The genetic variation of Indonesian native chicken crossbred between *Putih* and *Wareng* chicken based on PRL gene exon 4

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Abstract. The PRL gene in chickens is one gene that plays a role in hatchability and egg production. Therefore, knowing the variation of the PRL gene can be used to predict the performance of egg productivity of native chickens. This study observed the genetics of Indonesian native chickens, *White* and *Wareng* chickens and their crossbred based on the Prolactin (PRL) gene in exon 4. Chicken samples were kept at the Experimental Farm, Faculty of Agriculture and Animal Science, University of Muhammadiyah Malang (UMM), Indonesia. DNA isolation and PCR reactions were carried out at the UMM Biotechnology Development Laboratory. Meanwhile, PRL gene exon four sequencing was carried out at the Macrogen Asia Pacific Pte. Ltd Laboratory, Singapore. Gene sequence analysis showed that local Indonesian *Putih* and *Wareng* chickens and their crosses were closely related to *Gallus gallus* and *Gallus gallus* strain *White Leghorn* based on the phylogenetic tree with a bootstrap value of 91%. The average genetic distance between native chicken samples was 0.036 and the Tajima test was not significant ($p > 0.01$) with a value of -1.35379. For further research, it is necessary to research the correlation between gene variation and egg production performance in Indonesian native chickens and their crossbreeds.

1 Introduction

Native chickens are one of the genetic resources of Indonesia's poultry diversity. Contrasted to commercial chickens, native chickens have more additive values, like being easy and simple to keep, having lower management costs, and being more resistant to disease. In Indonesia, native chicken is also more popular among people since this chicken has a specific

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taste and aroma characters of meat and eggs, which is more likely compared to commercial chicken [1, 2]

However, native chickens have weaknesses, like slow growth, low production, broodiness trait, late sexual maturity, long laying intervals and low genetic quality [3]. Also, its characteristics is non-uniform, like the phenotype and genotype are still very varied, such as the colour of the feathers, namely black, Columbian pattern, and striped feathers. This is represented that the genetic of native chicken is more divers. Hence, to produce or obtain reliable and standard genetic abilities of native chickens, genetic quality

must be graded.

Cross-breeding is the crossing of mates between males and females from different strains. While reciprocal crossing is the re-mating of the cross-breeding but by changing the chicken's sexes, this cross-breeding model expected can increase the chicken offspring performance, especially in egg production with the same genotype ratio. Also, crossbreeding is expected to obtain the offspring or parents' stock that has a good heritability value. Thus, the crossbred expected will express the productivity match and sustain with their ancestor that have a good trait.

The prolactin gene (PRL) in chickens is one of the genes that controls of brooding, laying and reproduction in chicken. [4, 5]. The traits that are often found in local chickens are brooding and moulting. This trait will reduce egg production and disrupt the reproductive system in local chickens. [6]. Brooding activity is controlled by the hormone prolactin, one of the gonadotropin hormones produced by the anterior pituitary to stimulate the reproductive tract glands to produce sex hormones, namely estrogen, progesterone and androgen, so that prolactin has an effect on chicken egg production. [7].

This study was carried out to analyze the variation of PRL gene on exon 4 from the Indonesian native crossbred between *Putih* and *Wareng* chickens. The information on PRL gene exon four variation is not only for the enrichment of Indonesian native chicken genetic data but also to use as a model for the genetic selection of native chicken layers that are higher in egg production.

2 Materials and Methods

2.1. Chicken blood samples

The chicken whole blood samples for DNA genome sources were taken from the parental and crossbred chicken of *Putih* (P) and *Wareng* (W) chickens. The age of parent chicken were around 12 months old and the crossbred chickens were around three months old. PW is the crossbred chicken between *Putih* (male) and *Wareng* (female), the parents. While WP is the reciprocal of those crossbreeding. The chickens were reared at the hen-house of the Experimental Farm Area, Faculty of Agriculture and Animal Science, University of Muhammadiyah Malang (UMM). In this study, 14 chickens were used as whole blood sources.

