≪Research Note≫

Determination of Polymorphisms in Pituitary Genes of the Native Afghani Naked Neck Chicken

Sadequllah Ahmadi[1,2], Misa Takeda[1] and Takeshi Ohkubo[1]

[1] College of Agriculture, Ibaraki University, 3-21-1 Chuo, Ami, Ibaraki 300-0393, Japan
[2] Faculty of Animal Science, Afghanistan National Agricultural Sciences and Technology University (ANASTU), Kandahar, Afghanistan

We investigated means to improve the production of the indigenous Naked Neck chicken in Afghanistan. Specifically, we analyzed single nucleotide polymorphisms (SNPs) in the prolactin (*PRL*) (24 bp indel), growth hormone (*GH*) (T185G), and pituitary specific transcript factor 1 (*PIT-1*) (intron 5) genes. Blood samples were collected from 52 birds and genomic DNA was extracted. Polymorphisms in the mentioned loci were analyzed by PCR, allele-specific PCR, and PCR-restriction fragment length polymorphism (RFLP) using *Taq*I and *Msp*I endonucleases. Cloning followed by DNA sequencing was performed to ascertain the accuracy of the PCR-RFLP analysis for *PIT-1*. Two alleles were found for the *PRL* 24 bp indel, *GH* (T185G), and *PIT-1/Taq*I, with the following respective allelic frequencies: *PRL-In* 0.64 and *PRL-Del* 0.36, *GH*-T 0.91 and *GH*-G 0.09, and *PIT-1*-A 0.64 and *PIT-1*-B 0.36. Regarding the *PIT-1/Msp*I polymorphism, three novel *Msp*I recognition sites, as well as two reported *Msp*I recognition sites, were detected in intron 5. Moreover, during sequence screening, two novel SNPs were found that generated restriction sites for *Mse*I. Therefore, our results suggest that the *PRL* indel, *GH* T185G, and *PIT-1/Taq*I polymorphisms may be used as selection markers for Afghanistan Naked Neck chickens. Intron 5 of *PIT-1* in the Afghani Naked Neck chicken was highly polymorphic compared to the reported *Gallus gallus PIT-1* gene (GenBank accession no. NC_006088.4).

Key words: genetic polymorphism, Naked Neck, *PIT-1*, pituitary hormone

Introduction

The Naked Neck chicken breed is widespread and indigenous to numerous African and Asian countries (DAD-IS and FAO, 2012). This breed is native to Afghanistan and is well known for its egg production and resilience to disease and hot climates. An autosomal dominant gene (*Na*) controls the bared neck trait, which results in heat reduction and improvement in thermoregulation. The *Na* gene is known to be associated with heat tolerance and *Na/na* birds are superior in carcass yield, laying rate, mean egg weight, eggshell strength, and egg mass (Merat, 1990; Njenga, 2005). Furthermore, in high ambient temperatures and unfavorable

environments, the dominant *Na* allele positively affects breast weight and growth rate, resulting in minimal loss of body weight during heat stress, high levels of heat shock protein 70 (HSP70), good feed conversion ratio, desirable carcass traits, and reduced effects of high ambient temperatures on fertility (Njenga, 2005; Islam and Nishibori, 2009). Thus, the Naked Neck chicken is a valuable breed for hot climates, including Afghanistan, and its characteristics can be further genetically investigated to enhance traits. Molecular markers are useful tools for improving production traits in farm animals and, to increase selection efficiency, marker-assisted selection linked to quantitative trait loci allows for the direct selection of genotypes in a population (Lamont *et al*., 1996).

The body weight and reproductive performance of vertebrates are regulated by the coordinated action of multiple factors (Sharma *et al*., 2008), including pituitary specific transcription factor 1 (*PIT-1*) that regulates vertebrate pituitary development, pituitary cell proliferation, and hormone expression. Moreover, PIT-1 also regulates the mRNA expression of growth hormone (*GH*), prolactin (*PRL*), and thyroid-stimulating hormone beta subunit (*TSHB*) in the

pituitary gland (Bodner *et al*., 1988; Castrillo *et al*., 1991; Cohen *et al*., 1996). In the pituitary gland of post-hatched chicks, mRNA expression of *PIT-1* was shown to correlate with *GH* and *PRL* expression (Tanaka *et al*., 1999). Additionally, PIT-1 reportedly transactivates the chicken *GH* and *PRL* promoters *in vitro* (Ohkubo *et al*., 2000; Ip *et al*., 2004), thus influencing growth and development. Consequently, mutations in *PIT-1* may result in altered expression levels of PRL, GH, TSHB, and PIT-1 itself (Li *et al*., 1990; Cohen *et al*., 1996).

Polymorphisms in *PRL*, *GH*, and *PIT-1* have been widely studied in domestic chickens with regard to reproduction, growth, and metabolism. For example, a 24 bp insertion and deletion in the promoter region of chicken *PRL* significantly affected egg production and occurrence of broodiness (Cui *et al*., 2006). Single nucleotide polymorphisms (SNPs) in this gene have also been associated with egg production in chickens (Kulibaba and Podstreshnyi, 2012; Li *et al*., 2012). Researchers have confirmed that chicken *GH* (c*GH*) has essential roles in growth, reproduction, body composition, aging, and egg production, and SNPs in c*GH* are associated with egg production and disease resistance (Kuhnlein *et al*., 1997), as well as growth and carcass traits (Yan *et al*., 2003; Wei *et al*., 2009). It was recently reported that the T185G substitution in c*GH* is significantly linked with growth and egg production (Su *et al*., 2014). Seventeen synonymous SNPs have been identified in chicken *PIT-1*, two of which were significantly associated with partial carcass traits (Xu *et al*., 2012). Restriction fragment length polymorphism (RFLP) analysis for *Taq*I and *Msp*I in intron 5 of chicken *PIT-1* in a cross of White Recessive Rock with Chinese Xinghua chickens (Nie *et al*., 2005, 2008) revealed an association with growth performance. Since the phenotypic and genotypic characteristics of any domestic bird, including the Naked Neck chicken, have not yet been characterized in Afghanistan, the present study aimed to determine whether economically valuable polymorphisms found in the *PRL*, *GH*, and *PIT-1* genes of other chicken breeds are retained in Naked Neck chickens to address the possibility of using these polymorphisms to improve the traits of this breed.

