Origin, Phenotypes, and Plumage Coloration of Golden *Pelung* Chicken Progenies (*G. gallus*, Linn.1758)

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Research Article

Keywords: MAS, Pelung chicken, Modern breeders, TYR gene, Plumage colouration

Posted Date: May 21st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-411024/v1

Abstract

Background

Pelung chicken has extensively been studied through selective breeding and used by the local poultry sector for ornamental purposes and occasionally as meat-type chicken. However, a well-documented and detailed description of its origins, genealogical backgrounds, unique traits, and diagnostic genotyping of its unique plumage colouration has never been compiled. Therefore, this study aimed to provide a detailed description of *Pelung* chicken and conduct a diagnostic genotyping of the *TYR* gene associated with golden plumage colouration accompanied with direct visual observations in *Pelung* chicken.

Results

Direct visual observations of GK resulted in dominant white shank governs by two autosomal loci and one sex-linked locus. Plumage colours were divided into four variants: black-brown barred, brown-golden barred, brown, and white. Each plumage colour group governs either by both the autosomal loci and recessive sex-linked locus or only autosomal locus. The diagnostic genotyping detected the presence of intron 4 retroviral sequence insertional mutation of tyrosinase (*TYR*) gene in both F_1 *Kamper* and GK. Full-length retroviral insertional mutation of the *G. gallus TYR* gene is associated with the appearance of recessive white (*C*C*/ *C*C*) chickens, with pigmented eyes.

Conclusions

The golden *Pelung* chicken was originated from inbreeding crossings between F_1 *Kamper*, the progenies of crossbreeding between Layer Lohmann Brown-Classic and *Pelung* chicken. Historical accounts suggested the first documentation about the possible origin of *Pelung* chicken located in several villages surrounding Warungkondang, Cianjur, West Java. Genealogical background of *Pelung* chicken referred to Thailand RJF (*G. g. gallus*) and the possible contribution of Javanese fowl (*G. g. bankiva*) to the formation of domesticated chicken breeds. The genealogical background resulted in two different taxonomical terms, *G. gallus* and *G. gallus domesticus* as a subspecies of RJF. The unique and distinctive characteristics of *Pelung* chicken are crowing duration and plumage colour composition. Our findings provide essential information to assist the development of MAS and conservation initiative of the *Pelung* chicken germplasm. Our findings provide essential information for modern chicken breeders to assist the development of MAS and conservation initiative of the *Pelung* chicken germplasm.

Background

The domestication of chicken affects productivity, phenotypic traits, performance, ornamental traits, and other genetic-related aspects. A large repertoire of genetic mutation in domesticated birds displays a high degree of phenotypic variation [13] including plumage colour [55, 92, 58], as can be observed in 34

distinguishable Indonesian indigenous chicken breeds [28]. Among them, *Pelung* chicken can be distinguished by its growth performance, body posture, and meat characteristics. *Pelung* chicken is the potential candidate for the meat-type chicken breed to support a more sustainable and greener approach in developing the local chicken poultry sector [9]. For years, the local protein source in Indonesia especially from chicken poultry relies heavily on foreign sources (e.g., chicken stocks, eggs, meat, etc.). Therefore, one of the objectives of the research is to become independent by identifying and empowering the untapped biodiversity resources of indigenous chicken germplasm.

The exploration of indigenous chicken biodiversity itself is not a new trend. The emergence of integrating indigenous chicken breeds into the local poultry sector, especially in developing tropical countries has been reported by numerous studies including a study on Korean native chicken [48], Nigerian native chicken [61], and Mazandaran indigenous chicken [60]. Modern breeders have utilized the basic Mendelian genetics and also molecular genetics including what is known as marker-assisted selection (MAS). The MAS using several major genes to improve productivity in smallholder poultry breeding programmes has been studied in various tropical countries including Indonesia, Malaysia, Thailand, Bangladesh, Bolivia, India, Cameroon, and Nigeria [18].

In the development, studies were performed to create a solid footprint and evaluative instruments for selective breeding of *Pelung* chicken. The breed itself has been used in a wide array of studies [63, 64, 46, 6, 45], however, a well-documented and detailed description of its genealogical origin, phenotype traits, and unique characteristics have never been compiled. The studies about selective breeding programmes of *Pelung* chicken have been focusing on three variables including effectiveness, reliability, and minimum ethical cost. Hence, the identification of its unique genetic markers is necessary to assist the actual field-testing as a guideline consist of several selective parameters. Selective parameters act as a preemptive measure to optimize efficiency and avoid unnecessary chicken sacrifice, unethical rearing, cost requirement, and error probability. Selective parameters can be utilized in the screening stage to select potential mating chickens or the improvement of the rearing system.

The development of reliable genetic-based selective parameters is important in selective breeding programmes. The genetic-based selection proves to be simple, rapid, and consistent while still being assisted by conventional phenotypic trait observation as it showed a highly significant correlation with performance traits (i.e., meat), productivity traits (i.e., egg), and commercial viability [56, 92, 46]. In terms of plumage colouration, as an observable-adaptive trait, it distinguishes descending origin, provides means of communication, and intraspecies interaction through a well-developed visual perception among individuals, which later translates into higher survivability against environmental dynamics. Etiological studies suggest that golden plumage colouration is directly affected by the expression of the *TYR* gene and its mutation, among other related genes through the intricate process of melanogenesis. Therefore, from diagnostic genotyping of the chicken *TYR* gene accompanied by direct visual observations, a more comprehensive and reliable selective parameter can be developed. This study also discussed the origins, genealogical backgrounds, unique traits, and diagnostic genotyping of the *TYR* gene responsible for the golden plumage colouration of golden *Pelung* chicken.

Methods

Animals and rearing systems

The study was conducted at 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. Animals reared and maintained under the strict regulation of the Animal Welfare Act of Indonesia and all procedures involving the handling of animals were according to the ethics and biosecurity guidelines approved by the Institution of Animal Care and Use Committee, DI Yogyakarta, Indonesia.

Parentals and progenies respectively consisted of the golden *Pelung* chickens (F_1 *Kamper*) and inbred golden *Pelung* chickens (GK). The grandparent stock of *Pelung* chicken was purchased from a local breeder located in Cianjur, West Java, Indonesia. Local breeders specializing in *Pelung* chicken breeding have a strict, rigid, and consistent record of the breeding programmes directly under the supervision of HIPPAPI, Indonesia (hippapi.or.id). Under a semi-intensive rearing system, F_1 *Kamper* hens and roosters were mated in a ratio of two to one, respectively. Both parentals and progenies reared under a semi-intensive rearing system with an ad-libitum standard feed diet (PT. Japfa Comfeed, Indonesia) of AD-II and BR-1, respectively. Parentals of each breeding group were fed with ad-libitum AD-II (15% Crude Protein) with the administration of the vaccine and prophylactic medications to ensure the optimal health of chickens. Progenies or DOCs of each breeding group were reared intensively in insulated bamboo pens. DOCs were fed ad-libitum BR-1 (22% Crude Protein, 3,050 Kcal ME/kg). Four-wk-old chickens of each breeding group were then transferred into the larger shed ($8m^2$) under a semi-intensive rearing system with an ad-libitum BR-1 diet for eight weeks.

Direct visual observations, phlebotomy, and DNA isolation

Direct visual observations were conducted to characterize the shank colour and plumage colour of parentals and progenies. Progenies and parentals went through the screening phase and were then selected for molecular analysis. A total of thirty selected progenies and ten selected parentals whole blood samples were venipuncture with sterilized syringes (1 mL) via brachial wing vein and stored in EDTA-ready-tube vacutainers (1.5-3 mL). The brachial wing vein is located between the biceps and triceps muscle on the underside of the wing [36]. Whole genomic DNA was isolated through the chelex method [17] from each blood sample. In total, 10 µL of blood sample mixed along with 1 mL of TE buffer and centrifuged at 13,000 rpm for 3 min. The supernatant was removed and 200 µL of 5% chelex solution along with 18 µL of 0.05 M DTT, and 2 µL of 10 mg/mL proteinase K were added to the mixture. The homogenization using vortex followed by incubation at 56 °C for one hour, accompanied by stages of homogenization with 15 min intervals. The sample was further incubated at 100 °C for 8 min and centrifuged at 13,000 rpm for 3 min. The supernatant containing DNA isolates transferred to 1.5 mL microtube DNA was later preserved with the addition of TE buffer (pH 8.0) and stored in the freezer at -20 °C for further use.