2.2. DNA genome isolation and PCR reactions

The isolation of chicken DNA genome was done by using isolation kit of DNeasy® Blood & Tissue Kit (Qiagen, Germany). The 50µL of whole blood sample was used in the DNA genome isolation processed. The isolation processed was carried out at the Laboratory of Center for Biotechnology Development UMM. The separation of DNA isolates was done on 0.8% of agarose gel for DNA yields confirmation. While, the DNA sample loading for electrophoresis was 2µL.

The PCR reactions using a pair of primers, which consisted of Forward Primer (F) 5'-TGT GGA CCA GCA TGA AGA CCT A-3' and Reverse (R) 5'-AGA TCC AGT CCC TTC ACA TGG T-3' [8]. PCR reaction was done by a total volume of 25 µL consisting of 3 µL DNA sample, 15 µL Master Mix, 3 µL nuclease-free water, and two µL forward primer and two µL reverse primer. PCR reaction began with initial pre-denaturation at 94°C for three minutes, followed by denaturation at 94°C for one minute, annealing at 44°C for one minute, and elongation at 72°C for one minute, and finally post-elongation at 72°C for five minutes. PCR cycles were carried out as many as 33 cycles. The results of the PRL gene amplification fragments were detected by separating the PRL band pattern using an electrophoresis machine on a 2% agarose gel, which was stained by Ethidium Bromide and recorded using a Gel Documentary apparatus (UV Transilluminator). The PRL gene exon 4 sequence of native chicken was done using a TruSeq PCR-free Library kit using a NovaSeq6000 DNA sequencing machine. The sequencing processed was caried out at Macrogen Asia Pacific Pte. Ltd. Laboratory, Singapore. The number of PCR products from PRL gene on exon 4 that proper for sequencing was 13 samples. The references of the PRL gene on exon 4 for genetic variation analysis were referred to the GeneBank database.

2.3. Data analysis

The data analysis aims to determine the genetic variation of the PRL gene exon 4 of native chickens resulting from a cross between 2 pure lines, *White* and *Wareng* chickens. The results of the PRL gene exon four sequence were then analyzed using Molecular Evolutionary Genetics Analysis (MEGA) V.11 and DNA Sequence Polymorphism (DNAsp) V.6 software. The PRL gene in the exon 4 sequence was first compared with the GenBank database and then analyzed using the Basic Local Alignment Search Tool (BLAST) available online at https://blast.ncbi.nlm.nih.gov. The PRL gene sequence of native chickens was then edited and aligned using the ClustalW DNA Matrix (1.6) MEGA V.11 software. Phylogenetic analysis and genetic distance analysis were carried out using MEGA V.11 software with the neighbour-joining (NJ) method for the Kimura 2-parameter evolution model and 1000x bootstrap [9]. Genetic variation and evolutionary rate in samples were analyzed using DNAsp V.6 software, which includes the number of haplotypes (Hn), haplotype diversity (Hd), and nucleotide diversity (π) [10], and the evolutionary rate of samples was analyzed using Tajima's D test statistical analysis [11]. The data obtained were then analysis using quantitative descriptive analysis.

3 Results and Discussions

3.1. DNA genome isolations

The important purpose of DNA extraction is to obtain good quality DNA, which means that the DNA extracted has a high level of purity since the good quality or purity of DNA is essential for the success of future analysis, especially in the DNA amplification through PCR (polymerase chain reaction) processes. Since, the cell and protein residues can inhibit the DNA amplification process. Hence, the key to successful DNA extractions is obtaining a high level of purity, which can be determined through further work, such as DNA amplification and sequencing, and we are becoming more effective
. The DNA isolation procedure followed the DNeasy® Blood & Tissue Kit (Qiagen, Germany) protocol. All blood samples were successfully isolated. The success of DNA isolation was tested qualitatively using 0.8% agarose gel electrophoresis in TBE 1X solution. The electrophoresis visualization showed that the DNA bands were presence from the 14 of

chicken blood samples. The visualization of DNA genome isolates can be seen in Figure 1. DNA fragments can be identified, separated, and purified by agarose gel for electrophoresis [13, 14]. Gel electrophoresis is one method to determine the purity of DNA from RNA contaminants and the integrity of isolated DNA [15].