Materials and Methods

Animals and DNA Extraction

Blood samples were collected from 52 Afghani Naked Neck chickens, comprising 25 females and 27 males reared in Kandahar, Afghanistan, and placed on FTA^{TM} ELUTE Micro Cards (GE Healthcare, Buckinghamshire, UK). Genomic DNA was extracted from the FTA^{TM} ELUTE Micro Cards according to the manufacturer's protocol, and DNA concentrations were measured using a BioSpec-Nano (Shimadzu, Kyoto, Japan).

PCR Detection of a PRL Polymorphism

A set of primers was designed for the *PRL* 24 bp indel (insertion/deletion) as previously reported by Cui *et al*. (2006) (Table 1) and used to amplify either a 130 bp or a 154 bp fragment containing the 24 bp indel in the chicken *PRL* promoter. Each PCR reaction (20 μL) contained 10 μL of 2× Green Master Mix (Promega, WI, USA), 1 μL of each forward and reverse primer (10 pmol/μL), 40−70 ng of genomic DNA as a template. PCR amplification was carried out for 35 cycles with an initial denaturation at 94℃ for 5 min, denaturation at 94℃ for 30 s, annealing at 60℃ for 30 s, and extension at 72℃ for 30 s, with a final extension for 5 min at 72℃. Finally, the PCR products were electrophoresed on 3% agarose gels and stained with ethidium bromide to visualize the DNA under UV light.

Allele-specific PCR Determination of a GH Polymorphism

Allele-specific PCR was used to detect the T185G polymorphism in the 5′ non-coding region of the chicken *GH* gene using a primer set (cGH_Common, cGH_T185, and cGH_G185) listed in Table 1. The PCR was performed in 20 μL reactions consisting of genomic DNA (40−70 ng), 10 pmol cGH_Common, 10 pmol cGH_T185 or cGH_G185, and 2× Green Master Mix (Promega). Amplification was performed for 35 cycles with an initial denaturation at 94℃ for 5 min, then denaturation at 94℃ for 30 s, annealing at 60℃ for 30 s, and extension at 72℃ for 30 s, with a final extension of 5 min at 72℃. Finally, the PCR products were electrophoresed on 2% agarose gels that were stained with ethidium bromide to visualize the DNA.

PCR-RFLP Analysis of PIT-1

The polymorphic intron 5 of the chicken *PIT-1* gene was

Table 1. **Primers used in the analysis for PRL, GH and Pit-1**

Primer Name	Primer sequence (5′ to 3′)	Reference
PRL 24bp Indel F	TTTAATATTGGTGGGTGAAGAGACA	Cui *et al*., 2006
PRL 24bp Indel R	ATGCCACTGATCCTCGAAAACT	
cPIT-1F	GGACCCTCTCTAACAGCTCTC	Nie *et al*., 2008
cPIT-1R	GGGAAGAATACAGGGAAAGG	
cGH_T185	GGTGGATTTTCTACCTGCGT	
cGH_G185	GGTGGATTTTCTACCTGCGG	
cGH_Common	AATGCAGATGTGTTCGCCAT	
cPIT-1/SEQ1	TCTCTAACAGCTCTCTGTCT	
cPIT-1/SEQ2	ATACAGGGAAAGGCCGCAGA	

amplified by PCR in 20 μL reactions consisting of genomic DNA (40−70 ng); reverse and forward primers (10 pmol each of Pit-1R and cPit-1F; Table 1), dNTPs (5 nmol each), and TaKaRa Ex Taq DNA polymerase (1 unit; TaKaRa Bio, Shiga, Japan). Amplification was carried out for 30 cycles with an initial denaturation at 94℃ for 5 min, denaturation at 94℃ for 30 s, annealing at 60℃ for 45 s, and extension at 72℃ for 1 min, with a final extension of 5 min at 72℃. Thereafter, 3 μL of each PCR product was separately digested with *Taq*I (New England Biolabs, Ipswich, MA, USA) and *Msp*I (TaKaRa Bio) incubated at 65℃ or 37℃, respectively for 4 to 8 h, and then electrophoresed on a 2% agarose gel stained with ethidium bromide to visualize the DNA.

Sequencing of PIT-1 Intron 5

After PCR-RFLP, amplified DNA that showed diverse digestion patterns for *PIT-1/Msp*I was selected for direct sequencing. The PCR products separated on 2% agarose gels were purified using the MonoFas DNA purification kit 1 (GL Sciences Inc., Tokyo, Japan). Purified DNA (200 ng) served as sequencing template using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with each strand primer (cPIT-1/SEQ1 and cPIT-1/SEQ2; Table 1), according to the manufacturer's instructions. The products were sequenced using the ABI 3130 Sequencer (Applied Biosystems).

To confirm the accuracy of the PCR-RFLP analysis and the results of direct sequencing, amplified DNA was sub-cloned into the pGEM-T Easy vector (Promega) followed by RFLP analysis for *Msp*I. Cloned DNA was also sequenced in both directions, using the ABI 3130 Sequencer (Applied Biosystems).