Primer designs for diagnostic genotyping using TP-PCR

Primer designs followed the results reported by [5] with three primer sets based on 1956 bp linear mRNA chicken (*G. gallus*) *TYR* gene coding sequences acquired from NCBI GenBank with the sequence accession number **D88349**. Designed primers as follow; the upstream primer Diag05-cc-up (5'-CCT CTG GCT CTA TTT GAG TAC ACA GT-3') located in the retroviral sequence, the upstream primer Diag05-nor-up (5'-CAA AAC CAT AAA TAG GAG TGG AAA TAG-3') located in the normal sequence of intron 4, and the primary downstream Diagnostic05-dw (5'-TTG AGA TAG TGG AGG TCT GAA ATG-3') located in exon 5 of chicken *TYR* gene. Primers were produced by Integrated DNA Technologies (IDT, Malaysia) with third-party associate Perseroan Terbatas (PT) Genetika Science Indonesia. The expected amplified products were 481 bp between Diag05-nor-up and Diagnostic05-dw and 345 bp between Diag05-cc-up and Diagnostic05-dw.

TP-PCR and agarose gel electrophoresis

TP-PCR was performed using Bio-Rad PCR Thermal Cyclers (Bio-Rad Laboratories, Inc). The TP-PCR amplification was performed in 25 µL vol reaction containing 10 pmol primers with the pre-denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 15 s closed with a final extension at 72 °C for 5 min. Bands visualization using electrophoresis on 2% agarose gel with 4 µL florosafe DNA stain (BIO-5170, 1st BASE, Malaysia) submerged in 0.5x TAE buffer at 100 V for 20 min. The agarose gel was prepared by dissolving 0.8 g agarose powder (BIO-1000, IDT, Malaysia) in 40 mL of 1x TAE. Each sample was amplified along with a 5 µL molecular marker of 100 bp Bench Top Ladder (BIO-5170, 1st BASE, Malaysia). The agarose gel electrophoresis was performed using the Submarine Electrophoresis System (Mupid-EXU) device. Electrophoresis results were observed and documented under ultraviolet light (λ_{260} nm) using AnalytikJena™ gel imaging system and GelDoc™ Documentation System. Images of electrophoresis gel were analyzed with ImageLab (Version 6.0.1) to identify each band based on molecular weight analysis (base-pair length alignment) with ImageLab 6.0.1 Bio-Rad 100 bp PCR Molecular Ruler using linear (semi-log) regression method and Grey image colour mode adjustment.

Results

Direct visual observations of golden Pelung chicken progenies

The selective breeding programmes for golden *Pelung* chicken are depicted in (Fig. 1). Close inbreeding crossings of the golden *Pelung* chickens (F_1 *Kamper*) produced inbred golden *Pelung* chickens (GK). Unlike the quantitative nature of polygenic characteristics, qualitative properties are controlled by one or more interrelated genes. Phenotype trait observation of shank colours and plumage colours on inbred progenies indicated a multigenic expression governing the melanogenesis activity displayed as colour variants (Table 1).

Table 1 Plumage and shank colour of inbred golden *Pelung* chicken progenies (GK) (see also Fig. 1)

Phenotypic traits	Colour variants (♂/♀; n = 30)											
Shank colour	White						Yellow				Greenish			
Individual	17						5				8			
Loci[a]	W^{+}, e^{+}			*Id*			*w*, e^{+}			*Id*	*w*, e^{+}		id^{+}	
Chromosomes[a]	A			chr*Z*			A			chr*Z*	A		chr*Z*	
Plumage colour	Group A				Group B						Group C		Group D	
Individual	6				10						10		4	
Loci[a]	*E*, e^{b}, *Db*, *mo*			*s*	*E*, e^{wh}, *Db*, *mo*			*s*			*E*, e^{y}	*s*	*C*C*, c^{a}	
Chromosomes[a]	A		chr*Z*		A		chr*Z*				A	chr*Z*	A	

A= autosomal; chr*Z*= *Z*-linked. The phenotype groups: A) black-brown barred chicken, B) brown-golden barred chicken, C) brown, and D) recessive white with pigmented eyes. The identification of loci and chromosomes is based on literature references stated with superscript [a] [38, 39, 4, 54, 79, 34, 47].

Plumage colour and shank colour are important to be determined as pigments are deposited throughout the body of the chicken including eyes, beak, legs, earlobe, and foot. Based on direct visual observation, shank colour is dominated by white variant governs by two autosomal loci and one sex-linked locus. Plumage colour can be divided into four variants as follows: Group A (black-brown barred), Group B (brown-golden barred), Group C (brown), and Group D (white). Each group governs either by both the autosomal loci and recessive sex-linked locus or only autosomal locus. Recessive *s*-allele was expressed in Group A, Group B, and Group C responsible for the golden plumage in the population of inbred progenies.

The diagnostic genotyping of the G. gallus TYR gene

Following phenotypic traits observation, phlebotomy was performed to diagnose intron 4 retroviral insertion of *G. gallus TYR* gene in samples of F_1 *Kamper* and GK chickens. In Fig. 2 (see also Supplemental File 1), the amplified products from each sample can be differentiated from the length and number of bands. TP-PCR produced the expected fragment length of 481 bp based on the recognition site between Diag05-nor-up and Diagnostic05-dw and 345 bp based on the recognition site between Diag05-cc-up and Diagnostic05-dw. In Fig. 2_A, the diagnostic genotyping of F_1 *Kamper* samples revealed the dominant homozygous chicken (5 samples; *C*N*/*C*N*) with single-band fragment 481 bp and the heterozygous (5 samples; *C*N*/*C*C*) with dual-band fragment 481 bp and 345 bp. In Fig. 2_B, the

diagnostic genotyping of GK chickens revealed the homozygous dominant (17 samples; *C*N*/*C*N*) with single-band fragment 481 bp, the heterozygous (9 samples; *C*N*/*C*C*) with dual-band fragment 481 bp and 345 bp, and recessive white phenotype (4 samples; *C*C*/*C*C*) with single-band fragment 345 bp. In Fig. 3, the DOCs of GK chickens show early plumage variation, despite future alteration during its lifespan. As described in Fig. 3_A, the wild-type individual possesses normal intron 4 while in Fig. 3_B, the recessive white individual possesses retroviral sequence insertion, with pigmented eyes.

Discussion

Fowls, its origins, and genealogical backgrounds

The earliest documentation about *Pelung* chicken was reported in a study by [21] about the matriarchy ancestor of all domesticated chicken breeds. Based on restriction digest analysis and 400 bases nucleotide sequencing, [21] reported that subspecies of the Thailand RJF (*G. g. gallus*) as the ancestor of all domestic breeds. Further, based on mtDNA sequences of the D-loop regions of *G. gallus*, *G. g. gallus*, *G. g. spadiceus*, *G. g. bankiva*, *G. lafayettei*, *G. sonneratii*, and *G. g. domesticus*, [22] solidified *G. g. gallus* as the sole matriarchic origin of all the domestic chicken breeds. Findings suggest that Javanese fowl (*G. g. bankiva*) contributed to the domestication event and formed a distinct entity in the phylogenetic tree, while *G. g. gallus* and *G. g. spadiceus* formed a single monophyletic cluster [21, 22]. Four chicken breed lineages (laying-type, game, meat-type, and Bantam), the laying-type of Mediterranean roots, and/or true Bantams were the earliest chicken breeds with the closest similarity with *G. gallus* [53].

A report of South American chicken breeds also found that *G. gallus* was the ancestor of Brazilian fighting roosters (*G. g. domesticus*) [68]. Researchers have always considered that the sport of cockfighting had tremendous influence not only on the domestication of the chicken but also the fowl dispersal rate and reach throughout the world. The agricultural innovations in East Asia around the early Holocene were the major reason behind chicken domestication and were followed by Neolithic poultry husbandry of chicken along with other species of domesticated animal [65]. The idea that habitat preference and historical, ritual, and leisure activities of ancient man might have resulted in Jungle Fowl being recruited for domestication [12]. In Indonesia, RJF domestication is indirectly affected by human community involvement either by purchasing or hunting in the forest and plantations [77].

