Genet. Sel. Evol. 37 (2005) 339–360

DOI: 10.1051/gse:2005005

Original article

Identification and characterization of single nucleotide polymorphisms in 12 chicken growth-correlated genes by denaturing high performance liquid chromatography

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(Received 6 May 2004; accepted 17 December 2004)

Abstract – The genes that are part of the somatotropic axis play a crucial role in the regulation of growth and development of chickens. The identification of genetic polymorphisms in these genes will enable the scientist to evaluate the biological relevance of such polymorphisms and to gain a better understanding of quantitative traits like growth. In the present study, 75 pairs of primers were designed and four chicken breeds, significantly differing in growth and reproduction characteristics, were used to identify single nucleotide polymorphisms (SNP) using the denaturing high performance liquid chromatography (DHPLC) technology. A total of 283 SNP were discovered in 31 897 base pairs (bp) from 12 genes of the growth hormone (*GH*), growth hormone receptor (*GHR*), *ghrelin*, growth hormone secretagogue receptor (*GHSR*), insulin-like growth factor I and II (*IGF-I* and *-II*), insulin-like growth factor binding protein 2 (*IGFBP*-2), *insulin*, leptin receptor (*LEPR*), pituitary-specific transcription factor-1 (*PIT*-1), somatostatin (*SS*), thyroid-stimulating hormone beta subunit (*TSH-β*). The observed average distances in bp between the SNP in the 5'UTR, coding regions (non- and synonymous), introns and 3'UTR were 172, 151 (473 and 222), 89 and 141 respectively. Fifteen non-synonymous SNP altered the translated precursors or mature proteins of *GH*, *GHR*, *ghrelin*, *IGFBP-2*, *PIT-1* and *SS*. Fifteen indels of no less than 2 bps and 2 poly (A) polymorphisms were also observed in 9 genes. Fifty-nine PCR-RFLP markers were found in 11 genes. The SNP discovered in this study provided suitable markers for association studies of candidate genes for growth related traits in chickens.

chickens / genes / SNP / DHPLC

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1. INTRODUCTION

Several quantitative traits for production such as growth, egg laying, feed conversion, carcass weight and body weight at different day-ages are important in domestic animals. These traits are controlled by genetic factors, also called quantitative trait loci (QTL). Progress has been made in mapping QTL for production traits by using microsatellite markers [29–31, 36, 38, 39], but fine mapping of QTL requires a much higher density of informative genetic markers. Due to the apparent lower complexity of the chicken, as compared to mammalian genomes, there seems to be lower numbers of microsatellite DNA markers present in the genome.

SNP are a new type of DNA polymorphism, mostly bi-allelic, but widely distributed along the chicken genome [40]. In humans, several high resolution SNP maps have been created for several chromosomes or even the whole genome, providing useful resources for studies on haplotypes associated with human diseases [2, 23, 28]. Furthermore, an SNP map of porcine chromosome 2 has been reported [18], however such studies have not been performed in the chicken yet. Nevertheless the results of the Chicken Genome Project, which ended in February of 2004, (http://genome.wustl.edu/projects/chicken/) enable the utilization of the draft sequence to identify SNP.

The candidate gene approach is an interesting way to study QTL affecting traits in chickens. As in mammals, the growth and development of chickens are primarily regulated by the somatotropic axis. The somatotropic axis, also named neurocrine axis or hypothalamus-pituitary growth axis, consists of essential compounds such as growth hormone (*GH*), growth hormone releasing hormone (*GHRH*), insulin-like growth factors (*IGF-I* and *-II*), somatostatin (*SS*), their associated carrier proteins and receptors, and other hormones like insulin, leptin and glucocorticoids or thyroid hormones [7, 26]. SNP markers in genes for this network could function as candidate genes for the evaluation of their effects on chicken growth traits [5].

Previous studies have shown that some SNP of the somatotropic axis genes indeed affected (economic) traits or diseases either in domestic animals or in humans [7, 26]. In chickens, certain SNP of *GH* [11], *GHR* [11, 12], *IGF-I* and *-II* genes [3, 41] have been reported to be associated with chicken growth, feeding and egg laying traits. The SNP in the porcine pituitary-specific transcription factor-1 (*PIT-1*) gene are also significantly related to carcass traits [33]. In humans, point mutations in *ghrelin*, *PIT-1* and thyroid-stimulating hormone beta subunit (*TSH-β*) genes have significant relationships with obesity [37], congenital hypothyroidism or pituitary dwarfism [4, 27], and TSH-deficiency hypothyroidism [9], respectively. Until now, only limited SNP

have been identified in these and other important genes of the chicken somatotropic axis. In part because the sequence of these genes was unknown, and since few efficient methods are available to identify SNP in chromosomal regions spanning 100 kb or even 1 Mb.

The present study was conducted to identify SNP in the complete sequences of 12 chicken genes of the somatotropic axis in four chicken populations that were significantly different in growth and reproduction characteristics. The 12 selected genes are *GH*, *GHR*, *ghrelin*, growth hormone secretagogue receptor (*GHSR*), *IGF-I* and *-II*, insulin-like growth factor binding protein 2 (*IGFBP-2*), *insulin*, leptin receptor (*LEPR*), *PIT-1*, *SS*, *TSH-β*. The sequences were obtained from Genbank [25] and were used to design gene specific primers for the identification of SNP. Denaturing high-performance liquid chromatography (DHPLC) was used to identify SNP because it is an efficient way for screening sequence variation. The SNP identified with DHPLC were also confirmed by direct sequencing. In addition, the possible effects of these SNP on growth and laying traits were analysed. Potential PCR-RFLP markers were also deduced when looking for restriction sites within sequences explored for SNP.

