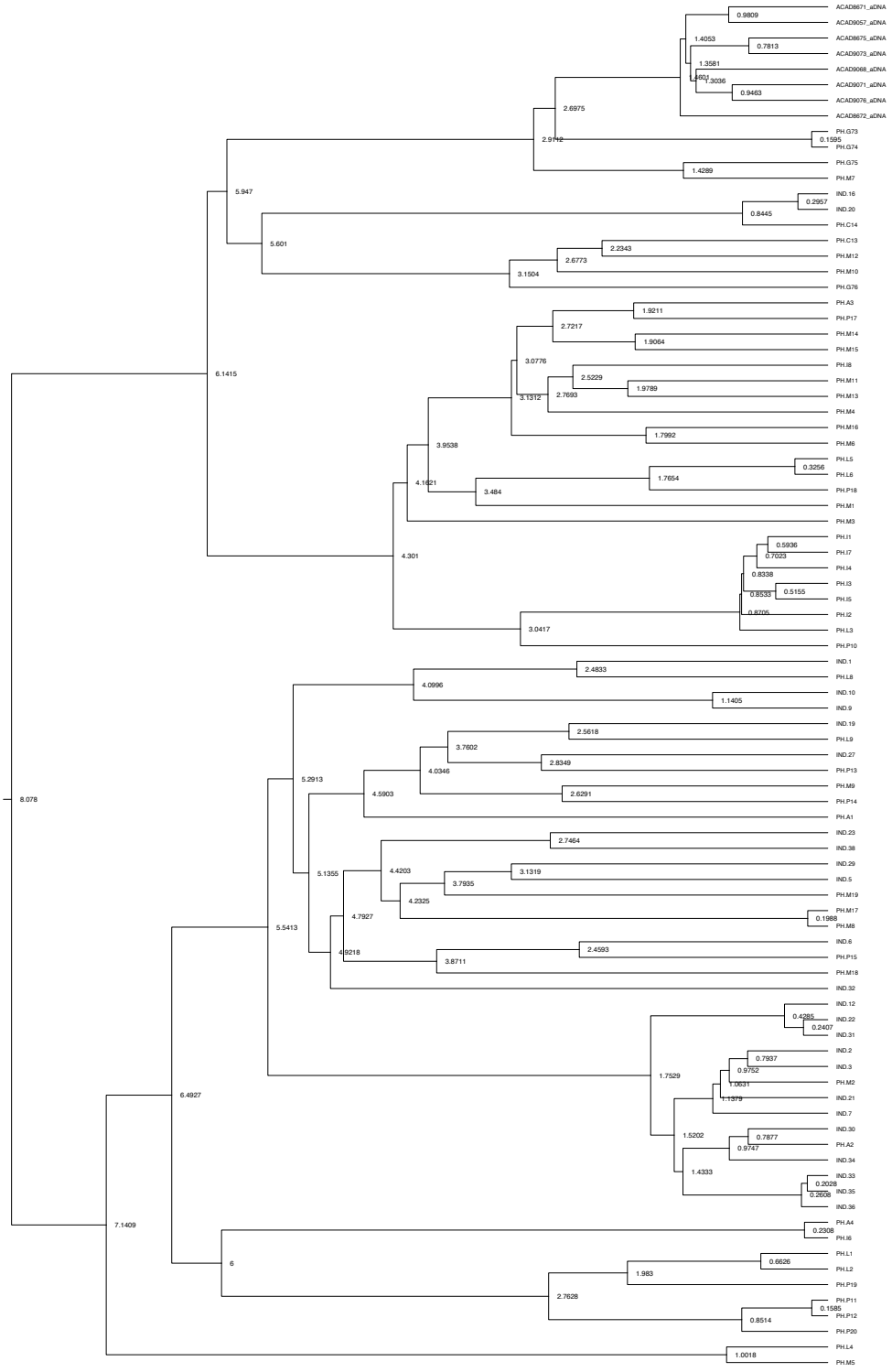
Accession number	Species/subspecies	Reference
AP003322	*Gallus gallus gallus*	Nishibori *et al.,* 2005
AP003318	*Gallus gallus:* White Plymouth Rock	Nishibori *et al.,* 2003
AB007723	*Gallus gallus gallus*: - White Leghorn	Miyake, 1997
NC_007239	*Gallus lafayetii*	Nishibori *et al.,* 2005
AP003324	*Gallus varius*	Nishibori *et al.,* 2005
NC_007241	*Gallus sonneratii*	Nishibori *et al.,* 2005
GU261690	*Gallus gallus spadiceus*	Miao *et al.,* 2013
GU261709	*Gallus gallus murghi*	Miao *et al.,* 2013
GU261696	*Gallus gallus jabouillei*	Miao *et al.,* 2013
AP003323	*Gallus gallus bankiva*	Nishibori *et al.,* 2005

