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Phenotypes, Performance, and Insulin Gene (*INS*) Single Nucleotide Polymorphism (SNP) C1549T Genotyping of Indonesian Meat-Type Chicken Breed

Research Article

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ABSTRACT

Pelung chicken, as one of the Indonesian indigenous chicken breeds, is known for its distinct characteristics. Pelung chicken has been an object of selective breeding programs due to its slow-growing performance, particularly in live weight gain. Through selective breeding, the backcross and its reciprocal generations have been produced. This study was aimed to identify the phenotypes, performance in live weight gain, and insulin gene (*INS*) single-nucleotide polymorphism (SNP) C1549T genotyping. The phenotypes consisted of morphometrics and morphological traits. The tetra-primer amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR) method was used to detect the SNP C1549T of the *INS* gene. The live weight gain of reciprocal backcross (RBC1) outperformed the first backcross (BC1) chickens. Morphometrics and morphological traits of BC1 and RBC1 indicated a directional selection effect towards pelung chicken. The transition mutation of SNP C1549T was only detected in RBC1 chickens as CT genotype (44.44%) and TT genotype (55.56%). The presence of SNP C1549T might have a significant association with the live weight gain of RBC1. T-ARMS-PCR method is suitable for the rapid detection of single nucleotide polymorphisms. Additional studies are required to confirm the association of *INS* gene polymorphism and live weight gain with a larger population size.

KEY WORDS genotyping, indigenous breed, insulin gene, phenotypes, selective breeding.

INTRODUCTION

Domestic demand in Indonesia for animal protein sources (such as meat, milk, and eggs) is increasing rapidly per capita each year, as evidenced by poultry consumption (Ferlito and Respatiadi, 2018; Nugroho, 2020). The domestic poultry industry, as one of the sectors in animal protein production, relies on foreign value chains (Prayugo *et al*. 2012) to support its operations, starting from broodstock, day-old-chickens (docs), chicken feeds, and vaccines (Nataamijaya, 2010). Since Indonesia has numerous unexplored local biodiversity, including 34 Indonesian indigenous chicken breeds (Henuk and Bakti, 2018; Mahardhika and Daryono, 2019), it opens up a new possibility for research and development. Pelung chicken stands in distinction among the others due to its body posture (Iskandar and Susanti, 2007), live weight gain (Iskandar, 2006; Daryono *et al*. 2010; Daryono and Muammar, 2013; Saragih *et al*. 2017), appealing plumage colors/appearances (Nataamijaya, 2010; Hidayat and Asmarasari, 2015; Fitriani *et al*. 2019), and its unique crowing ability (Daryono *et al*. 2020; Daryono *et al*. 2021). The unique meat flavor and texture (Suhita *et al*. 2015; Mahmud *et al*. 2017) of pelung chicken are more preferable for Indonesian taste than the regular-imported

meat-type chicken (Suprijatna, 2010). In the first stage of the selective breeding program, crossbreeding between the pelung chicken and the commercial meat-type chicken breed was conducted. Daryono *et al*. (2012) reported a promising result of this crossbreeding. In the second stage, backcross and reciprocal generations were produced.

In relation to living weight gain, there are numerous reports about significant genes related. The previous studies reported *INS* gene polymorphism as one of the possible causes for early growth rate in chicken (Qiu *et al*. 2006; Thinh *et al*. 2020). Besides the *INS* gene, several genes, including insulin-like growth factors (*IGF-I* and *IGF-II*) (Anh *et al*. 2015; Al-Anbari and Mohammad, 2016), dopamine receptor gene (*DRD1*) (Gholami *et al*. 2019), insulin-like growth factor-binding protein 2 (*IGFBP2*) (Furqon *et al*. 2018), growth hormone receptor (*GHR*) (Khoa *et al*. 2013; Al-Khatib and Al-Hassani, 2016), and chicken growth hormone (*cGH*) (Lei *et al*. 2007) have been associated with growth performance, meat quality, and productivity in chickens. Al-Anbari (2018) and Al-Anbari (2019) suggested that economic traits and insulin genes could not be separated, and its genotyping may provide a useful tool for the early selection of chicks for breeding.

This study aims to measure the phenotypes and performance in live weight gain of first reciprocal backcross (RBC1) and first backcross (BC1) chickens. This study was also accompanied by the genotyping of SNP C1549T of the *INS* gene.

