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Full Length Research Paper

Single nucleotide polymorphisms in ghrelin gene and the resulting genetic variants at ghrelin locus in different strains of indigenous Tswana chickens

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Ghrelin is a growth hormone releasing peptide which also affects feed intake in chickens. Ghrelin is encoded by chicken ghrelin gene (cGHRL) found in chromosome 7. Single nucleotide polymorphisms (SNPs) have been reported in cGHRL in Chinese native chickens, but such studies have not been carried out in chickens native to Africa. The aim of this study was therefore to identify SNPs in cGHRL in three strains of indigenous Tswana chickens. DNA was isolated from the blood of 60 indigenous Tswana chickens and three pairs of primers were used for amplification of cGHRL. The resultant plymerase chain reaction (PCR) products were sent to Inqaba Biotec (Pretoria, South Africa) for DNA sequencing. A total of 25 SNPs were found in cGHRL gene in different strains of Tswana chickens. Fourteen of the SNPs have been previously reported and the rest are novel SNPs, which might be unique to Tswana chickens. Two non-synonymous SNPs (A429G and A2355G) were found in the coding region of cGHRL gene in the normal strain and only the A2355G SNP was found in dwarf and naked neck strains. The SNPs in the coding region resulted in three alleles in the normal strains and two alleles in the dwarf and naked neck strains.

Key words: Single nucleotide polymorphisms (SNPs), ghrelin gene, Tswana chickens.

INTRODUCTION

Ghrelin (GHRL) is an endogenous ligand for growth hormone secretagogue receptor and is a novel growth hormone (GH) releasing peptide with reported effects on feed intake in chickens (Fang et al., 2007). In mammals, ghrelin has been reported to stimulate feed intake, while in chickens it inhibits feeding of neonatal chicks. The positive effect of ghrelin on growth hormone secretion is however conserved between chicken and mammalian species (Kaiya et al., 2002). In chickens, ghrelin is predominantly expressed in the proventriculus but not in the gizzard (Kaiya et al., 2002).

Chicken ghrelin (cGHRL) gene has been mapped to chromosome 7 and comprises five exons and four introns (Nie et al., 2004). The first exon of cGHRN is part of the 5'UTR and therefore does not encode any amino acid (Richards et al., 2005). cGHRN gene is approximately 2706 bp and the mature peptide contains 26 amino acids (Genbank Accession no. AY303688). A total of 19 single nucleotide polymorphisms (SNPs) have been reported in cGHRN gene and most of these SNPs are scattered in the 4 introns (Nie et al., 2004; Richards et al., 2005). GenBank Accession no. AY299454 and Nie et al. (2004) reported a non synonymous A2355G SNP in exon 5 of cGHRN that results in an amino acid change (Gln113Arg). Nie et al. (2004) further reported the presence of C223G SNP in the 5'UTR of the cGHRN gene that determined the presence or absence of a specific transcription factor (serum response factor) binding site that might influence expression levels of cGHRN gene. SNPs occurring within candidate genes for

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Table 1. Primers used for selective amplification of chicken ghrelin gene.

Primer name	Forward primer (5' – 3')	Reverse primer (5' – 3')
GHRN 1	CATTTCTAAGCTTTTGCCAGTT	CACTGTTATTGTCATCTTCTC
GHRN 2	ATAAAGTGAATGCAAGAATAGT	TGTGTGGTGGGAGTTACTAC
GHRN 3	GTCAAGATAACAGAAAGAGAGT	GGAAATAAAATAAGCCTACACGT

specific traits may be responsible for different allelic forms of the candidate gene and ultimately for different phenotypes or performance in traits of economic importance.

Indigenous Tswana chickens have not been artificially selected for any trait of economic importance and thus exhibit greater variability in growth performance. Moreki (1997) and Badubi et al. (2006) reported the existence of the normal, dwarf, naked neck, frizzled and rumpless strains within the indigenous Tswana chicken population. A preliminary study by our group (results unpublished) revealed significant differences in growth performance between the normal, dwarf and the naked neck strains. Differences in growth performance between the three strains thus provide an excellent opportunity for identifying causative SNPs within genes of the somatotropic axis. The objectives of this study were therefore to determine SNPs in cGHRN gene and based on the SNPs in the open reading frame of cGHRN gene, to determine the alleles and estimate allele frequencies at cGHRN locus in the normal, dwarf and naked neck strains of indigenous Tswana chickens.