Genotyping and Statistical Analysis

Based on the genotypes visualized on agarose gels, allelic frequencies were calculated via the maximum likelihood formula (Kulibaba and Podstreshnyi, 2012):

$$P_A = \frac{2n_{AA} + n_{AB}}{2N}; \quad P_B = \frac{2n_{BB} + n_{AB}}{2N}$$

where, P_A and P_B are the frequencies of the existing alleles, n_{AA} and n_{BB} are the numbers of homozygous birds, n_{AB} is the number of heterozygous birds, and 2N is the number of alleles (twice the number of individuals in the study group). Moreover, the Chi-square test ($\chi^2 = \Sigma(\frac{O}{E})^2$, where O is the observed value and E is the expected value) was used to confirm whether this population was in Hardy–Weinberg equilibrium.

Results

Frequency of the PRL 24 bp Indel Polymorphism in the Afghani Naked Neck Chicken

For the *PRL* 24 bp indel, two alleles, *In* and *Del*, were distinguishable with three genotypes, *In/In*, *In/Del*, and *Del/Del* (Fig. 1). The gene frequencies for *In* and *Del* were 0.64 and 0.36, respectively, and the genotypic frequencies for *In/In*, *In/Del*, and *Del/Del* were 0.40, 0.48, and 0.12, respectively (Table 2). The χ^2 test indicated that the genotypic distribution for the indel polymorphism was not in Hardy–Weinberg equilibrium.

Frequency of the GH T185G Polymorphism in the Afghani Naked Neck Chicken

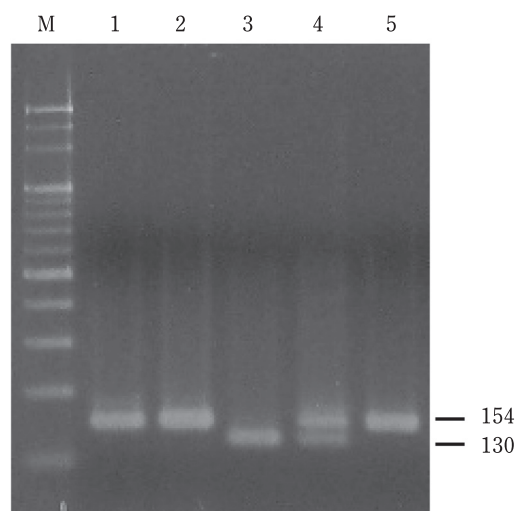For the *GH* T185G polymorphism, two alleles, T and G,

Fig. 1. Determination of the *PRL* 24 bp indel mutation by agarose electrophoresis. The 154 bp band represents individuals with the *In/In* genotype (lanes 1, 2, and 5), 154 and 130 bp bands represent individuals with the *In/Del* genotype (lane 4), and the 130 bp band represents individuals with the *Del/Del* genotype (lane 3). M shows the molecular weight marker (100 bp ladder).

Table 2. **The genotype and genetic frequency of PRL 24 bp indel, GH T185G and Pit-1/*Taq*1 in Afghani Naked Neck chicken population**

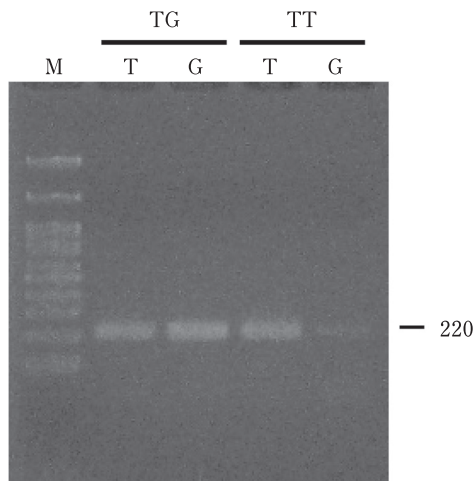| Population	Polymorphism	Genotypic frequency			Genetic frequency		Locus equilibrium χ^2 test
Naked Neck (n=52)	PRL 24 bp indel	**In/In** (n) 0.40 (21)	**In/Del** (n) 0.48 (25)	**Del/Del** (n) 0.12 (6)	**In** 0.64	**Del** 0.36	P<0.05
	GH T185G	**TT** (n) 0.83 (43)	**TG** (n) 0.17 (9)	**GG** (n) 0.0 (0)	**T** 0.91	**G** 0.09	
	PIT-1/*Taq* I	**AA** (n) 0.38 (20)	**AB** (n) 0.52 (27)	**BB** (n) 0.10 (5)	**A** 0.64	**B** 0.36	

Fig. 2. **Representative profile of the *GH* T185G mutation by agarose electrophoresis.** M shows the molecular weight marker (100 bp ladder).

Fig. 3. **The electrophoretic profile of *PIT-1* intron 5 digested with *Taq*I.** Lanes 1 and 4 are heterozygous (AB) genotypes, lanes 2 and 3 are homozygous (BB) genotypes, and lanes 5 and 6 are homozygous (AA) genotypes. M shows the molecular weight marker (100 bp ladder).

were recognized via allele-specific PCR, in which two genotypes, TT and TG, were detected (Fig. 2). The genotypic frequencies of T and G alleles were 0.91 and 0.09, respectively (Table 2). The GG genotype was absent in this population (Table 2). The χ^2 test indicated that the genotypic distribution of *GH* T185G was not in Hardy–Weinberg equilibrium.

PCR-RFLP Analysis of PIT-1

For the *PIT-1/Taq*I polymorphism, two alleles (A and B) with three genotypes (AA, AB, and BB) were clearly distinguished (Fig. 3). The fragment size generated for allele A was 599 bp without the *Taq*I restriction site, while for allele B, containing the *PIT-1/Taq*I restriction site, digestion yielded fragment sizes of 456 and 143 bp. The frequencies of the A and B alleles were 0.64 and 0.36, respectively, and the genotypic frequencies of AA, AB, and BB were 0.38, 0.52, and 0.10, respectively (Table 2). Based on the χ^2 test, this population was not in Hardy–Weinberg equilibrium. Notably, the allelic frequency for allele A (0.64) was significantly higher than for allele B (0.36) (Fig. 3; Table 2).

