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Polymorphic Analysis of the *GH* Gene (Exon 3 and 4) and its Association with Growth Traits in Pakistani Quail (*Coturnix japonica* PK)

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ABSTRACT

Genetic markers, such as single nucleotide polymorphisms (SNPs) in *growth hormone* (*GH*) genes, can play a significant role in selection for improving growth traits in poultry birds. The data on SNPs of *GH* genes of *C. japonica* in Pakistan is limited. Therefore, this study was conducted to look into the SNPs in *GH* gene exon 3, 4 and partial sequence of intron 3, 4, and 5 of Pakistani quail *Coturnix japonica* PK (*C. japonica* PK) through polymerase chain reaction (PCR). Five body weight categories (upper outliers body weight; > 250g, higher body weight; 211 to 250g, medium body weight: 171 to 210g, small body weight: 130 to 170g and lower outliers body weight; <130g) of birds were made, and *GH* gene was amplified, sequenced, and screened for detection of SNPs. Furthermore, polymorphisms and their association with different growth traits such as body weight (BW), body length (BL), wing spread (WS), shank length (SL), shank circumference (SC), drumstick length (DL), drumstick circumference (DC), breast width (BD) and keel length (KL) of *C. japonica* PK were also evaluated. A total of eighteen SNPs were detected in the *GH* gene. Genotypes of three SNPs were significantly associated with BW. Genotype TC at position c.31T>C (exon 3) and genotype AA at position c.161A>C (exon 3) had greater BW as compared to the other genotypes (P=0.000). Whereas n.703G>A (intron 4) revealed a very strong association of genotype (AA) with breast width (BD) (P=0.001) and n.12C>T (intron 5) revealed a very strong association of genotypes (CC and TT) with large body weight category and upper outlier body weight category (P=0.008) and DC (P=0.038). c.31T>C (exon 3) revealed a very strong association of genotype (TC) with upper outlier body weight categories (P=0.000), BL (P=0.000) and, DL (P=0.004). c.161A>C (exon 3) revealed a very strong association of genotype (AA) with upper outlier body weight categories (P=0.000), SC (P=0.040) DC (P=0.032) and BW (P=0.007). Thus, *GH* gene may be used as a candidate gene, to improve growth traits of *C. japonica* PK. These results demonstrated that these three SNPs of the *GH* gene might be exploited further as a candidate genetic marker for the genetic improvement of growth-related traits in *C. japonica* PK.

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Authors' Contribution
AM and ARA planned experiments. AM conducted the experiments and collected relevant data. HJ provided the samples. AM and FS interpreted the results. AM, WM and TM wrote the manuscript. AS and RZ design the experiments and statistically analyzed the results. ARA and AS reviewed the manuscript.

Key words
Polymorphisms, Growth hormone gene, *Coturnix japonica* PK, Single nucleotide polymorphism

INTRODUCTION

In Pakistan, *Coturnix japonica* is commonly known as the "BETAIR" and its farming has become increasingly popular over the last few years. It has become a highly profitable business with low investment and high returns (Jatoi *et al*., 2013). Quail farming in some villages of Pakistan has impact on economy. Due to increasing population demand of animal protein also increases (Zahid *et al*., 2018). However, farmers are facing several challenges during *C. japonica* farming including disease management (Mohammed and Ejiofor, 2015), feed management (Nasar *et al*., 2016), housing and space requirements (Razee *et al*., 2016), and selection of genetically improved breed stock (Khaldari *et al*., 2010). The main challenges in commercializing *C. japonica* farming in Pakistan are the birds' lower live body weight (BW), compounded by risks of uncontrolled mating,

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inbreeding, poor knowledge of selective breeding, and their adverse impact on economic production (Jatoi, 2012). Training program was started in collaboration with UVAS Department of Poultry Production and Small and Medium Enterprise Development Authority (SMEDA), to introduce quail farming as a source of income and means of alleviating poverty by creating employment opportunities especially in the rural areas (https://www.thepoultrysite.com/news/2011/02/quail-meat-processing-plant-to-open-shortly).

In the context of animal growth, both genetics and environmental factors play significant role. However, in *C. japonica*, genetic correlations were found to be more significant than those of phenotypic and environmental correlations (Akbas *et al*., 2004). Growth hormone (GH) is a polypeptide hormone. Pituitary gland is involved in the synthesis and secretion of GH (Thakur *et al*., 2009). The *GH* gene is highly polymorphic and involved in several physiological functions (Apa *et al*., 1994). Genetically, the growth of skeletal muscles is controlled by a group of genes, among which the *GH* gene is the most important. In *C. japonica*, the *GH* gene not only plays a crucial role in growth but also promotes other secondary functions such as egg production, reproduction, aging, and BW (Kadlec *et al*., 2011; Nie *et al*., 2005a, b; Kansaku *et al*., 2003).