MATERIALS AND METHODS

Collection of blood samples

Blood samples were collected from the brachial vein of a total of 20 normal-feathered, 20 dwarf and 20 naked-neck strains of indigenous Tswana chickens obtained from different parts of the country using vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA). The blood samples were stored at -20°C until DNA extraction.

DNA extraction

Total genomic DNA was extracted using Quick-gDNA Mini Prep kit (Zymo Rwesearch Corporation, CA, USA) following the manufacturer's protocol. Briefly, 600 µL of Genomic Lysis Buffer was added to 150 µL of blood and the mixture was vortexed completely for 4 to 6 s. The mixture was then allowed to stand for 10 min at room temperature before being transferred to a Zymo-spin column placed in a collection tube. The Zymo-spin columns were centrifuged at 10000 × g for 1 min and the collection tubes with the flow through discarded. The Zymo-spin columns were then transferred to a new collection tube and 200 µL of DNA Pre-Wash Buffer was added to the spin columns and the spin columns were again centrifuged at 10000 × g for 1 min. Afterward, 500 µL of g-DNA Wash Buffer was then added to the spin column and the spin columns centrifuged for 1 min at 10000 × g. The spin columns were then transferred to a clean micro centrifuge tube and 100 µL of DNA elution buffer was added to the spin columns, incubated for 2 min at room temperature and then centrifuged at 21000 × g for 30 s to elude the DNA.

Primers and PCR amplification

Three pairs of primers were used for selective amplification of cGHRN gene using reference sequence Genbank Accession No. AY303688 and Primer3 web program (Primer 3_www.cgi v0.2). The primers were synthesized by Inqaba Biotec (Hartfield, Pretoria, South Africa) and their sequences are shown in Table 1.

Selective amplification of different regions of ghrelin gene was performed using thermocycler GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) and PCR reagents synthesized by Fermentas Life Sciences (Opelstrasse, Germany). The PCR mixture contained 50 ng genomic DNA, 0.6 µM of each primer, 0.2 mM dNTPs mixture, 3 mM Magnesium chloride and 1.0 unit of Taq DNA polymerase in a final reaction volume of 50 µL. PCRs were performed in a programmable thermocycler with the following protocol: 94°C for 5 min; followed by 30 cycles of 94°C for 30 s, 58°C for 45 s and 72°C for 1 min; with a final extension step of 72°C for 10 min. Amplification of the different segments of cGHRN gene was confirmed by running the PCR products on 1% agarose gel and visualizing under UV rays. Forty microliters of each PCR product was sent to Inqaba Biotec (Hartfield, Pretoria, South Africa) for purification and sequencing using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the thermocycler GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Sequences were generated with both the forward and reverse primers and read on a 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Sequence analysis

Chromatographs generated from sequencing were processed using Chromas lite version 2.01 (http://www.technelysium.com.au) to verify the sequences and the SNPs in the three amplified regions of cGHRN gene. Both forward and reverse primer sequences were then aligned using the ClustalW multiple sequence alignment program (http://www.ebi.ac.uk/clusatlw/) to determine the presence of genetic polymorphisms.

Statistical analysis

Different nucleotide combinations at the polymorphic sites for all the individuals sequenced were used to determine the different sequence variants or haplotypes and alleles at ghrelin locus. Allele and genotypic frequencies observed and expected homozygotes and heterozygotes at ghrelin locus as well as tests for Hardy–Weinberg equilibrium were performed using GENEPOP Web version 3.4 program (http://wbiomed.curtin.edu.au/genepop/) (Raymond and Rousset, 2001). Observed and expected number of homozygotes and heterozygotes were computed using the algorithm of Levene (1949). To obtain the exact p-value of Hardy–Weinberg equilibrium, the Markov Chain method (Guo and Thompson, 1992) with a dememorization number of 1000, 100

Table 2. SNPs and their locations in ghrelin gene sequence in three strains of indigenous Tswana chickens.