The history of chicken domestication is presumed to have occurred more than once throughout Southeast Asia. Some evidence leads to the conclusion that India [33, 16, 81, 65, 12] is one of the birthplaces for present days chicken breeds. Four species of genus *Gallus* inhabit Southeast Asia: RJF (*G. g. gallus*), La Fayette's Jungle Fowl (*G. lafayettei*), Grey Jungle Fowl (*G. sonnerati*), and Green Jungle Fowl (*G. varius*) [75]. Although domesticated chicken is closely related to wild RJF, evidence suggests that endangered southern India's *G. sonnerati* [67] also plays an important role in the ancestral tree of domesticated chickens. Through MCMC simulation [75] it was discovered that domesticated chickens divaricate from RJF around 58,000 (±16,000) years ago, with *G. varius* as their shared common ancestor.

The polyphyletic or hybrid origin of the domestic chicken was proven by the identification of yellow skin genes [15, 16], which indicated the partly involvement of *G. sonneratii*.

Introgression and artificial selection affected the homozygosity, phenotypes, and shared haplotypes between domesticated chickens and Jungle Fowl [75]. The possible involvement of Indian Jungle Fowl or *G. g. murghi* was also discovered [75]. The Bangladesh Native Chicken is closely related to *G. g. murghi*, *G. g. bankiva*, and *G. g. gallus* thus proves the dispersal of domesticated chickens throughout Southeast Asia and India [33]. India was the original platform for the worldwide dispersal of chicken [16]. On the contrary, [59] identified a substantial variation of MHC B-locus of RJF and none of its haplotypes were found in a large sample of commercial and heritage chicken breeds. This phenomenon leading to a conclusion that the RJF population in Vietnam where the study was conducted may not directly the ancestor of domestic chickens or radical artificial selection that has affected the MHC B-locus in domestic chickens. Based on spatial genetic diversity and population structure, RJF in its natural habitats is widely distributed but tends to form small and isolated populations with strong spatial genetic patterns that occur at both local and regional scales [59]. Jungle Fowl as adaptive species displayed seasonal breeding, well-established social hierarchy, explorative behavior, territoriality, aggression, and short-ranged flight [12].

After centuries of selection and breeding, the variation of colours, shapes, and sizes of chicken has reached around 350 combinations. As a response, an organization was established in 1873 to set the standards of excellence and establishing ways of classification of various chicken breeds. The phenotypic variance described in [12] is the result of high phenotypic input from worldwide dispersal and adaptation to a wide range of management and breeding regimes. In the present day, poultry farms are separated into purebred poultry and industrialized poultry. Systematic breeding schemes focused on production traits (i.e., eggs or meat), overcome the negative genetic and phenotypic relationship between reproduction and growth [16]. As the evidence suggests, RJF showed distinct microsatellite alleles distribution and a high level of genetic divergence compared with commercial chicken breeds [81]. Human intervention by comparing microsatellite variations between RJF and commercial chicken breeds [81]. The purebred poultry is mainly driven by hobby and conservation, in which purposively selected and bred fowls for their natural conformity or distinctive unique ornamental traits. On the other hand, industrialized poultry is driven by commercial purposes and science, in which purposively developed and bred meat-type or laying-type breeds. The impact of industrialized poultry is visible in the egg-meat production ratio between commercial breed and purebred RJF [16]. The classification of chicken by the U.S. Department of Agriculture is *G. gallus*, whereas others classify chicken as *G. g. domesticus*, a subspecies of RJF. The different terms are mainly because taxonomists and ornithologists considered chicken as the domesticated form of wild RJF, its main ancestor. The widely progressive and pivotal importance of domesticated chicken has now reached approximately 60 medium-sized breeds mostly descended from the Javanese wild subspecies of RJF (*G. g. bankiva*, family Phasianidae, order Galliformes).

Pelung chicken: Indonesian indigenous chicken breed

Pelung or locally known as *Ayam Kampong* is classified as a meat-type chicken breed among 34 breeds of Indonesian indigenous chicken. The possibilities of introgression and artificial selection in the selective breeding programme of *Pelung* chicken and the commercial breeds have been reported in several concurrent studies. *Pelung* chicken selection was based on the earliest reports about its potential as the candidate for Indonesian indigenous meat-type chicken breed [32, 9]. In the development, reports about the selective breeding programme of *Kampong*-Broiler-type chicken, the progenies of *Pelung* chicken, and commercial breed Broiler Cobb 500 were found to be promising [46, 6, 51]. The possibilities were also investigated in phenomics studies including the heterosis effect on the bodyweight of backcross II progenies [64], *IGF-1* expression [63], myostatin gene polymorphism [82], and intron 3 growth hormone gene (*Gh*) in backcross I progenies [85]. Other related studies about the selective breeding programme of *Pelung* chicken including phenotypic characteristics of Exotic-Broiler [73], meat quality of *Pelung Sentul Kampung* Broiler crossbreed chicken [7], and as early as 2001 about the intercross between *Pelung* chicken and *Kampung* chicken [8]. The MAS using the *IGFBP2* gene in *Kampong* chickens by [23] reported its association with growth performance and body composition traits.

Besides its bodyweight performance, another unique trait of *Pelung* chicken is the ability to sing or produce acoustic melody [70, 32, 66]. *Pelung* chicken based on bioacoustics analysis can also be classified as ornamental chicken or long crow fowl. Bioacoustics among other economic-related traits have been investigated in different varieties of Indonesia indigenous chicken, for example, *Gaga* chicken [1] and *Kokok Balenggek* chicken [71]. The economic value of *Pelung* chicken is not only its beautiful voice [95] but also as a source of local chicken meat [32]. Appealing ornamental characteristics and relative bodyweight ratios may correlate with the conservation of indigenous chicken germplasm as an attractive factor. In the case of *Pelung* chicken, the bioacoustics element consists of four phases: initial-crow (*tetelur*), middle-crow (*kukulur-kukudur*), end-crow (*kukulir*), and closing-crow (*kook*) [32, 95]. In Sundanese culture, crow or local term called *malewung* or *melung* means rhythmic sounds that could be heard from a long distance and an indication of the end-crow when *Pelung* extends their neck into a curve shape [3]. Unique morphological traits of *Pelung* chicken based on comb shape are classified into single-comb with four different variants, including *Bajing Turun* (squirrels' tail-type comb), *Ngabaret* (tilted comb), *Ngaplek* (pendulous comb), and *Gobed Nyarande* (leaned saw comb) [3]. Based on pigmentation, mostly have yellow eyes, black beak, and black shank, while plumage colour is dominated by black [3].

Pelung chicken purebred poultry on local farms with medium-sized housing under a semi-intensive rearing system has high potential commercial benefits. The average yearly revenue from thirty *Pelung* chicken farmers around Cianjur, West Java reaching 782.71 million rupiahs/yr [69]. *Pelung* chicken purebred poultry is highly concentrated in Warungkondang, Cianjur, West Java, Indonesia. Cianjur is located in longitude 106° 42' – 107° 25' E and latitude 6° 21' – 7° 25' S with an elevation range from 7 – 2,962 meters above sea level [3] (Fig. 4). The average temperature in Cianjur is 24.4 °C with an annual rainfall of around 2,610 mm [3]. Cianjur, West Java is known as the natural habitat of *Pelung* chicken where it is first documented. The earliest historical accounts regarding the possible origin of *Pelung* chicken point out several villages in Warungkondang, Cianjur, West Java, including Jambu Dipa village, Bumi Kasih village, Songgom village, and Tegal Lega village [32]. Based on local folklore around 1850

Pelung chicken has been nurtured as an animal with its distinct vocal character and strong appearance/body posture. In 1978, the local government set up a breeding center for *Pelung* chicken as part of preserving local chicken germplasm and cultural heritage. The rich and deeply rooted cultural philosophy behind the *Pelung* chicken based on sociocultural, historical events, and old literature sources are unquestionable [66]. From the economic perspective, a sustainable breeding programme, continuous funding support, and local feed supply can significantly improve the *Pelung* chicken poultry business [19]. However, these strategies have proven to be conventional and are not sufficient to cope with ever-growing demands for rapid and reliable solutions. Hence, the necessity for a creative and integrative approach must be taken based on multidisciplinary scientific innovation including molecular genetics, genetic engineering, and bioinformatics to assist the breeding programme of *Pelung* chicken.