The recent surge in DNA sequencing technology has identified a vast number of single nucleotide polymorphisms (SNPs) in the avian *GH* gene, which include deletions, insertions, and substitutions. These SNPs have a remarkable effect on the transcription and translation of genes and are widely distributed throughout the avian genome (El-Bayomi *et al*., 2016). The impact of *GH* gene polymorphisms on poultry growth performance has been extensively studied. Where, specific polymorphisms have been identified in chickens and goose that are associated with growth traits and carcass traits, respectively (Ghelghachi *et al*., 2013; Zhao *et al*., 2011). Overexpression of the *GH* gene has been shown to significantly increase BW and growth rate in broiler chickens (Jia *et al*., 2018). Although limited studies have been conducted on *C. japonica*, significant associations have been found between specific *GH* gene polymorphisms and growth traits in *C. japonica* (Ghahramani *et al*., 2017). *GH* gene polymorphisms have also been associated with BW and breast muscle weight in *C. japonica* (Nasirifar *et al*., 2018). Additionally, the effects of *GH* gene manipulation on *C. japonica* growth performance have been investigated. Studies have reported that *GH* gene overexpression increases the growth rate and feed conversion rate in *C. japonica* (Teshfam *et al*., 2011), while knockout of the *GH* gene resulted in reduced BW and growth rate (Donahue and Beamer, 1993). These findings suggest that the *GH* gene plays a pivotal role in *C. japonica* growth and development, and *GH* gene polymorphisms and manipulation have a considerable effect on the growth performance of these birds. Since 2007, a selective breeding program for a higher BW of *C. japonica* is in progress at Avian Research and Training Centre, University of Veterinary and Animal Sciences (UVAS), Pakistan, where the live BW of birds has been increased from 100 g to 314 g by primarily focusing on genetic selection using the selective breeding approach (https://uvas.edu.pk/academics/faculties/FAPT/PP/ART.htm#gsc.tab=0). Recently, genetic marker technologies including gene introgression, parentage identification, and marker-assisted selection have been extensively used to assist the selective breeding programs in birds and livestock due to their higher efficiency, genetic diversity, and precision (Davis and DeNise, 1998). Therefore, this study was designed to amplify the *GH* gene and to elucidate any possible role of *GH* gene polymorphism (*C. japonica* PK) in association with phenotypic growth traits of birds. To this end, a 887 bp region of *GH* gene (including the complete sequence of exon 3 and 4 and partial sequences of intron 3, 4, and 5) was amplified and further scrutinized for any polymorphic association linked with growth traits to find an appropriate genetic marker for selection of higher category weight birds.

MATERIALS AND METHODS

Experimental birds and their body measurements

A total of 150 (male and female) locally evolved *C. japonica* PK were divided into five body weight categories, with each category containing 30 birds. The body weight categories were as follows: Upper outliers body weight category (>250g), higher body weight category (211-250g), medium body weight category (171-210g), small body weight category (130-170g), and lower outliers body weight category (<130g). Body measurements associated with the *GH* gene including BW, body length (BL), wing spread (WS), shank length (SL), shank circumference (SC), drumstick length (DL), drumstick circumference (DC), breast width (BD) and keel length (KL) were determined following the methodology as described in our earlier study (Adil *et al*., 2022).

Blood collection and DNA extraction

Blood samples (150 birds) were collected from the brachial vein of the birds at the age of 30 days in ethylenediaminetetraacetic acid (EDTA) containing vacutainer tubes. Theses blood samples were stored at -20 °C for future use. Genomic DNA was extracted from these blood samples. Organic method of DNA extraction

was used with little modifications (Sambrook and Russell, 2001).

Briefly, 250 µL blood was mixed with 1mL wash buffer (Tris-EDTA; 10mM Tris and 1mM EDTA, pH=8.0) and vortexed for 30 seconds then, centrifuged at 13,000 rpm (20 min at 4°C). After centrifugation supernatant was discarded and the pellet was mixed with 300 µL buffer A1 (1M Tris, 1M NaCl, and 0.5 M EDTA, pH=8.0), 100 µL SDS (10 %), and 100 µL proteinase K (20mg/ mL), followed by overnight incubation at 50 °C. Next, phenol: chloroform: isoamyl alcohol (25:24:1) (Carl Roth) 500 µL mixture was added in the cell pellet, followed by centrifugation at 13,000 rpm (20 min at 4 °C). The supernatant was transferred to a new microcentrifuge tube with 2 volumes of 100% isopropanol and incubated at 25 °C for 5 min for nucleic acid precipitation followed by centrifugation at 8,000 rpm (4 °C for 7 min). Twice washings using 80% ethanol (Merck), was given to nucleic acid pellet. The nucleic acid pellet was kept at room temperature until dry. Then, the pellet was dissolved in 100 µL nuclease-free water, and stored for further use at -20 °C. The genomic DNA was subjected to electrophoresis using 0.8 % agarose gel to determine the property of the genome. The DNA quantity and quality were also checked using NanoDrop (2000/2000c, Thermo Fisher Scientific, Wilmington, USA).

