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Polymorphisms of Palu Sheep IGF-1 Gene and Their Relationship with Skeletal Growth

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ABSTRACT

This study aims to examine the polymorphisms of the IGF-1 gene associated with body weight and size. A total of 60 sheep from different farm locations in Palu City (Villages of Taipa, Poboya, Kawatuna, and Petobo), Central Sulawesi, Indonesia, were used. The variables observed were adult body weight and measurements, such as shoulder height, body length, humerus length, radius-ulna length, metacarpus length, hip height, femur length, tibial length, and metatarsus length as phenotypic characteristics. IGF-1 gene polymorphisms were analyzed using the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) with Bsp143II as a restriction enzyme. These gene polymorphisms were calculated using the allele and genotype frequency approach, as well as the Polymorphic Informative Content (PIC). Genotype associations with body weight and bone size were analyzed using the General Linear Model analysis method. The PCR-RFLP analysis results showed the presence of polymorphisms with the frequency and genotypes of GG (21.7%), GC (68.3%), and CC (10%). Furthermore, the frequencies of G (55.8%) and C (44.2%) genes with a 49.32% polymorphism rate (PIC) were not in the Hardy-Weinberg equilibrium ($p<0.05$). In conclusion, the IGF-1 gene polymorphism was detected in Palu sheep with an insignificant effect on body weight and bone size.

Keywords: Palu sheep; IGF-1 gene; PCR-RFLP; body weight; body size

INTRODUCTION

Optimizing livestock utilization is necessary due to the current increase in meat production. Sheep is a small ruminant that significantly contributes to meat production because it is effortless to maintain, does not require a large maintenance space, and unqualified feed is often changed to their living needs. Many of them were raised in rural and suburban areas (Widiati & Kusumastuti, 2017). According to Budisatria *et al.* (2010), sheep in Indonesia are generally raised for meat production or saving purposes.

Palu sheep as a germplasm asset in Central Sulawesi has a good potential to be developed as a meat source, and the fat-tailed type has been raised from one generation to another in the Palu Valley of this province. The animal was initially known as Donggala and later designated as the Palu Sheep Clump based on the Minister of Agriculture Decree Number 697/Kpts/PD.410/2/2013. Both the male and female sheep are processed as meat for the community (Malewa, 2014).

However, the main challenge in the sheep-farming business is the low productivity of livestock (Petrović *et al.,* 2011) and low sustainability of superior young sheep supply with high and efficient production and affordable prices in the breeder's levels (Montossi *et al.,* 2013). Furthermore, the young sheep, which acts as the cultivation process initial capital, directly affects profits, causing its quality to be needed in sufficient quantities, easy to obtain, and with guaranteed sustainability (Udo & Budisatria, 2011).

Superior young sheep requires a sufficient population, with phenotypic and genotypic diversities used to generate more significant selection opportunities. One significant problem associated with the Palu breed is a deficient population compared to the other regions in Indonesia, as it decreases continuously per year due to the narrowing of grazing areas by settlement developments. For instance, in 2014, the population decreased to 8164 heads (Badan Pusat Statistik Provinsi Sulawesi Tengah, 2015). Also, there is no extensification program for sheep farming for breeders in the other areas with an adequate feed environment.

The existence of livestock products based on the consumer-quality demand is acquired from young sheep with adequate technical requirements (Peraturan Menteri Pertanian Nomor :19/Permentan /Ot.140/2/2010 Tentang Pedoman Umum Program Swasembada Daging Sapi 2014, 2010). This was obtained due to the established guidelines for good goats and sheep breeding (good breeding practice). The criteria enabled the possibility of young-sheep maintenance at experimental sta-

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tions or by livestock companies specialized in inbreeding, and they are vulnerable to be sold when conflicting with the interests of breeders. At the same time, the potential of young sheep is also expected to be obtained from the community's farms through genetic improvement selection (Haile *et al.*, 2019; Yousefi & Azari, 2012).

The growth trait controlled by multiple genes is an economic trait for livestock. Hence Molotsi *et al.* (2017) stated the selection of sheep with high growth characteristics greatly benefits breeders. Furthermore, advances in molecular biology have enabled the efforts of scientists to increase the accuracy and efficiency of conventional selection through marker-assisted selection. Therefore, the diversity of genes that significantly influence economic properties is very useful information.