During the research, Gama Ayam Research Team developed, bred, and performed studies of the F_1 *Kamper* [84, 45]. The selective breeding programme of golden *Pelung* chicken or first-generation *Kampong*-Layer (F_1 *Kamper*) consisted of continuous crossbreeding between the *Pelung* chicken grandparent stock with commercial breed Layer Lohmann-Brown Classic. The idea was to improve the reproductive performance and acquired the appealing golden colouration of plumage. The commercial breed Layer Lohmann-Brown Classic was chosen due to the high laying productivity of around 380 eggs/yr. The bodyweight performance of F_1 *Kamper* chickens could reach approximately 911-1100 g for 49 d of rearing. Inbreeding crossings were performed to increase the homozygosity of progenies, however, the risk of heterosis was also high. The inbreeding crossings produced inbred golden *Pelung* chickens with a wide range of phenotypic variance, including plumage colour and shank colour. Based on plumage colour and shank colour, the heterozygous state of the inbred progenies GK (Fig. 1) can still be observed as early as DOCs (Fig. 3).

The selective breeding programme has achieved numerous variances in colouration among and within individual progenies, compared to their wild ancestors as the result of human intervention to gain specific preference or novelty [16]. The phenotypic variance can be reduced through selecting with MAS. Diagnostic genotyping of retroviral sequence insertion in the intron 4 of *G. gallus TYR* gene is the preliminary stage in developing a reliable and robust MAS.

Diagnostic genotyping of *G. gallus TYR* gene

Besides the shapes and sizes, plumage colour is also the key identifier in underlying origins and basic traits of certain species or breeds. Plumage colour and other related aspects around it are deemed as a suitable model to study bird species [16, 57, 58]. Genetic variations in the GK chickens assessed with diagnostic genotyping of retroviral sequence insertion of intron 4 *TYR* gene are observable as the phenotypic variance. The GK chickens, progenies of F_1 *Kamper* still show phenotypic variance, one of which displayed in plumage colours, plumage patterns, and shank colours, despite continuous introgression and artificial selection of the previous generation. The increase in phenotypic variance can be the result of segregated allelic distribution based on Mendelian genetics. The existence of phenotypic variance can be traced to the selection of hens and roosters from F_1 *Kamper* chickens. The selection was

solely relied on phenotype preferences through visual observation, including posture, plumage similarity, bodyweight performance, and egg productivity, without any solid footprint of molecular genetics observations. However, these results indicate that golden *Pelung* has a unique trait quality compared with the purebred *Pelung* chickens. As stated in [3] a decrease in phenotypic variance specifically the plumage colour and the absence of unique colour or pattern were observed in purebred *Pelung* chickens. Thus, the selective breeding programme succeeded in altering the phenotypic and genotypic variability of purebred *Pelung* chickens.

Despite the probability of selection errors, other highly considered factors are genetic variability and external factors, including the possibility of mutation or nutrition. The complex interplay between genetic variability and environmental factors [14] are two probable cause for the high degree of variations occurred in GK chickens. In the grandparent stock of *Pelung* chicken, considerable phenotypic variance appeared, and for generations, local breeders have selected the plumage colour based on their preferences. Local breeders of *Pelung* chicken in Cianjur, have slightly different preferences when selecting chicken especially hens, based on plumage colour, some preferred black and yellow, while others brown [3]. Genetic inheritance derived from a different state of the allelic structure in the parental generation, especially genes related to qualitative traits, together with environmental pressure (i.e., nutrition, temperature, and lighting) is known to cause phenotype alterations in the inbred progenies. Although remain untouched, the phenomenon of epigenetic changes in wild populations is suspected to underlie certain colour patterns in domestic or laboratory animals [72]. Spatial and temporal modularization of gene expression via transcription factors or epigenetic changes is also expected to be of great importance to differently use the same genetic machinery at distinct body parts [72]. Nonetheless, the domestication has achieved phenotypic changes in present-day chickens, including external and internal morphology, physiology, development, and behavior [16].

Avian species exhibits a diversity of plumage colour among different species and uniformity of plumage colour within the same species. Plumage colour and patterns correlate with the adaptive ability and environmental awareness assist by a well-developed visual perception among avian species. Plumage colour and patterns in some ways act as the species-recognition mechanism [20], the ability in which birds belong to a certain species can differentiate between birds from similar species or outgroup birds. Sensitivity towards the environment dynamics or changes influence the survivability rate, for example in mating rituals and behavior, and provides means of intra-or interspecific communication. The pigmentation serves as camouflage, mimicry, intraspecific communication, protection against ultraviolet radiation, and mate attraction [16, 58]. Environmental awareness is displayed from an intraspecific variation which highly correlates with chromatic variability and plumage colour conspicuousness [11]. Thus, the diversity of plumage colour, in this case, can affect the behavior, physical condition, and performance of chicken breeds. During the rearing under a semi-intensive system, GK chickens belong to group A were observed to have aggressive and dominant behavior than the other group (i.e., group B; C; D). The GK chicken belongs to group D were observed to be non-aggressive or more submissive and simply lack the ability of aggression due to smaller body size and agility. Thus, gradually group D started to show a decrease in health state as the result of unproportionate feed intake. As the result, during the

observation, GK chickens with the same age group were divided into four different solitary pens corresponds to each phenotype group to avoid cannibalism or aggression. Overall welfares of recessive white chicken (group D, Fig. 1) showed lower health conditions than wild-type chicken, indicated by slower growth performance and smaller posture. At the observation during day old period, the DOCs of group D had indicated a similar condition. Despite these observation results, group D chickens were still able to reproduce and grow normally, furthermore, recessive white allele appeared to have not directly correlated with a physical condition or physiological state. Several studies only indicated a tendency for a phenotypic variance to correlate directly with the physiological state, physical condition, and genetic quality of chickens attributed to certain plumage colour, patterns, and shank colour [16, 40]. The possibility of correlation between aspects of a multiple plumage ornamentation system may reflect together some aspects of individual quality, thereby functioning as a composite signal [40]. In the case of golden *Pelung* chickens, the plumage colour, crowing duration, volume, and rhythm have been used by breeders to select potential hens and roosters [3].

Multiple allelic interactions appeared in GK chickens (Table 1), in this case, displayed as colour variants of plumage and shank. Shank skin colour appeared to have governed by three different loci, including *W*, e^{+}, and *Id* with two first loci are autosomal and the last one is sex-linked to *Z*-chromosome. The W^{+} locus is located on GGA24 and had previously been described in studies [39, 15, 26, 79, 34] to be responsible for shank skin colour, with two alleles, one being autosomal dominant W^{+} for white shank skin colour and the other being autosomal recessive *w* or *W*Y* for yellow shank skin colour. The W^{+} locus controls the number of xanthophylls in the skin and dependent on its deposition [26]. Feed with a high among of carotenoid enhance yellow pigmentation in the skin, and this phenotype expression only become observable until the chicks are 10 to 12-wk [26]. The *BCDO2*/*CMO2* of W^{+} locus inhibition from cis-acting and tissue-specific regulatory mutation(s) caused the yellow skin phenotype in chicken [15, 26, 16, 34]. The sequence divergence between haplotype of *BCDO2* gene among white- and yellow skin alleles implied the polyphyletic origin of the domestic chicken, as it should have been inherited from the Grey Jungle Fowl [26]. Through genome-wide analysis found additional major pigment determining gene candidates for yellow-feathered [31] or triple-yellow (yellow beak, feathers, and feet) [30] chicken breeds besides *BCDO2*, including *SLC23A2*, *RALY*, *LGR4*, and *SLC2A14*.

The *Id* locus is located on the distal end of the long arm chr*Z*/*Z*-chromosome and has a causal relationship with dermal melanization. The *Id* locus consists of two alleles, the dominant *Id* for dermal-black pigment, and the recessive id^{+} for no pigment [34, 88]. Together with the *W* locus, the *Id* locus regulates the pigmentation through melanins, and carotenoids deposited in epidermis and dermis tissue, respectively [88]. The position of the Id locus is proximal with the centromere from another sex-linked gene, barring (*B*) controlled by *CDKN2A*/*B* locus and highly associated with *Id* locus [88]. The responsible gene, which controls the *Id* locus based on gene expression profile revealed *GRAMD3* as the most likely candidate, with the possible flanking region containing mutation [88].