Figure 4a shows the PCR-RFLP analysis for *PIT-1/Msp*I digestion. Although *Msp*I did not digest some samples completely (i.e., lanes 3, 4, and 6), multiple digestion patterns were observed in the PCR-RFLP analysis for *Msp*I. Two *Msp*I sites were found in intron 5 of the *PIT-1* gene in the Iranian commercial broiler line (Rodbari *et al.*, 2011), generating 599 bp (A allele), 500 bp, and 99 bp (B allele); and 321 bp and 278 bp (C allele) DNA fragments. Naked Neck chickens carrying the B allele (lane 2; BB homozygote) and C allele (lane 1; CC homozygote) were identified, but AA homozygotes were absent in the population. However, unexpected PCR-RFLP patterns from a previous study were observed (lanes 3 to 9); one pattern was speculated to represent a homozygote of unknown genotype (lane 8) and heterozygotes of an unknown allele and the B allele (lanes 5
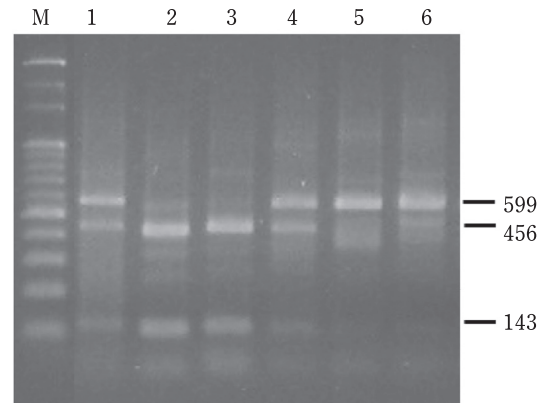
and 7) or and the C allele (lane 9). Therefore, direct sequencing was performed to validate the accuracy of our observed PCR-RFLP results.

Sequencing of the PIT-1 Polymorphic Region

The DD homozygote was confirmed by direct sequencing (lane 8 in Fig. 4a). The sequencing results revealed an A-to-G transition at position 280 of the amplified DNA fragment, creating a new *Msp*I recognition site (Fig. 4b). Therefore, cloning and sequencing were performed for the unexpected digestion patterns observed by PCR-RFLP (Fig. 4a; lanes 3, 4, and 6). As a result, the observed individuals possessed two new point mutations, A-to-G and T-to-C transitions, which generated two new *Msp*I restriction sites at nucleotide positions 500 and 519, respectively. We named this allele E (Fig. 5a, lane 1 and Fig. 5b, panel 2). Three point mutations were detected for one individual (Fig. 4a, lane 6), in which T-to-C, A-to-G, and T-to-C transitions generated three *Msp*I recognition sites at nucleotide positions 280, 500, and 519, respectively. This allele was called F (Fig. 5a, lane 4 and Fig. 5b, panel 3).

Moreover, while screening the *PIT-1* intron 5 sequence, we also found a double point mutation resulting in a CG-to-TA transition and a single point mutation resulting in an A-to-T transversion compared to the reported *Gallus gallus PIT-1* sequence (GenBank accession no. NC_006088.4). These mutations generated new *Mse*I restriction sites (Fig. 6).

Discussion

Even in environments that are poor and unfavorable for animal production, the Naked Neck chicken breed in Afghanistan still provides high-quality protein in the form of meat and eggs to the rural population, which comprises 75% of the total population of Afghanistan. The resilience of the Naked Neck chicken breed in high ambient temperatures promotes productivity (Islam and Nishibori, 2009). Genetic
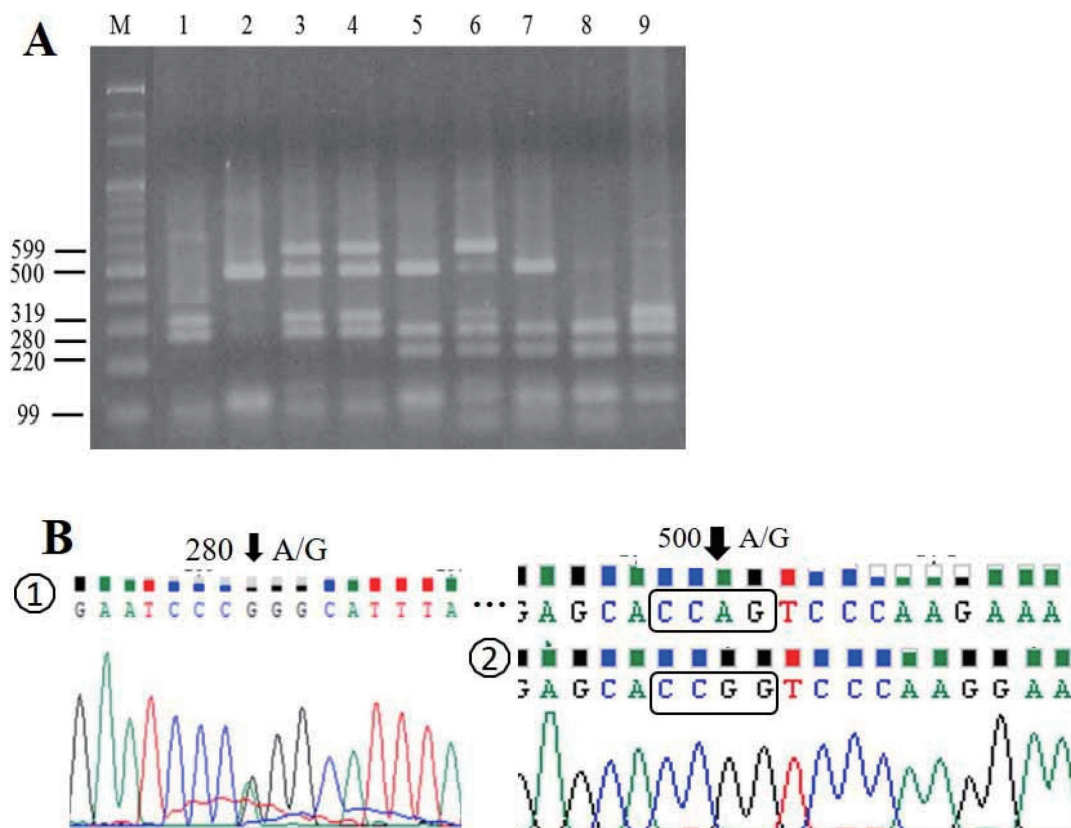
Fig. 4.   **RFLP analysis of *PIT-1* intron 5 using *Msp*I and confirmed
by sequencing.** (a) Photograph of the digest patterns of PCR-RFLP for
*Msp*I in *PIT-1* intron 5. Lanes 1 and 2 represent the expected digestion
pattern from a previous report (Rodbari *et al*., 2011). Lane 8 represents
a homozygous genotype (DD) newly identified in the Afghanistan Naked
Neck breed. Lanes 3, 4, and 6 are the unclear genotypes. M shows a
100 bp ladder marker. (b) Sequence of the polymorphic site of a novel
D allele in which panel (1) shows the *Msp*I recognition site at nucleotide
position 280 and panel (2) shows the *Msp*I recognition site at nucleotide
position 500. Therefore, the gel shows that novel patterns (320, 280,
and 99 bp) were generated.