According to a previous study, the growth rate and weaning weight of Palu and East Java sheep are determined by analyzing the effects of GH I HaeIII polymorphism (Malewa, 2014). The IGF-1, as one of the genes involved in controlling growth traits, is expected to complement the information used in the MAS-based Palu sheep selection program (Marker-Assisted Selection). The IGF-1 gene polymorphisms analyzed using the PCR-RFLP Bsp143ll method showed their relationship to the phenotypic diversity (body weight and size of sheep) as potential candidate genes controlling growth traits. This study aims to examine the polymorphisms of the IGF-1 gene associated with body weight and size.

MATERIALS AND METHODS

A certificate of Research Ethics Feasibility was received from the Animal Ethics Commission for Animal Research, Faculty of Animal Husbandry and Fisheries, Tadulako University with number: 9675A/UN28.1.31/PT/2018.

This field and laboratory study is used to determine the relationship between the IGF-1 gene polymorphism and the body weight and size of 37 male and 23 female sheep aged 1, 2, and 3 years. A total of 60 sheep from the farm locations in Palu City (Villages of Taipa, Poboya, Kawatuna, and Petobo) were used. The weight correction factor included Age 1 year= 1.08, 2 years= 1, and 3 years= 0.86.

The data collected were body weight (BW) in adult sheep (kg), shoulder height (SH), body length (BL), humerus length (HL), radius-ulna length (RuL), metacarpus length (ML), hip height (HL), femur length (FL), and tibia length (TL) in cm.

Blood Sampling

The tools used for blood sampling were a vacutainer needle no 21G, a 6 mm Vacutainer + EDTA tube, an ice-box, and an ice jelly pack. Blood samples were collected from sheep in the morning at 7.00-9.00 before meals after measuring their body weight and bone size, making approximately 6 mL obtained from the neck's jugular vein through a needle and then dropped into a vacutainer tube containing 1 mL of 10% EDTA. This was followed by storage in an ice-box prepared and filled with an ice jelly pack while in the field before being isolated in a freezer.

Measurements were made using a measuring stick/ruler with a scale on SH, BL, HL, RuL, ML, HH, FL, TL, and MtL in centimeter (cm). Shoulder height (SH) was the size of the body measured from the highest point of the shoulder (*Os vertebra thoracalis*) until the ground level was perpendicular (Tyasi *et al.*, 2015). Body length (BL) was from the anterior shoulder edge to the posterior edge of the ischium (Sabioni *et al.*, 2020). Hip height (HH) was measured from the highest point of the hip to the ground level perpendicularly (Tyasi *et al.*, 2015). Humerus length (HL) was measured from the humerus tip head to the bottom end, femur length (FL) was measured from a distance between both ends of the femur, radius-ulna length (RuL) was measured from the olecranon to the styloid process of the ulna near the carpus, metacarpus length (ML) was measured from carpus to prox sesamoids, tibial length (TL) was measured from the tibia top to the bottom, and metatarsus length was measured from a distance between both ends of the metatarsus (Putra *et al.*, 2012).

Total DNA Isolation

DNA isolation included the extraction and purification process were carried out with whole blood. Total DNA isolation was conducted using a modified phenol method and a Genomic Blood Kit manufactured in NORGREN Canada (Sambrook *et al.*, 1989). Furthermore, DNA, PCR, and RFLP isolations were carried out at the Laboratory of Animal Molecular Genetics, Faculty of Animal Husbandry, Bogor Agricultural University (IPB University). Sequencing was performed to determine the similarities or mutations in Palu sheep samples by comparing them with the *Ovis aries* gene Bank. Furthermore, the sequence (ABI trace file) was analyzed molecularly with Evolutionary Genetic Analysis (MEGA) 6.0. Basic Local Alignment Search Tool (BLAST) used to identify similarities (homology) with gene data in GenBank (www.ncbi.nhl.nih.gov./BLAST) (Eric *et al.*, 2014).