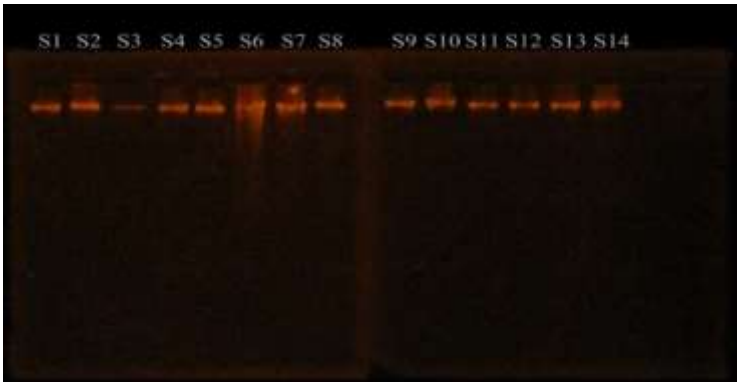

Fig. 1. Genome DNA isolates from *Putih* and *Wareng* chickens and its crossbred.

Notes: S1, S4 = *Wareng* pure-line chicken; S2, S3 = *Putih* pure-line chicken; S6, S7, S8, S10, S11 = crossbred *Putih* (male) X *White* (female) chickens; S5, S9, S12, Sl3, S14 = crossbred *Wareng* (male) X *White* (female) chickens.

DNA isolation is one of the important techniques in molecular biology. Isolation of high molecular weight DNA is becoming important with the increasing demand for DNA fingerprinting, Restriction Fragment Length Polymorphism (RFLP), genomic library construction or sequencing, also PCR analysis in research and industrial laboratories. In addition, DNA isolation is the first step in studying specific DNA sequences in complex DNA populations and analyzing genome structure and gene expression. DNA's quantity, quality, and integrity will directly affect these results [16].

The DNA isolated was showed good, which is indicated by the appearance of clear bands from all wells. The thicker and brighter of the visible band, the more DNA is obtained. The thick and bright bands qualitatively indicated a high concentration of isolated DNA, while thin bands indicated a low concentration of DNA yield [17]. DNA purity can be known qualitatively from the presence or absence of smears formed [18]. Currently, most commercial kits for DNA isolation are silica-based. In this regard, new strategies are being developed with the aim of optimizing silica-based DNA purification protocols. [19]

3.2. PCR reactions

The recent technique for amplifying DNA segments is PCR (Polymerase Chain Reactions). The DNA sample that will be amplified is first heated, so the DNA is denatured or separated into two single-stranded DNA parts. Then, a *Taq polymerase* enzyme can synthesize two copies of new DNA strands by using the original strand as a template. This process produces duplicates of the original DNA, with each new molecule containing one strand of old DNA and one strand of new DNA. Then each strand can be used to make two new copies of the DNA strand, and so forth. The cycle of denaturation and new DNA synthesis can be repeated 30 or 40 times, producing more than a billion exact copies of the original DNA segment. The entire PCR cycle process is automated and can be completed in just a few hours. The process can be controlled by a machine called a thermocycler, which is programmed to change the

reaction temperature every few minutes to allow the denaturation and synthesis of DNA to run according to the program [20].

Amplification of the PRL gene exon four from the fragment has been successfully amplified at an annealing temperature of 44°C for 60 seconds. A total of 14 samples have been successfully amplified at that temperature and time. The PCR cycle for amplification of chicken DNA samples was carried out in 33 cycles. According to Wetton et al. (2002), the number of cycles generally ranges from 20-40 cycles. In the chicken PRL gene, the PCR reactions can be 35 cycles [21], while in the CHD gene on the Z and W chromosomes in chickens, the PCR cycle is 35 times [22]. The PCR product was then verified by 2% agarose gel electrophoresis. The electrophoresis of the PCR product is shown in Figure 2 and Figure 3.