2. MATERIALS AND METHODS

2.1. Chicken populations

Four chicken breeds with different growth-rates, morphological characteristics, and laying were used in this study: Leghorn (L), White Recessive Rock (WRR), Taihe Silkies (TS) and Xinghua (X). Genomic DNA of 10 animals per breed were isolated from the blood. The Leghorn is a layer breed and has been bred as a laying-type for dozens of years, whereas WRR is a fast-growing broiler line that has also been bred as a meat-type for many generations. Both TS and X chickens are Chinese native breeds with the characteristics of being slow-growing, and having lower reproduction and favorable meat quality. They have not been subjected to dedicated or intensive breeding programs.

2.2. Primer design and PCR amplification

The sequences of the 12 chicken candidate genes of the somatotropic axis are obtained from Genbank (http://www.ncbi.nlm.nih.org). The accession numbers are given in Table I. Primers were designed using the GENETOOL program (http://www.biologysoft.com/).

Table I. Details of 75 pairs of primers used for SNP identification in the 12 selected candidate genes.

Primer	Gene	Nucleotide constitutes Forward primer (5'-3') / Reverse primer (5'-3')	Sequence ID[1]	Length (bp)	Temp[2] (°C)	Temp[3] (°C)
101	*GH*	gccctggcagccctgttaacc / caccccaccatcgtatcccatc	AY461843	518	62	58.4
102	*GH*	atgggatacgatggtggggtgt / ccttcctgagcagagcacggtac	AY461843	689	65	60.4
103	*GH*	cgcgccaaagagtgtaccgtg / gcacggtcctggaggcatcaag	AY461843	412	62	62.5
104	*GH*	gggctcagcacctccacctcct / cgcagcctgggagtttttgttgg	AY461843	546	65	60.5
105	*GH*	tcccaggctgcgttttgttactc / acgggggtgagccaggactg	AY461843	429	62	59.8
106	*GH*	gctgcttcggttttcactggttc / gcccaaccccaacccactcc	AY461843	396	68	60.0
107	*GH*	gcgggagtgggttggggttg / ggggcctctgagatcatggaacc	AY461843	538	65	57.7
108	*GH*	cccaacagtgccacgattccatg / tgcgcaggtggatgtcgaacttg	AY461843	483	62	61.1
109	*GH*	ccgcagccctctcgtcccacag / cgccccgaacccgccctatat	AY461843	366	55	63.2
201	*GHR*	cccttccattatgcattttatc / gggggtacactctagtcacttg	AJ506750	576	58	56.1
202	*GHR*	gcaacatcagaatcgctttt / tcccatcgtacttgaatatcc	AJ506750	544	58	54.5
203	*GHR*	tcacctgagctggagacattt / ctgcctctgaattcctccact	AY500876	529	60	55.8
204	*GHR*	gaacccaggctctcaacagtg / tggaggttgaggtttatctgtc	AY468380	457	60	56.7
205	*GHR*	tgccaacacagatacccaacagc / cgcggctcatcctcttcctgt	M74057	336	64	59.0
206	*GHR*	ctccagggcagaaatccaaggtg / gcacccaacccaagctgactctg	M74057	453	60	58.9
207	*GHR*	tgctgaaacccaaaatgagg / tttcatgctcagttcccaattac	***a***	332	64	55.1
208	*GHR*	attgggaactgagcatgaaag / aaccagaatttgatgaagaacag	***a***	447	60	51.6
209	*GHR*	tgcagcaaaaattaaaaacag / ccgtattcaattcctgtgttt	***a***	522	54	53.0
210	*GHR*	tgaaacacaggaattgaatacg / cgttctgaatcgtaaaaatcc	***a***	423	53	56.5
211	*GHR*	catgaatgctctctttgtgac / gggacagatcaaagacaatac	***a***	416	56	55.1
701	*ghrelin*	catttctaagcttttgccagtt / gcattattctgacttttacctg	AY303688	431	55	55.2

Table I. Continued.

Primer	Gene	Nucleotide constitutes Forward primer (5'-3') / Reverse primer (5'-3')	Sequence ID[1]	Length (bp)	Temp[2] (°C)	Temp[3] (°C)
702	*ghrelin*	tctggctggctctagtttttt / gcagatgcagcaaattagttag	AY303688	486	56	55.3
703	*ghrelin*	ataaagtgaatgcagaatagt / cactgttattgtcatcttctc	AY303688	323	55	56.1
704	*ghrelin*	atttttcactcctgctcacat / cttctccagtgcttgtccatac	AY303688	532	62	55.0
705	*ghrelin*	gtcaagataacagaaagagagt / tgtgtggtgggagttactac	AY303688	354	58	57.3
706	*ghrelin*	gagcaacggaagtatctgatgt / caggcactcaaatgaagaaag	AY303688	458	60	56.8
707	*ghrelin*	agctttatctttcttcatttgag / ggaaataaaataagcctacacgt	AY303688	340	58	56.5
1402	*GHSR*	gtcgcctgcgtcctcctctt / acgggcaggaaaaagaagatg	AB095994	533	61	62.8
1403	*GHSR*	ctccagcatcttctttttcct / tgtgggtttagaggttagt	AB095994	523	59	57.1
1404	*GHSR*	cccacaaagttagctgcagac / cacctctccatctggctcatt	AB095994	537	60	58.0
1405	*GHSR*	ggcagaggtgaagggctaatg / gcactgggctgttttcatatg	AB095994	500	69	57.9
1406	*GHSR*	gcagatgaaaacagcccagtg / catcttcctgagcccaacact	AB095994	525	59	58
1407	*GHSR*	aggtggaaaaactgcaaaaag / aggcaccccataacttttcag	AB095994	534	59	57.2
1408	*GHSR*	tggttgaaaagagagaatgct / ccacacgtctccttttatattc	AB095994	598	59	59.4
301	*IGF-ćñ*	ctgggctacttgagttactacat / cacggaaaataagggaatg	M74176	480	59	57.7
302	*IGF-ćñ*	gccacccgaaagttaaccagaat / ttccattgcggctctatct	M74176	361	60	61.3
303	*IGF-ćñ*	ggagagagagagaaggcaaatg / agcagacaacacacagtaaaat	M74176	401	58	63.3
305	*IGF-ćñ*	agaatacaagtagagggaacac / gcaaataaaaaaacaccactt	AY331392	457	59	55.7
306	*IGF-ćñ*	ggagtaattcatcagccttgt / ggccagacccttttcatataac	AY331392	515	58	54.1
307	*IGF-ćñ*	caagggaatagtggatgagtgct / gcttttggcatatcagtgtgg	M32791	97	58	54.1
308	*IGF-ćñ*	tgaaagggtctggccaaaaca / gggaagagtgaaaatggcagagg	AY253744	387	62	53.3
309	*IGF-ćñ*	agctgttcgaatgatggtgtttt / gccccagcattctctttcctt	AY253744	583	63	54.5