Amplification of GH *gene, its purification, and sequence analysis*

A total of two primers (Table I) were designed. Primer 3 software was used for designing the primers (Untergasser *et al*., 2012). Partial sequence of the *C. japonica GH* gene (Accession No: NC_029542.1), which spans 23,525 bp from position 1156600 to 1159853 (compliment) was amplified. These primers were designed under the guidelines reported earlier (Ye *et al*., 2012) specifically to amplify a total 887 bp region from position 1158084 to 1157953 (GHJE3_F and GHJE3_R) and 1157242 to 1156756 (GHJE4_F and GHJE4_R), for the detection of polymorphisms in *GH* gene of *C. japonica* PK (Fig. 1). The designed primers were able to amplify the complete sequence of exon 3 and 4, and partial sequence of the intron, 3, 4 and 5. Primer-Blast (Ye *et al*., 2012) tool was further utilized to confirm the specificity of all the designed primers against the reference sequence (Accession No: NC_029542.1). Default parameters were used for all the software/tools until otherwise mentioned.

For PCR amplification with an expected 400 bp and 487 bp product size against the exon 3 and 4 respectively, all the reagents purchased from Thermo Fisher Scientific, were used. Reaction volume prepared was 20 µL, consisting of 2µL of template DNA (50 ng/ µL), $(NH_4)_2SO_4$ buffer (10X) 2 µL, dNTP (2.5 mM) 2 µL, $MgCl_2$ (25 mM) 1.5 µL, 50 pmol of each, forward and reverse primers (1 µL each primer), *Taq* polymerase (5 U/ µL Thermo Scientific) 0.4 µL and nuclease-free water (10.1 µL). The PCR profile consisting of an initial denaturation step (94°C for 5 min), followed by 30 cycles of; denaturation (94 °C for 1 min), annealing 59 °C for 40 sec for Exon 3 and 70 °C for 30 sec for Exon 4, and extension at 72°C for 50 sec for Exon 3 and 72 °C for 40 sec for Exon 4. The final extension was performed at 72 °C for 10 min for both exon 3 and 4.

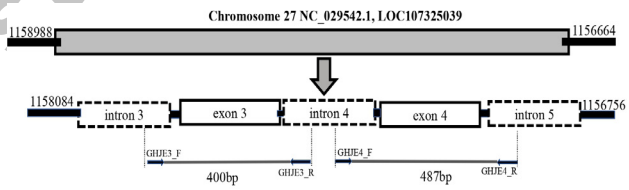

Fig. 1. Primer binding sites of *C. japonica* PK *GH* gene (exon 3 and exon 4).

The amplicons were purified using GenElute PCR Clean-Up Kit (Thermoscientific) following the manufacturer's protocol. The amplicons were eluted in 50 µL of elution buffer provided with the kit and were sequenced using the dideoxy chain termination method (Sangers and Coulson, 1977) with genetic analyzer ABI_3100 (Applied Biosystems, Inc., Foster City, CA).

Table I. Primer sequence, target sequence position, expected PCR product size and target region of *C. japonica* PK *GH* gene (exon 3 and 4).

Primer	Nucleotides sequence (5'-3')	Target sequence position	Product size(bp)	Target region	Source
GHJE3_F	TATCACACCTGAGAAGGAGAGC	1158084 to1158062	400	Exon 3	This study
GHJE3_R	GCCGTGGGCTGGGTTT	1157953 to1157969			
GHJE4_F	CGTCCAGACTTTGCTGTCCA	1157242 to1157223	487	Exon 4	This study
GHJE4_R	GGGAGATGTGCCCGTCAG	1156773to1156756			

The data obtained were analysed using Chromas Version 1.0 (Technelysium Pty Ltd, Unit 406, 8 Cordelia St, South Brisbane QLD 4101, Australia). The FASTA sequences obtained were aligned individually using BLAST (Basic Local Alignment Search Tool) with reference *GH* gene sequence (Accession No: NC_029542.1) (Ye *et al*., 2006). The variations in the sequence were confirmed from chromatogram peaks. These sequences were aligned and compared separately according to the body weight categories using Clustal Omega (Sievers and Higgins, 2014) and BLAST (Ye *et al*., 2006). Genotypic and allele frequency were calculated at each locus to confirm the specific region amplification. Variations in the amplified region were compared with reference sequence (Accession No: NC_029542.1) as well as with body weight categories of *C. japonica* PK.