MATERIALS AND METHODS

Rearing systems and bird management

This study was approved by the Local Animal Experiments and Ethical Committee of Laboratorium Penelitian dan Pengujian Terpadu, Universitas Gadjah Mada (number 00038/04/LPPT/VI/2018). The animals were reared with a semi-intensive rearing system in Pusat Inovasi Agroteknologi (PIAT) Berbah, Sleman, DI Yogyakarta, Indonesia. Berbah is located between latitude 7˚47'45.1"-S and longitude 110˚27'55.0"-E at the elevation of 489 m above sea level. The analysis was conducted in the Laboratory of Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada, DI Yogyakarta, Indonesia. Eggs were hatched in Hartono-Tirto hatchery, Sugeng Jeroni, DI Yogyakarta, Indonesia.

Feeding regiments consisted of corn-concentrates-rice sifting mixture with a one-to-one ratio for broodstock. Standard feed BR-1 (22% crude protein, 3050 Kcal ME/kg) produced by PT Japfa Comfeed, Indonesia for progenies (0 to 7 weeks). Both broodstock and progenies were fed on an *ad-libitum* diet. Prophylactic supplements and dissolvable vitamins were administered in drinking water.

DOCs were reared in an insulated battery bamboo cage (1×0.5 m^2) equipped with a 25-watt incandescent lamp as an incubator for four weeks before being transferred into chicken sheds (3×2 m^2) for another four weeks. Broodstock and progenies (older than 4-weeks-old) were reared in a semi-intensive system that allowed free-roaming and open access into an outdoor area. Administrations of antibiotics and prophylactic supplements were not mandatory.

Measurement of morphological traits, morphometric characteristics, and live weight gain

Observable morphological traits as qualitative parameters consisted of comb shape, plumage color, and shank color for progenies of BC1. Photographs of morphological traits were documented. Morphometric characteristics were measured as quantitative parameters as described in Mahardhika and Daryono (2019). Live weight gain per week was measured using the digital scale KrisChef™ EK9350H with an accuracy of 0.01-gram for eight weeks.

Blood sampling, DNA isolation, and T-ARMS-PCR of the *INS* gene

Progenies and broodstock were screened and selected for molecular analysis before the blood collection. A total of 23 chickens consisted of three pelung females, six BC1 chickens (one male and five females), four F_1 chickens (one male and three females), and consisted of five males RBC1 and five females RBC1. Whole blood samples were collected with a syringe (1 mL) via brachial wing vein venipuncture and stored in an ethylenediaminetetraacetic acid (EDTA)-ready-tube vacutainer (1.5-3 mL).

Genomic DNA isolation was performed by preparing a 500 mL TKM 1/Low Salt Buffer composed of 0.605 g Tris-HCL 10 mM pH 7.6 added with 0.372 g KCl 10 mM, 1.016 g $MgCl_2$ 10 mM, 0.372 g EDTA, in 500 mL sterile single distilled water. Triton™-X reagent 10 mL mixed with 9.9 mL sterile single distilled water added with 0.1 mL 100% Triton™-X. The TKM 2/High Salt Buffer 100 mL was made by mixing 0.121 g Tris-HCL 10 mM pH 7.6 with 0.074 g KCl 10 mM, 1.203 g $MgCl_2$ 10 mM, 0.074 g EDTA 2 mM, 0.467 g NaCl 0.4 M, dissolved in sterile single distilled water 100 mL. SDS 10% was made from 1 g sodium dodecyl sulfate dissolved in 10 mL sterile single distilled water. NaCl 6M was made by mixing 8.765 g NaCl in 25 mL sterile single distilled water. Buffer Tris-EDTA (TE) was made by mixing 0.030 g Tris-HCL 10 mM pH 8 and 0.009 EDTA 1 mM dissolved in 100 mL sterile single distilled water.

Red blood cells (RBC) were lysed by combining 900 L TKM 1 buffer and 50 L Triton™-X with 300 L whole blood sample in a sterilized 1.5 mL microtube and incubating for 5 min at 37 ˚C. The blood and reagent mixture was

centrifuged for 3 min to separate the supernatant. These steps were repeated 2 to 3 times until Triton™-X depleted. White blood cells (WBC) would be left out after RBC was lysed. Cell lysis was performed by adding TKM 2 buffer and 40 µL 10% SDS followed with homogenization. Followed by 5 min of incubation at 37 ˚C, 100 L NaCl 6M was added and vortexed to precipitate the protein at the end. Followed by centrifugation at 12000 rpm for 5 min.