DNA extraction was carried out manually using the phenol-chloroform method. About 100 µL from each sheep sample was put into 1.5 mL tubes, making the number of blood samples used to be 60 pieces. A total of 1,000 µL of 0.2% NaCl was added to the tube, then shaken with a vortex until homogeneous, and left to stand for 5 minutes at room temperature. After that, the mixture was centrifuged at a speed of 8,000 rpm for 5 minutes. The supernatant was removed, and 350 µL x STE (sodium tris-EDTA), 40 µL of 10% SDS (sodium dodecyl sulfate), and 10 µL of protK were added. The solution was shaken with a titler at 55 °C for 2 hours, then 40 µL of 5M NaCl, 400 µL of phenol solution, and 400 µL of CIAA (chloroform isoamyl alcohol) were added and shaken again with a titler for 1 hour at room temperature. The solution formed was centrifuged at a speed of 12,000 rpm for 5 minutes. About 400 µL of the supernatant (clear top part) was dropped into a new 1.5 mL tube with a pipette, and 800 µL of absolute ethanol (EtOH) was added, then shaken until homogeneous

and allowed to stand overnight at -20 °C. Afterward, the solution was centrifuged at 12,000 rpm for 5 minutes, and the supernatant was removed. A total of 800 µL of 70% ethanol was added and again centrifuged at 12,000 rpm for 5 minutes. The DNA molecule precipitate at the bottom of the tube was dried for 2-3 hours, followed by adding 100 µL of 80% TE buffer (tris EDTA), then shaken until it became homogeneous and the DNA was ready for use.

DNA Amplification with Polymerase Chain Reaction (PCR)

DNA analysis was performed using the PCR-RFLP method and electrophoresis based on instructions (Moradian *et al.,* 2013). In addition, a specific oligonucleotide primers' pair was used to prepare a 5' to 3' end hybrid of the target DNA strand, which was amplified to the desired sequence. The primers used for PCR were designed using oligo explorer software and a part of the insulin-like growth factor-I (IGF-I) gene sequence in *O. aries* with access number GenBank x17229 (Dickson *et al*., 1991). The primers used were F: 5'-TGA GGG GAG CCA ATT ACA AAG C-3, and R: 5'-TCG TGG AGG ATA GGT GAG CA-3', with an amplicon length of 238 bp. Also, the composition of the solution for PCR was as follows: 2 µL DdH2O, 1 µL Primer F, 1 µL Primer R, 5 µL PCR mic (Promega), and 1 µl Whole Genome.

PCR products from the Palu sheep DNA genome along 238 bases (bp) are based on the *O. aries* gene sequence located in the first intron (1436-1674) of the IGF1 gene (GenBank X17229). The SNP target position for the IGF-1 gene based on the primer used is at position "1511bp" according to the GenBank x17229 gene reference. The composition of the solution for PCR was as follows: 10 µL Nuclease Free Water, 0.5 µL Primer F, 0.5 µL Primer R, 2x 12.5 µL MyTaq HS Red Mix, and 2 µL DNA Template in a 0.2 mL tube.

Statistical Analysis

Polymorphism analysis in the population utilized % heterozygosity of DEG using the following formula:

$$PICi = 1 - \sum p^2_{ij} \text{ or } PICi = 1 - (p^2 + q^2)$$

where PICi was *Polymorphic Information Content* for i locus, i was genotype (GG, GC, and CC), j was allele (gene), Yij was average of body weight and body size, and pij was the frequency of the j allele from the i locus.

The mathematical model for allele frequencies is represented as follows:

$$Xi = (2nii + \sum nij) / 2N$$

where Xi was frequency of the ith allele, nii was number of individuals with genotype ii, nij was number of individuals with genotype ij, and N was number of individual samples

The formula for calculating the genotype frequency is represented as follows:

$$Xi = (Gi / N) \times 100\%$$

where Xi was the frequency of the ith genotype, Gi was the number of individuals with genotype i, and N was the number of individual samples.

Also, Hardy-Weinberg equilibrium (HWE) was determined using the Popgene32 program, while the Chi-Square equation is as follows:

$$\chi 2 = \sum \{(O\text{-}E)\ 2) / E\},$$

where $\chi 2$ was HWE test, O was the observed number of genotypes, E was the expected number of genotypes, df was the genotype probabilities number of alleles.