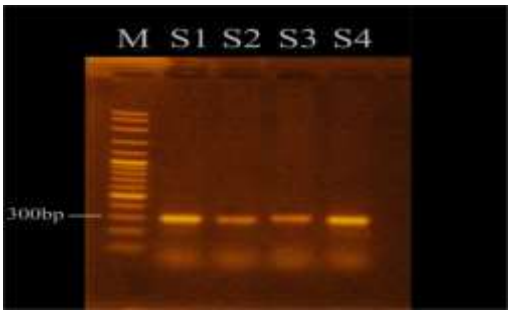

Fig. 2. Sample PCR products from the DNA of native chicken parents separated in 2% agarose gel. M= Marker; S1, S2 = *Putih* chicken Parents; S3, S4 = *Wareng* chicken Parents

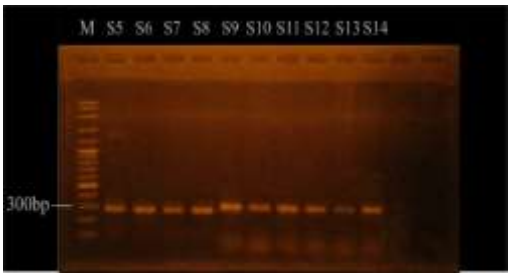

Fig. 3. Sample PCR products from the DNA of crossbred native chicken separated in 2% agarose gel. M= Marker; S6, S7, S8, S10, S11 = *Putih* (male) X *Wareng* (female) crossbred; S5, S9, S12, Sl3, S14 = *Wareng* (male) X *Putih* (female) crossbred.

Although agarose gel electrophoresis was introduced almost 50 years ago, this method is still a reliable method that is commonly applied for DNA separation, for example PCR products. When applying agarose gel electrophoresis to evaluate the success of a PCR reaction, the brightness of the bands provides additional information than simply indicating the success or failure of the reaction. Evaluation of band brightness can be used to assess the relative quantity of amplicons of the same length. This can be useful for post-PCR applications that require PCR product concentrations within a specific range to function correctly since the additional quantification step can be omitted. [23].

The primers successfully amplified target DNA fragments with a length of approximately 259 bp. These results are by the study of Osman et al. [8], that the PRL4F and PRL4R primers successfully annealed at locations 5816 bp-5825 bp in intron 3 and locations 6053 bp-6074 bp in intron 4 of the chicken prolactin gene. Both of these primers resulted in

the amplification of PCR products of approximately 259 bp at nucleotide positions 5816-6074bp of the prolactin gene, which respectively represent the intron 3 (10 bp), exon 4 (183 bp) and intron 4 (66 bp) of the chicken prolactin gene. The DNA bands from each sample in Figures 2 and 38 are in the same row position. This indicates that the DNA isolation sample has high DNA specificity [24].

3.3. Basic Local Alignment Search Tool (BLAST)

Currently, one of the most commonly used tools to examine DNA and protein sequences is the Basic Local Alignment Search Tool, known as BLAST. BLAST is a computer algorithm available for use online on the National Center for Biotechnology Information (NCBI) website, as well as many other sites. BLAST can quickly align and compare query DNA sequences with sequence databases, making it an important tool in today's genomics research. In fact, the initial paper describing the program, published in the Journal of Molecular Biology and titled "Basic Local Alignment Search Tool," was the most cited publication in the 1990s [25]. In recent years, the parallel development of large-scale sequencing projects, bioinformatics tools such as BLAST have enabled scientists to study the genetic blueprint of life in many species, and have also helped connect biology and computer science in the emerging field of bioinformatics [26].