The *E* locus is located on GGA11 [34] and in GK chickens the effect was not only seen in shank skin colour but also plumage colour. The Extension (*E*) locus is classified as the primary pigmentation of plumage and consists of two alleles, one being autosomal dominant *E* is responsible for the extension of melanic pigmentation for plumage and shank skin colours, while the autosomal recessive *e* is responsible for the non-extension of the black colour [38, 39, 54, 80, 26, 34, 76, 47]. The polyallelic Extension locus *E* determines the basic or zonal distribution of black eumelanin across the body of a chicken and depends on the *MC1R* gene [10], sex, and other interacting loci [34, 76, 47]. The *E* locus expression is sex-dependent and consists of eight alleles, including *E*-extended black; E^{R}- birchen; e^{Wh}- dominant wheaten; e^{+}-wild-type; e^{b}-brown; e^{bc}-buttercup; e^{s}-speckled; and e^{y}-recessive wheat [27, 76, 47]. The *MC1R* gene interacts with other colouration loci, including dark brown (*Db*), Columbian (*Co*), and Mahogany (*Mh*), interplays in restricting eumelanin to a certain degree [76, 86].

In GK chickens, genes-modifiers were observed including dark brown (*Db*) and golden (*s*). The autosomal *Db* locus is located on GGA1 [26, 25, 47], and its mutation in chickens reduces the expression of black eumelanin and increases the expression of pheomelanin, in certain parts of the plumage. The *Db* phenotype is highly associated with *SOX10* which regulates a shift towards pheomelanin, as displayed in altered nature the pigmentation, not in the absence or presence of pigmentation [47]. The *Db* expression is classified as sex-dependent, in females (recessive; db^{+}) the pheomelanin appears as orange-tan while in males (dominant; *Db*) as red-brown breasts [26]. The *Db* locus shows a stronger pheomelanistic expression in the breast (ventral) than in the tail (dorsal) part of the birds [26]. The *Db* locus is linked to the autosomal barring/penciling (*Ab*/*Pg*) locus and is affected by the *MC1R* gene encoded by the *E* locus {26, 76]. The recessive Golden (*s* or s^{+}) is an allele of sex-linked Silver (*S*) locus located on chr*Z* and consists of a total of three alleles, including *S* and sex-related imperfect albinism (s^{Al}) [26, 47]. In its mode of inheritance, the *S* locus is quite complex due to the strong influence of gene-modifiers. The *SLC45A2* gene on chr*Z* of *Al* locus regulates the sorting of vesicles in melanocytes and involves specific inhibition of red pheomelanin in silver chickens [49, 47]. Secondary pigmentation of plumage in GK chickens caused by the expression of the motley colour of the plumage by the autosomal mottle (*mo*) locus associated with the *EDNRB2* gene located on GGA4 [37]. The *mo* locus consists of two alleles, the recessive white (mo^{w}) and the mottled (*mo*). The mottled (*mo*/*mo*) chicken from some studies appeared to express six different phenotypes based on a different combination of the gene *mo* with other colour genes, for example, the rare and endangered chickens expressing black-and-white Australorp, Millefleur breed chickens, and Pushkin breed [47].

The chicken *TYR* gene cDNA clones suggested 73% amino acids sequence similarity with human tyrosinases [52], and a six-nucleotide deletion (−ΔGACTGG) at a Cu-binding site of tyrosinase cDNA sequence is speculated to be responsible for albinism in chickens [83]. The insertion of a complete avian retroviral sequence in intron 4 of the *TYR* gene causes aberrant transcripts lacking exon 5 and is the causal mutation for the recessive white mutation in chickens [5]. Melanin biosynthesis and types of melanin including eumelanin and pheomelanin [24] that involves in plumage, skin, and coat colouration in avian and mammalian species depend on the activity and mutation of the *TYR* gene [74, 92, 58, 47].

One of the key genes that correspond to plumage colouration in chicken is the *TYR* gene [47], thus its potential to be implemented as the MAS is highly promising.

Diagnostic genotyping of retroviral sequence insertion in intron 4 *G. gallus TYR* gene found a similar result described in [5, 44]. In GK chickens, the presence of different phenotype groups, particularly group D (white plumage) indicated different expressions of the *TYR* gene. The heterozygous state of parentals F_1 *Kamper* based on diagnostic genotyping confirmed the possibility of mutational inheritance into the inbred progenies. The *C* locus is autosomal multiallelic locus located on GGA1 and associated with *TYR* gene, consists of four alleles, including dominant full pigmentation wild-type *C*N* or C^{+}, recessive white *C*C* or *c* due to retroviral sequence insertion, autosomal albino *C*A* or c^{a} due to deletion of six nucleotides (−ΔGACTGG), and the red-eye white *C*RE* or c^{re} [83, 5, 74, 47]. The day-old chicks may exhibit a lightly pigmented down hatch in homozygous carriers of the *C*C* mutation [5]. This explained the characters exhibited by DOCs of GK chicken (Fig. 3). The lack of pigment formation in recessive white GK chickens indicated insertion mutation of the *TYR* gene. In chicken breeds, a mutation in any gene in the pigment synthesis pathway can disrupt pigment formation [58].

In GK chickens, the retroviral sequence insertion mutation of the *TYR* gene caused recessive white progenies with pigmented eyes. The retroviral insertion causes aberrant mRNA by modifying the splicing procedure, thus affects the transcription pattern [5]. Exon 5 (Fig. 3) involves the proper positioning of the tyrosinase enzyme in the melanosomes, thus any defect may cause significant consequences in the biosynthesis of melanosomes. As described in [5], the recessive white mutation could affect the translation of the membrane-spanning domain due to the lack of the exon 5, which in turn disturb the melanogenesis despite the transcript being absent of any stop codon before the polyadenylation. The pigmented eyes in the recessive white chicken (Fig. $3_{A\text{-}B}$) can be the result of different precursor cells and pigment transfer, thus indicated the tyrosinase activity.

In Fig. 2_A, the diagnostic genotyping of F_1 *Kamper* shows the *C*N*/*C*N* and *C*N*/*C*C* alleles, translates into wild type with dominant full pigmentation and carrier for recessive white. Both the recessive white and albino could produce tyrosinase-like molecules that are inactive or silenced due to functional, antigenic, and electrophoretic change [83, 5]. In GK chickens, the carriers produced inbred individuals with homozygous recessive white *C*C*/*C*C* and more carrier individuals with heterozygous wild type *C*N*/*C*C*. However, the number of homozygous wild-type *C*N*/*C*N* was larger than the rest. Age is also a valuable consideration regarding the ideal selection period for the expression of the *TYR* gene. In the observation of plumage colour and shank colour between DOCs (Fig. 3) and the eight-wk-old (Fig. 1) GK chickens, the appearance might gradually transform, as it corresponded with the expression level of tyrosinase. The expression levels of *TYR* declined dramatically according to age, and expression at hatch was the highest, while the expression of *MC1R* gene was the highest during 28-d of age than the younger and older ages, also the expression of *TYR* in chickens carrying *E*/*E* and *E*/*e* alleles on *MC1R* loci were higher than those carrying *e*/*e* alleles from hatch to 28-d of age [43]. The obtained findings in GK chickens

confirmed that the retroviral sequence insertion in the *C* locus is stable and heritable in a Mendelian way, both progenies and parentals genotypes were consistent.

The molecular genetics observation confirmed the reliability of genetic-based selective parameters to screen the individuals for a future breeding programme. The problem with recessive white and carrier individuals is due to the fact it may cause inconsistency with the aim of producing golden *Pelung* chicken through a selective breeding programme. The effect can be traced back to the biosynthesis of pigments regulated by the *C* locus as the structural tyrosinase gene locus. The *TYR* gene together with the *MC1R* gene has been reported in numerous studies as the major genes involved in the plumage pigmentation of chickens [43, 42, 27, 91, 29, 24, 90]. The plumage pigmentation depends on several pigments, including carotenoid, melanin (eumelanin and pheomelanin), porphyrins, flavins, psittacofulvins, pterins, purines, and turacin [26, 16, 24, 76, 20, 94]. The two most common and widely found among birds, in particular chickens, are melanin and carotenoid. Carotenoid is regulated by the expression of the *BCDO2/ CMO2* gene and responsible for yellow skin and beak phenotype in the domestic chicken. Carotenoid is highly influenced by nutritional content from feed intake, for that reason, it is worth being put into consideration as one of the factors of phenotypic variance. The most talked-about pigment and directly related with *TYR* and *MC1R* genes is melanin and its derivates, the brown-black-eumelanin and reddish-yellow-brown-pheomelanin, both are synthesized in the melanocyte-specific organelle, the melanosome [26, 16, 76, 47, 35, 78].