SNP Position	Nucleotide changes	Position	Codon changes	Strains found
173	T →C	Intron 1	None	Naked neck
242	T →A	Intron 1	None	Normal
328	C →A	Exon 2 (5'UTR)	None	Normal
429	A→ G	Exon2	ACA→GCA (Threonine→Alanine)	Normal
638	C → T	Intron 2	None	Naked neck
641	A → T	Intron 2	None	Naked neck
911	C→ T	Intron 2	None	Naked neck, Normal and dwarf
1167	T →A	Intron 3	None	Naked neck, Normal and Dwarf
1215	G→ A	Intron 3	None	Naked neck, Normal and Dwarf
1268	G→ A	Intron 3	None	Naked neck, Normal and Dwarf
1274	T →C	Intron 3	None	Naked neck and Normal
1345	C →T	Inron 3	None	Naked neck, Normal and Dwarf
1363	A →G	Intron 3	None	Naked neck, Normal and Dwarf
1403	C →T	Intron 3	None	Naked neck, Normal and Dwarf
1494	C →T	Intron 3	None	Naked neck and Dwarf
1784	A →T	Intron 4	None	Naked neck
1810	G →A	Intron 4	None	Naked neck
1864	G →A	Intron 4	None	Naked neck, Normal and Dwarf
1875	C →T	Intron 4	None	Normal
1931	C →T	Intron 4	None	Naked neck, Normal and Dwarf
2100	T →C	Intron 4	None	Naked neck, Normal and Dwarf
2101	C →T	Intron 4	None	Dwarf
2157	G →T	Intron 4	None	Naked neck, Normal and Dwarf
2355	A→ G	Exon 5	CAG→ CGG (Glutamine→Arginine)	Naked neck, Normal and Dwarf
2466	C →T	3'UTR	None	Naked neck and Normal

batches and 1000 iterations per batch was used. The p-value returned by this method is calculated as the sum of the probabilities of all tables and its standard error.

RESULTS AND DISCUSSION

SNPs at ghrelin locus

Different SNPs found in indigenous Tswana chickens and their locations according to GenBank No. AY303688 are shown in Table 2. A total of 25 SNPs were found in cGHRN gene in the three strains of indigenous Tswana chickens with 20, 18 ad 14 SNPs occurring in the naked neck, normal and dwarf strains, respectively. SNPs C911T, T1167A, G1215A, G1268A, C1345T, A1363G, C1403T, G1864A, C1931T, T2100C, G2157T and A2355G were found in all the three strains of indigenous Tswana chickens. SNPs T173C, C638T, A641T, A1784T and G1810A were found only in the naked neck strain and SNPs T242A, C328A, A429G and C1875T were found only in the normal strain and SNP C2101T was unique to the dwarf strain. The occurrence of many SNPs in cGHRN gene in indigenous Tswana chickens is consistent with Nie et al. (2004, 2005) who reported 19 and 25 SNPs, respectively in the white leghorn, white recessive rock and Chinese native chicken breeds. Out of the 25 SNPs reported in the current study, SNPs C328A, C911T, T1167A, G1215A, G1268A, C1345T, A1363G, C1403T, A1784T, C1931T, T2100C, G2157T, A2355G and C2466T have also been reported by Nie et al. (2004) and Nie et al. (2005) in cGHRN gene in Chinese native chickens. Eleven SNPs reported in the current study (T173C, T242C, A429G, C638T, A641T, T1274C, C1494T, G1810A, G1864A, C1875T and C2101T) are novel SNPs at cGHRN locus and might be unique to indigenous Tswana chickens.

Nineteen of the SNPs found in indigenous Tswana chickens led to transitional exchanges(A/G or C/T) and only six SNPs led to transversional exchanges (A/C or C/T). More transitional than transversional exchanges found in the current study is consistent with the study of Nie et al. (2005) who reported more transition SNPs (74.2%) than transversion SNPs (11.3%) in 12 chicken growth correlated genes. SNPs C328A and C2466T occurred in the 5'UTR and 3'UTR of cGHRN gene, respectively, while SNPs A429G and A2355G occurred in the coding region of cGHRN gene. The C328A SNP found in the 5'UTR and the C2466T SNP found in the

Table 3. Different sequence variants or haplotypes of the ghrelin gene in different strains of indigenous Tswana chickens.

