Scientific Papers. Series D. Animal Science. Vol. LXV, No. 1, 2022
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

GENETIC CHARACTERISTICS OF WERIS (*GALLIRALLUS PHILIPPENSIS*) FROM MINAHASA BASED ON MITOCHONDRIAL-DNA *CYTOCHROME-B* GENES

Lucia LAMBEY[1], Ronny NOOR[2], Wasmen MANALU[2], Dedy SOLIHIN[2], Ben TAKAENDENGAN[1]

[1]Sam Ratulangi University, Jalan Kampus Unsrat, Manado 95115, North Sulawesi, Indonesia
[2]IPB University, Jalan Raya Dramaga, Bogor, West Java, Indonesia

Corresponding author email: juvarda@unsrat.ac.id

Abstract

Utilization of animal genetic resources that live wildly needs to be maintained as well as one of the conservation efforts in a sustainable manner. This study aims to obtain information on genetic characteristics and kinship of Weris (local name) Gallirallus philippensis in several locations in Minahasa through molecular analysis using the Cyt-b gene. Gallirallus philippensis in Minahasa seems to have considerable genetic differences with their relatives from the Philippines and Australia where the results of the analysis show that the species found in Minahasa (Papontolen, Ranoyapo, Tondano, and Wusa) although still have a high genetic diversity based on the existence of 7 different haplotypes and form several branches but still in the same cluster. Alleged Weris in Minahasa may be a separate species (Gallirallus celebensis) need to be considered.

Key words*: cytochrome-B, Gallirallus philippensis, genetic, Minahasa birds.*

INTRODUCTION

The Weris (*Gallirallus philippensis*) known as the "Buff Banded Rail" belongs to the genus *Gallirallus*, the Rallidae family (Allen *et al*. 2004), its widespread distribution includes the Philippines, Indonesia, New Guinea, Australia and New Zealand. In Indonesia it was known by the name of the "Mandar Padi Kalung Kuning" and in Minahada, North Sulawesi was known as the "Weris Bird". In Sulawesi there were three species namely *Gallirallus torquatus*, *Gallirallus striatus* and *Gallirallus philippensis* (GP) which live wild in nature. In addition, Coates & Bishop (1997) stated that *Gallirallus hilippensis* was also found in the regions of Maluku and Nusa Tenggara.

The three species of the genus *Gallirallus* had a diversity that distinguishes one species from another, with several key factors of each species, besides that each species was monomorphic which can be the main marker of differences of each species. As a comparison of the Rallidae family, for example the species *Gallirallus torquatus* had a dark beak and legs, a dark brown back, face, cheeks, and neck that was black and there was a prominent white line on the cheeks under the eyes, while the bottom was a black and white zebra stripe. *Gallirallus striatus* had a yellow horn beak and the base was pink, the legs were gray, the sides of the face and chest were light gray, the back was brown (Taylor 1998; Kennedy et al. 2000). *Gallirallus okinawae* had a red beak and legs, a brown back, face, cheeks and a black neck, with prominent white lines on the cheeks under the eyes and at the bottom were black and white.

The existence status of each of these species varies according to Bird Life International (2004) where *Gallirallus torquatus, Gallirallus striatus*, and *Gallirallus philippensis* were in the "least concern" status, while *Gallirallus okinawae* was in the "endangered" category. Some groups of birds were often difficult to distinguish morphologically despite occupying a variety of habitats and spread over diverse geographies, especially variations in intraspecies. However, molecularly Fain et al. (2007) stated that mitochondrial DNA had many advantages as a molecular marker at the level of vertebrate intraspecies because, according to Avise (1994) the form of the pattern of inheritance of Mitochondrial DNA through the maternal line causes no recombination and high

mutation rates. The encoding area of Cytochrome B (*Cyt b*) in the bird group (*Gallus gallus*) had a size of 1143 nucleotides (nt) located between ND5 and tRNA (Desjardins & Morais 1990). *Cyt-b* in each level of species had a relatively high variation so that this encoding can be used as a comparison for phylogenetic analysis at the level of the same species, genus or family (Bretagnole et al. 1998).