Analysis of the association of GH polymorphism with growth traits

A multiple linear regression model was used for association analysis of *GH* gene SNPs and growth traits by SPSS (Statistical Package for Social Sciences) software (Version 20.0; International Business Machines Inc., Chicago, IL, USA).

Multiple linear regression model used was as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_6 X_6 + \beta_7 X_7 + \beta_8 X_8 + \beta_9 X_{9+} \epsilon$$

Y is SNP genotypes (dependent variable), β_0= Intercept, $\beta_{(1\text{-}9)}$= Partial regression coefficients, $X_{(1\text{-}9)}$= body measurements (independent variables), ϵ is Error term.

$$R^2 = 1 - \frac{\text{Unexplained variation}}{\text{Total variation}}$$

Genotype frequencies and allele frequencies were calculated by following formula (Enayati and Rahimi-Mianji 2009).

$$P = \frac{2(AA) + (AB)}{2N}$$

$$q = \frac{2(BB) + (AB)}{2N}$$

P is the gene frequency of allele A, q is the gene frequency of allele B, N is the total number of birds.

RESULTS

GH *gene and confirmation of SNPs*

PCR amplified 400 bp and 487 bp DNA fragments (Fig. 2) by using the two primers described earlier. These amplified fragments were comprised of the complete sequence of exon 3 and exon 4, along with a partial sequence of intron 3, intron 4, and intron 5 of *GH* gene in *C. japonica* PK. Amplified fragments were purified, sequenced and were aligned with the reference sequence of *C. japonica* (Accession No: >NC_029542.1:c1158988-1156664). Variations were observed with reference sequence as well as between different body weight categories. Total of eighteen SNPs were identified in the amplified regions. Among these eighteen SNPs two SNPs were located in exons 3 (c.31T>C; c.161A>C); one SNP was located in exon 4 (c.82G>A); two SNPs were located in intron 3 (n.79T>C, n.95T>C); twelve SNPs were located in intron 4 (n.68C>T, n.94T>G, n.103T>C, n.104A>T, n.105T>C, n.106G>C, n.703G>A, n.732C>T, n.775G>C, n.818G>A, n.842T>A, n.843C>A); and one SNP was located in intron 5 (n.12C>T) were identified by sequencing directly. SNPs in the *GH* gene of *C. japonica* PK associated significantly with body weight categories has been illustrated in Figure 3.

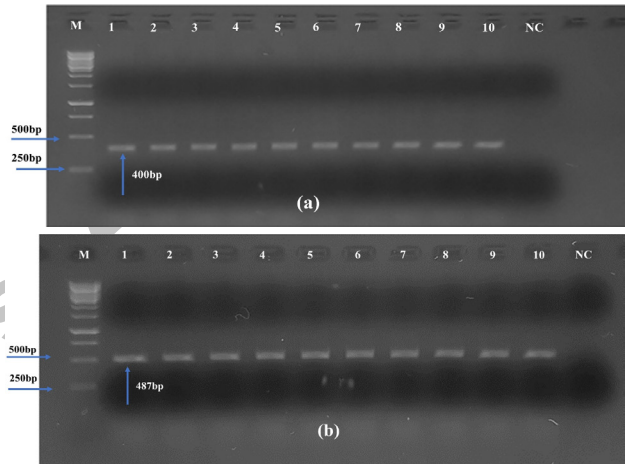

Fig. 2. (a) amplicons of *C. japonica* PK *GH* gene (a) exon 3. Lane M: DNA marker 1kb Fermentas. Lane 1-10: Amplicons of *C. japonica* PK exon 3 (400 bp) and NC: Negative control exon 3. (b) exon 4; Lane M: DNA marker 1kb Fermentas, Lane 1-10: Amplicons of *C. japonica* PK exon 4 (487 bp) and NC: Negative control exon 4.

Fig. 3. *C. japonica GH* gene significant SNPs (a) Exon 3 (c.31T>C) genotype TT; (b) Exon 3 (c.31T>C) genotype TC; (c) Exon3 (c.161A>C) genotype AA; (d) Exon3 (c.161A>C) genotype AC; (e) Intron 5 (n.12C>T) genotype CC; (f) Intron 5 (n.12C>T) genotype TT and (g) Intron 5 (n.12C>T) genotype CT.

Table II. Significant SNP number, target region, position, nucleotide change and observed genotypes of *C. japonica* PK *GH* gene (exon 3 and 4).

S. No.	SNP No.	Target region (Exon/Intron)	Position	Nucleotide change	Genotypes observed
1.	12	Exon 3	31	T>C	TT and TC
2.	13	Exon 3	161	A>C	AA and AC
3.	27	Intron 5	12	C>T	CC, CT and TT

Genotype and allele frequency

Genotypes, genotype frequency and allele frequency were identified at each variable region (Table III). Briefly, Genotype TC frequency at position c.31T>C (exon 3) was highest in upper outlier body weight category. Genotype AA frequency at position c.161A>C (exon 3) was highest in large body weight category and upper outlier body weight category. Genotype CC and TT frequency at position n.12C>T (intron 5) frequencies were highest in medium body weight category, large body weight category and upper outlier body weight category. Frequency of allele A is higher in large body weight category and upper outlier body weight category at position c.161A>C (exon 3).