Table I. Continued.

Primer	Gene	Nucleotide constitutes Forward primer (5'-3') / Reverse primer (5'-3')	Sequence ID[1]	Length (bp)	Temp[2] (°C)	Temp[3] (°C)
310	*IGF-ćñ*	agtgctgcttttgtgatttcttg / gctgcagtgagaacatcccttaa	***b***	503	61	54.6
311	*IGF-ćñ*	atgtgaatgtgaaccaagaatact / tccacatacgaactgaagagc	***c***	300	62	59.6
902	*IGF-ćò*	ggtagaccagtgggacgaaat / cctttgggcaacatgacatag	AH005039	470	60	58.2
903	*IGF-ćò*	gggcgagcagcaatgagtagagg /c cggagcggcgtgatggtg	AH005039	448	68	61.8
904	*IGF-ćò*	atcccactcctatgtcatgttgc / gggaagggagaacaacacagtg	AH005039	469	61	59.7
811	*IGFBP-2*	tcggtgaatgggcagcgtggag / acggggcgaggagcaaaaaagac	U15086	421	68	62.1
812	*IGFBP-2*	tttggttgagtcctaggcttg / aggcgtactacactgcagagg	***i***	527	62	61.8
813	*IGFBP-2*	aggcgtactacactgcagagg / gggaaaaagggtgtgcaaaag	AY326194	540	60	61.3
815	*IGFBP-2*	gggcatttatatctgaggaacac / ggcaaagagcaacccaacac	AY326194	379	61	59.1
816	*IGFBP-2*	tggcgaggcgttattttc / gctgctttgcctgttccttagag	AY326194	468	58	61.9
817	*IGFBP-2*	gggcaacctttccagtgtgtc / gggccacagcaagcaggac	AY331391	504	65	63.1
818	*IGFBP-2*	agcccatgagcaggaggacc / ggggacaggcaggacacaaga	U15086	490	60	62.1
819	*IGFBP-2*	ccccgagaccaaagactgtaaat / aagcgaaaatggagggacaagag	U15086	482	59	61.5
820	*IGFBP-2*	gctgctcttgtccctccattt / cggcggcagggaagttattt	U15086	300	59	59.5
1301	*insulin*	cgtgtctcctttgcttcctac / tggagctttctgtgacaattc	AY438372	462	60	58.1
1302	*insulin*	ggcaagcagggaaaggagatt / tgggccaaatgcagaacagtt	AY438372	546	60	56.4
1303	*insulin*	tgttctgcatttggcccatac / gcagaatgtcagctttttgtcc	AY438372	530	59	58.7
1304	*insulin*	ctccatgtggcttccctgta / aatgctttgaaggtgcgatag	AY438372	419	58	60.4
1208	*LEPR*	atgctgcttgattcttcctcct / ccctaggcaaatggtaatgaac	AF222783	501	58	58.9
1209	*LEPR*	cctgctcctctgccctat / aatcatttggactcttacctact	AF222783	468	58	56.5

Table I. Continued.

Primer	Gene	Nucleotide constitutes Forward primer (5'-3') / Reverse primer (5'-3')	Sequence ID[1]	Length (bp)	Temp[2] (°C)	Temp[3] (°C)
501	*PIT-1*	tgaggatggctgaggggcttaat / tgaaggcacagcacagggaaact	AF029892	444	62	56.6
502	*PIT-1*	gcctgacccctgcctttat / ccagcttaattctccgcagttt	AF029892	243	60	60.9
503	*PIT-1*	ctggagaggcactttggagaac / ttaggccttcaacagtccaaat	AF029892	407	60	56.2
504	*PIT-1*	tttgctgcctttctctggac / cccacttgttctgcttcttcc	***d***	384	60	58.6
505	*PIT-1*	tgctgctgatgaggggaaagt / atggtggttctgcgcttcctctt	***e***	391	62	55.4
506	*PIT-1*	ttttgtacccttgaattctgac / gaaagctcccacaggtaatat	***f***	540	55	55.4
507	*PIT-1*	aggggactgtacatatttctgc / ccccataggtagaggcttgat	***g***	435	60	57.8
1002	*SS*	ggggccgagcaggatgaagt / cacgcaagaaccggtcagaaatc	X60191	357	65	57.6
1003	*SS*	ccctgctctccatcgccttg / ggatgtgctggaagggtggtc	***j***	466	60	63.1
601	*TSH-β*	cccttcttcatgatgtctctcc / ggtccttagttccatctgtgc	AY341265	521	60	57.3
602	*TSH-β*	gagcacggtgagcattactgg / ggaggtacatttctgccacgt	***h***	485	60	59.0
603	*TSH-β*	tgcacagatggaactaaggac / aactgtagtgccaagggatct	AY341265	528	62	58.0
604	*TSH-β*	cagcagcttgtctccatctag / ccgtgctctgtggttttaaat	AY341265	544	59	58.6