Appendix table 4.6.5. Percent similarity of the PH and IND RJFs with *G.g. gallus* and *G.g. bankiva.*

IND RJF	*G.g. gallus (%)*	*G.g. bankiva (%)*	PH RJF	*G.g. gallus (%)*	*G.g. bankiva (%)*
IND.1	99.76	99.92	PH.M1	99.76	99.59
IND.2	99.84	100	PH.M2	99.92	99.76
IND.3	99.84	100	PH.M3	99.68	99.51
IND.5	99.68	99.84	PH.M4	99.84	99.68
IND.6	99.76	99.92	PH.M5	99.59	99.59
IND.7	99.84	100	PH.M6	99.84	99.68
IND.9	99.59	99.76	PH.M7	100	99.84
IND.10	99.68	99.84	PH.M8	99.68	99.84
IND.12	99.68	99.84	PH.M9	99.68	99.84
IND.16	99.84	99.84	PH.M10	99.92	99.76
IND.19	99.76	99.92	PH.M11	99.92	99.76
IND.20	99.84	99.84	PH.M12	99.92	99.76
IND.21	99.84	100	PH.M13	99.92	99.76
IND.22	99.76	99.92	PH.M14	99.92	99.76
IND.23	99.68	99.84	PH.M15	99.92	99.76
IND.27	99.76	99.92	PH.M16	99.76	99.59
IND.29	99.68	99.84	PH.M17	99.59	99.76
IND.30	99.84	100	PH.M18	99.68	99.84
IND.31	99.76	99.92	PH.M19	99.76	99.92
IND.32	99.68	99.84	PH.A1	99.51	99.68
IND.33	99.76	99.92	PH.A2	99.76	99.92
IND.34	99.84	100	PH.A3	99.84	99.68
IND.35	99.76	99.92	PH.A4	99.35	99.51
IND.36	99.76	99.92	PH.I1	99.76	99.59
IND.38	99.76	99.92	PH.I2	99.59	99.76
			PH.I3	99.76	99.59
			PH.I4	99.76	99.59
			PH.I5	99.76	99.59
			PH.I6	99.27	99.43
			PH.I7	99.76	99.59
			PH.I8	99.84	99.68
			PH.C13	99.76	99.59
			PH.C14	99.84	99.84
			PH.G73	99.76	99.59
			PH.G74	99.76	99.59
			PH.G75	99.84	99.68
			PH.G76	99.76	99.59
			PH.L1	99.51	99.68
			PH.L2	99.43	99.59
			PH.L3	99.76	99.59

PH.L4	99.76	99.76
PH.L5	99.76	99.59
PH.L6	99.76	99.59
PH.L8	99.68	99.84
PH.L9	99.76	99.92
PH.P10	99.84	99.68
PH.P11	99.68	99.84
PH.P12	99.68	99.84
PH.P13	99.68	99.84
PH.P14	99.76	99.92
PH.P15	99.76	99.92
PH.P17	99.84	99.68
PH.P18	99.51	99.35
PH.P19	99.68	99.84
PH.P20	99.43	99.59