Association analysis between GH *gene and growth traits*

Multiple linear regression model was applied to eighteen variable regions respectively for association analysis of SNPs of *GH* gene and growth traits. It suggested three significant SNPs; c.31T>C (exon 3), c.161A>C (exon 3) and n.12C>T (intron 5) among total of eighteen variable regions. These three SNPs genotypes were significantly associated with body weight categories (Supplementary Tables II, III and IV). In the tested *C. japonica* PK population, birds with TC genotype were heavier than those with TT genotype at locus c.31T>C of exon 3. Birds with AA genotype were heavier than those with AC genotype at locus c.161A>C of exon 3. Birds with CC and TT genotypes were heavier than those with CT genotype at locus n.12C>T of intron 5. n.703G>A (intron 4) revealed a very strong association of genotype (AA) with BD (P=0.001). n.12C>T (intron 5) revealed a very strong association of genotype (CC and TT) with DC (P=0.038). c.31T>C (exon 3) revealed a very strong association of genotype (TC) with BL (P=0.000) and DL (P=0.004). c.161A>C (exon 3) revealed a very strong association of genotype (AA) with SC (P=0.040), DC (P=0.032) and BW (P=0.007) (Supplementary Tables II, III and IV).

The frequency of TT genotype at locus c.31T>C (exon 3) was found predominant in the overall population of the bird under study while the frequency of AC genotype at

Table III. *C. japonica* PK *GH* gene genotypes, genotypic frequency and allelic frequency.

S. No	Polymorphism position	Body weight category	Genotypes	Genotypic frequency	Allelic frequency
1.	c.31T>C (Exon 3)	Lower outlier body weight	TT = 29 TC = 1 CC = 0	TT = 0.96 TC = 0.03 CC = 0	T = 0.98 C = 0.02
		Small body weight	TT = 30 TC = 0 CC = 0	TT = 1 TC = 0 CC = 0	T= 1 C= 0
		Medium body weight	TT = 30 TC = 0 CC = 0	TT = 1 TC = 0 CC = 0	T = 1 C = 0
		Large body weight	TT = 28 TC = 2 CC = 0	TT = 0.93 TC = 0.06 CC = 0	T = 0.97 C = 0.03
		Upper outlier body weight	TT = 0 TC = 30 CC = 0	TT = 0 TC = 1 CC = 0	T = 0.50 C = 0.50
2.	c.161A>C (Exon 3)	Lower outlier body weight	AA = 0 AC = 30 CC = 0	AA = 0 AC = 1 CC = 0	A = 0.50 C = 0.50
		Small body weight	AA = 0 AC = 30 CC = 0	AA = 0 AC = 1 CC = 0	A = 0.5 C = 0.5
		Medium body weight	AA = 0 AC = 30 CC = 0	AA = 0 AC = 1 CC = 0	A = 0.5 C = 0.5
		Large body weight	AA = 30 AC = 0 CC = 0	AA = 1 AC = 0 CC = 0	A = 1 C = 0
		Upper outlier body weight	AA = 30 AC = 0 CC = 0	AA = 1 AC = 0 CC = 0	A = 1 C = 0
3.	n.12C>T (Intron 5)	Lower outlier body weight	CC = 0 CT= 29 TT = 1	CC = 0 CT =0.96 TT = 0.03	C = 0.48 T = 0.52
		Small body weight	CC = 1 CT= 29 TT = 0	CC = 0.03 CT = 0.96 TT = 0	C= 0.52 T= 0.48
		Medium body weight	CC = 21 CT= 1 TT = 8	CC = 0.70 CT = 0.03 TT = 0.26	C = 0.72 T = 0.28
		Large body weight	CC = 18 CT= 1 TT = 11	CC = 0.60 CT = 0.03 TT = 0.36	C = 0.62 T = 0.38
		Upper outlier body weight	CC = 22 CT= 0 TT = 8	CC = 0.73 CT = 0 TT = 0.26	C = 0.73 T = 0.27

Table IV. Effect of *GH* gene genotypes on body weight, body length, wing spread, shank length, shank circumference, drumstick length, drumstick circumference, breast width and keel length. Values are Mean ± SD.