[1] Sequence accession numbers used for primer designing. ***a***: A sequence published by Burnside *et al*. [6]; ***b***: Forward (M32791), Reverse (unpublished intron sequence); ***c***: Forward (unpublished intron sequence), Reverse (M32791); ***d***: Forward (AY299400), Reverse (AF089892); ***e***: Forward (AY324228), Reverse (AF089892); ***f***: Forward (AF089892), Reverse (AY324229); ***g***: Forward (AY324229), Reverse (AF089892); ***h***: Forward (AY341265), Reverse (AF033495); ***i***: Forward (AY326194), Reverse (AY331391); ***j***: Forward (X60191), Reverse (AY555066). [2] Annealing temperature for PCR amplification. [3] Column temperature for DHPLC detection.

The twenty-five μL PCR reaction mixture contained 50 ng of chicken genomic DNA, 1 × PCR buffer, 12.5 pmol of each primer, 100 μM dNTP (each), 1.5 mM $MgCl_2$ and 1.0 Units Taq DNA polymerase (all reagents were from the Sangon Biological Engineering Technology Company; Shanghai, China). The PCR conditions were 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 45 s at certain annealing temperatures (ranged from 55 °C to 68 °C for each

primer), 1 min at 72 °C, and a final extension of 5 min at 72 °C in a Mastercycler gradient (Eppendorf Limited, Hamburg, Germany). The PCR products were analyzed on a 1% agarose gel to assess the correct size and quality of the fragments.

2.3. SNP identification with the DHPLC method and sequencing confirmation

Mutation analysis was conducted with the DHPLC method on a WAVE® DNA Fragment Analysis System (Transgenomic Company, Santa Clara, USA). Eight μL PCR products from each pair of primers were loaded on a SaraSep DNASep column, and the samples were eluted from the column using a linear acetonitrile gradient in a 0.1 M triethylamine acetate buffer (TEAA), pH = 7, at a constant flow rate of 0.9 mL per min. The melting profile for each DNA fragment, the respective elution profiles and column temperatures were determined using the software WAVE Maker (Transgenomic Company, Santa Clara, USA). Chromatograms were recorded with a fluorescence detector at an emission wavelength of 535 nm (excitation at 505 nm) followed by a UV detector at 260 nm. The lag time between fluorescence and UV detection was 0.2 min.

According to the DHPLC profiles, the representative PCR products with different mutation types were purified and sequenced forward and reverse by BioAsia Biotechnology Co. Ltd (Shanghai, China). The sequences obtained were analyzed using the DNASTAR program (http://www.biologysoft.com/) for SNP confirmation.

2.4. Calculations

In order to obtain an estimate of nucleotide diversity, the normalized numbers of variant sites (θ) was calculated as the number of observed nucleotide changes (K) divided by the total sequence length in base pairs (L) and corrected for sample size (n), as described by Cargill *et al.* [8]. The formula is as follows:

$$\theta = K \Big/ \sum_{i=1}^{n-1} i^{-1} L.$$

2.5. Locating genes on chromosomes

The chicken genome sequence draft could be obtained from http://genome.ucsc.edu/cgi-bin/hgBlat and http://genome.wustl.edu/projects/chicken/. By BLAST analysis, the locations of all 12 genes in the chromosomes were made clear, which was consistent with the original mapping results of some genes [10, 16, 32, 34, 42].

3. RESULTS

3.1. Characterizations of the primers

Ninety-two primer pairs were tested in this study, of which seventy-five successfully amplified specific fragments. There were 9 primer pairs for *GH*, 11 for *GHR*, 7 for *ghrelin*, 7 for *GHSR*, 10 for *IGF-I*, 3 for *IGF-II*, 9 for *IGFBP-2*, 4 for *insulin*, 2 for *LEPR*, 7 for *PIT-1*, 2 for *SS* and 4 for the *TSH-β* gene. The details of these 75 primers, including their nucleotide constituents, length of PCR products, annealing temperature for PCR and column temperature for DHPLC, are shown in Table I. These primers spanned 31 897 bp of the genomic sequence, including 1543 bp of the 5' regulatory region (5'-flanking and 5'UTR), 7095 bp of the coding region, 17 218 bp of the introns and 6041 bp of the 3' regulatory region (3'-flanking and 3'UTR).

3.2. PCR amplification, DHPLC profiles and sequencing confirmation

In 40 animals from the four divergent breeds used for SNP identification, good quality PCR products were obtained using each of these 75 pairs of primers. After PCR products were analyzed with the WAVE® DNA Fragment Analysis System, different DHPLC profiles were observed among 40 individuals (example shown in Fig. 1). Different nucleotides among individuals with different DHPLC profiles were identified, and their sites and nucleotide mutations were determined by direct sequencing (Fig. 1). In addition, three genotypes in each SNP can also be easily determined by direct sequencing (Fig. 1).