DNA precipitation was performed with 300 µL isopropanol in a microtube (1.5 mL) centrifuged at 12000 rpm for 10 min. The supernatant was disposed of, followed by the addition of ethanol 70% and homogenization. Centrifugation at 12000 rpm for 5 min to induce additional DNA precipitation. The supernatant was separated and air-dried, followed by the addition of 50 µL buffer TE to dissolve and preserve the DNA. Agarose electrophoresis and spectrophotometry (see Supp. File) were used to determine the quality and quantity of DNA isolates.

Primers for conducting the Tetra Amplification Refractory Mutation System Polymerase Chain Reaction (T-ARMS-PCR) were designed with a web-based service (http://primer1.soton.ac.uk/primer1.html). Primers consisted of forward outer primer (5'—3') 295-TCTTTCCTATTCCTTGATGAACAGCATC-322, *forward inner primer* (C allele), 420-CTTATTTCACGGTGGTAAATGGATTATCC-448, *reverse outer primer* (5'—3') 619-TTTCCTGCATTACCGATTTACAGAAGAG-592, and reverse inner primer (T allele) 474-ACACTGGCTGCATCACAACTTACTACA-448. Primers were designed based on *INS* gene GenBank ID AY438372 (Qiu *et al*. 2006) 4074 bp DNA complete coding sequence. Modification in reagents for the T-ARMS-PCR stage consisted of two tubes. The first tube contained KAPA Taq PCR mix (12.5 µL), forward outer primer (1.25 µL), reverse outer primer (1.25 µL), ddH_2O (7 µL), and genomic DNA (5 µL). The second tube contained the same contents with replacement of forward inner primer C allele (1.25 µL) and reverse inner primer T allele (1.25 µL).

Following the optimization, the optimum annealing temperature was found to be 59 ˚C. T-ARMS PCR was performed in 35 cycles, which included denaturation for 3 min at 95 ˚C, a 15 s denaturation stage, annealing for 15 s at 95 ˚C, extension for 24 s at 72 ˚C, and a final extension for 5 min at 72 ˚C.

T-ARMS-PCR results were followed by 2% agarose electrophoresis using the Submarine Electrophoresis System (Mupid-EXU) device with SYBR™ Safe DNA gel stain. Electrophoresis results were visualized with UV-transilluminator AnalytikJena™ and documented with the GelDoc™ Documentation System.

Statistical analysis

Morphometric measurements were analyzed using the IBM® SPSS® Statistics program (SPSS, 2011). Electrophoresis gel images were analyzed according to each band based on molecular weight analysis (base-pair length alignment) with ImageLab (Version 6.0.1) under gray image colors mode adjustment and Bio-Rad 100 bp PCR Molecular Ruler using linear (semi-log) regression method.

RESULTS AND DISCUSSION

In this study, the genetic improvement in the live weight gain of pelung chicken was conducted through a series of crossbreeding with the parental stock of commercial meat-type chicken breeds. Intercross between slow-growth and fast-growth rate meat-type chicken breed, pelung chicken and Broiler chicken produced first filial generation (F_1) of Kambro, an abbreviation of kampong and broiler chicken (Figure 1).

In a recent selective breeding program, the backcross and reciprocal backcross between F_1 and pelung chicken were performed. The presence of backcross and reciprocal backcross effects are important in deciding upon the use of either the sire (male) or dam (female) line in crosses (Haunshi and Sharma, 2006). In selective breeding, genetic introgression and artificial selection are two main underlying principles that affect homozygosity, phenotypic modifications, and shared haplotypes between backcross progenies and parentals. Amusan *et al*. (2020) added that backcross is a well-known and long-established breeding method for the introgression of desirable traits from a donor parent into the genetic architecture of a recurrent parent. In this case, the BC1 and RBC1 populations were developed for introgression of major genes associated with fast-growth and live weight gain gains from the Broiler population to the pelung population. The live weight gain of BC1 chickens from wk-0 and wk-7 was 29.17 ± 0.75 g and 519.33 ± 64.04 g, respectively. In comparison, based on internal previous studies, the average live weight gain of RBC1 chickens from wk-0 and wk-7 was 33.5 ± 3.71 g and 882.2 ± 125.6 g, respectively.