The results of the BLAST analysis showed that 13 sequence data from the research sample (one sample number 4 was not eligible) were highly similar to the *Gallus gallus* chicken species. The PRL gene sequence of native chickens that was used as a reference in the BLAST analysis was sample no. 5, namely the F1 crossbred chicken between *Putih* X *Wareng*. The BLAST result sequence was selected from among the top 7 nucleotide sequences with the highest similarity and used as the basis for constructing a phylogenetic tree. The BLAST results can be seen in Table 1. There are several parameters that can be seen in the BLAST analysis, namely Max score, Query cover, E-value and ident. Max score shows the value of base pair similarity, Query cover shows the percentage of samples used in the BLAST analysis, and E-value shows the statistical probability level of an item. From the four parameters, the most accurate is seen from the E-value [27]. The smaller the E-value, the higher the level of homology. Conversely, the higher the Max score and ident values, the higher the level of homology [28].

Table 1. BLAST analysis of PRL gene exon 4

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Yangshan	333	333	80%	7e-87	97.45	AH013784.3
Taihe Silkies	333	333	80%	7e-87	97.45	AH013785.3
White Recessive Rocks	333	333	80%	7e-87	97.45	AH013786.3
White Leghorn	333	333	80%	7e-87	97.45	AH013783.3
Gallus gallus	389	389	86%	3e-76	98.80	AB011438.2

The results of the BLAST analysis showed that the sequence similarity (identity) between the GenBank database and the PRL gene from the sample was 97.45% - 98.80%, or higher than GenBank. The percentage of sequence similarity with GenBank data is said to be

significant if it reaches 97% - 100%. While the percentage of similarity between 92% and 96% is included in the sufficient category, less than 91% is not significant. The query cover value is 80% - 86%, which means that 91% - 100% of the sequences in the sequence in the BLAST analysis are used [29]. Thus, in this study, BLAST proves that the sequence analyzed is part of the PRL gene. The BLAST analysis can be determined from the score parameters that are more than 150 and the E value is less than 10^{-4} or close to zero (0). Thus, the level of homology is good. The higher the score (bit), the better the level of homology, and the lower the E value, the better the level of homology [30]. The BLAST results show that the PRL gene sample in this study has the highest similarity to the *Gallus gallus* sequence, with the same Max score and total score of 389, an E value of < 0.0, and a Similarity value of 98.80%. This shows that the results of the sequence alignment are significant. Therefore, the sample specimens are identical because they are suspected to be in the same genus and even at the species level. They also have a high level of homology with the *Gallus gallus* sequence in the GenBank database.

3.4. Phylogenetics and genetics distances

Phylogenetics is the scientific study of phylogeny. Phylogenetics studies the evolutionary relationships among different groups of organisms based on their evolutionary history, similarities, and differences. Phylogenetics uses molecular sequencing data (such as homologous sequences, protein sequences, nucleotide sequences, etc.) and morphological data matrices to understand and analyze the evolution of proteins and genes from genetically related groups of organisms [31].

In this study, phylogenetic analysis was performed using 13 DNA sequences from local chicken samples and seven sequences from the GenBank database information. The phylogenetic tree was constructed using MEGA 11 software [9] with the Neighbor-Joining (NJ) method based on the Kimura 2-parameter model, with a bootstrap value 1000x. The results of the phylogenetic analysis are shown in Figure 4.