Melanosomes are differentiated into eumelanosomes and pheomelanosomes, derived from stages of morphogenesis from unstructured and round vesicles from the ER [26]. The rate of melanin biosynthesis is limited by the synthesis of tyrosinase and the tyrosinase-related proteins *TYRP1* [41] and *TYRP2/ DCT*, which at a high level induce the formation of black eumelanin. Pheomelanin is produced by the addition of cysteine and dopaquinone whenever the low activity of tyrosinase present. The cytotoxic dopaquinone and DOPA [92] is derived from the initial oxidation step of tyrosinase and acts as an intermediate substance used for both the production of eu- and pheomelanin [76]. As described in [76], pheomelanin depends on environmental cysteine levels, acidic pH, *TYR* concentration, expression of *RAI14* [2], and inhibition via agouti signaling pathways [91] of eumelanin production. Melanogenesis pathways of eumelanogenesis (Raper-Mason) or pheomelanogenesis (Prota-Rorsman) consist of enzymatic-catalyzed oxidation of phenolic precursors to quinones, and unregulated polymerization of phenols and their related quinones [24].

In a more detailed explanation, melanogenesis [16] involves two primary signaling proteins, the *α-MSH/ ASIP* and the *MC1R* located in the membrane of the melanocytes [16]. Together *α-MSH* and *MC1R* can elevate the cAMP levels, leads to the activation of *CREB* and *MITF* [87, 47, 89]. The process continues with the transcription of the *TYR* gene and the *TYRP1* and *TYRP2/ DCT*. The tyrosinase catalyzes the conversion of L-tyrosine or L-dopa to dopaquinone, the precursor of eumelanin and pheomelanin, which in eumelanogenesis the *TYRP1* and *TYRP2/ DCT* involves in catalyzing the dopaquinone to produce brown/black eumelanin. In pheomelanogenesis, the interaction between *ASIP* and *MC1R* reduces the cAMP levels and induces pheomelanin production using only cysteine and dopaquinone. Higher *MC1R*

activity usually results in darker pigmentation [47]. Eumelanosomes can be affected by the *I* locus associated with the *PMEL17* gene on GGA33 [5, 47]. Besides *TYR* and *MITF* genes, [93] reported other genes, including four homeobox genes, two *GSH*, and *TGF-β*. The eumelanin pigment deposition in the chicken plumage involves the migration of melanoblast from the neural crest to the epidermis and plumage follicles, where the synthesis is gene-controlled by *NUAK1* and *SHH* [90].

Most interspecies diversity is caused by regulatory changes affecting gene expression involved in pigment synthesis and pigmentation patterns, which vary more significantly between species than in the ability to produce pigments.

The future selective breeding programme

The future is here as it has been proven by numerous pioneering studies in the chicken selective breeding programme around the world with a various approach such as phenomics, sequencing analysis [78, 35], transcriptome analysis [57, 94], QTL mapping of complex traits [14, 30, 20], and genome-wide association study [62, 2, 30, 50]. This study demonstrated the possibility of using a genetic marker in the selective breeding programme of golden *Pelung* chicken with the addition of conventional approaches like morphometrical analysis and phenotypes observation. The inbreeding crossings of F_1 *Kamper* were conducted to increase homozygosity, therefore, produce progenies with uniform phenotypic variations. However, misconduct and errors still present, thus with genetic-based observation, it is expected to be realized in a future breeding programme. This research provides a genetic marker specified for MAS of plumage colour in the GK progenies.

Conclusions

Direct visual observations of GK resulted in a dominant white shank governs by two autosomal loci and one sex-linked locus. Plumage colours were divided into four variants: black-brown barred, brown-golden barred, brown, and white. Each plumage colour group governs either by both the autosomal loci and recessive sex-linked locus or only autosomal locus. The diagnostic genotyping detected the presence of intron 4 retroviral sequence insertional mutation of tyrosinase (*TYR*) gene in both F_1 *Kamper* and GK. Full-length retroviral insertional mutation of the *G. gallus TYR* gene is associated with the appearance of recessive white (*C*C*/ *C*C*) chickens, with pigmented eyes.

In this study, we also concluded that golden *Pelung* chicken was originated from inbreeding crossings between F_1 *Kamper*, the progenies of crossbreeding between Layer Lohmann Brown-Classic and *Pelung* chicken. Historical accounts suggested the first documentation about the possible origin of *Pelung* chicken located in several villages surrounding Warungkondang, Cianjur, West Java. Genealogical background of *Pelung* chicken referred to Thailand RJF (*G. g. gallus*) and the possible contribution of Javanese fowl (*G. g. bankiva*) to the formation of domesticated chicken breeds. The genealogical background resulted in two different taxonomical terms, *G. gallus* and *G. gallus domesticus* as a subspecies of RJF. The unique and distinctive characteristics of *Pelung* chicken are crowing duration and

plumage colour composition. Our findings provide essential information to assist the development of MAS and conservation initiative of the *Pelung* chicken germplasm.

Declarations

Ethical approval and consent to participate: Animals reared and maintained under the strict regulation of the Animal Welfare Act of Indonesia and all procedures involving the handling of animals were according to the ethics and biosecurity guidelines approved by the Institution of Animal Care and Use Committee Universitas Gadjah Mada, DI Yogyakarta, Indonesia (Ethical Clearance Commission of Laboratorium Penelitian dan Pengujian Terpadu, Universitas Gadjah Mada, Yogyakarta No: 00038/04/LPPT/VI/2018).

Consent for publication: Not applicable.

Availability of data and material: The data generated or analyzed during this study are included in this published article [Supplemental File 1].

Competing interest: The authors declare that they have no competing interest.

Funding: Stranas Research Grant -No. 1734/UN1/DITLIT/DIT-LIT/2018 and Applied Research Grant -No. 2830/UN1.DITLIT/DIT-LIT/LT [from 2019 until 2021].

Authors' contributions: IWSM and IH performed the research, analyzed the results, and prepared the manuscript in consultation with ABIP, T, and BSD. ABIP, T, and BSD conceived the idea and supervised the experiments. All authors have read and approved the final manuscript.

Acknowledgements: We would like to thank the Gama Ayam Research Team and the Ministry of Research, Technology, and the Higher Education of the Republic of Indonesia. We also thank Pusat Inovasi Agroteknologi (PIAT) and Laboratory of Genetics and Breeding Faculty of Biology Universitas Gadjah Mada (UGM) DI Yogyakarta Indonesia for their continuous support in facilitating our research.

Abbreviations

MCMC: Markov chain Monte Carlo; HIPPAPI: Himpunan Peternak Penggemar Ayam Pelung Indonesia; cDNA: complementary DNA; *TYR*: tyrosinase gene; DOC: day-old chicks; GK: *Golden Kamper*, EDTA: ethylenediaminetetraacetic acid; DNA: deoxyribonucleic acid; TAE: tris-acetate EDTA; TE: Tris-EDTA; DTT: dithiothreitol; TP-PCR: three primers-polymerase chain reaction; F_1 *Kamper*: first filial generation *Ayam Kampong*-Layer; RJF: red jungle fowl; MHC B-locus: major histocompatibility complex B-locus; MAS: marker-assisted selection; GGA: *Gallus gallus* autosome; *BCDO2*/ *CMO2*: beta-carotene dioxygenase 2/carotenoid-monooxygenases 2; *GRAMD3*; GRAM domain containing 3 gene; *MC1R*: melanocortin 1-receptor gene; chr*Z*: chromosome Z; *EDNRB2*: endothelin receptor B2; DOPA: dihydroxyphenylalanine; *α-MSH*/ *ASIP*: α melanocyte-stimulating hormone/agouti signaling protein; cAMP: cyclic adenosine monophosphate; *CREB*: cAMP response element-binding protein; *MITF*: microphthalmia-associated

transcription factors; *TYRP1*: tyrosine-related protein 1; *TYRP2*/*DCT*: tyrosine-related protein 2; *GSH*: glutathione; *TGF-β*: transforming growth factor-beta; *NUAK1*: NUAK1 family kinase 1; *SHH*: sonic hedgehog; QTL: quantitative trait loci; *IGFBP2*: insulin-like growth factor binding protein 2; ER: endoplasmic reticulum; *RAI14*: retinoic acid-induced protein 14