Traits	c.31T>C (Exon 3), c.161A>C (Exon 3), n.12C>T (Inton 5)
Weight categories	1.00-5.00±1.41
Genotypes	
	(T T, TC) 1-2±0.41 (AA, AC) 1-2±0.49 (TT, CT and CC) 1-3±0.743
Body weight	75.00-314.00±56.42
Body length	23.40-34.00±2.39
Wing spread	13.60-35.00±2.52
Shank length	1.90-6.10±0.62
Shank circumference	0.90-2.70±0.31
Drumstick length	3.90-9.20±1.05
Drumstick circumference	1.00-5.60±1.34
Breast width	1.40-6.70±1.19
Keel length	3.00-8.80±1.27

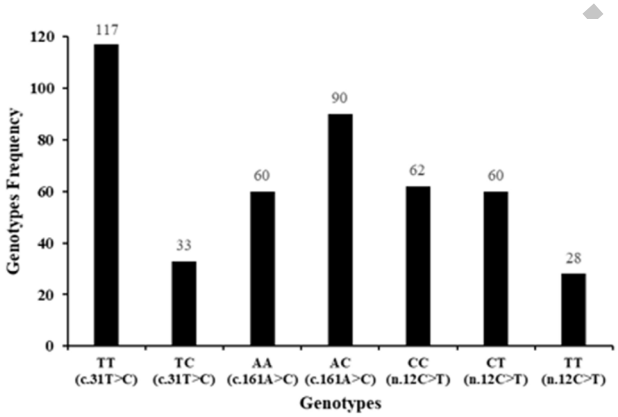

Fig. 4. Frequency of significant SNPs; c.31T>C (Exon 3), c.161A>C (Exon 3) and n.12C>T (Intron 5) genotypes in population of *C. japonica* PK under study.

locus c.161A>C (exon 3) was found predominant in overall population of bird under study (Fig. 4). The coefficient of determination R^2 of polymorphism at c.31T>C (Exon 3), c.161A>C (Exon 3) and n.12C>T (Intron 5) implied that 68%, 81% and 2% variation in BW was accounted for the specific genotypes respectively (Supplementary Table I). The multiple linear regression model also revealed that variation in three loci of the selected *GH* gene of *C. japonica* PK c.31T>C (Exon 3) p value =0.000, c.161A>C (Exon 3) p value = 0.000 and n.12C>T (Intron 5) p value = 0.008 were significantly associated with the body weight categories (Supplementary Tables II, III and IV).

DISCUSSION

The *GH* gene is of paramount importance among all growth-related genes as it plays a major role in the production of growth hormones. This hormone significantly contributes to the pre-and post-hatching growth rates in birds (Scanes, 2009). *GH* gene and its higher degree of polymorphism have been observed in various poultry and livestock species and showed a positive correlation with different growth traits (Zhang *et al*., 2007; Hua *et al*., 2009; Balogh *et al*., 2009; Zhao *et al*., 2011; Dettori *et al*., 2013). In birds, *GH* gene is the functional candidate gene for regulating growth, development, and metabolism (Vasilatos-Younken and Scanes, 1991). However, no scientific studies have been documented with reference to *C. japonica* PK *GH* gene amplification and evaluation of its possible polymorphic association linked with growth traits. Therefore, the present study aimed to amplify the *GH* gene *C. japonica* PK, and investigate any potential SNPs and their possible role in the growth and development of *C. japonica* PK.

In this study, a 887 bp region of the *GH* gene (comprising a complete sequence of exons 3 and 4 and the partial sequence of intron 3, 4, and 5) was amplified, sequenced, and screened for SNPs by extracting DNA from 150 birds (*C. japonica* PK). Several researchers have reported the amplification of the *GH* gene in quails and exploited it further to aid in the genetic selection of heavy body weight birds. For example, in Iran, intron 1 of *GH* gene (776 bp fragment) from 346 birds (*C. japonica*) was amplified using PCR technique. The amplicons were digested by restriction fragment length polymorphism (RFLP) (Nasirifar *et al*., 2018); in Iraq, a 776 bp fragment was amplified and digested by PCR-RFLP technique from 363 quails (Ahmed and Al-Barzinji, 2020); in Turkey, intron 2 of *GH* gene was amplified and sequenced from 50 birds for the selection higher body weight birds (El-Bayomi *et al*., 2016); and in Vietnam, *GH* gene from two populations of laying (6-26 weeks old) *C. japonica* was amplified and further evaluated (Lan *et al*., 2017).

Association was observed among genotypic profile of *GH* gene and *C. japonica* growth traits. Genetic profile could potentially be used as genetic marker for selection of heavy weight birds (Lajan and Al-Barzinji, 2022). Similar associations between *GH* gene and morphometric traits have been studied. Association study observed among other poultry species includes ducks (Mazurowski *et al*., 2015) and chickens (Okafor *et al*., 2019; Nie *et al*., 2005a). Mazurowski *et al*. (2015) observed morphological traits

(BW, SL, length of breast bone, chest girth, length of trunk and length of trunk with neck) and their association with *GH* gene (intron 2) in ducks (Mazurowski *et al*., 2015). Okafor *et al*. (2019) also found a correlation between *GH* gene-associated morphological traits, such as BW, SL, BD, and breast girth, in Nigerian chickens. In this study, we also reported that morphological traits of *C. japonica* PK are associated with *GH* gene. SNPs were observed in *GH* gene exon 3 and exon 4 (complete sequences) and partial sequences of intron 3, 4 and 5 whereas in ducks, only variations in the *GH* gene's intron 2 region were observed (Mazurowski *et al*., 2015).