Strain	Haplotype	Nucleotide combinations at SNP loci	Resulting allele
Naked Neck	H1	TTC**A**CACTGGTCACCAGACCTCG**A**C	A1
	H2	TTC**A**CATAAGTTGCTAGACCCCG**G**T	A2
	H3	TTC**A**CACTGGTCACCAGACTTCT**A**C	A1
	H4	TTC**A**CACTGACCATCAGACCTCG**A**C	A1
	H5	TTC**A**CACTGGTCACCAGACTTCT**A**C	A1
	H6	CTC**A**TTTAAGTTGCCTAACCCCG**G**C	A2
	H7	TTC**A**CACTGATCATTAGACCTCG**A**C	A1
	H8	TTC**A**CATAAGTTGCCAGACTCCT**G**C	A2
Normal	H1	TTC**A**CACTGGTCACCAGACCTCG**A**C	A1
	H9	TTC**A**CACTGGTCACCAGGCCTCG**A**C	A1
	H10	TTA**A**CATAAATTGCCAGATTCCG**A**C	A1
	H11	TAC**G**CATAGACCACCAGACCCCG**A**T	A3
	H12	TTC**A**CACTGGTCACCAGACCTCG**A**C	A1
	H13	TTC**A**CACTGGTCATCAGACTTCT**A**C	A1
	H14	TTC**A**CATAAGTTGTCAGACCCCG**G**T	A2
	H16	TTC**A**CATAGACCACCCAGACCCCG**A**T	A1
	H17	TTC**A**CACTGATCACCAGATTTCT**G**C	A2
Dwarf	H1	TTC**A**CACTGGTCACCAGACCTCG**A**C	A1
	H18	TTC**A**CATAAGTTGCCAGACCCCG**G**C	A2
	H19	TTC**A**CACTGATCATTAGACTTCT**A**C	A1
	H12	TTC**A**CACTGGTCACCAGACCTC**G**AC	A2
	H21	TTC**A**CACTGGTCATTAGACTTTT**A**C	A1

3'TR of cGHRN gene have also been reported by Richards and Poch (2003) and Nie et al. (2004). Although these SNPs are not in the protein coding region, they can affect the expression of cGHRN gene by modifying the transcription factor binding sites and thus the rate of transcription and the mRNA stability and consequently the protein turnover rate (Mignone et al., 2002; Chabanone et al., 2004). The A429G and A2355G SNPs were predicted to cause amino acid substitutions in the resulting ghrelin pre-protein from threonine to alanine and from glutamine to arginine, respectively. The A2355G SNP was reported by Nie et al. (2004, 2005) in Chinese native chickens, while the non-synonymous A429G SNP in the coding region of cGHRN gene is being reported for the first time in this paper.

In total, eighty-four percent (84%) of the reported SNPs in the current study occurred in the introns of cGHRN gene. Few SNPs in the coding regions than non-coding regions is meant to minimize reading frame mutations and codon changes and to maintain the integrity and functionality of the resulting proteins (enzymes and hormones) so as to minimize disturbances to important physiological processes influenced by the enzymes or hormones (Conne et al., 2000). SNPs in the non-coding region may however, still exert a significant influence on gene expression or protein turnover rate through their consequences on gene splicing, transcription factor binding and interactions with the translational machinery of the cell (Wang et al., 2006).

Haplotypes at ghrelin locus

The SNPs reported in Table 2 linked up in individual chickens to produce the different haplotypes at cGHRN locus are shown in Table 3. The haplotypes are characterized by the nucleotides at SNP loci, 173, 242, 328, 429, 638, 641, 911, 1167, 1215, 1268, 1274, 1345, 1363, 1403, 1494, 1784, 1810, 1864, 1875, 1931, 2100, 2101, 2157, 2355 and 2466, respectively, and the SNP locations are according to GenBank No. AY303688. A total of 21 unique haplotypes at cGHRN locus were found in the three strains of indigenous Tswana chickens. The H1 haplotype was the most frequent and was found in all the three strains of indigenous Tswana chickens. The H12 haplotype was common only to the normal and naked neck strains. A total of 8, 9 and 5 different haplotypes or sequence variants of cGHRN gene were found in the naked neck, normal and dwarf strains of indigenous Tswana chickens, respectively. Li et al. (2006) also reported a total of 8 different haplotypes in 12 Chinese indigenous chicken breeds.