The formula for calculating the polymorphism value (PIC) in each locus is as follows:

$$PIC= 1 - \sum_k^{i=1} Pi - \sum_k^{i=1} 2Pi2Pj$$

where Pi and Pj were frequency of the i and j alleles and k was the number of alleles.

The IGF-1 gene associations with body weight and bone size were analyzed using ANOVA with the GLM method of Minitab 16 software by including genotype effect after correction factors for age and gender were obtained by analysis of variance following statistical models.

$$Yij = \mu + \alpha i + € ij$$

where Yij was averages of body weight and size, µ was general average, αi was the influence of IGF-1 gene genotype, i was GG, GC, and CC, and € ij was error

RESULTS

IGF1 Gene Association with Body Weight and Bone Size of Palu Sheep

The association analysis showed that the IGF1 | gene Bsp143|| had no significant effect on body weight and bone size in Palu sheep. Besides, the associated body weight and bone size are presented in Tables 1 and 2.

IGF-1 Gene Amplification

The products of IGF-1 gene amplification determined in the two primary locations were annealed at 60.3 °C for 45 seconds. The results were PCR products with a length of 238 bases (bp) obtained using a personal Eppendorf master cycler PCR machine (Figure 1).

DISCUSSION

PCR products from the Palu sheep DNA genome along 238 bases (bp) were based on the *O. aries* gene sequence located in the first intron (1436-1674) of the IGF1 gene (GenBank X17229) (Dickson *et al.,* 1991; Curi *et al.,* 2005). The primers were F: TGAAGAACACAAGTAGAGGG, R: GGGTCATTTTTGCAAGGTG, while the restriction enzyme used was Bsp143|| (*Bacillus* sp. RFL143) with 5'-RGCGC↓Y-'3 as the cut point.

The Restriction Fragment Length Polymorphism (RFLP) method was used to determine the IGF-1 5'-flanking region's genotype. According to Farajallah (2007), this is the easiest, cheapest, and most accurate

Table 1. Body weight and body size of different IGF-1 genotype in Palu Sheep

Body weight and body size	Genotype (heads) (value ± sd)		
	GG	GC	CC
	N= 13	N= 41	N= 6
Body weight (kg)	26.08±1.82	26.05±1.08	25.00±1.95
Shoulder height (cm)	58.23±0.98	57.05±0.67	56.33±1.45
Body length (cm)	55.92±1.01	54.63±0.53	55.67±1.43
Humerus length (cm)	13.30±0.30	13.78±0.18	13.50±0.44
Radius-ulna length (cm)	15.55±0.36	15.76±0.18	15.73±0.41
Metacarpus length (cm)	13.22±0.21	13.20±0.14	12.75±0.27
Hip height (cm)	62.54±1.22	61.27±0.74	62.17±1.35
Femur length (cm)	15.60±0.23	15.55±0.22	15.58±0.57
Tibia length (cm)	23.83±0.42	23.37±0.23	23.35±0.67
Metatarsus length (cm)	15.73±0.21	15.45±0.14	15.45±0.37

Note: N= number of sample.

Table 2. Genotype and allele frequencies, polymorphic informative content (PIC), and test χ2 of IGF-1 gene in Palu sheep

Sample	N	Genotype frequency			Frequency allele		PIC (%)	χ2
		CC	GC	GG	C	G		
Palu sheep	60	0.10(6)	0.68(41)	0.22(13)	0.44	0. 56	49.32	8.92