Daryono and Muammar (2013), recorded the average live weight gain gains for BC1 chickens was 811.63 ± 116.63 g higher than the current BC1 chickens. Utama *et al*. (2018) also reported 919.9 g as the average live weight gain of BC1. Daryono *et al*. (2012) further recorded the average live weight gain gains for BC1 and RBC1 chickens respectively were 1129.6 g and 901.33 g. In the previous study, the BC1 outperformed the RBC1 chickens. A different gain was expected to be caused by different parental generations of F_1 kambro chicken.

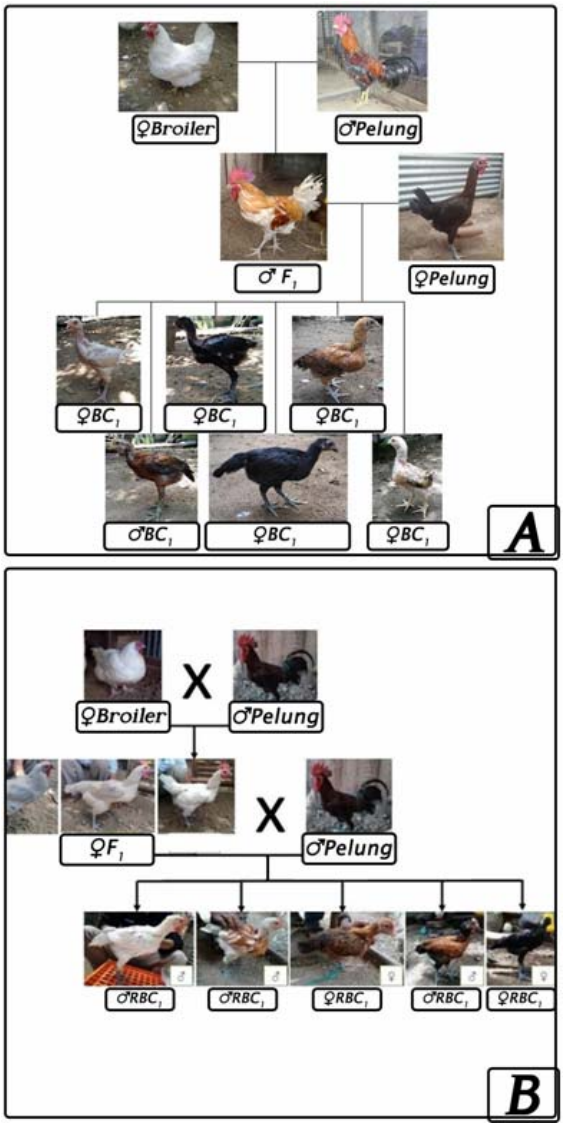

Figure 1 Breeding schematic to acquire **a**) BC1 and **b**) RBC1 chickens
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Thus, the effect of backcross and reciprocal backcross depends on the pedigree structure of the parental generation. This statement was proven by Daryono and Muammar (2013), who reported the average live weight gain gains of second backcross chicken to be 563.83 ± 109.527 g lower than 1124 g reported by Perdamaian *et al.* (2017). Utama *et al.* (2018) found that the feed conversion ratio (FCR) for BC1, pelung chicken, and Broiler chicken to be 2.32, 3.35, and 1.55, respectively. This FCR value was lower than the reports for pelung chicken.

As described in Table 1, the RBC1 significantly outperformed the BC1 chicken in almost thirteen characters measured except for comb height, comb length, and wingspan.

In comparison, pelung chicken was significantly different from the BC1 chicken in several characters, including body height, head length, body width, and wingspan.

The percentage of similarity between BC1 with RBC1 and BC1 with pelung chicken respectively was 23.08% and 69.23%. Utama *et al.* (2018) reported the percentage of similarity between BC1 with Broiler chicken and BC1 with pelung chicken as 47% and 33%. Daryono and Muammar (2013) performed a second backcross between BC1 chickens and demonstrated the minor morphometric characteristics of their offspring. Based on morphometric characteristics and live weight gain gains, the RBC1 chickens derived from reciprocal backcrosses between female F_1 and male pelung chicken were proven to be the ideal candidate for the future selective breeding program.