Table 3. Different sequence variants or haplotypes of the ghrelin gene in different strains of indigenous Tswana chickens.

Strain	Haplotype	Nucleotide combinations at SNP loci	Resulting allele
Naked Neck	H1	TTC**A**CACTGGTCACCAGACCTCG**A**C	A1
	H2	TTC**A**CATAAGTTGCTAGACCCCG**G**T	A2
	H3	TTC**A**CACTGGTCACCAGACTTCT**A**C	A1
	H4	TTC**A**CACTGACCATCAGACCTCG**A**C	A1
	H5	TTC**A**CACTGGTCACCAGACTTCT**A**C	A1
	H6	CTC**A**TTTAAGTTGCCTAACCCCG**G**C	A2
	H7	TTC**A**CACTGATCATTAGACCTCG**A**C	A1
	H8	TTC**A**CATAAGTTGCCAGACTCCT**G**C	A2
Normal	H1	TTC**A**CACTGGTCACCAGACCTCG**A**C	A1
	H9	TTC**A**CACTGGTCACCAGGCCTCG**A**C	A1
	H10	TTA**A**CATAAATTGCCAGATTCCG**A**C	A1
	H11	TAC**G**CATAGACCACCAGACCCCG**A**T	A3
	H12	TTC**A**CACTGGTCACCAGACCTCG**A**C	A1
	H13	TTC**A**CACTGGTCATCAGACTTCT**A**C	A1
	H14	TTC**A**CATAAGTTGTCAGACCCCG**G**T	A2
	H16	TTC**A**CATAGACCACCCAGACCCCG**A**T	A1
	H17	TTC**A**CACTGATCACCAGATTTCT**G**C	A2
Dwarf	H1	TTC**A**CACTGGTCACCAGACCTCG**A**C	A1
	H18	TTC**A**CATAAGTTGCCAGACCCCG**G**C	A2
	H19	TTC**A**CACTGATCATTAGACTTCT**A**C	A1
	H12	TTC**A**CACTGGTCACCAGACCTC**G**AC	A2
	H21	TTC**A**CACTGGTCATTAGACTTTT**A**C	A1

The high diversity of haplotypes observed in the current study attest to the fact that indigenous Tswana chickens have not been artificially selected in traits of economic importance and hence exhibit greater variability in performance (phenotype) and the underlying genotypes. Although there are many sequence variants or haplotypes at cGHRN locus, only the nucleotides at the SNP loci 429 and 2355 (bolded SNPs in Table 3) define the alleles at cGHRN locus (shown in Table 4) because they form part of the coding region of cGHRN gene. The 8 and 5 different haplotypes found in the naked neck and dwarf strains, respectively result in two different alleles, A1 (defined by A_{429} A_{2355} nucleotides) and A2 (defined by A_{429} G_{2355} nucleotides) at cGHRN locus due to the degeneracy or the redundancy of the genetic code. The 9 haplotypes or sequence variants found in the normal strain result in alleles A1, A2 and an additional A3 allele (defined by G429 A2355 nucleotides) at cGHRN locus. Most studies on cGHRN gene have, however, not reported the different sequence variants or haplotypes at cGHRN locus.

Gene and genotypic frequencies at ghrelin locus

The different alleles at cGHRN locus and their characteristic open reading frame SNPs are shown in Table 4. Sequence alignment shows indigenous Tswana chickens to have similar open reading frame nucleotide sequences at cGHRN locus with reference sequence AY303688 except at polymorphic sites 429 and 2355 where they have the sequences shown in Table 4. Two alleles (A1 and A2) and 3 alleles (A1, A2 and A3) were found in the naked neck/dwarf and normal strains of indigenous Tswana chickens, respectively. The two alleles (A1 ad A2) found in all the three strains have been implied by Nie et al. (2004) and Nie et al. (2005) and were due to lack of SNP at nucleotide location 429 and the A2355G polymorphism. Moreover, the A3 allele found in the normal strain is due to the novel A429G polymorphism found only in the normal strain of indigenous Tswana chicken.