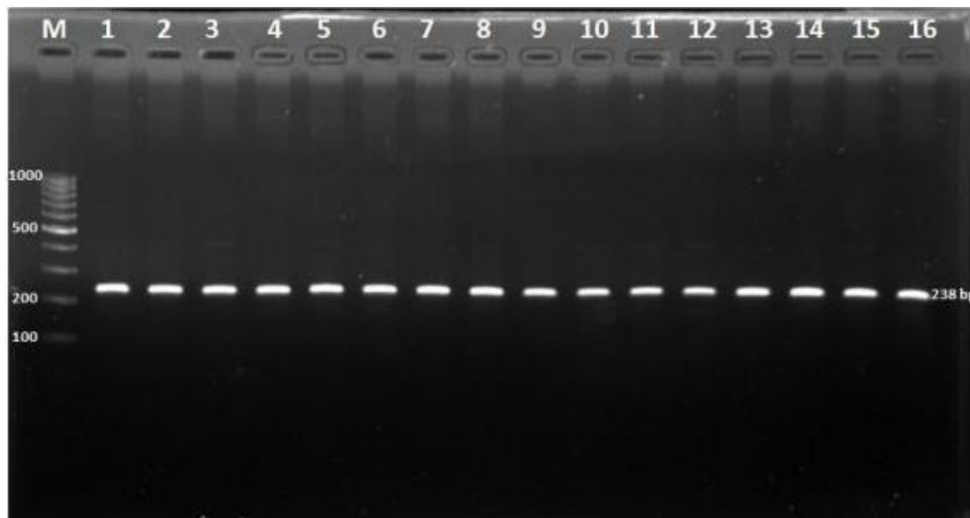

Figure 1. PCR amplification result for the IGF-1 gene with agarose 1.5%. M= 100 bp ladder size standard and Line 1-16= individual sample of sheep.

method for detecting substitution mutations in several livestock populations. Besides, the genotyping results showed 3 DNA fragments in bands with a length of 238 bp in all individual samples because the restriction enzyme recognizes the cut site on the amplicon. These results were visualized on 2% agarose gel, as shown in Figure 2.

The Palu sheep IGF1 gene was successfully amplified using a pair of primers at an annealing temperature of 58°C for 15 seconds (Figure 2). The length of the PCR product was 238 bp. Genotyping using Bsp143II yielded three genotypes, namely CC (238 bp), GC (238, 138, and 100 bp), and GG (138 and 100 bp).

Genotype Frequency, Gene Frequency, and PIC Percentage

The genotype frequencies of CC, GC, and GG were 0.10, 0.683, and 0.217, respectively, as shown in Figure 1. The C and G alleles frequencies in this population were equal to 0.442 and 0.558, with a PIC value of 49.32%, and X^2 of 8.92, as shown in Figure 2. PIC is a measure of marker informativeness and ranges from 0 to 1. According to Botstein *et al.* (1980), a locus with a PIC value of 1 or close to 1 and many alleles is usually desirable for genetic diversity studies at a polymorphism (PIC) level of 49.32%. The PIC ranged from 0.35 to 0.37 in different populations, suggesting the usefulness of the marker in population studies since, in a bi-allelic marker system, the maximum possible value is 0.375

(Tsehay *et al.*, 2020). WuJun *et al.* (2010) reported the PIC value of 36% is classified as high information above 0.5. Furthermore, it has sufficient information when the value is 0.25-0.5 and below 0.25. Therefore, the sample IGF-1 gene of Palu sheep had a moderate diversity that is usable for genetic studies. The PCR-RFLP is a useful method for evaluating variations in this selected population.

The IGF1 gene polymorphisms in Palu sheep are presented in Figure 3. The genotype frequencies were 0.10 (CC), 0.683 (GC), and 0.217 (GG), respectively. C and G alleles frequencies in the population were 0.442 and 0.558 with a PIC value of 49.32% and χ^2 of 8.92. The genetic diversity was due to a point mutation at the 97th base. Therefore, the PCR product was cut into three fragments, namely 100, 138, and 238 bases. The results of PCR-RFLP laboratory analysis with Bsp143II showed polymorphism with the genotype frequencies of GG (21.7%), GC (68.3%), and CC (10%), and allele frequencies of G (55.8%) and C (44.2%). A similar type of polymorphism at the IGF-1 locus was discovered using the RFLP method in Egyptian small ruminant breeds, where the genotype frequencies were 53.66%, 30.49%, and 15.85% for GG, CG, and CC with allele frequencies of G (68.9%) and C (31.1%). Sebastiano *et al.* (2020) identified allele and genotype frequencies with the SNPs method in the Sarda sheep breed at position g184028491. The result showed C and G allele frequencies were 90 and 10%, and the genotype frequencies of CC, CG, and GG were 85%, 11%, and 4%, respectively. WuJun *et al.* (2010) stated that the A and B allele frequencies were 0.395 and 0.61, respectively, while AA, AB, and BB genotypes were 0.277, 0.237, and 0.486. In addition, Darwish *et al.* (2017) detected three genotypes in Egyptian and Iranian sheep through the PCR-SSCP method in Barki, at frequencies of GG (25%), GA (58%), and AA (17%), while the G and A alleles frequencies were 54.4% and 45.6%.