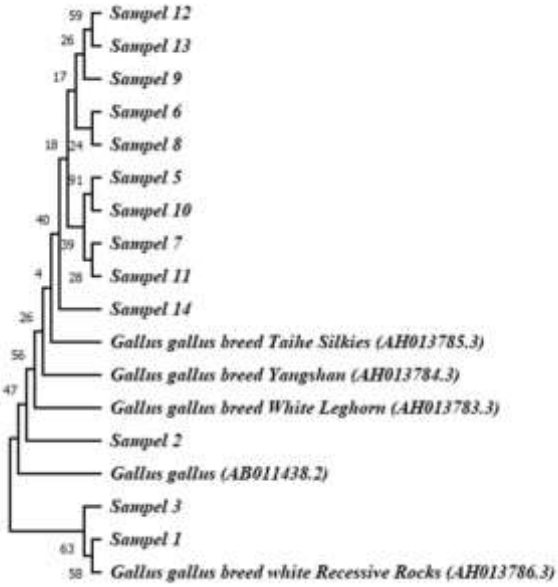

Fig. 4. The phylogenetic tree of 20 sequences of PRL gene exon 4 of chickens

The phylogenetic tree shows that all four sequences of the PRL gene exon have a close genetic relationship, which is indicated by one branch of the phylogenetic tree. Sample no. 2, namely the *Putih* male chicken, has the closest genetic relationship with *Gallus gallus* and *Gallus gallus* breed *White Leghorn*, and sample no. 1, namely the *Wareng* male chicken, has the closest genetic relationship with the *White Recessive Racks* chicken. This shows that the chickens used as the parents of the chickens in this study are closely related to the sequence in the GenBank database that has been studied previously.

Putih chicken is a local Indonesian chicken that is often found in the Kedu area, Central Java, which is a crossbred from Dorking and native chicken in the Dieng area [32], while *Wareng* chicken is also one of the local Indonesian chicken that is often found and recognized by the Tangerang community as a Local Genetic Resource for their area. *Wareng* chicken is also called *Tangerang Wareng* chicken or *Russian* chicken. Initially, this chicken came from eggs owned by Mr. Armin in Pasir Gadung Village, Cikupa District, Tangerang Regency, in the early 1980s. The eggs came from his friend who sent them from Russia. Mr Armin's chickens were further bred and spread throughout Tangerang Regency, West Java [33].

In the reconstruction of the phylogenetic tree, the highest bootstrap value was 91%, and the lowest was 4%, indicating that the phylogenetic tree structure has a high level of confidence because the resulting branching has a value of more than 65% [34]. Evaluation of the bootstrap value on each cladogram shows that the Prolactin exon 4 DNA sequence sample data have a range of too weak to moderate bootstrap values with 4%-91%. The bootstrap value categories include high (>85%), moderate (70–85%), weak (50–69%), or too weak (<50%) [35]. The data used is good if the bootstrap value ranges between 70%-95% [36].

Bootstrapping measures how consistently the data support a bipartition of taxa. It is not a test of how accurate a phylogenetic tree is; it simply provides information about the stability of the tree topology (branching order) and helps assess whether the sequence data are adequate to validate the topology. A high bootstrap value (close to 100%) indicates uniform support; if the bootstrap value of a particular clade is close to 100%, nearly all informative characters agree that it is a group [37].

Table 2. The genetics distance between the sample Indonesian native chicken and reference sequence from GeneBank