Appendix figure 4.6.1 Feather samples from different countries and species.
NP: Nepal; MY: Malaysia; ID: Indonesia; GJF: Green junglefowl; PH: Philippines



Appendix figure 4.6.2. Coalescent time estimate between PH and IND RJFs with reference to ancient Polynesian chickens. *Divergence time in kilo years (ky) is indicated on the branch node. IN= Indonesia; PH=Philippines*

CHAPTER V

General Discussion

5.1 Diversity and Conservation of RJFs in the Philippines: Addressing the United Nations Sustainable Development Global 15- Life on Land

Majority of the world's biodiversity occurs in the tropics, but human actions in these regions have precipitated an extinction crisis due to habitat degradation, overexploitation, and climate change. Understanding which ecological, biogeographical, and life-history traits predict the risk of extinction is critical to conserving species (Kittelberger *et al.,* 2021).

Red junglefowls (RJF), the ancestors of today's domesticated chickens were scattered in the forested areas of the different South and Southeast Asian countries. Some of the RJF subspecies were even considered endemic to certain countries like the *G.g.bankiva* in Indonesia and the *G.g.lafayette* in Sri Lanka. Currently, Thailand is considered as the center of chicken domestication with G.g. spadiceus as the maternal origin (Wang *et al.,* 2020), a deviation from the previously known domestic chicken ancestor, the G.g.gallus (Fumihito *et al.,* 1994; 1996).

Philippines is one of the most biodiverse countries in the world (Posa *et al.,* 2008), an archipelago composed of more than 7000 islands, and a country with diverse habitats which formed a global hotspot of species diversity and endemism (Heaney, 1993; Oliver and Heaney, 1996; Stattersfield *et al.,* 1998; Myers *et al.,* 2000). Though RJFs were still sighted in the forest areas in the Philippines, habitat destruction and fragmentation, and rampant hunting threaten their population size and genetic diversity. Some RJFs were captured for trade, recreational sports, captive breeding, and food. Also, male RJFs were crossed with female chickens of fighting cock genetic descent for breed improvement. Fears have been expressed that wild RJF may be genetically contaminated, leading to the inference that there

may not be any pure RJF left in the wild (Fernandes *et al.,* 2007). The coexistence of the RJFs and native chickens in the wild or in a controlled environment could result in the admixture of the wild and domestic chicken gene which may lead to the depletion and jeopardization of the genetic integrity of the wild population.

Current approaches to biodiversity conservation were largely based on geographic areas, ecosystems, ecological communities, and species, with less attention paid to genetic diversity and the evolutionary continuum from population to species (Coates *et al.,* 2018). In relation to this, preliminary studies must be conducted to address concerns on RJF diversity and conservation in the Philippines for us to know and assess its ecological status which is essential in the formulation of conservation programs or the proper implementation of the existing biodiversity laws. Thus, in this study, diversity assessment was conducted from basic taxonomic classification to molecular analysis through genotyping and phylogenomic analysis.

The ecological status of the RJFs was first assessed through actual fieldwork in mountainous areas in the Philippines. The initial assessment was conducted through morphology evaluation in comparison and reference with the previous studies on PH RJFs (Hachisuka, 1939; Nishida and Masangkay, 1978; Nishida *et al.,* 1985; Masangkay *et al.,* 2010). The analysis revealed that the morphology of the Philippine RJFs, many years ago, is still the same as today. This was evident on the plumage and shank color, and the expression of eclipse plumage that was only evident in wild junglefowl. Moreover, the previously reported white earlobe color which was inherently a characteristic of a *G.g.gallus*, is now less evident among the RJFs sampled. However, the genetic analysis of this study suggested that earlobe color should not be used in the subspecies classification of RJFs. It was also

observed that the shank color was observed to be the clearest deviation of wild and domestic chickens, with the latter having the yellow-colored shank which was influenced by the beta-carotene dioxygenase 2 gene (*BCD02*) (Eriksson *et al.,* 2008). This trait was observed on the hybrid RJF, which also expressed other wild RJF physical features, except for the shank color. However, hybridization through human intervention is not desirable if the main goal is to conserve the genetic identity of the RJFs.