selection, in addition to livestock management such as
feeding and veterinary care, plays an important role in further
improving the productivity of local breeds (Padhi, 2016). In
the present study, PCR, allele-specific PCR and PCR-RFLP
were used to scrutinize known mutations in somatotropic
axis-related genes (*PRL*, *GH*, and *PIT-1*) that are signifi-
cantly associated with growth and reproduction in diverse
chicken breeds (Cui *et al*., 2006; Nie *et al*., 2008; Su *et al*.,
2014), to determine the utility of adapting these polymor-
phisms to enhance the productive performance of the Naked
Neck chicken in Afghanistan. Among these genes, *PIT-1*
regulates not only its own transcription but also that of *PRL*,
GH and *TSHB*. In the pituitary gland, *PIT-1* mRNA has been
detected in somatotropic, thyrotrophic, and lactotrophic cells
(Simmons *et al*., 1990).

The *PRL* gene is known to be responsible for egg pro-
duction and broodiness in chickens (Shimada *et al*., 1991).

Polymorphisms in *PRL* were significantly correlated with
egg production in the Muscovy duck (Zhang *et al*., 2015) and
egg quality and number in chickens (Cui *et al*., 2006;
Bhattacharya *et al*., 2012). Moreover, the indel polymor-
phism of *PRL* is also associated with egg laying and broodi-
ness (Cui *et al*., 2006; Bagheri *et al*., 2013). The *In* allele
has significant effects on egg production and the frequency
of the *In* allele was higher (0.98) than that of the *Del* allele
(0.02) in the White Leghorn chicken that produces more than
300 eggs in a year. The *In* and *Del* frequencies in Taihe
chickens were 0.14 and 0.86, respectively, and this breed
shows strong incubation behavior and reduced egg produc-
tion (Cui *et al*., 2006). This group further concluded that the
Del allele might also be associated with broodiness, which
results in the loss of egg production (Cui *et al*., 2006). In
this study, we found that the allelic frequency of *In* was
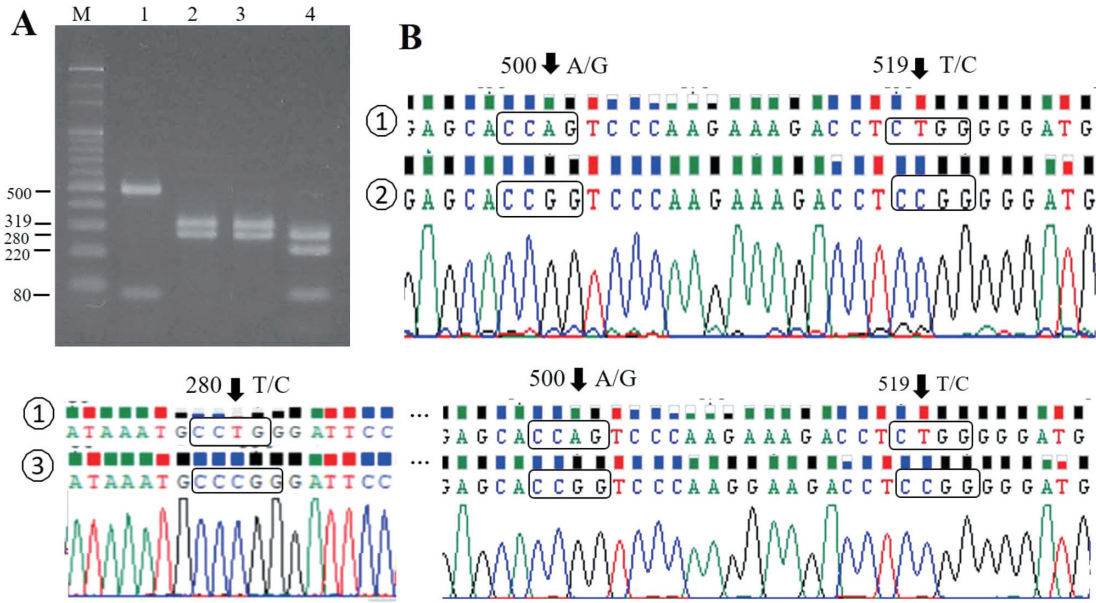higher than that of *Del* in Naked Neck chickens. This result