The lack of information and data regarding phylogeny and morphology of birds in the genus *Gallirallus* makes the study of phylogeny (kinship) that plays a very important role in animal management, especially to regulate breeding strategies to avoid extinction, needs to be done. The existence of Weris in their habitat serves as a reservoir of germplasm wealth, both as a collection and conservation of biodiversity and for breeding material. Animal genetic resources had an important role in the formation of superior strains, while genetic diversity can only be known through continued characterization and evaluation. In terms of the utilization of existing animal genetic resources, diversity needs to be maintained as well as one of the preservation efforts in a sustainable manner. In addition, this genetic information will be very useful and valuable for ecological, behavioral and physiological studies of Weris and other genus *Gallirallus*.

Obtaining maps and phylogenic relationships and genetic variations of Weris *(Gallirallus philippensis*) in several locations in Minahada (Papontolen, Ranoyapo, Tondano, Wusa) were the main objectives of this study.

MATERIALS AND METHODS

Research activities carried out in two stages, namely field activities for blood sampling (DNA analysis) and activities in the laboratory. Sampling was done purposively by selecting several locations in Minahada, namely Tondano (1º 17' 31.60 "N 124º 54' 03.94" E 681.5 m asl), Wusa (1º 34 '01, 24 "N 124º 55 '37.97" E 81.82 m asl), Papontolen (1º 16' 22.17 "N 124º 37 '27.73" E 13.3 m asl) and Tompaso Baru (0º 54 '47.95 ”N 124º 28' 22.25” E 346.7 m asl) (Figure 1). While the molecular analysis was conducted at the Laboratory of Molecular Biology Research Center for Biological Resources and Biotechnology, IPB University.

The equipment used in sampling was to use 40 m x 2 m green trawlers, GPS, digital hygrometer thermometers and DSLR cameras. Trawl was installed in accordance with the area of rice fields Trapped birds were usually adults. A total of 150 birds were obtained from the catcher, and 30 blood Weris were taken for DNA samples. A total of 25 samples were sequenced, and those successfully dialeded were 16 samples (Four samples in each at Papontolen, Tondano, Wusa and Ranoyapo sites.

Blood samples were taken by using a syringe on a wing vein (jugular vein) of 0.5 mL and put in a micro tube (1.5 mL) that had been partially filled with absolute alcohol and shaken until homogeneous. Then the absolute alcohol sample was added again until the eppendorf tube was full, closed and stored at room temperature.

DNA extraction was carried out by modification of the phenol purification method developed by Sambrook et al. (1989).

Total isolated DNA was then electrophoresed on 1.2% agarose gel using 1 X TBE buffer solution (89 mM Tris, 89 mM Boric Acid and 2 mM EDTA, pH 8.0) in a submarine electrophoresis (Hoefer) device. DNA was visualized using UV Transluminator (λ = 260 nm).

The primary was used to amplify the Cyt b region, which was based on Siahaan (2006), the results of extraction in the form of total DNA are used as a template to amplify the Cyt b region. A pair of primers are used to amplify the Cyt b region, namely the forward primer M101 5'-CAA ATC CTC ACA GGC CTA TTC CTA GC-3 ', and reverse primer M102 5'-TAG GCG AAT AGG AAA TAT CAT TCG GGT TGA T-3' . The PCR was performed using the AB system system 9700. The composition for each PCR reaction consisted of 2 times the Gotaq green (promega) 12.5 µl master mix, total DNA 3 µL (10-100 ng), Enhancer 2 µL, 50 mM MgCl¬2 1.5 µL, each as much as 1 µL of 20 µm primary M101 and M102 and dw (ddH 2 O) sterile water as much as 4 µL to a total volume of 25 µL. DNA Cyt b PCR products were traced using the automatic DNA machine ABI Prism version 3.4.1. (USA). The tracking process was carried out at PT. Genetic Science, Singapore. Traceability data obtained were then aligned using MEGA software version 4.0 (Tamura et al., 2007). As a comparison of the ingroup in this study the complete nucleotide of Buff Banded

Rail (*Gallirallus philippensis*, from Australia with access number Gen Bank DQ485907), and from the Rallidae family out group namely Rallus longirostris (Access number Gen bank DQ485908) (Fain et al., 2007). Analysis of phylogenetic reconstruction using MEGA software version 4.0 (Tamura et al., 2007). The results of the tracing were seen the close relationship with each other based on the genetic distance of Kimura 2 parameters with the phylogeny tree construction using the Bootstrap Neighboor Joining method 1000 repetitions.