3.3. Single nucleotide polymorphisms in 12 chicken candidate genes

In total, 283 SNP were identified in 31 897 bp of sequence within the 12 selected genes. The SNP markers are summarized in Table II. Considering the

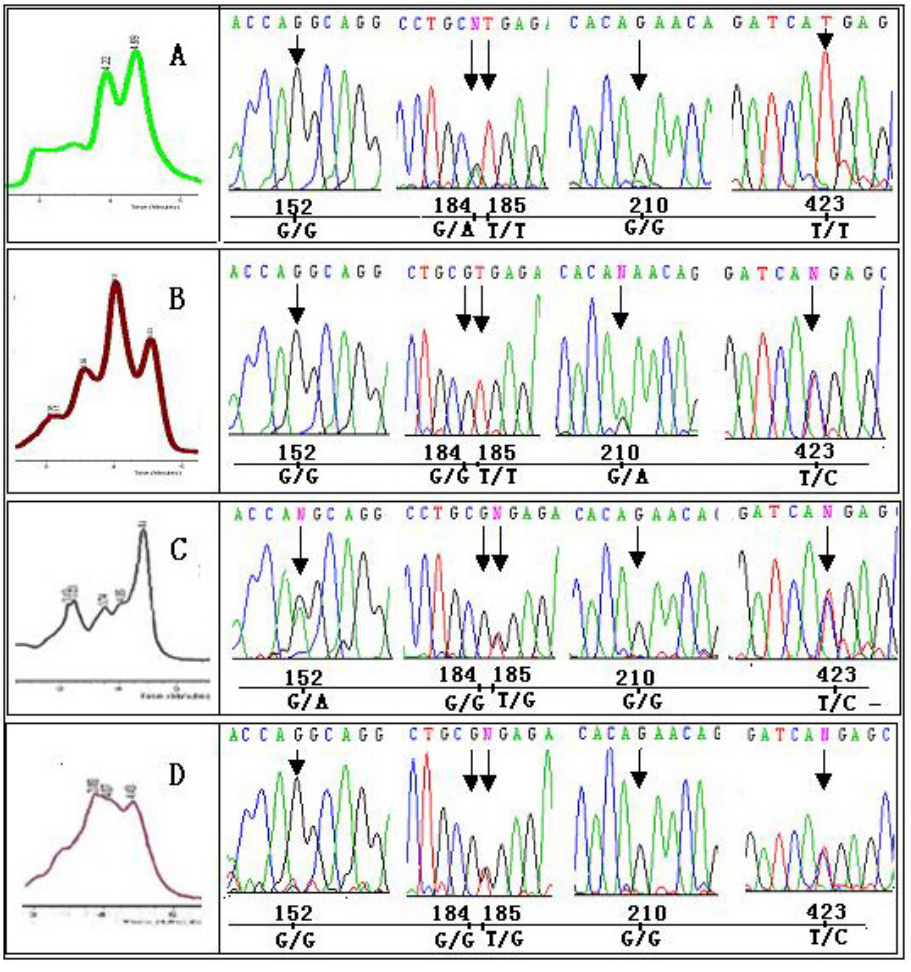

Figure 1. Example of a DHPLC-plot and sequencing confirmation in the 5'UTR of the chicken *GH* gene. Profiles A, B, C, and D indicate four mutation types identified by DHPLC method, and their corresponding nucleotides in five SNP sites are marked by the arrowhead. "N" represents two nucleotides existing in this site, and the SNP location (152, 184, 185, 210 and 423) was given according to the chicken *GH* gene sequence published (Genbank accession number: AY461843).

12 genes as a whole, every 113 bps generated one SNP on average, giving rise to its corresponding θ value of 2.07×10^{-3}. The average spread in bps per SNP and per gene region is presented in Table III.

The 283 SNP identified contained 74.2% of transitions (210 SNP), 11.3% of transversions (15), and 1.8% of indel (5). All SNP obtained were bi-allelic

Table II. Summary of 283 SNP in the 12 selected candidate genes.

Gene	Chrom[1]	Bps scanned	Primer pairs	Total SNP	SNP numbers[2]			
					5'UTR	Syn/non-	Intron	3'UTR
GH	1	3945	9	46	4	3/2	36	1
GHR	Z	4007	11	33	0	3/5	17	8
ghrelin	7	2536	7	25	1	1/1	21	1
GHSR	9	3628	7	27	0	9/2	25	1
IGF-I	1	3578	10	15	3	0/0	1	11
IGF-II	5	1681	3	4	0	1/0	3	0
IGFBP-2	7	4311	9	35	0	4/1	18	12
insulin	5	1793	4	24	1	0/0	22	1
LEPR	8	1070	2	9	0	3/0	6	0
PIT-1	1	2400	7	23	0	2/2	16	3
SS	9	944	2	11	0	1/2	3	5
TSH-β	26	2004	4	31	0	5/0	26	0
In total	-	31897	75	283	9	32/15	194	43

[1] The chromosomes containing the chicken *GH*, *GHR*, *IGF-II*, *insulin*, and *LEPR* gene were confirmed by previous studies on physical mapping of each gene [10,16,32,34,42], and those of the rest of the genes were determined according to the draft sequence of the chicken genome recently released (http://genome.ucsc.edu/cgi-bin/hgBlat). [2]5'UTR = 5' untranslation region; Syn = synonymous; non- = non-synonymous; 3'UTR = 3' untranslation region.

Table III. The estimates for different classes of polymorphic sites.

Polymorphic sites[1]	bp screened	SNP No.	Density (SNP/bp)	Individual No.	θ value
5'UTR	1543	9	1/172	40	1.35×10^{-3}
Coding	7095	47	1/151	40	1.55×10^{-3}
-Syn	7095	32	1/222	40	1.05×10^{-3}
-Non-syn	7095	15	1/473	40	4.9×10^{-4}
Introns	17218	194	1/89	40	2.63×10^{-3}
3'UTR	6041	43	1/141	40	1.65×10^{-3}
Total	31897	283	1/113	40	2.07×10^{-3}

[1] 5'UTR = 5' untranslation region; Syn = synonymous; Non-syn = non-synonymous; 3'UTR = 3' untranslation region.

Table IV. Non-synonymous SNP that led to the changes of amino acids.