Fig. 5.   **RFLP analysis and sequencing result for *PIT-1* intron 5 after cloning.**   (a) Lanes 1, 2 (cloned sample of lane 3 from Fig. 1a), 3, and 4 (cloned sample of lane 6 from Fig. 1a) represent alleles E, B, B, and F, respectively, in *PIT-1* intron 5.   The 19 bp fragment could not be visualized in this gel.   M shows the molecular weight marker (100 bp ladder).   (b) Panels (2) (named allele E) and (3) (named allele F) are the Naked Neck cloned sequences (of lane 3 or 4 and lane 6, respectively, from Fig. 1a) and panel (1) is the original sequence released by Gen-Bank.
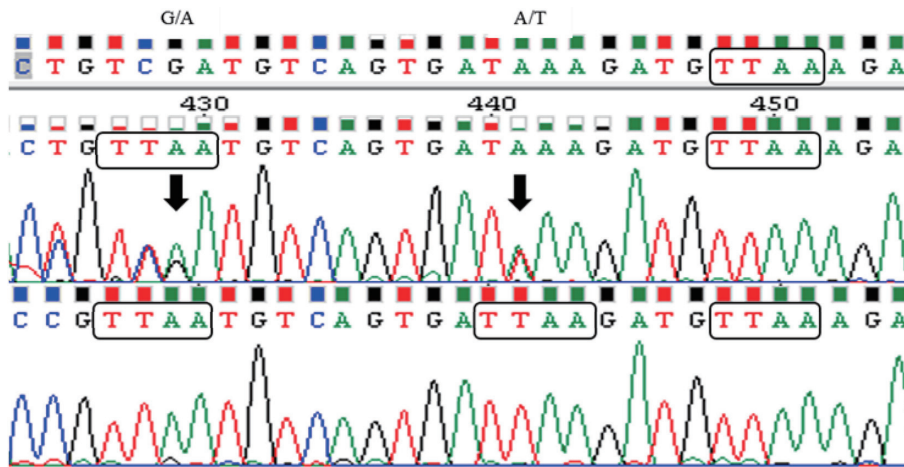

Fig. 6.   **Novel restriction sites for the *Mse*I endonuclease in intron 5 of the *PIT-1* gene in the Afghani Naked Neck chicken.**   Arrowhead indicates the SNPs in intron 5 of *PIT-1* of the Naked Neck chicken.   The upper sequence shows the original sequence reported by GenBank that contains only one *Mse*I restriction site (GenBank accession no. NC_006088.4).   The middle and lower sequences exhibit more than one *Mse*I recognition site.
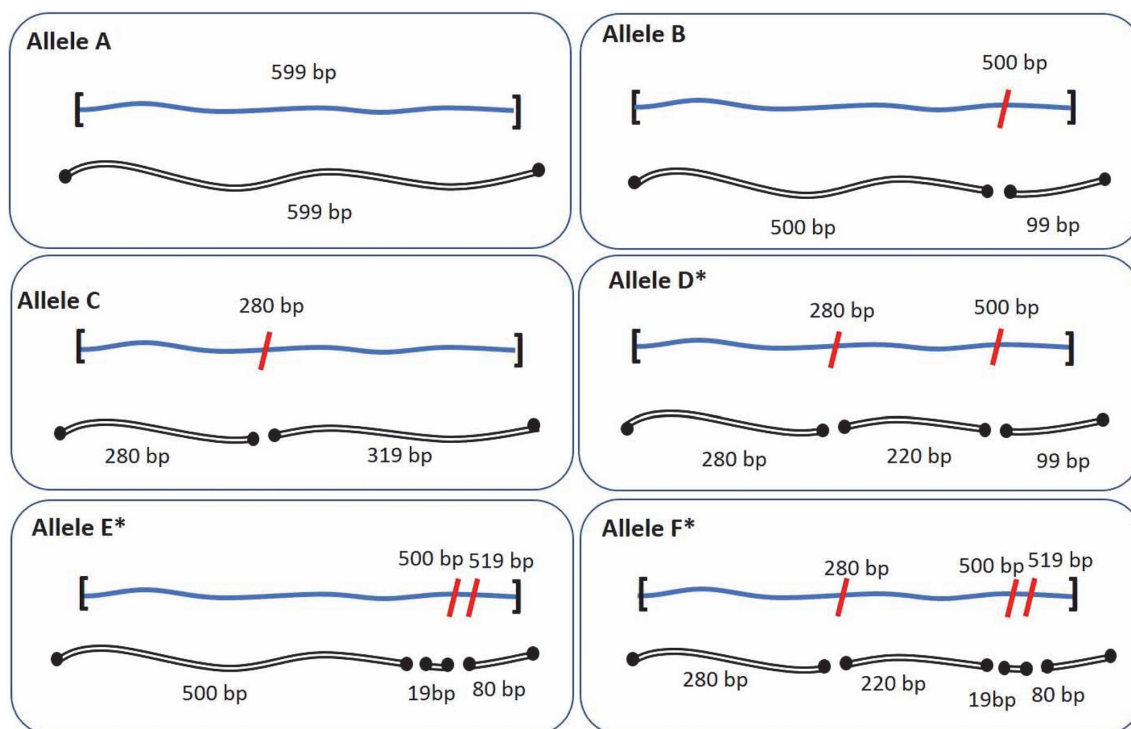
Fig. 7.   **Schematic representation of *Msp*I recognition sites in *PIT-1* intron 5 that are generated by SNPs in the Naked Neck chicken of Afghanistan.** Each box shows a different allele (A, B, C, D*, E*, and F*); the solid horizontal line in parentheses represents the 599 bp sequence of *PIT-1* intron 5 in which / is the recognition site for *Msp*I created by a point mutation. The double line between the solid circles represents the agarose gel fragment sizes visualized after digestion with *Msp*I in agarose gel. Asterisk (*) indicates a new allele.

may indicate that the *PRL* genetic background is a limiting factor for productivity in Naked Neck chickens in Afghanistan.