The detailed studies on avian *GH* gene also reported the presence of different SNPs controlling the birds BW (Okafor *et al*., 2019). For example, Okafor and colleagues amplified chicken *GH* gene by PCR and sequenced. Two SNPs linked with growth traits were identified in the amplified region (Okafor *et al*., 2019). Four divergent breeds of chicken were selected. *GH* gene SNPs were identified by two techniques denaturing high-performance liquid chromatography then sequencing. Total forty-six SNPs were detected in *GH* gene of which four SNPs were in the 5′ untranslated region (UTR) and one in the 3′ UTR. Thirty-six SNPs were in intronic region and five were in the exonic region. These SNPs were associated with BW and SL (at different ages) (Nie *et al*., 2005a). In chicken *GH* gene SNPs are associated with growth traits. The results demonstrated that SNP G662A of *GH* gene may be used as a candidate marker gene for genetic improvement of growth traits in Mia chicken breed (Thinh *et al*., 2019). Chicken *GH* gene SNPs were associated with BW in chickens. A total of 300 birds from three strains (100 birds from each strain) *GH* gene was investigated (El-Sayed *et al*., 2022). In this study, a total of eighteen SNPs were identified in *C. japonica* PK three of which are significantly associated with body weight categories. Among significant SNPs two SNPs were at exon 3 (c.31T>C and c.161A>C) and one at intron 5 (n.12C>T).

The allelic frequency distribution of SNPs provides valuable information about genetic variability within a population and its implications for growth-related characteristics (Li *et al*., 2020). At locus *GH/Bsm*FI of ducks two alleles C and T were observed. Three genotypes CC, CT and TT were observed at this locus. Frequency of allele C and T in duck population was as follows; Pekin (0.53 and 0.47), Muscovy-CK (0.01 and 0.99), Muscovy-CRAMMLCFF (0.00 and 1.00) and Mulard (0.18 and 0.82). Frequency of genotype TT, TC and CC in population of ducks was as follows; Pekin (0.21, 0.51 and 0.28), Muscovy-CK (0.98, 0.02 and 0.00), Muscovy-CRAMMLCFF (1.00, 0.00 and 0.00) and Mulard (0.64, 0.36 and 0.00). Pekin duck with genotype TT had higher BW ($P<0.01$) than Pekin ducks with CC and CT genotypes. All the evaluated traits recorded in Mulards ducks with genotype TT were higher in values as compared to Mulards ducks with genotype CT and CC. Polymorphism of the *GH* gene (intron 2) only markedly impacted SL in males (Mazurowski *et al*., 2015). In this study allelic frequency linked with higher BW was allele A at position c.161A>C (Exon 3). Allele A linked with large body weight category and upper outlier body weight category.

In different breeds of chickens, a significant association was identified between morphometric and growth traits (Okafor *et al*., 2019). In this study *C. japonica* PK with TC and AA genotype at position c.31T>C (exon 3) and c.161A>C (exon 3) respectively were characterized by higher BW value than those with other genotypes ($P=0.000$). *C. japonica* PK with TC genotype at position c.31T>C (exon 3) significantly associated with morphometric traits such as; BL ($P=0.000$) and DL ($P=0.004$). Genotype AA at position c.161A>C (exon 3) significantly associated with morphometric traits; SC ($P=0.040$) DC ($P=0.032$) and BD ($P=0.007$). Genotype CC and TT at position n.12C>T (intron 5) significantly associated with DC ($P=0.038$). Genotype AA at position n.703G>A (intron 4) was significantly associated with only one morphological trait BD ($P=0.001$). No significant association of this SNP found with BW categories. *C. japonica* PK morphometric traits that influenced significantly includes BW, BL, SC, DL, DC and BD.

CONCLUSION

In conclusion, SNPs in the *GH* gene (*C. japonica* PK) at exon 3 (c.31T>C and c.161A>C) and intron 5 (n.12C>T) are associated with growth traits and could serve as genetic markers for marker-assisted selection in *C. japonica* PK breed improvement programs. However, further investigation about the genetic interactions between the *GH* gene and other candidate genes involved in growth regulation in *C. japonica* PK are also needed to fully understand these interaction at farm level.