RESULTS AND DISCUSSIONS

Habitat Description of Weris (*Gallirallus philippensis*)

The Minahasa region in general had mountainous topography and there were stretches of rivers, lakes and rice field which were widely available in Indonesia.

Rice fields can be divided into tidal paddy fields (influences to obtain water), non-tidal paddy fields (water from rivers or from irrigation channels) and rain-fed (only utilize rainwater for processing), while lebak type were made on the edge of a swamp or lake at low tide (Davies et al., 1996).

The type of rice field Minahada was non-tidal rice fields which obtain water from rivers or from irrigation channels, generally had 2 growing seasons. In this study, other bird species occupying habitat (based on morphological identification) that had been caught by trawlers were *Gallirallus torquatus, Gallinulla chloropus*, and *Porphyrio porphyrio*.

DNA Isolation and PCR *Cyt b* Gene

From 20 blood samples analyzed, 16 total DNA results were obtained with a good enough yield. From all analyzed samples successfully amplified by M101 forward primer and M 102 revers reversal DNA fragment at 695 bp. At locations W (Wusa), R (Ranoyapo), P (Papontolen), and T (Tondano), only 16 samples successfully amplified the complete *Cyt b* partial gene.

Genetic Variation *Cyt b* Gene

The DNA sequencing of the Polytase Chain Reaction (PCR) product of the *Cyt-b* gene of the *Gallirallus philippensis* bird sample from 4 locations in Minahada produces an alignment of DNA along 695-nt (nucleotides). Of the 695 nucleotides of Weris that were aligned, there was the same nucleotide (conserve) of 685 while there were 10 different nucleotides (10) with 3 parsimony sites and 7 singleton sites. There were 685 different sites, showing differences in intraspecies. Different nucleotides or (variable) had parsimony properties (Parsimony informative sites). This means that the results of the nucleotide sequence (at least two sequences) were observed and compared with other sequential data shows the difference from the other two sequential data. Parsimony occurs at sites 55, 180, and 507. However, the singleton site occurs at sites 107, 141, 156, 161, 290, 320, and 661. Nucleotide changes that cause transition substitution (pyrimidine and pyrimidine) namely Cytosine (C) to Thymine (T) in the amount of 12.03% or vice versa Thymine (T) to Cytosine (C) in the amount of 14.58%, (purines and purines) Adenine (A) to Guanin (G) a total of 5.73%, or conversely Guanin (G) to Adenine (A) a total of 12.56%. Nucleotide changes that cause subversion (purine and pyrimidine) substitution, namely Adenine (A) to Cytosine (C) of 8.79% or conversely Cytosine (C) to Adenine (A) 7.9%, Adenine (A) to Thymine (T) of 7.25% % or vice versa Thymine (T) to Adenine (A) of 7.9%. Guanin (G) to Cytosine (C) was 8.79%, or conversely Cytosine to Guanin was 3.6%. The difference in nucleotides that occur was the transition substitution was greater than the transversion substitution. Transition mutations generally occur during DNA replication whereas transversion was more related to DNA repair systems that are prone to errors (Burn & Bottino 1988; Sofro 1994). The results of the alignment along the 695 nucleotides were the most common nucleotide fragments C (31.9%), followed by A (28.7%), T (26.3%) and the least was G (13.1%). This proportion was in accordance with the opinion of Kocher et al. (1989) that for the group of birds and fish, the most nucleotides were C followed by A, T, and G (Table 1).

The data in Table 1 shows the different nucleotide sequences of all *G. philippensis* bird samples. Of the four locations, one location consisting of four samples had the same nucleotide. This similarity of nucleotides was also found in every other location was represented by 1 sample.

Table 1. Difference between nucleotide and haplotype results from 16 samples (4 locations) *G. philippensis*

Samples	1 1	1 1 1	2 3 5	6	Haplotip	Location			
	5 0 4	5 6 8	9 2 0	6					
	5 7 1	6 1 0	0 0 7	1		P	T	W	R
P3	A C C	C G A	C T T	A	1	1			
P4	. . .	. . C	. C .	.	2	1	2		
P5	G . .	. . C	. C .	C	3	1			
P7	G . .	. . C	. C .	.	4	1	1	4	2
T1	. . .	. . C	. C .	.	2				
T3	. . .	. . C	. C .	.	2				
T4	G . .	. A C	. C C	.	5		1		
T8	G . .	. . C	. C .	.	4				
W3	G . .	. . C	. C .	.	4				
W9	G . .	. . C	. C .	.	4				
W7	G . .	. . C	. C .	.	4				
W8	G . .	. . C	. C .	.	4				
R4	G . .	. . C	. C .	.	4				
R6	G . .	. . C	. C .	.	4				
R8	G G G	G . C	G C .	.	6				1
R5	G . .	. . .	. C C	.	7				1
Sum of haplotip					7	4	3	1	3