Regarding the T185G polymorphism in *GH*, the genotypic frequency was significantly biased; TT and GT genotypes were found, but the GG genotype was not detected in the studied population (Table 2). The T185G polymorphism is known to be associated with increased total egg number in 300-day-old birds (EN 300), as well as egg and body weight in Recessive White chickens (RW) and Qingyuan partridges (QY). Birds possessing the TT and GT genotypes in QY and RW strains showed good performance in EN 300 (Su *et al*., 2014). Our results suggest that the T185G polymorphism in the *GH* gene has been almost fixed to T and further consideration of this SNP may not be relevant.

Polymorphism in intron 5 of the *PIT-1* gene is also associated with productivity in highly selected exotic chicken breeds (Xu *et al*., 2012). In this study, PCR-RFLP and sequence analyses were carried out on the 599 bp fragment of intron 5 in the Naked Neck chicken. The results for the *PIT-1/Taq*I mutation were in accordance with both Nie *et al*. (2008) and Rodbari *et al*. (2011). However, the frequency of the B allele was significantly lower (36%) than that of the A

allele (64%) in the Naked Neck chicken, which is an economically important locus for early growth rates in chickens. In the studied population, five novel polymorphisms were identified that generated three novel restriction sites for *Msp*I and two for *Mse*I. Only one restriction site for *Msp*I was found in the originally reported sequence of the chicken *PIT-1* gene and one recognition site for *Mse*I (Nie *et al*., 2008). In the same region, Rodbari *et al*. (2011) found an extra allele, named C, in an Iranian line of broiler chicken by PCR-RFLP for *Msp*I. It was previously shown that the *PIT-1/Msp*I locus (BB genotype) had a positive correlation with average daily gain at 4−8 weeks of age in chickens ($P <$ 0.05). According to the different band patterns in the PCR-RFLP analysis, two alleles (A and B) with three genotypes (AA, AB, and BB) were identified for the *PIT-1/Msp*I locus in Chinese broiler chickens (Nie *et al*., 2008). Remarkably, in Naked Neck chickens, the A allele for *PIT-1/Msp*I was absent; this allele had previously been shown to have negative impacts on the early growth rate in Chinese native chickens and Iranian broiler chickens (Nie *et al*., 2008; Rodbari *et al*., 2011). In addition, the C allele found in Iranian broiler chickens was significantly associated with early growth rate (Rodbari *et al*., 2011). The absence of the

A allele and presence of the C and D alleles in the Naked Neck chicken population were valuable observations noted in this study (Fig. 1a, lane 2). Importantly, the DD genotype found in the Naked Neck chicken comprised two different growth-associated alleles (B and C) that might influence the early growth rate of this breed (Fig. 4d). Additional research is needed to confirm this result. None of these SNPs have been associated with fat deposition and carcass traits in chickens (Nie *et al*., 2008).

The sequencing results after cloning and PCR-RFLP analysis revealed three novel SNPs that generated *Msp*I recognition sites in intron 5 of Naked Neck chicken *PIT-1* (Figs. 1b and 2), named alleles D, E, and F (Fig. 4). The mutations in the E and F alleles resulted in different fragment sizes after digestion with *Msp*I (500 bp + 19 bp + 80 bp and 280 bp + 220 bp + 19 bp + 80 bp, respectively) (Fig. 2a). It was difficult to determine the allelic frequency for the *PIT-1/Msp*I locus of intron 5 in our studied population, as also demonstrated in other breeds (Nie *et al*., 2008; Rodbari *et al*., 2011). Two novel *Mse*I sites were found in intron 5 of Naked Neck chicken *PIT-1* that have yet to be reported in other chicken breeds and their association with productive traits have yet to be identified. Our results support the idea that nucleotide diversity in native chickens is greater than in commercial breeds (Nie *et al*., 2005).

In conclusion, the *PRL* 24 bp indel, *GH* T185G, and *PIT-1/Taq*I polymorphisms are fixed in Afghani Naked Neck chickens and may be viable selection markers to improve the breed. In addition, the 599 bp fragment from intron 5 of the *PIT-1* gene in the Naked Neck chicken was highly polymorphic, and five novel SNPs were found in the population, generating three novel restriction sites for *Msp*I and two for *Mse*I. Further investigation is required to characterize these SNPs that may improve the productivity of this breed and thus enhance the economic value of the Naked Neck chicken in Afghanistan.

Acknowledgments

References

Bagheri S, Niazi ASA, Zamiri MJ and Dadpasand TM. Polymorphisms of prolactin gene in a native chicken population and its association with egg production. Iranian Journal of Veterinary Research, 14: 113−119. 2013.

Bhattacharya TK, Chatterjee RN and Priyanka M. Polymorphisms of Pit-1 gene and its association with growth traits in chicken. Poultry Science, 91: 1057−1064. 2012.

Bodner M, Castriilo JL, Theill LE, Deerinck T, Ellisman M and Karin M. The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. Cell, 55: 505−518. 1988.

Castrillo JL, Theill LE and Karin M. Function of the homeodomain protein GHF1 in pituitary cell proliferation. Science, 253: 197−199. 1991.

Cohen LE, Wondisford FE and Radovick S. Role of Pit-1 in the gene expression of growth hormone, prolactin, and thyrotropin. Endocrinology and Metabolism Clinics of North America, 25: 523−540. 1996.