Table 1 Morphometrics results of BC1 in comparison with RBC1 and pelung chicken at 8-weeks-old

Morphometrics (cm)	Filial groups		
	BC1	RBC1	Pelung
Chicken height	25.92±1.69[a]	39.00±2.73[b]	30.63±1.69[a]
Body height	15.08±1.16[a]	25.40±2.22[b]	21.67±0.76[b]
Head length	2.92±1.39[a]	4.60±0.55[b]	6.53±0.25[b]
Head width	2.40±0.94[a]	4.00±0.71[b]	3.33±0.29[a]
Comb height	1.57±1.55[a]	2.00±0.00[a]	12.00±0.00[a]
Comb length	2.72±1.24[a]	4.25±0.50[a]	24.67±1.15[a]
Body length	13.08±1.66[a]	18.56±2.13[b]	17.50±1.80[a]
Body width	6.08±0.38[a]	9.14±1.21[b]	7.92±1.59[b]
Chest circumference	14.50±1.58[a]	26.00±2.08[b]	22.75±5.20[a]
Dorsal length	16.92±1.91[a]	19.50±1.05[b]	15.00±2.29[a]
Wingspan	13.53±0.91[a]	15.80±1.30[a]	9.02±0.65[b]
Neck length	7.17±0.82[a]	9.88±1.64[b]	6.33±0.58[a]
Tibia length	8.0±1.14[a]	11.38±1.06[b]	8.75±0.25[a]

BC1: first backcross and RBC1: reciprocal backcross.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

Another purpose of backcrossing is that it can be used to isolate a gene or chromosomal region in different genetic backgrounds to better understand the genetic architecture of qualitative traits (Amusan *et al.* 2020). As described in Figure 2A, the comb shape of BC1 progenies was 100% single-shaped comb (rrpp). Plumage colors of BC1 progenies consisted of three variations, including black (33.33%), brown (33.33%), and white (33.33%) as depicted in Figure 2B. As depicted in Figure 2C, the shank colors of BC1 progenies were black/gray/greenish (50%) and white/yellow (50%).

Previous studies (Daryono *et al.* 2012; Daryono and Muammar, 2013; Perdamaian *et al.* 2017; Utama *et al.* 2018) recorded almost similar percentage and morphological traits composition to this current study. The inclination toward directional selection was responsible for the similar appearances and composition of colors observed in pelung chicken (Nataamijaya, 2005). Based on the morphological traits, a single-shaped comb is expressed dominantly. Comb shape in particular could be associated with reproductive performance (Dong *et al.* 2019) and live weight gain (Moro *et al.* 2015).