Remarks: P (Papontolen), T (Tondano), W (Wusa), R (Ranoyapo), G (Guanin), A (Adenine), T (Thymine), C (Cytosine).

Changes in nucleotides that cause transition substitution (purine bases to other purines), namely from Adenine (A) to Guanin (G) amounting to one site, namely the 55th nucleotide and vice versa from Guanin (G) to Adenine (A) amounting to one site namely the 161st site, and the transition substitution (pyrimidine base to another pyrimidine) from Thymine (T) to Cytosine (C) amounted to two sites namely 320th and 507 nucleotides. Transitional substitution (pyrimidine base to purine) from Cytosine (C) to Guanin (G) consists of four sites, namely the 107th, 141st, 156th, and 290th nucleotides or from the purine base to pyrimidine, namely from Adenine (A) to Cytosine (C) totaling two sites, namely the 180th site and 661st. The number of haplotypes produced from 16 samples of Cyt-b sequences along 695-nt from four locations amounted to 7 haplotypes. The Papontolen had 4 haplotypes, the Tondano had 3 haplotypes, the Wusa had 1 haplotype and the Ranoyapo had 3 haplotypes. These results indicate that Papontolen locations had the most diverse haplotype diversity compared to Tondano, Wusa and Ranoyapo populations. From the data shows that the Wusa location only had one haplotype (uniform).

The genetic distance was used to see the close genetic relationship between birds of Werist through the use of pairwise distance calculation analysis. With the Kimura 2 Parameter model, it can be shown a genetic difference matrix in paired nucleotides that take into account the degree of substitution of transitions and transversions. The kimura 2 parameter genetic distance value from the smallest (null) to the largest 0.009.

This shows that that *G. philippensis* cannot be distinguished between locations. A very close relationship occurs in several birds in four different locations (inter-location) because it had a genetic distance of null, but also in the intra-species relationship had a genetic distance of null or had a nucleotide similarity. Samples P4, T1, and T3 had the same nucleotide sequence. The main differences with samples P3, P5, P7, T4, T8, W3, W9, W7, W8, R4, R6, R8, and R5 were shown by the 55th nucleotide sequence (A-G). In other groups that had the same nucleotide sequence were samples of Weris, P7 T8, W3, W9, W7, W8, R4, and R6. The main difference from the T4 sample was the 161th nucleotide sequence (G-A). The R8 sample differs from the other 15 samples in the nucleotide sequence 107, 131, 147, and 290 (C-G). A close association arises in intra-species that was the species in W3, W8, W7, W9 and aware in one branch with the sample R4, R6, T8, and P7.

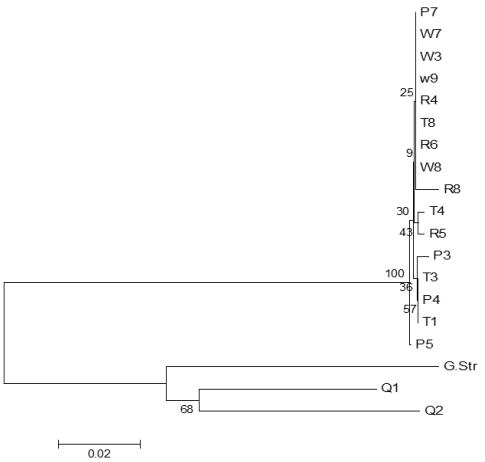

Figure 1. The phylogeny of *G. philippensis* between locations in Minahasa (P, R, T, W) and *G. philippensis* from Australia (Q1), compared to the *R. longirostris* (Q2) outgroup, *G. striatus*

The phylogeny tree construction of *G. philippensis* uses the neighbor joining method with a genetic distance of 2 kimura bootstrap parameters 1000x delivered in Figure 1, which was divided into two large clusters. Cluster A consists of 16 samples and cluster B only represented by