DECLARATIONS

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Ethical statement and IRB approval

The study was approved from Animal Ethics Committee of UVAS, Lahore, Pakistan (DR/ 71 Dated: 08-02-2016). All blood samples were carried according to instructions of Animal Ethics Committee of University of Veterinary and Animal Sciences (UVAS), Lahore Pakistan.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20230731171437

Statement of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Material

Polymorphic Analysis of the *GH* Gene (Exon 3 and 4) and its Association with Growth Traits in Pakistani Quail (*Coturnix japonica* PK)

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Supplementary Table I. Level of variation in the dependent variable that can be explained by the independent variable *C. japonica* PK.

Genotypes	R	R square	Adjusted R square
c.31T>C (Exon 3)	0.82	0.68	0.66
c.161A>C (Exon 3)	0.90	0.81	0.79
n.12C>T (Inton 5)	0.51	0.26	0.20

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Supplementary Table II. Linear regression equation coefficients of *C. japonica* PK *GH* gene position c.31T>C (Exon 3).

Body measurements	Unstandardized coefficients		Standardized coefficients	t	Sig.	95.0% confidence interval for B		Collinearity statistics	
	B	Std. Error	Beta			Lower bound	Upper bound	Tolerance	VIF
	3.223	.429		7.507	.000	2.374	4.072		
Weight categories	.242	.062	.825	3.911	.000	.120	.364	.050	19.836
Body weight	.002	.002	.290	1.293	.198	-.001	.005	.045	22.335
Body length	-.078	.018	-.449	-4.412	.000	-.113	-.043	.216	4.619
Wing spread	-.006	.009	-.036	-.690	.491	-.023	.011	.806	1.240
Shank length	.003	.052	.004	.053	.958	-.099	.105	.377	2.652
Shank circumference	.002	.108	.001	.016	.987	-.212	.216	.326	3.071
Drumstick length	-.102	.035	-.259	-2.926	.004	-.171	-.033	.286	3.500
Drumstick circumference	-.023	.030	-.075	-.778	.438	-.082	.036	.243	4.115
Breast width	-.018	.035	-.053	-.526	.599	-.088	.051	.222	4.501
Keel length	.004	.028	.012	.143	.886	-.051	.059	.305	3.274

Supplementary Table III. Linear regression equation coefficients of *C. japonica* PK *GH* gene position c.161A>C (Exon 3).

Body measurements	Unstandardized coefficients		Standardized coefficients	t	Sig.	95.0% confidence interval for B		Collinearity statistics	
	B	Std. Error	Beta			Lower bound	Upper bound	Tolerance	VIF
	2.305	.396		5.813	.000	1.521	3.089		
Weight categories	-.304	.057	-.879	-5.334	.000	-.417	-.192	.050	19.836
Body weight	-.001	.002	-.066	-.380	.704	-.004	.002	.045	22.335
Body length	.011	.016	.055	.696	.487	-.021	.044	.216	4.619
Wing spread	-.010	.008	-.052	-1.252	.213	-.026	.006	.806	1.240
Shank length	-.038	.048	-.048	-.801	.425	-.132	.056	.377	2.652
Shank circumference	-.207	.100	-.135	-2.075	.040	-.405	-.010	.326	3.071
Drumstick length	.034	.032	.074	1.068	.287	-.029	.098	.286	3.500
Drumstick circumference	.059	.027	.162	2.163	.032	.005	.114	.243	4.115
Breast width	.088	.032	.214	2.722	.007	.024	.152	.222	4.501
Keel length	-.015	.026	-.038	-.570	.570	-.066	.036	.305	3.274

Supplementary Table IV. Linear regression equation coefficients of *C. japonica* PK *GH* gene position n.12C>T (Intron 5).

Body measurements	Unstandardized coefficients		Standardized coefficients	t	Sig.	95.0% confidence interval for B		Collinearity statistics	
	B	Std. Error	Beta			Lower bound	Upper bound	Tolerance	VIF
	1.501	1.182		1.270	.206	-.836	3.838		
Weight categories	-.460	.170	-.878	-2.701	.008	-.796	-.123	.050	19.836
Body weight	.007	.005	.549	1.592	.114	-.002	.016	.045	22.335
Body length	.009	.049	.030	.189	.851	-.087	.105	.216	4.619
Wing spread	-.021	.024	-.070	-.861	.390	-.068	.027	.806	1.240
Shank length	-.104	.142	-.087	-.734	.464	-.385	.177	.377	2.652
Shank circumference	-.007	.298	-.003	-.024	.981	-.596	.582	.326	3.071
Drumstick length	.162	.096	.229	1.679	.095	-.029	.352	.286	3.500
Drumstick circumference	-.172	.082	-.311	-2.099	.038	-.333	-.010	.243	4.115
Breast width	.176	.097	.283	1.829	.070	-.014	.367	.222	4.501
Keel length	-.029	.077	-.050	-.379	.705	-.181	.123	.305	3.274