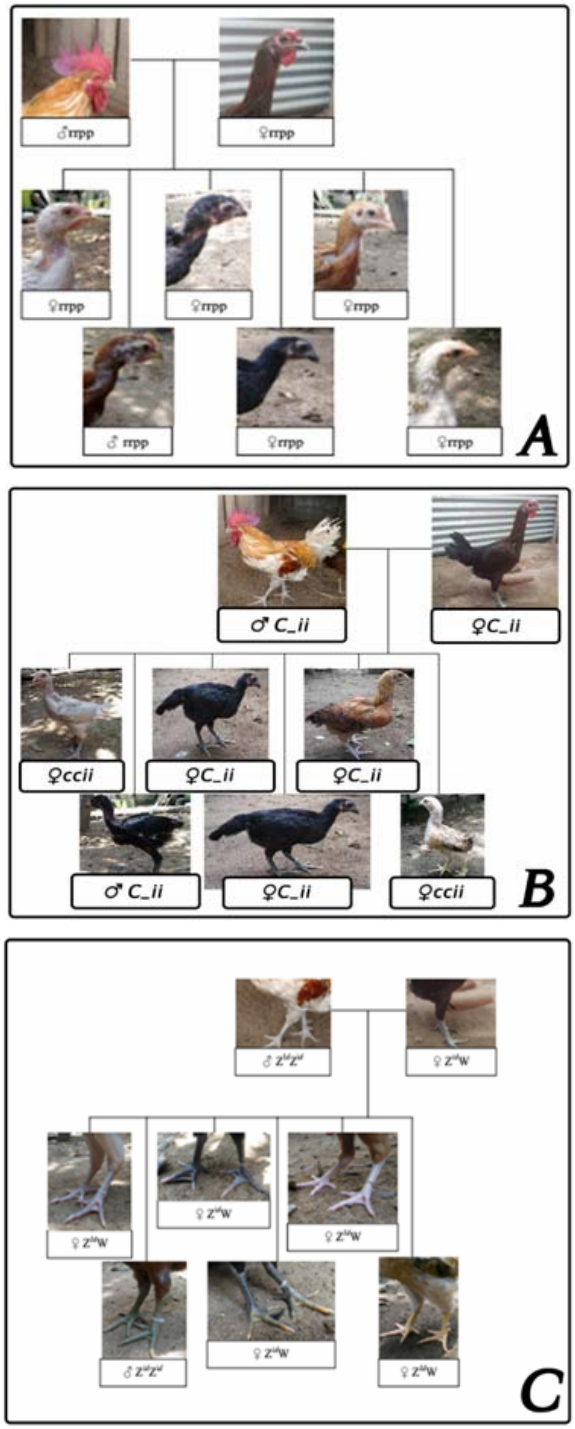

Figure 2 Schematics of morphological traits of BC1 chickens at 8-weeks-old

a) observation showed the expression of rrpp genotype responsible for single comb shape in chicken; **b**) observation showed the expression of *C_ii* and *ccii* genotypes responsible for colored plumage and white color plumage in chicken, respectively and **c**) observation showed the expression of *Z*-linked (Z^{Id} and Z^{id}), genotypes responsible for white/yellow color and black/gray/greenish color shank in chicken, respectively

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As live weight gain and histological aspects of chicken were highly correlated, concurrently, besides genetic (Jayasena *et al*. 2013; Suhita *et al*. 2015) and feeding composition factors (Del Bosque *et al*. 2020), these suggest the association between carcass and meat quality of progenies. Daryono *et al*. (2012) concluded based on carcass and meat quality analysis that progenies of crossbred and backcross were acceptable for consumers. Darwati *et al*. (2017) reported a similar result for carcass and meat quality of pelung and Broiler crossbreds.

Qiu *et al*. (2006) identified four SNPs position of *INS* gene (Figure 3A) in the chicken population of crossbreds between Chinese native Xinghua chickens and White Recessive Rock chickens. *INS* gene polymorphisms in vertebrates were located across three exons (E1, E2, and E3) as the following (A+428G, C+1549T, T+3737C, and A+3971G) (Qiu *et al*. 2006).

The results of the T-ARMS-PCR method (Figure 3B-C) revealed that the genotype of BC1 chickens for the *INS* gene was heterozygous for the CT genotype (100%). The amplification of the *INS* gene from BC1 chickens and their parentals revealed in Figure 3C that the genotype of the parental generation of RBC1 was heterozygous except for one parental female RBC1 (lane 3) with a homozygous CC genotype. For the RBC1 chickens, the genotype was heterozygous CT genotype and homozygous TT genotype. Four of the RBC1 chickens (44.44%) possessed the heterozygous CT genotype, consisting of three males and one female. The homozygous TT genotype was possessed by five chickens (55.56%) consisted of two males and three females, while the homozygous CC genotype was undetected.

A transition mutation of SNP C > T affected the performance of RBC1 chickens with the presence of CT genotype (44.44%) and TT genotype (55.56%), whereas in BC1 chickens the genotypes were undetected. Qiu *et al*. (2006) found that a similar SNP C > T was associated with early live weight gain in chicken.

In humans, insulin and insulin-like growth factors are presumed to be responsible for cancer in diabetes (Froesch and Zap, 1985; LeRoith *et al*. 2011). Selection for rapid growth in Broiler chickens affected the adipose expansion (Ji *et al*. 2012) which can lead to obesity and type 2 diabetes in humans (LeRoith *et al*. 2011). Rapid growth selection in F_1 kambro, which affects live weight gain gains, can also be associated with muscle and myofibers (Saragih *et al*. 2017; Tanjung *et al*. 2019). Several growth-related genes in chicken, including insulin-like growth factor 2 (*IGF-2*) (Nurcahya *et al*. 2020), growth hormone gene (*GHR*), and insulin-like growth hormone type I (*IGF-1*) (Kadlec *et al*. 2011; Jawasreh *et al*. 2019) have been investigated. Qiu *et al*. (2006) explained that insulin is a peptide hormone secreted by the β cells of the pancreatic islets of Langerhans and its association with early growth in chicken was not similar to fat deposition.