Gene	Sequence ID[1]	SNP	Codon change	Amino acid change[2]	Region[3]
GH	AY461843	G1494A	GCT→ACT	A13T	Pre
	AY461843	G2075A	CGC→CAC	R59H	Mat
GHR	M74057	G1359A	GCT→ACT	A442T	Mat
	M74057	G1475C	CAG→CAC	Q480H	Mat
	M74057	G1507T	AGC→ATC	S491I	Mat
	M74057	A1512T	ACA→TCA	T493S	Mat
	M74057	G1599C	GAG→CAG	E522Q	Mat
ghrelin	AY303688	A2355G	CAG→CGG	Q113R	Pro
IGFBP-2	U15086	G645T	ATG→ATT	M205I	Mat
GHSR	AB095994	A1071T	AAC→TAC	N227Y	Mat
	AB095994	C3833T	GCC→GTC	A323V	Mat
PIT-1	AJ236855	A499G	ATG→GTG	M167V	Mat
	AJ236855	A761G	AAT→AGT	N254S	Mat
SS	X60191	A275G	CAG→CGG	Q79R	Pre
	X60191	A370G	AAA→GAA	K111E	Mat

[1] Refers to Genbank accession number of each sequence. [2] Indicates the changes of amino acids.
[3] Pre = precursor; Mat = mature protein; Pro = procursor.

polymorphisms except in two cases: a tri-allelic SNP was observed in the *insulin* gene (T/C/A, nt 1295 of AY 438372) and the other in the *LEPR* gene (T/G/A, nt 885 of AF 222783). For these two tri-allelic SNP, sequencing artefacts were excluded by performing repetitive sequencing for several individuals with different genotypes.

3.4. Non-synonymous SNP

Fifteen non-synonymous SNP were identified in the present study, most of which (12 of 15) affected the translated mature proteins (Tab. IV). In the *GH* gene, G1494A and G2075A changed the signal peptide (A13T) and mature protein (R59H) respectively. Five SNP of G1359A (A442T), G1475C (Q480H), G1507T (S491I), A1512T (T493S) and G1599C (E522Q) all occurred in the intracellular region of the *GHR* gene, but they had no influence on the conserved features of 5 cysteine residues in this domain. A1071T and C3833T altered the mature protein of the *GHSR* gene with the amino acid changes of N227Y and A323V. Transitions A499G and A761G in the *PIT-1* gene led to the changes of M167V and N254S, however, the conserved POU domain was not affected. A2355G was located in the coding region of preproghrelin. A275G (Q79R) and A370G (K111E) of the *SS* gene changed the precursor and mature somatostatin-14 (or -28) respectively.

3.5. Other sequence variations identified

Seventeen DNA sequence variations, other than SNP, were identified in 9 genes: *GH*, *GHR*, *ghrelin*, *GHSR*, *IGFBP-2*, *insulin*, *PIT-1*, *SS* and *TSH-β*. These changes included 15 cases of indel polymorphisms of no less than 2 bp and 2 cases of polymorphic numbers of continuous A nucleotide in the present study. Most of these variations were polymorphisms with minor allelic frequencies over 1% (Tab. V). These variations occurred in non-coding regions of each functional gene, and did not change the terminal products of translated precursors.

3.6. PCR-RFLP DNA markers

From the 283 SNP and 17 other variations, 58 SNP and one case of a 6 bp indel polymorphism, led to the presence or absence of some restriction sites. As a result, 59 PCR-RFLP markers were developed, but they were not validated experimentally. The numbers of markers developed for the 12 genes are summarized in Table VI. All these PCR-RFLP markers were located in either coding regions (synonymous and non-synonymous) or non-coding regions such as 5'-flanking, 5'UTR, intron and 3'UTR. Furthermore, the choice of a PCR-RFLP marker was also based on the cost of the restriction enzyme.

Table V. Other sequence variations identified in the 9 chicken growth-correlated genes.

Gene	Sites	Sequence ID[1]	Variations	Region	Frequency (%)[2]	Comment[3]
GH	3308-3357	AY461843	50 bp lost	Intron 4	0.5	Nie *et al*. [25]
GHR	2180	M74057	GTGA indel	3'UTR	7.5	
	71-72	AY303688	CC indel	5'UTR	2.5	
	79-86	AY303688	CTAACCTG indel	5'UTR	5	AB075215; AY299454
ghrelin	643-662	AY303688	(A)n	Intron 2	2.5	
	1418-1419	AY303688	TG indel	Intron 3	2.5	
GHSR	3407-3412	AB095994	GGTACA indel	Intron	30	
IGFBP-2	965	AY326194	CCAGGTG indel	Intron 2	7.5	
	783-794	AY438372	12 bp indel	Intron	12.5	
insulin	1295-1296	AY438372	TC indel	Intron	5	
	1589-1593	AY438372	ATTTT indel	Intron	2.5	
PIT-1	586	AY396150	57 bp indel	Intron 2	30	SSC 1(+) 86752736~86752792
SS	270	X60191	81 bp insertion	Exon 1	0.5	
	394-405	AY341265	(A)n, n = 12,13,15	Intron 2	2.5	
	423-424	AY341265	CA indel	Intron 2	10	
TSH-β	1120-1123	AY341265	TTGT indel	Intron 2	5	
	1662	AY341265	GT indel	Intron 2	25	

[1] Refer to Genbank accession number. [2] Indicates minor allele frequencies. [3] Means some results were proven by previous studies; AB075215 and AY299454 are Genbank accession numbers; "SSC 1(+) 86752736~86752792" refer to the inserted 57 bp sequences were nt 86752736~86752792 of chromosome 1(+) published by the Chicken Genome Project (http://genome.wustl.edu/projects/chicken/).

4. DISCUSSION

In this study DHPLC was successfully used to discover SNP in functional chicken genes. As a highly sensitive and automated method, DHPLC is mainly based on the capability of ion-pair reverse-phase liquid chromatography

Table VI. Fifty-nine PCR-RFLP DNA markers in the 11 chicken genes.