	Sampel_1	Sampel_2	Sampel_3	Sampel_5	Sampel_6	Sampel_7	Sampel_8	Sampel_9	Sampel_10	Sampel_11	Sampel_12	Sampel_13	Sampel_14	Yangshan	white Recessive Rocks	Tahe Sikes	White Leghorn	Gallus gallus
Sampel_1		0,024	0,026	0,024	0,029	0,029	0,026	0,029	0,024	0,027	0,031	0,029	0,024	0,017	0,015	0,017	0,017	0,014429189
Sampel_2	0,091		0,024	0,018	0,018	0,021	0,015	0,019	0,018	0,015	0,021	0,021	0,017	0,013	0,013	0,013	0,013	0,016727973
Sampel_3	0,098	0,092		0,022	0,027	0,027	0,024	0,025	0,022	0,023	0,029	0,026	0,022	0,021	0,017	0,021	0,021	0,016823897
Sampel_5	0,096	0,050	0,073		0,014	0,013	0,011	0,014	0,000	0,009	0,016	0,015	0,008	0,010	0,009	0,010	0,010	0,016727973
Sampel_6	0,122	0,056	0,108	0,039		0,015	0,008	0,012	0,014	0,010	0,011	0,014	0,011	0,013	0,017	0,013	0,013	0,008845778
Sampel_7	0,135	0,077	0,109	0,034	0,045		0,013	0,015	0,013	0,013	0,016	0,017	0,013	0,015	0,018	0,015	0,015	0,015783865
Sampel_8	0,109	0,040	0,091	0,024	0,014	0,035		0,011	0,011	0,010	0,011	0,015	0,008	0,010	0,013	0,010	0,010	0,008845778
Sampel_9	0,121	0,061	0,096	0,039	0,029	0,045	0,024		0,014	0,012	0,013	0,014	0,013	0,014	0,012	0,014	0,014	0,016727973
Sampel_10	0,096	0,050	0,073	0,000	0,039	0,034	0,024	0,039		0,009	0,016	0,015	0,008	0,010	0,009	0,010	0,010	0,016727973
Sampel_11	0,111	0,046	0,081	0,015	0,020	0,030	0,020	0,030	0,015		0,013	0,015	0,010	0,012	0,013	0,012	0,012	0,012893229
Sampel_12	0,134	0,071	0,114	0,044	0,024	0,050	0,024	0,034	0,044	0,035		0,011	0,012	0,014	0,018	0,014	0,014	0,016727973
Sampel_13	0,122	0,072	0,096	0,039	0,039	0,055	0,040	0,039	0,039	0,040	0,024		0,013	0,013	0,017	0,013	0,013	0,012919438
Sampel_14	0,097	0,050	0,079	0,014	0,024	0,035	0,014	0,024	0,014	0,020	0,024	0,029		0,006	0,010	0,006	0,006	0,008845778
Gallus_gallus_breed_Yangshan_(AH013784.3)	0,045	0,027	0,063	0,016	0,027	0,039	0,016	0,033	0,016	0,022	0,032	0,027	0,005		0,011	0,000	0,000	0,008845778
Gallus_gallus_breed_white_Recessive_Rocks_(AH013786.3)	0,033	0,027	0,044	0,016	0,044	0,056	0,027	0,027	0,016	0,028	0,049	0,044	0,016	0,021		0,011	0,011	0,008845778
Gallus_gallus_breed_Tahe_Sikes_(AH013785.3)	0,045	0,027	0,063	0,016	0,027	0,039	0,016	0,033	0,016	0,022	0,032	0,027	0,005	0,000	0,021		0,000	0,008845778
Gallus_gallus_breed_White_Leghorn_(AH013783.3)	0,045	0,027	0,063	0,016	0,027	0,039	0,016	0,033	0,016	0,022	0,032	0,027	0,005	0,000	0,021	0,000		0,008845778
Gallus_gallus_(AB011438.2)	0,032	0,018	0,045	0,018	0,012	0,038	0,012	0,018	0,018	0,025	0,018	0,025	0,012	0,012	0,012	0,012	0,012	

Notes: 1-14= chicken samples; 15= Yangshan (AH013784.3); 16= White Recessive Rocks (AH013786.3); 17= Tahe Sikes (AH013785); 18= White Leghorn (AH013783.3); 19= *Gallus gallus* (AB011438.2).

The analysis of genetic relationship can be determined from the genetic distance between each individual. The analysis using MEGA 11 software [9] showed that genetic variation between 13 sample sequences of native chicken had a value range 0.012-0.135 with an average of 0.054, while in 19 total sequences had a value range 0.012-0.135 with an average of 0.036. These results mean that in 13 sequence samples, there is an average of 1 different base pair for every 1000. In 18 sequences, there are two different base pairs. According to Nei and Kumar [10], the genetic distance is close if the value ranges from 0.010-0.099, stated as moderate ranges from 0.100-0.990 and far with the range of 1-2. The genetic distance's closeness indicates the genetic relationship between sequences, which is indicated by the low variation or difference in base pairs between the sequences. Hence, it can be stated that the sequences have a low level of diversity [38]. The low matrix values indicate that a close relationship and are also indicated by the shape of the phylogenetic tree. Thus, the genetic distance between each individual has a low level of genetic difference [39].