Phylogenomic analysis was conducted using the mtDNA D-loop region to elucidate the matrilineal origin of PH RJFs and their evolutionary history with RJFs from other countries. The result of this analysis revealed that the Philippines has no endemic RJF subspecies. This also showed that the PH RJFs shared the same haplogroup with the wild chickens of China and Indonesia and the domestic chickens of India and Laos. This genetic relationship also suggested multiple maternal origin of the PH RJFs. However, the haplotype analysis only showed haplotype sharing of the PH RJFs with the reference RJFs from Indonesia and the Philippines which provided evidence on the wild chicken ancestry of the new current PH RJF samples. The haplotype sharing of the PH RJFs with the domestic chickens of Laos and India also provided information on the introduction of the domestic chicken gene into the wild RJF population in the Philippines. This was supported by the haplotype sharing of the known hybrid RJF sample in this study with the commercial chickens of India and China.

Though with the presence of wild RJFs in the Philippines with high haplotype and nucleotide diversity, the haplotype sharing of the PH RJFs with domestic and commercial chickens observed in this study is of biodiversity concern.

This could mean genetic admixture of the wild and domestic chicken population, thereby jeopardizing the genetic integrity of the RJFs. Moreover, several factors could explain this result. First, it was previously reported that the Philippines might have accepted introduced junglefowl brought by human settlements (Peterson and Brisbin, 1998). These junglefowls could possibly be no longer pure and were already introgressed with domestic chicken gene before it arrived in the Philippines. Second, introgression through natural selection which was possibly caused by the coexistence and breeding of wild and domestic chicken in one area. Habitat fragmentation could also be the reason for their coexistence in the same area. Third, introgression and hybridization through human intervention which was observed, documented and verified in this research. This hybridization was validated by the haplotype sharing of the morphologically known hybrid PH RJF with the commercial chicken reference sequences.

Furthermore, the genomic matrilineal sharing of PH RJFs and IND RJFs prompts the need to conduct coalescent time divergence analysis. Determining the estimated year of divergence, the result of this study showed that the PH RJFs is older as compared to the IND RJFs. However, it was also observed that some RJF samples from the two populations shared the same divergence period; thus, supporting the matrilineal and haplotype sharing of PH and IND RJFs observed in this study. This result also supported the presence of two RJF subspecies (*G.g.gallus and G.g.bankiva*) present in the Philippines.

Moreover, genetic diversity is the basis of the evolutionary potential of species to respond to environmental changes, this becomes an essential pillar in conservation genetics (Toro and Caballero, 2005). Therefore, the result of this

study's genetic diversity of the PH RJF through haplotype diversity analysis suggested that the RJFs in the Philippines are not at risk of extinction. This is supported by the population expansion of the PH RJFs based on the negative neutrality test analysis and Bayesian Skyline Plot analysis result of this study. However, though the results suggested that the genetic diversity of the PH RJFs is not alarming, the need to protect the genetic integrity of the RJFs in the wild must be addressed based on the haplotype sharing of the PH RJFs with the commercial chickens.

Translating the result of this study in addressing global concerns, one of the seventeen Sustainable Development Goals (SDG) of the United Nations (UN) that this research wants to address is the SDG15: Life on Land. This goal is focused on the protection, restoration, and promotion of the sustainable use of terrestrial ecosystems. In addition, this goal focuses on sustainable forest management to prevent if not stop biodiversity loss.

The first SDG15 target that this research aimed to address is target 15.6 which focuses on the promotion of genetic resources access and fair sharing of benefits. Promote fair and equitable sharing of the benefits arising from the utilization of genetic resources and promote appropriate access to such resources, as agreed internationally. In this research, all DNA sequences was made public through the GenBank submission. All independent experiments were and will also be published to serve as basis for future RJF studies with the aim of providing information that would benefit the RJFs in the Philippines, and in Asia in general. Furthermore, this study's objective was to connect the genetic information collected from PH RJFs with the complex genetic matrilineal diversity of RJFs in Asia.