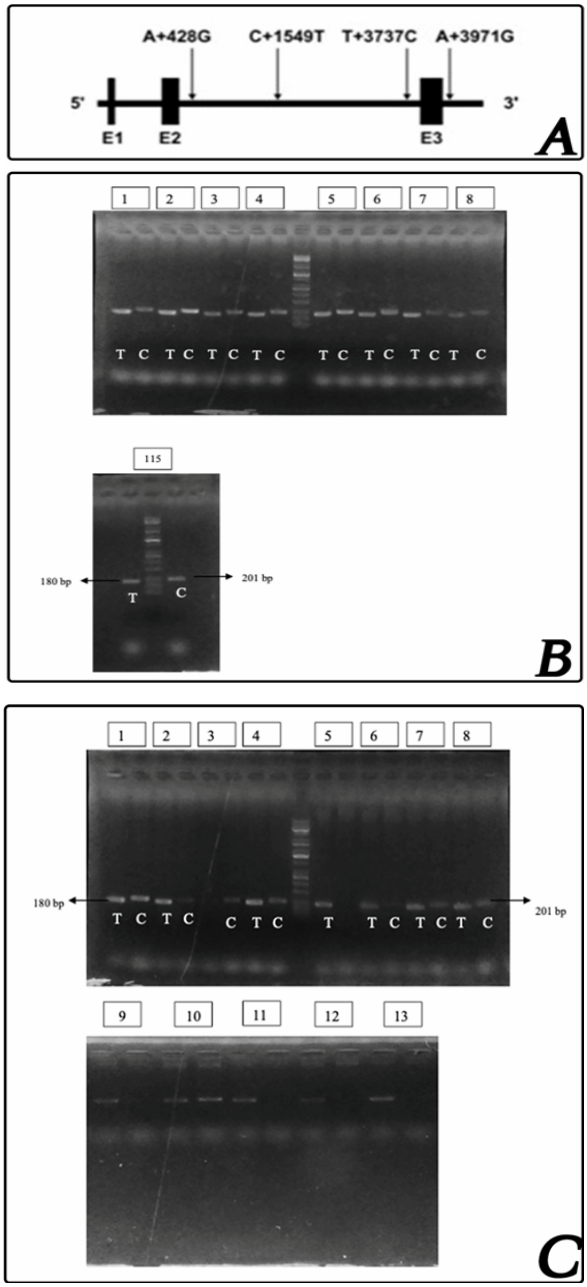

Figure 3 Genotyping of SNP C+1549T in chicken insulin (*INS*) gene
a) an illustration of the position of chicken's *INS* gene SNPs associated with growth at an early age. Reproduced from (Qiu *et al.* 2006). Dotted lines indicated four positions as the following A+428G, C+1549T, T+3737C, and A+3971G; **b**) the electrophoresis products of *INS* gene amplification using the T-ARMS-PCR method in female lanes: 1, 2, and 3; male hybrid lane 4; female hybrid lanes 5, 6, 7, 8, and 9. Band lengths of C and T were 201 bp and 180 bp, respectively and **c**) the electrophoresis products of *INS* gene amplification using the T-ARMS-PCR method produced parental male RBC1 lane: 1; parental female RBC1: 2, 3, and 4; male RBC1 progenies: 5, 6, 7, 8, and 9; female RBC1 progenies: 10, 11, 12, 13, and 14
Band lengths of C and T were 201 bp and 180 bp, respectively
INS: insulin gene; E: exon and SNPs: single nucleotide polymorphisms
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Saragih and Daryono (2010) reported that the number of pancreatic β cells in Broiler Cobb 500 chicken was 2770 ± 58.31, significantly higher than pelung chicken 652.4 ± 32.77. The crossbred F_1 kambro derived from the parent stock of Broiler Cobb 500 chicken and pelung chicken inherited enough pancreatic β cells as the result of the genetic combination. Insulin is closely related to cellular glucose uptake, regulating carbohydrate, lipid, and protein metabolism as well as promoting cell division and growth (Khoa *et al.* 2013). Based on the findings, SNP C1549T might have a significant correlation with average live weight gain gains of RBC1 and BC1. However, additional studies were required to confirm this assumption with a larger population size.

Additional studies possibly genomic selection with HD marker panel can be performed to create a comprehensive understanding of the correlation between production traits and its respected gene (Salvian *et al.* 2020). Genomic selection requires linkage disequilibrium (LD) as the basic requirement for estimating the breeding value and exploring quantitative traits (Nosrati, 2016).

CONCLUSION

Reciprocal backcross was found to be the most suitable breeding pathway for effective introgression of major genes associated with growth performance in the selective breeding program of pelung chicken. This study found that the *INS* gene was polymorphic and only detected in RBC1 chickens. Later confirmation of association between SNP C1549T of *INS* gene and live weight gain of RBC1 chickens is required. T-ARMS-PCR method could accommodate a rapid detection of SNP with optimization.

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