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Figures

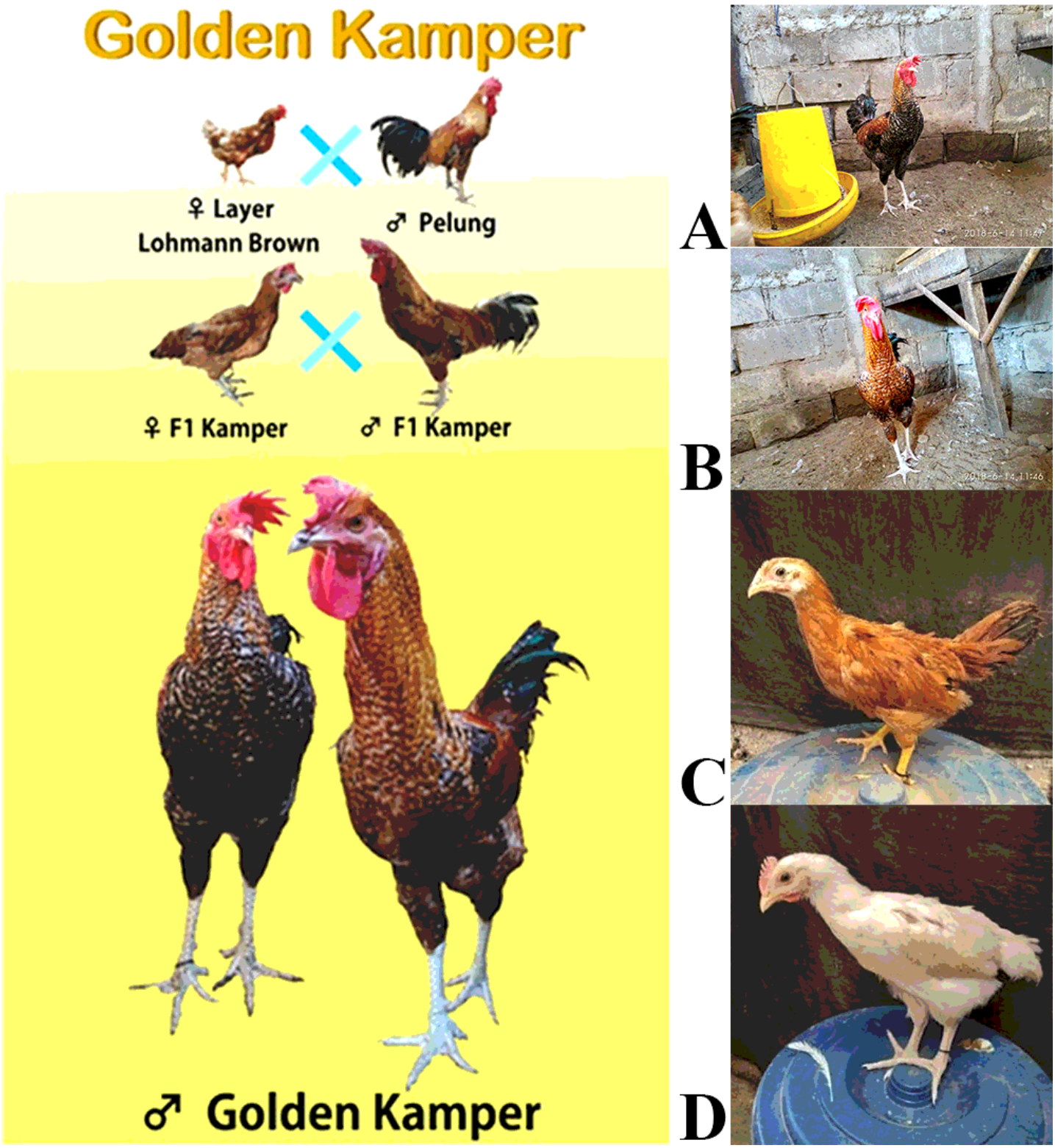

Figure 1

The selective breeding programme of golden Pelung chickens. Parentals and progenies respectively consisted of the golden Pelung chickens (F1 Kamper) and inbred golden Pelung chickens (GK). The depicted individual of GK chickens represented four groups as follows: A) black-brown barred chicken, B) brown-golden barred chicken, C) brown chicken, and D) recessive white with pigmented eyes.

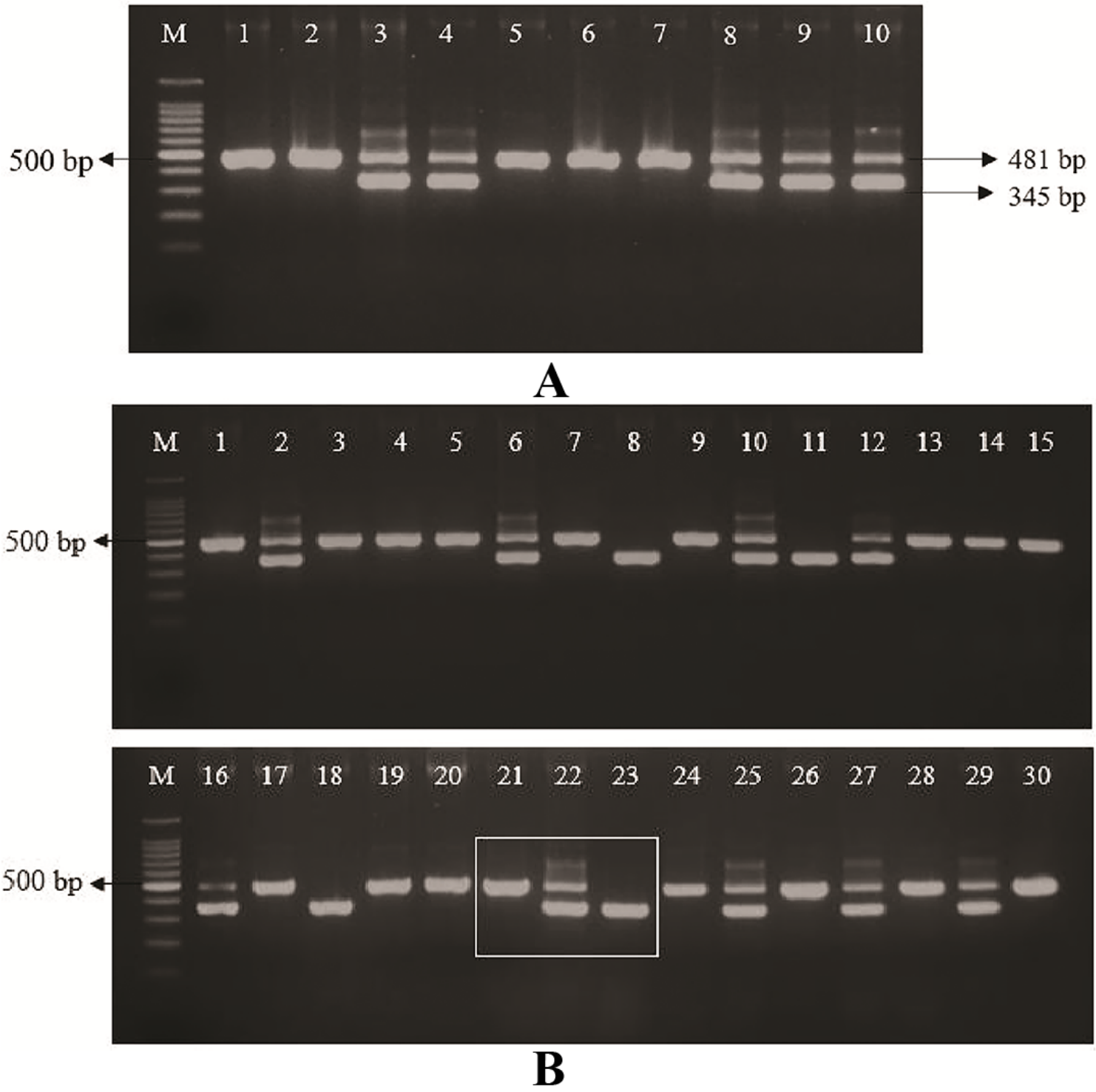

Figure 2

Diagnostic genotyping results of A) F1 Kamper and B) GK chickens. M: 100 bp ladder Bench Top Ladder (BIO-5170, 1st BASE, Malaysia). Lane 21: homozygous coloured chicken (single-band 481-bp; C*N/C*N); Lane 22: heterozygous coloured chicken (dual-band 481-bp and 345-bp; C*N/C*C); Lane 23: recessive white chicken (single-band 345-bp; C*C/C*C). Fragment length measurement based on the gel image analysis using ImageLab (V. 6.0.1) under Grey image colours mode adjustment. Molecular weight

analysis standard used ImageLab 6.0.1 Bio-Rad 100 bp PCR Molecular Ruler with linear (semi-log) regression method.