The estimated frequencies of the identified alleles at cGHRN locus in the three strains of indigenous Tswana chickens are shown in Table 4. The A1 allele is by far the most frequent allele in the normal, naked neck and dwarf strains of indigenous Tswana chickens (0.85, 0.80 and 0.90, respectively) followed by the A2 allele (0.10, 0.20 and 0.10 in the normal, naked neck and dwarf strains, respectively). The A3 allele was not identified in the naked neck and dwarf strains of indigenous Tswana

Table 4. Alleles, their characteristic SNPs and allele frequencies at ghrelin locus in different strains of indigenous Tswana chickens.

Allele	Characteristic SNPs	Normal	Naked neck	Dwarf
A1	A_{429} A_{2355}	0.85	0.80	0.90
A2	$A_{429}G_{2355}$	0.10	0.20	0.10
A3	G_{429} A_{2355}	0.05	0	0
Total		1.00	1.00	1.00

Table 5. Observed and expected genotypic frequencies at ghrelin locus in different strains of indigenous Tswana chickens.

Genotype	Normal		Naked neck		Dwarf	
	Observed number	Expected number	Observed number	Expected number	Observed number	Expected number
A1A1	14	14.32	12	12.63	16	16.11
A1A2	4	3.58	8	6.74	4	3.79
A2A2	0	0.11	0	0.63	4	0.11
A1A3	2	1.79	0	0	0	0
A2A3	0	0.21	0	0	0	0
A3A3	0	0	0	0	0	0

Table 6. Observed and expected number of homozygotes and heterozygotes and test for Hardy-Weinberg Equilibrium (p-value) at ghrelin locus in different strains of indigenous Tswana chickens.

Strain	Sample size	Observed homozygotes	Expected homozygotes	Observed heterozygotes	Expected heterozygotes	p-values
Normal	40	0.70	0.72	0.30	0.28	0.98
Naked neck	40	0.60	0.66	0.40	0.34	0.45
Dwarf	40	0.80	0.81	0.20	0.19	0.81

chickens and is therefore, assumed to be non-existent in the two strains. Furthermore, the A3 allele occurs at a very low frequency in the normal strain. Although the actual allele frequencies in the whole population of normal, naked neck and dwarf strains of indigenous Tswana chickens might differ from the frequencies reported in the current study due to our small sample size, the order of the frequencies of the different alleles is most likely to remain unchanged. The high frequency of the A1 allele in all the three strains of indigenous Tswana chickens suggests that the A1 allele may be the wild type at cGHRN locus. While the relatively low frequency of the A3 allele in the normal strain and its total absence in the naked neck and dwarf strains also suggests that this might be a relatively new allele resulting from recent mutations in cGHRN gene.

The alleles at cGHRN locus are expected to pair up within individuals resulting in the different genotypes as shown in Table 5. Table 5 also shows the observed genotypic frequencies and the expected genotypic frequencies at cGHRN locus in different strains of indigenous Tswana chickens assuming Hardy-Weinberg equilibrium. With only two alleles (A1 and A2) at cGHRN locus in the naked neck and dwarf strains, only three genotypes (A1A1, A1A2 and A2A2) were expected in the two strains, while all the six genotypes as shown in Table 5 were expected in the normal strain. The absence of some genotypes in the normal strain could be due to the small number of individuals sequenced and genotyped in the current study. Despite the sample sizes, the observed and expected genotypic frequencies under Hardy-Weinberg equilibrium for all the three strains were in complete agreement (p-values in Table 6), suggesting random mating and no intentional selection at cGHRN locus. Selection and mating decisions can alter both allele and genotypic frequencies in the population and inbreeding in particular can increase homozygosity with a concomitant decrease in heterozygosity. The observed and expected levels of both homozygosity and heterozygosity in the three strains of indigenous Tswana chickens are shown in Table 6.

In general, the observed and expected levels of both homozygosity and heterozygosity in the three strains of indigenous Tswana chickens were in complete agreement with Hardy-Weinberg equilibrium, suggesting a lack of intensive selection effect for growth traits at this

locus in the naked neck, normal and dwarf strains of indigenous Tswana chickens.

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