3.5. The genetic variation analysis

The analysis of the Genetic Diversity and Polymorphism Index on sequences using DNASp software showed that 18 sequences have 15 sites, while 13 native chicken sample sequences have 13. The sequence diversity value in 18 sequences was 0.01016, while in the 13 native chicken sequences, it was 0.01220. Furthermore, the haplotype diversity value in the total of sequence analysis was 0.56863, while in the native chicken was 0.64103. The results of the analysis are shown in Table 3.

Table 3. Genetic diversity and polymorphism index

Sequence	N	NHap	S	Pi	Hd	Tajima's Test
Native chicken sequences	13	6	13	0,01220	0.64103	-1.35379[NS] (p >0.10)
Total sequence	18	7	15	0.01016	0.56863	-1.63151[NS] (p >0.10)

Note: N= Sequence number; NHap= Haplotype number; S= Site number; Pi= Sequence diversity; Hd= Haplotype diversity, and NS=Not significance

The results of genetic diversity of all chicken sequences showed low numbers with the number of haplotypes (NHap) was 7 with haplotype diversity (Hd) was 0.56863 and nucleotide diversity (Pi) was 0.01016. Sulandari study [40] based on polymorphic site of the mitochondrial D-loop HV1 region has found 69 haplotypes identified on Indonesian indigenous chicken, two haplotypes from the gen *Gallus* which sequence is taken from GenBank: *Gallus gallus gallus* (GenBank accession number AB007720) and *Gallus gallus bankiva* (GenBank accession number AB007718) and 7 Clade reference haplotypes (Clade I, II, IIIa, IIIb, IIIc, IIId, and IV). A study by Godinez [41] in South East Asia (SAE) chicken showed that the analysis of 519 complete mitochondrial DNA control region sequences identified 133 haplotypes with 70 variable locations. Then, 82.7% of haplotypes are geographically unique and distributed across the main haplogroups, except for haplogroup C, which shows high polymorphism among the studied individuals. Mainland South East Asia chickens (MSEA) have higher overall genetic diversity than island SEA chickens (ISEA).

Haplotype diversity values range from 0.80000 - 1.00000 in the high category, 0.50000 - 0.70000 in the medium category and 0.10000 - 0.40000 in the low category [10]. The higher of the diversity of the haplotype (Hd), the higher of the genetic diversity level in a population and vice versa. Haplotypes will increase with the increasing number of samples analyzed [42]. Haplotype variation greatly determines the level of genetic diversity which is a key factor in the survival of a species [43]. Changes in haplotype variation are greatly influenced

by various factors such as natural selection, migration, mutation, and gene flow that lead to the formation of new species [44].

The Tajima D test can determine genetic evolution in a DNA sequence [11]. The Tajima D test shows a non-significant negative result in the reference sequence (-0.90920) and all native chicken sequences (-1.53473). Non-significant negative values identify that genetic diversity in the sequence data set used is low (Adimaka et al., 2019) [45]. Two factors influence genetic diversity: genetic diversity increases factors, including mutation and migration, and genetic diversity decreases factors, including natural selection and genetic drift [46]. These factors are generally occurrence in the evolution of population [47]. Hence, this means that the low genetic diversity will result a low level of evolution, hence the current chicken sequence is not too different from the previous chicken.

4 Conclusions

The genetic variation of Indonesian native chicken *Putih* and *Wareng* chickens and its crossbred based on the PRL exon four gene is low, which refers to a low genetic distance value ranging from 0.000-0.135 with an average of 0.054, as well as haplotype diversity (Hd) of 0.64103 and nucleotide diversity (Pi) of 0.01220 which is also low. The existence of low genetic diversity also shows that the level of evolution in Indonesian native chickens related to this study is low. Thus, the current Indonesian native chickens are not genetically diverse from the previously genetic chickens.

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