The chi-square test results ($\chi 2$) provided a more significant value of 8.92 compared to the Chi-square table at a 5% confidence level (5.99). Therefore, the genotype distribution was not in the Hardy-Weinberg equilibrium ($p<0.05$) due to non-random mattings in the population. This is in line with the study conducted by Nazari *et al.* (2016), using a rural local sheep breed on Locus IGF-1 in Zandi, which showed the population was not in Hardy - Weinberg equilibrium ($p<0.01$).

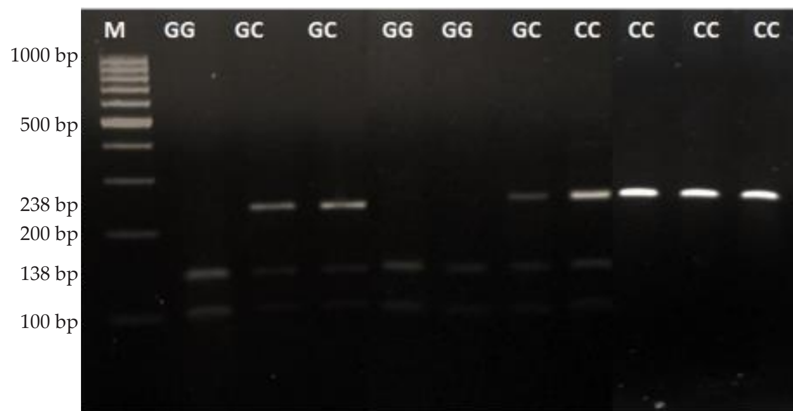

Figure 2. The PCR-RFLP pattern for IGF1 gene with Bsp143II restriction enzyme and 2% agarose. M=100 bp markers; 1, 4, 5= GG genotype; 2, 3, 6= GC genotype; and 7-10= CC genotype.

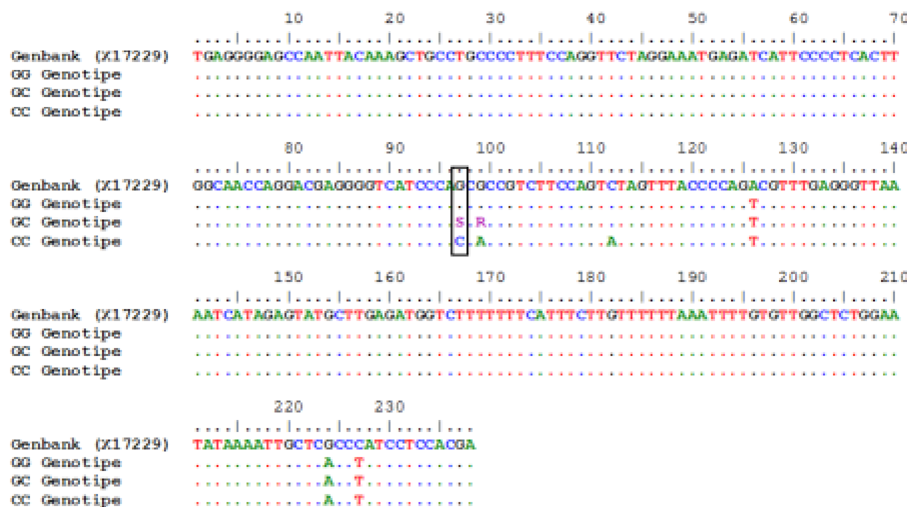

Figure 3. The nucleotide sequence alignment between the two different alleles C and A. Single nucleotide polymorphism (SNP) (C/A) at position 1511bp.

Similarly, Sankhyan *et al*. (2020) stated Locus IGF-1 in the Himachal Pradesh sheep, and goat breeds deviated significantly from the expected ratio.