Another target that this research aimed to address is the SDG15 target 15.9, which talks about the integration of the ecosystem and biodiversity in governmental planning. Molecular and genetic identification and phylogenetic analysis result in this study could be used as bases for the improvement of the existing conservation programs and laws in the Philippines. Currently, the ruling law that deals with wildlife animals in the Philippines is the Republic Act No. 9147, an act for the conservation and protection of wildlife resources and their habitats, appropriating funds therefore and for other purposes. However, though with the existence of this law, poaching and hunting of RJFs are still rampant on a wide scale. This is evident in the sampling of the RJFs used in this study from farmers and hunters. Hunting of RJFs was probably rooted on the poor government information drive on wildlife conservation issues. Furthermore, the information generated in this research could serve as basis of the government in the formulation of policies for the protection of RJFs in the wild. In defense, most of the PH RJFs in this study was used with the approval from the Department of Environment and Natural Resources under R.A. 9147. In this sense, through proper information drive on the need to preserve the genetic integrity of RJFs in the wild and halting purposeful hunting, could lead to the achievement of the SGD15 target 15.C. This target tackles on the fight against global poaching and trafficking by increasing the capacity of local communities to pursue sustainable livelihood opportunities.

As human activities continue to cause global declination and losses in avian biodiversity, assessment and possible prediction of extinction risk in wildlife will be even more important as a preemptive conservation strategy (Kittelberger *et al.,* 2021). Moreover, the need to understand the scope of climate change, especially to

vulnerable countries like the Philippines, will help us pivot the programs that would safeguard the biodiversity of the avian species in the Philippines, including the RJFs.

In general, though RJFs are considered as least concerned (LC) species of the International Union for Conservation of Nature (IUCN) (BirdLife International, 2021), the result of this study suggested that aside from conservation of species due to population size declination, consideration based on phylogenomics, genetic diversity, and genetic integrity must be incorporated as well for a more structured conservation and endangerment classification. Moreover, given the genetic resource importance of RJF and its role in our biodiversity as well as its role in our future food security, it is therefore suggested that the Philippine RJFs will be given equal conservation initiatives by drafting conservation policies with the aim of protecting them in the wild. Doing such initiative will greatly help in the conservation of RJFs, development of future conservation strategies as well as the improvement of valuable genetic resource and its possible role in the Philippine food security.

CHAPTER VI

Conclusion

This study concluded that differentiation of wild RJFs from domestic chicken gene introgressed RJFs is possible through qualitative and quantitative morphology assessment. Using the morphology assessment indexes used in this study, preliminary identification of the taxonomic classification of RJFs can be carried out. However, some body traits were very difficult to utilize as an identification index since some body parts of the animals used in this study were intentionally removed (spur and earlobe). Thus, to address this limitation, development of genetic biomarkers that would reveal the phenotype of the RJF is necessary.

Though the diversity and ecological status of PH RJFs through morphology assessment could be used as an initial evaluation approach, the need for DNA analysis could provide a better understanding of the taxonomic classification, biodiversity status, and the evolutionary relationship of the PH RJFs with the RJFs from other Asian countries. Thus, this study highlighted the phylogenomic advantage of mtDNA in determining the past, present, and future of the PH RJFs through phylogenetic studies and population demography analysis. Using mtDNA, this study illuminated the classification of the PH RJFs under *G.g.gallus* and *G.g.bankiva*, as supported by the haplotype sharing of some PH RJF samples with the subspecies mentioned. This study also prompted the possibility of admixture between these two subspecies as evident on the clustering and haplotype sharing of the *G.g.bankiva* classified IND RJFs with the *G.g.gallus* classified PH RJFs. Divergence time analysis between the RJFs of Indonesia and the Philippines also supported this result.