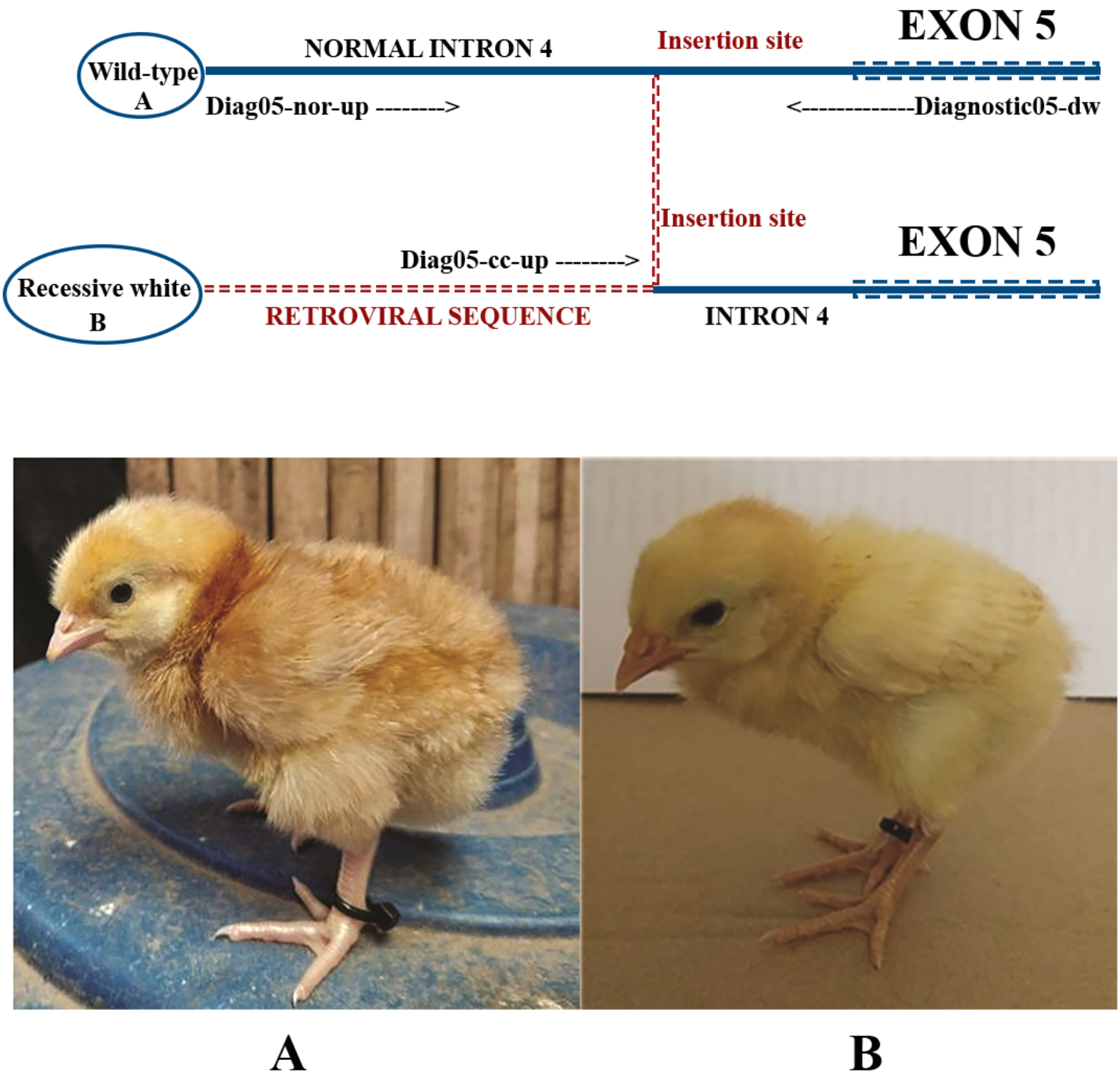

Figure 3

The primers and recognition site of intron 4 retroviral sequence insertional mutation G. gallus TYR gene (NCBI GenBank with the sequence accession number D88349) based on [5]. The TP-PCR-amplified region corresponding to the retroviral sequence on intron 4 is indicated with the red dotted line. The DOCs of GK chickens A) wild-type and B) recessive white with pigmented eyes.

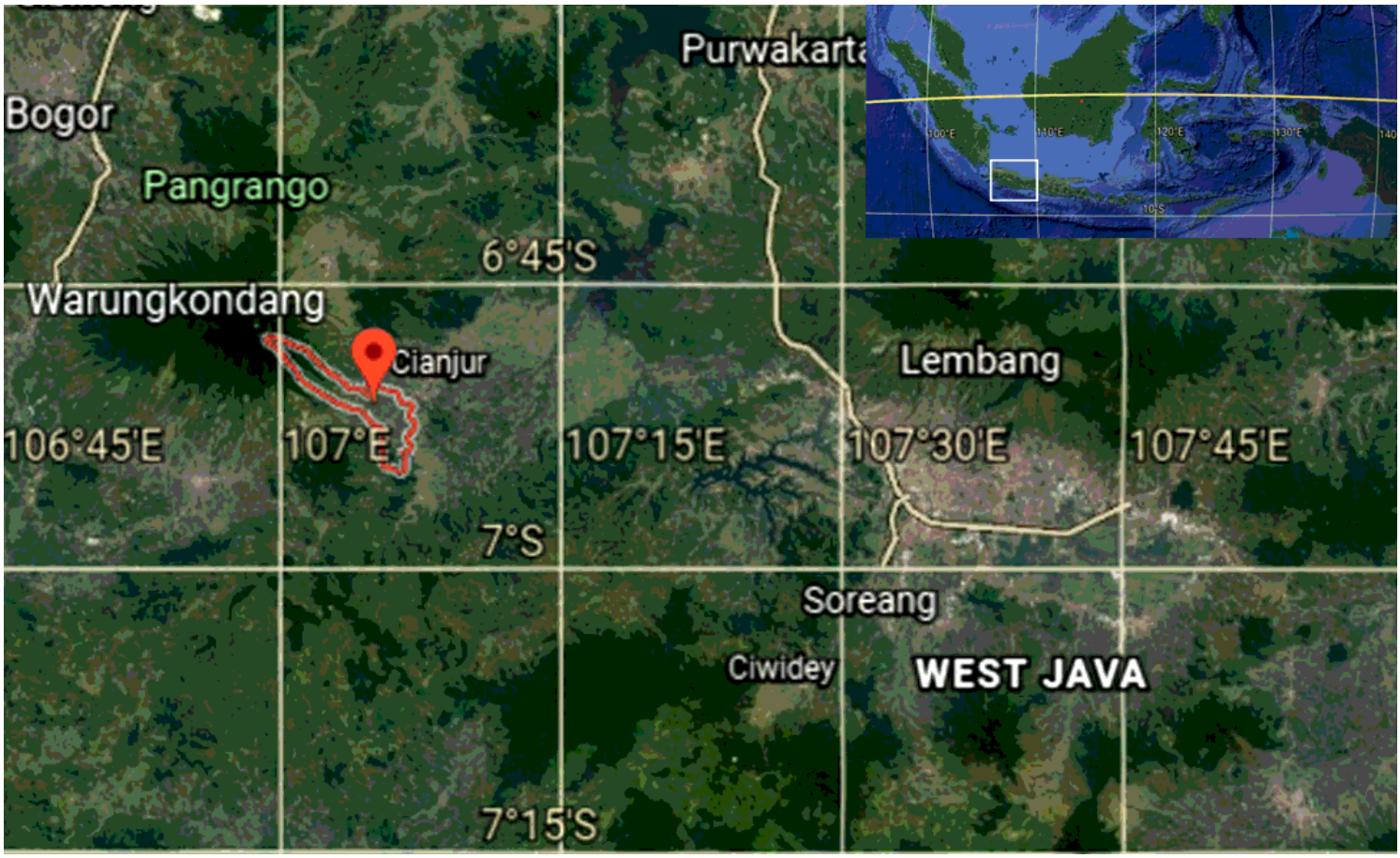

Figure 4

The satellite image of Warungkondang, Cianjur, West Java the region in which Pelung chicken originated. Pelung chicken or locally named Ayam Kampong was first documented and identified from the region which is known as Warungkondang village in 1850 and continues up until the present days. The image was taken and edited from Google Earth Version 9.125.0.0- https://earth.google.com/web Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

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