2 samples based on differences in nucleotides and genetic distance from each sample from four different locations. Even more clearly there were differences in interpopulation and intrapopulation, as well as similarities in intraspecies and interspecies. Samples that form clusters had slight differences (no by location). This can be explained by the fact that the four habitat sampling sites were the same even though the height of the sea level and the average location were different. The difference was only because the elders were too far away. The four sampling locations were quite far apart, but it was still possible for mating to occur between birds from one location, with another location as a population in one island typically. Another possibility that can occur was the accidental introduction by farmers or catchers. This can result in mating between birds in one location with another location. The phylogeny tree construction of *G. philippensis* originating from four populations in Minahasa compared to *G. philippensis* from Australia. and other groups of *R. longirostris*, were listed in Figure 1.

Based on the phylogeny tree above it appears that there were two main clusters, namely *G. philippensis* from Minahasa was in one cluster. The other cluster was *G. philippensis* from Australia, *R longirostris* and *Gallirallus striatus* in same branch.

A quite distant relationship between cluster Minahasa and Australia were limited distribution due to the flight range was close so that migration from Minahasa to Australia was not possible. This was supported by the theory of tectonic plate movements, namely Austaralia and Celebes (Minahasa) that were separated several thousand years ago, so that the Minahasa and Australian bird even though one species, but had different nucleotides caused by mutations resulting in evolution that can form new subspecies. The high variety of Weris haplotypes indicated cross-breeding between locations that had different haplotypes. Information about genetic diversity based on the variety of haplotypes makes Papontolen site was the most appropriate location for in-situ conservation. Conversely, by looking at a uniform haplotype, the ex-situ domestication of birds should take at the Wusa site. The breeding program through the domestication process was not only aimed at economic interests, but also to yield Weris bird that had phenotypes in accordance with the purpose of maintenance, for example producing birds for the production of meat, resistance to diseases, and so on. However, the results of captivity process was not returned to nature because it will affect the genetic purity of Weris in Minahasa. A uniform haplotype at also indicates that the ancestors did not originate from the Wusa due to this haplotype exists at the other three locations. Likewise be said that area had the most diverse haplotypes was the area of origin of the bird, in this case it should be assumed that the area was Papontolen.

If the alignment includes other species that were more closely related to *G. striatus* (Gen Bank JQ342144), *G. philippensis philippensis* (Gen Bank GJQ348003), *G. torquatus celebensis* (JQ347982), the number of nucleotides which was shorter is only 272 bp.

It can be seen (Figure 2) that *G. philippensis* from Minahasa formed a separate cluster different from *G. philippensis* from Australia and *G. philippensis philippensis*. This was likely a species of *G. celebensis* that was different from the two nearby species, based on large genetic distances. This was shown by cluster A, namely *G. philippensis* from Minahasa (1-16) with a genetic distance of 0.107, different from cluster B (17-21).

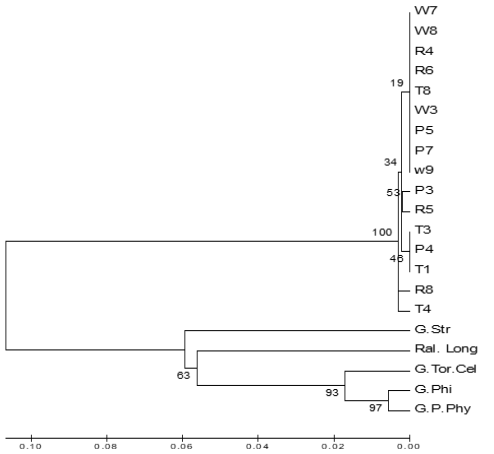

Figure 2. Phylogeny tree of Indonesia (Minahasa) *Gallirallus* sp. compared to 272 NT of Gen Bank Data

CONCLUSIONS

G. philippensis in four locations in Minahasa, namely Papontolen, Ranoyapo, Tondano and

Wusa, were still closely related but had genetic differences with *G. philippensis* from *Australia* and *G. philippensis philippensis* therefore reinforcing the notion that Minahasa's Weris bird possibly will be a separate species *(Gallirallus celebensis)* from two species from Australia and the Philippines

ACKNOWLEDGEMENTS

This research work was carried out with the support of Ministry of High Education, Cultural, Research and Technology, IPB University and also was financed from BPPS 2012.

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