Furthermore, by tracking the matrilineal origin of the PH RJFs, the result of this study provided evidence on the multiple maternal origins of the PH RJFs. The haplotype sharing of PH RJFs with the RJFs from other Asian countries raised the possibility that Laos, China, India and Indonesia is the maternal origin of PH RJFs. The clade sharing of PH RJFs with the RJFs from these countries also supported this conclusion. Moreover, mtDNA also revealed population expansion of the PH RJFs for each sampling area and the Philippines as a whole.

Moreover, the high haplotype and nucleotide diversity observed in PH RJFs provided information on its ecological status and conservation potential. This study also pointed out that species conservation must not just be based on its population size and genetic diversity but also on its evolutionary status. The point of concern that this research revealed is the phylogenetic clustering of wild RJFs with domestic and commercial chickens. This haplotype sharing provided evidence on the introgression of the domestic chicken gene into the wild chicken population. This clustering and introgression is not desirable if the main goal is to preserve the genetic integrity of the wild RJF population. This study also concluded the mix RJF subspecies ancestry of the RJFs in the Philippines around ~7.14 kya. Furthermore, this result also suggested that the PH RJFs analyzed were older than the IND RJFs.

In connection to the Sustainable Development Goals of the United Nations, the result of this study aimed to address SDG15, which is the protection of life on land. This study tried to address this goal through preliminary assessment of RJFs using the basic taxonomic classification method and deeper evaluation through molecular analysis. Thus, in conclusion, the results of this study can be used as a

basis in the formulation of conservation policies and the improvement of existing programs.

In general, molecular analysis validated the *G.g.gallus* and *G.g.bankiva* of the PH RJFs as initially identified through morphology evaluation. However, mtDNA also revealed the mixed ancestry of the PH RJF that the morphology analysis failed to determine. Thus, though morphology analysis can be used for initial evaluation, it should be proceeded with molecular analysis for result validation. This study also highlighted the role of mtDNA in tracing possible migration routes of Philippine and Indonesian chickens during the ancient times. Also, through mtDNA, adopting phylogenetic metrics using measures such as phylogenetic diversity and endemism makes it possible to highlight areas that can be targeted for protection and be given priority in any planning process for an improved biodiversity conservation.

CHAPTER VII

Research Summary

Red junglefowl (RJF) is an important animal resource domesticated long ago for the use of man in different aspects of life. Current reports suggested that the *Gallus gallus spadiceus* and not the *Gallus gallus gallus* is the maternal origin of the domestic chickens based on the 863 genomes sampled worldwide. In the Philippines (PH), RJFs still exist in the forests, however, their subspecific classification is still in question, given that the published results are often inconclusive and at times contradicting. To address this concern, an in-depth molecular study and analysis encompassing the whole Philippine archipelago could help us understand it genetic identity, maternal origin, and diversity, including their ecology. Furthermore, due to identification limitations, the need to use unconventional methods and DNA sources was explored as well. Connecting taxonomic classification and phylogenomic result of this study could help us address the possible conservation of this animal in the Philippines. This will also help us address one of the Sustainable Development Goals of the United Nations.

Initial identification of RJFs could serve as a vantage point towards a deeper understanding of this animals. Currently, due to the ambiguous subspecific classification of PH RJFs, the establishment of baseline data based on the morphological characteristics of the RJFs found in the different areas of the Philippines could provide us an initial perspective on the identity of these animals. Thus, evaluation of RJFs through genotyping of phenotypic morphological expressions and morphometric assessment was conducted. This study revealed that there are still RJFs in the Philippines that exhibited the wild-type morphology. Moreover, in comparing the wild-type RJFs from the hybrid

RJF, shank color, and eclipse plumage expression in male RJFs appeared to be the most important identification index. In determining the subspecific classification of RJFs, earlobe color, hackle, and mantle in males and females appeared to be the most significant parameters to assess. To bridge the gap between morphology and genetics, the genetic analysis conducted in this study supported the morphological classification of Philippine RJFs under *G.g.gallus* and *G.g.bankiva*. Consequently, this research supported the idea that identifying a wild RJF from a hybrid RJF is possible through morphological examination. Furthermore, the observed hybrid RJF in this study is a threat to the genetic diversity and integrity of RJFs.