Cui JX, Du, HL, Liang Y, Deng X M, Li N and Zhang XQ. Association of polymorphisms in the promoter region of chicken prolactin with egg production. Poultry Science, 85: 26−31. 2006.

DAD-IS and FAO (Domestic Animal Diversity Information System: Food and Agriculture Organization of the United Nations) Breed Data Sheet. 2012. Retrieved from http://dad.fao.org/. Accessed on July 10, 2017.

Ip SCY, Lau JS, Au WL and Leung FC. Characterization of the 5'-flanking transcriptional regulatory region of chicken growth hormone gene. Experimental Biology and Medicine, 229: 640−649. 2004.

Islam MA and Nishibori M. Indigenous naked neck chicken: a valuable genetic resource for Bangladesh. World's Poultry Science Journal, 65: 125−138. 2009.

Kuhnlein, U, Ni L, Zadworny D and Fairfull W. DNA polymorphisms in the chicken growth hormone gene: response to selection for disease resistance and association with egg production. Animal Genetics, 28: 116−123. 1997.

Kulibaba, RA and Podstreshnyi AP. Prolactin and growth hormone gene polymorphisms in chicken lines of Ukrainian selection. Cytology and Genetics, 46: 390−395. 2012.

Lamont SJ, Lakshmanan N, Plotsky Y, Kaiser MG, Kuhn M, Arthur JA, Beck NJ and O'sullivan NP. Genetic markers linked to quantitative traits in poultry. Animal Genetics, 27: 1−8. 1996.

Li DF, Liu WB, Liu JF, Yi GQ, Lian L, Qu LJ, Li JY, Xu GY and Yang N. Whole-genome scan for signatures of recent selection reveals loci associated with important traits in White Leghorn chickens. Poultry Science, 91: 1804−1812. 2012.

Li S, Crenshaw EB, Rawson EJ, Simmons DM, Swanson LW and Rosenfeld MG. Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene Pit-1. Nature, 347: 528−533. 1990.

Merat P. Pleiotropic and associated effects of major genes. In: Poultry Breeding and Genetics (Crawford RD ed.). pp. 442−448. Elsevier. Amsterdam. 1990.

Nie Q, Fang M, Xie L, Zhou M, Liang Z, Luo Z, Wang G, Bi W, Liang C, Zhang W and Zhang X. The PIT1 gene polymorphisms were associated with chicken growth traits. BMC Genetics, 9: 20. 2008.

Nie Q, Lei M, Ouyang J, Zeng H, Yang G and Zhang X. Identification and characterization of single nucleotide polymorphisms in 12 chicken growth-correlated genes by denaturing high performance liquid chromatography. Genetics Selection Evolution, 37: 339. 2005.

Njenga SK. Productivity and socio-cultural aspects of local poultry phenotypes in coastal Kenya. MSc Thesis. The Royal Veterinary and Agricultural University. Denmark. pp. 1−78. 2005.

Ohkubo T, Tanaka M and Nakashima K. Molecular cloning of the chicken prolactin gene and activation by Pit-1 and cAMP-induced factor in GH3 cells. General and Comparative Endocrinology, 119: 208−216. 2000.

Padhi MK. Importance of indigenous breeds of chicken for rural economy and their improvements for higher production performance. Scientifica, 2016: 2604685. 2016.

Rodbari Z, Alipanah M, Seyedabadi HR and Amirinia C. Identification of a single nucleotide polymorphism of the pituitary-specific transcription factor 1 (Pit 1) gene and its association with body composition trait in Iranian commercial broiler line.

African Journal of Biotechnology, 10: 12979‒12983. 2011.

Sharma P, Bottje W and Okimoto R. Polymorphisms in uncoupling protein, melanocortin 3 receptor, melanocortin 4 receptor, and pro-opiomelanocortin genes and association with production traits in a commercial broiler line. Poultry Science, 87: 2073‒2086. 2008.

Shimada K, Ishida H, Sato K, Seo H and Matsui N. Expression of PRL gene in incubating hens. Journal of Reproduction and Fertility, 91: 147‒154. 1991.

Simmons DM, Voss JW, Ingraham HA, Holloway JM, Broide RS, Rosenfeld MG and Swanson LW. Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. Genes & Development, 4: 695‒711. 1990.

Su YJ, Shu JT, Zhang M, Zhang XY, Shan Y, Li GH, Yin JM, Song WT, Li HF and Zhao GP. Association of chicken growth hormone polymorphisms with egg production. Genetic and Molecular Research 13: 4893‒4903. 2014.

Tanaka M, Yamamoto I, Ohkubo T, Wakita M, Hoshino S and Nakashima K. cDNA cloning and developmental alterations in gene expression of the two Pit-1/GHF-1 transcription factors in the chicken pituitary. General Comparative Endocrinology, 114: 441‒448. 1999.

Wei Y, Wang JY, Liu DL, Yu YB and Zhang GX. Correlation analysis on single nucleotide polymorphism of the GH gene and carcass traits in Jinghai Yellow chicken. China Poultry, 31: 15‒18. 2009.

Xu HY, Wang Y, Liu YP, Wang JW and Zhu Q. Polymorphisms and expression of the chicken POU1F1 gene associated with carcass traits. Molecular Biology Reports, 39: 8363‒8371. 2012.

Yan B, Deng X, Fei J, Hu X, Wu C and Li N. Single nucleotide polymorphism analysis in chicken growth hormone gene and its associations with growth and carcass traits. Chinese Science Bulletin, 48: 1561‒1564. 2003.

Zhang DX, Xu ZQ, He J, Ji CL, Zhang Y and Zhang XQ. Polymorphisms in the 5'-flanking regions of the GH, PRL and Pit-1 genes with Muscovy duck egg production. Journal of Animal Science, 93: 28‒34. 2015.