No	SNP site	Sequence No[1]	Gene	Region	Restriction enzyme
1	T185G	AY461843	*GH*	5'-flanking	Hin 6 I
2	C423T	AY461843	*GH*	5'-flanking	Pag I
3	G662A	AY461843	*GH*	Intron 1	Msp I
4	G2048A	AY461843	*GH*	Intron 2	Mph 1103 I
5	G2248A	AY461843	*GH*	Intron 3	EcoRćõ
6	T3094C	AY461843	*GH*	Intron 4	Msp I
7	C3199T	AY461843	*GH*	Intron 4	Msp I
8	G3581T	AY461843	*GH*	Intron 4	Bsh 1236 I
9	G565A	AJ506750	*GHR*	Intron 5	Eco 72 I
10	C895G	AJ506750	*GHR*	Intron 5	BsuRćõ
11	A387G	AY468380	*GHR*	Intron 7	Eco 1051
12	G2408A	M74057	*GHR*	3'-UTR	Hin 6 I
13	C2907A	M74057	*GHR*	3'-UTR	BsuR I
14	G687A	AY303688	*ghrelin*	Intron 2	KspA I
15	T1167A	AY303688	*ghrelin*	Intron 3	Nde I
16	T2100C	AY303688	*ghrelin*	Intron 4	Pag I
17	C2466T	AY303688	*ghrelin*	3'-UTR	Csp 6 I
18	G656A	AB095994	*GHSR*	Exon 1 (R synonymous)	Msp I
19	C842T	AB095994	*GHSR*	Exon 1 (A synonymous)	BsuR I
20	A1071T	AB095994	*GHSR*	Exon 1 (N→Y)	Csp 6 I
21	T1857C	AB095994	*GHSR*	Intron 1	Hin 6 I
22	A1965G	AB095994	*GHSR*	Intron 1	Nco I
23	A2044G	AB095994	*GHSR*	Intron 1	Hin 6 I
24	C2047T	AB095994	*GHSR*	Intron 1	BspT I
25	T2133A	AB095994	*GHSR*	Intron 1	Tas I
26	3407~3412 indel	AB095994	*GHSR*	Intron 1	Csp 6 I
27	C3678T	AB095994	*GHSR*	Exon 2 (F synonymous)	Bsp 119 I
28	C3753T	AB095994	*GHSR*	Exon 2 (S synonymous)	Hin 6 I
29	T159C	M74176	*IGF- I*	5'UTR	Tas I
30	C253T	M74176	*IGF- I*	5'UTR	Mph 1103 I
31	C570A	M74176	*IGF- I*	5'UTR	Hinf I
32	C664T	AY331392	*IGF- I*	3'UTR	Hinf I
33	C129T	AY253744	*IGF- I*	3'UTR	Bsp 119 I
34	G329A	S82962	*IGF-ćò*	Intron 2	Hinf I

Table VI. Continued.

No	SNP site	Sequence No[1]	Gene	Region	Restriction enzyme
35	G639A	U15086	*IGFBP-2*	Exon 2 (S synonymous)	Bsh 1236 I
36	G645A	U15086	*IGFBP-2*	Exon 2 (M→I)	BseG I
37	G1510A	U15086	*IGFBP-2*	3'UTR	Xho I
38	G1946A	U15086	*IGFBP-2*	3'UTR	Eco 72 I
39	G287A	AY326194	*IGFBP-2*	Intron 2	Alu I
40	A754G	AY326194	*IGFBP-2*	Intron 2	Alw 44 I
41	G809T	AY326194	*IGFBP-2*	Intron 2	Dra I
42	C1032T	AY326194	*IGFBP-2*	Intron 2	Eco 72 I
43	C173T	AY331391	*IGFBP-2*	Intron 3	Mva I
44	C206T	AY331391	*IGFBP-2*	Intron 3	Bsp 143 I
45	C208T	AY331391	*IGFBP-2*	Intron 3	Bglćò
46	C195T	AY438372	*insulin*	5'UTR	BsuR I
47	T409C	AY438372	*insulin*	Intron 2	Taq I
48	A428G	AY438372	*insulin*	Intron 2	Nde I
49	C1218A	AY438372	*insulin*	Intron 2	Nde I
50	C1549T	AY438372	*insulin*	Intron 2	Msp I
51	T3737C	AY438372	*insulin*	Intron 2	Msp I
52	A3971G	AY438372	*insulin*	3'UTR	Msp I
53	C352T	AF222783	*LEPR*	Intron 8	Bsh 1236 I
54	G427A	AF222783	*LEPR*	Intron 8	Bsh 1236 I
55	A660G	AF222783	*LEPR*	Intron 8	Tas I
56	G543A	AJ236855	*PIT-1*	Exon 3 (E synonymous)	EcoR I
57	C425G	AY341265	*TSH-β*	Intron 2	Csp 6 I
58	T1761C	AF341265	*TSH-β*	Exon 3 (S synonymous)	Hin 6 I
59	G1821A	AF341265	*TSH-β*	Exon 3 (P synonymous)	Msp I

[1] Refers to Genbank accession number of each sequence.

to resolve homoduplex from heteroduplex molecules under conditions of partial denaturation [15]. Currently, DHPLC seems to be limited in distinguishing different kinds of homoduplex and in genotyping individuals for each SNP, especially when several SNP are present in a DNA fragment [22]. For this reason, and due to the small sample size (10 individuals for each breed) used in this study, the allele frequency of each SNP in four chicken breeds was not calculated. Nevertheless allele frequency estimates would provide important information for a future evaluation of the potential effect of each SNP.