DNA sequencing is used to determine genes or different DNA fragments' identification and characteristics by comparing the series with different sequences (Wong *et al*., 2019). The current advances in DNA sequencing technology, from first-era sequencing (FGS) to third-generation sequencing (TGS), have continuously transformed the panorama of genome studies. This has generated huge, sparse, and heterogeneous sequencing records and remarkable throughput with several folds compared to past technologies. This leads to the rapid development of various data protocols and bioinformatics tools for handling sequencing data. On this evaluation, a historical snapshot of DNA sequencing is analyzed using information manipulation & tools, while the technological records are defined and reviewed in a thorough element. The information compression methods are highlighted and mentioned to offer readers a realistic perspective within the real-international setting. This method is further used in determining the pitfalls of prevailing DNA sequencing technologies to provide potential solutions (Wong *et al.,* 2019). The *O. aries* sequence is located in the first intron (at position 1436-1674) of the IGF1 gene. According to Dickson *et al.* (1991), no access to GenBank X17229 is needed to complete the IGF-1 gene of *Ovies aries.* The results of DIALIGN study sample sequencing were 230 bp in size, while the nucleotides were in the intron region of the gene in sheep. Furthermore, the presence of a point mutation in the nucleotide base is also the cause of polymorphism.

Figure 1 shows these nucleotide differences caused the polymorphism or DNA diversity. Nucleotide changes also occur in the intron region, as well as mutations at the DNA level due to changes in bases (A= Adenine, T= Thymine, G= Guanine, and S= Cytosine) in the form (type) of substitution (transition or transversion), deletion, insertion, and inversion. The mutations found in the study sample were caused by the transversion (G / C) of the 97 bp position. This is in line with Grochowska *et al.* (2017), which observed the effect of IGF gene in Merino sheep. The IGF-1 gene transversion (G / C) in sheep, the position of nucleotides based on DNA fragments, variations in base length, and sequencing results concluded that the study samples were in a polymorphic state.

Relationship of Genotype with Body Size

Insulin-like growth factor-1 (IGF-1) is a hormone that plays an important role in bone and muscle growth in animals. The gene of IGF-1 in Palu sheep was amplified successfully using PCR for all samples. According to the PCR-RFLP conducted using Bsp143ll as restriction enzyme, the region of IGF-1 gene was polymorphic. This was because the allele frequency obtained was more than 0.01. Three genotypes, namely CC, GC, and GG were detected with frequencies of 0.10, 0.683, and 0.217, while the allele frequencies for C and G were 0.442 and 0.558, respectively. The variations of IGF-1 gene in Palu sheep were not in Hardy-Weinberg equilibrium, and the $\chi 2$ value was 8.92 ($p<0.05$). Gunawan *et al*. (2017) stated that a population is in Hardy-Weinberg equilibrium when the dominant and recessive allele's genotype frequencies are constant from one generation to another with no selection, mutation, migration, or genetic drift.

The statistical analysis results showed the effect of IGF-1 gene genotype was insignificant on body weight and size. These results are in line with the results reported by Al Qasimi *et al.* (2019), studying the effect of IGF-1 and GH genes on body weight and size in Awassi sheep. Gholibeikifard et al. (2013) discovered no relationship between exon 3 IGF-1 polymorphism and birth, weaning, six-month, nine-month, and once-a-year weights for the Baluchi sheep. Similarly, Raji *et al.* (2018) stated that the polymorphisms IGF-1 gene did not significantly affect the height at withers of Makui sheep in Iran as well as the morphometric traits in Uda sheep. The three authors, however, observed genotypes AA, AB, and BB did not differ, but BB had higher body length measurements significantly ($p<0.05$) compared to AA and AB girth, height at withers, and body weight. Nazari *et al.* (2016) also reported a non-significant effect on early growth.

CONCLUSION

The IGF-1 gene was discovered to be polymorphic and not in the Hardy-Weinberg equilibrium. Three of its genotypes were detected, but their effects were insignificant on body weight and bone size. Therefore, selection cannot be carried out using the IGF-1 gene in Palu sheep.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to the material discussed in the manuscript.

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