Moreover, taxonomic classification through genotyping and morphometric analysis is not enough to verify the subspecific classification and determine the evolutionary history of the PH RJFs. Thus, phylogenetic analysis utilizing the genomic and matrilineal tracing advantage of mtDNA in addressing the past classification ambiguity of the PH RJFs, as well as evaluate its present ecological diversity status, and its complex evolutionary relationship with the RJFs in Asia was conducted. To address these concerns, we analyzed the mtDNA of the PH RJFs that were collected from the different mountainous areas in the Philippines. This study revealed that the Philippine RJFs analyzed were classified under haplogroups D, Y, and E. The result revealed multiple maternal origin of the PH RJFs, possibly from China and Indonesia. The haplotype sharing of the investigated RJFs with the domestic chickens of Laos, China, and India also showed prevalence of domestic chicken gene introgressed RJFs in the Philippines. It was also observed that two *Gallus gallus* subspecies were present

in the Philippines, the *G.g.gallus* and *G.g. bankiva*, thus confirming the morphological analysis result of this research. The genetic analysis also revealed high genetic and nucleotide diversity, and population expansion of the PH RJFs. Moreover, considering the close genetic relationship of RJFs from the Philippines and Indonesia as revealed in this study, the need to conduct phylogenetic analysis comprising the RJFs from these two countries could help us understand their complex evolutionary relationship.

Initial phylogenetic analysis suggested that the RJFs in the Philippines shared the same haplogroup and haplotype with a *G.g.bankiva* RJF from Indonesia. However, to further clarify the phylogenetic relationship of the RJFs from the Philippines and Indonesia, a phylogenetic and divergence time analysis was conducted. The results revealed that the Indonesian RJFs were solely classified under haplogroup D, in contrast to the haplogroups D, Y, and E classification of the PHRJFs. Moreover, Indonesian RJFs only shared the same haplotype with a *G.g.bankiva* from Indonesia, aside from the domestic chickens from Laos and China. Furthermore, both the Philippine and Indonesian RJFs shared the same haplotype, thus, supporting common maternal ancestry. The coalescent divergence time analysis of this study also supported the close genetic relationship of the RJFs from these two countries. The divergence analysis of this study also suggested that the RJFs from the Philippines diverge first from least most common RJF ancestor than the Indonesian RJFs.

Philippines is one of the most biodiverse countries globally, which forms a global hotspot of species diversity, including RJFs. The collective result of this study reported continued existence of RJFs in the wild. However, the haplotype

sharing of the Philippine RJFs with domestic and commercial chickens observed in this study is of biodiversity concern. This could mean genetic admixture of the wild and domestic chicken population, thereby jeopardizing the genetic integrity of the RJFs. Translating the result of this study in addressing global concerns, this study suggested that conservation consideration based on the phylogenomics must be incorporated for a more structured conservation and endangerment classification to address the SDG 15: Life on land.

In conclusion, this study revealed that differentiation of wild RJFs from RJFs introgressed with domestic chicken gene is possible through qualitative and quantitative morphological assessment. However, though the diversity and ecological status of Philippine RJFs through morphological assessment could be used as an initial evaluation approach, the need to conduct DNA analysis could provide us a better understanding of its the taxonomic classification, biodiversity status, and its evolutionary history. This study also highlighted the genomic advantage of mtDNA in determining the matrilineal ancestry, phylogenetic diversity, and endemism of the Philippine RJFs. mtDNA also highlighted the areas that can be targeted for protection and be given priority in any planning process for improved biodiversity conservation, leading to the sustainability of life on land.

CHAPTER VIII

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