In the present study, 283 SNP were identified in a total length of 31 897 bp of DNA, covering the 12 chicken genes in the somatotropic axis. The results provide basic information on the distribution and characteristics of SNP in chicken genes. The average bps per SNP in the 12 selected genes was very low (113 bp), consequently the nucleotide diversity seems to be much higher in chickens even when this is adjusted for the small sample size studied (40 individuals or 80 chromosomes) (Tab. III). In human SNP screening studies, the SNP density reported is much lower, and one SNP is reported to occur in every 1000–2000 bases when two human chromosomes are compared [2, 23, 28]. Another study analysing SNP incidence in 106 human genes, provided a higher density of one SNP per 348 bp, and their θ values of synonymous and non-synonymous SNP in coding regions were 1.0×10^{-3} and 1.96×10^{-4} when corrected for sample size. These θ values were quite comparable to our results [8]. The lower SNP density reported in humans might be due to the fact that fewer intronic SNP were identified and sequences of less individuals were compared. On the contrary, the chicken genome is much more compact than that of humans, since their genome size were almost 3.2 and 1.1 billion respectively (http://genome.ucsc.edu/cgi-bin/hgBlat). The higher SNP incidence in chickens seemed to compensate for its small genome size and much lower repetitive DNA (including microsatellite sequences) occurrence. A forthcoming paper that focuses on millions of SNP in the chicken genome will be available soon in Nature. In the pig, a recently developed SNP map of chromosome 2 showed that the SNP density is much higher [18], which is in accordance with the present study.

Among 283 SNP, 278 were single-base substitutions and only 5 were single base indels. Furthermore, over 74% of the SNP (210 of 283) were transitions, similar to the ratio (75%) obtained from 10 human genes [14]. Although most SNP were bi-allele polymorphisms, two tri-allelic variations were observed in the *insulin* gene (T/C/A, nt 1295 of AY 438372) and the *LEPR* gene (T/G/A, nt 885 of AF 222783), respectively. Since expected introns had higher SNP densities than coding regions and up- or down stream regions because of selection pressure on exons and flanking regions, the latter is likely to be related to the control of expression levels.

In this study, most 283 SNP of the 12 candidate genes identified are from TS and X chickens, which seems to indicate that the two Chinese native chicken breeds are more diverse than the two commercial breeds. It has previously been shown that the level of heterozygosity in commercial broilers and layers is lower than that observed in Chinese native chicken breeds in allozymes, random amplified polymorphism DNA and microsatellite DNA

polymorphisms [43]. The long-term and intense selection for growth and production traits has resulted in decreasing diversity of the Leghorn and WRR breeds. However, further study is needed on the effect of the observed variation and the differences in growth rate and egg production between these breeds. The SNP from the 12 candidate genes identified in the present study provides suitable genetic markers for the analysis of such differences.

The twelve functional genes studied are all key factors in the chicken somatotropic axis, and play crucial roles in growth and in the metabolism of the chicken. There might be certain underlying relationships between some of the SNP identified in these genes and quantitative traits like growth and carcass traits. The SNP or more specifically the 59 PCR-RFLP markers identified in this study provide a good opportunity to perform association studies for growth or reproduction related traits in the diverse breeds used.

A few SNP of these twelve genes have been reported previously, and some of them are related to growing, laying, meaty quality or disease-resistance traits. In the chicken *GH* gene, several SNP in introns have been identified and reported to be associated with growth, egg production and disease resistance [11, 13, 20]. Sex-linked dwarf chickens are just due to a mutation at an exon-intron splicing site of the *GHR* gene [17]. Another SNP that led to the presence or absence of a poly (A) signal in intron 2 was found to influence ages at first egg and egg production from 274 to 385 days [11, 12]. Two SNP in the *IGF-II* gene were significantly related to growth and feeding traits [3].

Fifteen non-synonymous SNP changed the translated precursor of the chicken *GH*, *GHR*, *ghrelin*, *IGFBP-2*, *PIT-1* and *SS*, and could affect the normal function of the mature proteins (Tab. IV). Other SNP in non-coding regions of 5'UTR, 3'UTR and introns, could also affect gene expression levels because of regulatory elements present in 5'UTR or 3'UTR regions [21]. These SNP with obviously different allelic frequencies between high reproduction (L) or fast-growing breeds (WRR) and slow-growing ones (TS and X) could contribute to their divergent growth performance (Tab. VI).

In seventeen other types of sequence variations (Tab. V), some of them were consistent with previous studies. A 50 bp deletion was reported to be present in the chicken *GH* gene of Chinese TS [24]. A 1773 bp deletion in exon 10 and 3'UTR of the *GHR* gene, however, have been proven to translate into a dysfunctional precursor and could explain the existence of sex-linked dwarf chickens [1]. For the *PIT-1* gene, a 57 bp indel polymorphism in intron 2 was quite frequent both in Chinese native chickens (TS and X) and commercial lines (L and WRR). This indel was confirmed by a Genbank sequence (AY396150) and the released genome sequence (nt 86752736~86752792 of Z chromosome)

of the Chicken Genome Project (http://genome.wustl.edu/projects/chicken). For the *SS* gene, many variations have been described in several species, including chickens, however, insertion or deletion of dozens of bps has not been reported before [35]. Since the *SS* gene consists of two exons in nearly all species, the 81 bp insertion in the chicken *SS* gene in the present study is remarkable. This might mean that the *SS* gene of the chicken contains 27 additional amino acids. Further study at the functional level is needed to asses the biological effects of this large insertion.

In conclusion, 283 SNP and 17 other variations in 12 chicken growth-correlated genes were identified in the present study. Some of these SNP could serve as useful markers for association studies for growth related traits, since there are indications that there are allele frequency differences among diverse chicken breeds.

ACKNOWLEDGEMENTS

This work was funded by projects under the Major State Basic Research Development Program, China, project no. G2000016102. We would like to thank Drs. Richard Crooijmans (Wageningen University, The Netherlands) and Changxi Li (University of Alberta, Canada), and the two referees for their comments on this manuscript.

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