Identification of Three Single Nucleotide Polymorphisms in the Chicken Insulin-Like Growth Factor 1 and 2 Genes and Their Associations with Growth and Feeding Traits

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ABSTRACT The chicken insulin-like growth factor *(IGF)1* and *IGF2* genes have been partially sequenced in six individuals of each of two chicken strains of the Black Penedesenca breed (PN and MN). These two strains are genetically diverse for growth traits. Sequence alignment revealed the existence of three single nucleotide polymorphisms (SNP) (IGF1-SNP1, IGF2-SNP2, and IGF2-SNP3). These three SNP and a fourth IGF1 polymorphism (IGF1-SNP4) were typed in 60 individuals from each strain by using PCR-RFLP or primer extension analysis. No significant associations among these four SNP, growth traits, and plasma IGF1 concentration were identified. In contrast, suggestive associations ($P \leq 0.05$) were found between IGF1-SNP1 and average daily gain at 107 d and feed efficiency at 44, 73, and 107 d. However, these associations were not simultaneously found in both strains suggesting that they might have been produced by linkage disequilibrium with another mutation located in the *IGF1* locus or another linked gene. Since the PN and MN strains differ very markedly on their feed intake, the chicken leptin gene was included in the sequence analysis. Unfortunately, attempts to amplify several regions of this gene were unsuccessful. Even when primers complementary to highly conserved regions were used, the PCR consistently failed. Other authors have reported similar problems when trying to amplify avian leptin sequences.

(*Key words*: chicken, growth, insulin-like growth factor, leptin, single nucleotide polymorphism)

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INTRODUCTION

Growth is a complex process that involves the regulated coordination of a wide diversity of neuroendocrine pathways. Leptin, insulin-like growth factors (IGF), and their receptors are essential players in this biological process by modulating intermediary metabolism and cell proliferation. Chicken insulin-like growth factors 1 and 2 (IGF1 and IGF2) are polypeptidic hormones that exert their function by binding to specific type 1 receptors (Zhou et al., 1995). The metabolic effects of avian IGF include an increased amino acid and glucose uptake and the upregulation of DNA and protein synthesis (McMurtry, 1998). Plasma levels of IGF1 and IGF2 decrease with fasting and increase with age (Beccavin et al., 2001). Moreover, there is ample evidence suggesting that IGF might influence growth rate, body composition, and lipid metabolism in poultry (McMurtry, 1998; Tomas et al., 1998; Beccavin et al., 2001).

Leptin that regulates energy imbalance, feeding behavior, and fertility is a molecule mostly secreted by the adipose tissue (Rosenbaum and Leibel, 1998). Mutations in the leptin and leptin receptor genes have been associated to hyperphagia and obesity in obese (*ob/ob*) and diabetic (*db/db*) mice, respectively (Strosberg and Issad, 1999). Biochemical and molecular studies indicate that the biological properties of leptin are conserved amongst mammalian and avian species (Denbow et al., 2000). According to Dridi et al. (2000), leptin plasma levels are higher in fed chickens than in fasted ones, showing that the expression of this signaling molecule is regulated by the nutritional status. Moreover, the intracerebroventricular injection of human recombinant leptin in Single Comb White Leghorn chickens decreased feed intake, a feature that demonstrates the existence of a functional link between leptin and satiety in chicken (Denbow et al., 2000).

The molecular characterization of the chicken *IGF1*, *IGF2*, and leptin genes has provided valuable clues for

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Abbreviation key: dNTP = deoxyribonucleotide; FE = feed efficiency; IGF = insulin-like growth factor; MN = maternal Black Penedesenca strain; PN = paternal Black Penedesenca strain; RT = reverse transcription; SNP = single nucleotide polymorphism.

understanding how they are regulated and expressed. The chicken *IGF1* gene maps to chromosome 1 and encompasses 50 kb (Kajimoto and Rotwein, 1991; Klein et al., 1996). Multiple alternative promoters and two different variants generated by alternative splicing have been reported (Kajimoto and Rotwein, 1991). The profile of *IGF1* mRNA expression is remarkably ubiquitous and includes liver, muscle, kidney, testes, heart, ovary, brain, intestine, and other tissues (Tanaka et al., 1996; McMurtry et al., 1996). The *IGF2* gene contains three exons and maps to chromosome 5 (Darling and Brickell, 1996; Yokomine et al., 2001). In therian mammals, the *IGF2* gene is paternally imprinted (DeChiara et al., 1991; Killian et al., 2001). In contrast, the chicken *IGF2* gene displays a biallelic pattern of expression (O'Neill et al., 2000; Nolan et al., 2001; Yokomine et al., 2001). The genomic structure and chromosomal location of the chicken leptin locus are still unknown, and, to the best of the authors' knowledge, there is no report describing polymorphisms in this gene. Taouis et al. (1998) and Ashwell et al. (1999) reported the successful cloning and sequencing of the chicken leptin cDNA. However, these results have been difficult to replicate by other scientific teams, and they are still a matter of debate (Friedman-Einat et al., 1999; Pitel et al., 2000; Dunn et al., 2001).

Association studies between mutations at the *IGF1*, *IGF2*, and leptin genes and productive traits are very scarce in chicken. Recently, Nagaraja et al. (2000) described one *Pst*I RFLP in the 5′ end of the *IGF1* gene that was associated to egg and egg shell weight in one White Leghorn chicken population. Moreover, Yan et al. (2002) reported an association between phenotypic variation at several growth and carcass traits with one polymorphism at exon 2 of the chicken *IGF2* gene. The main goal of the current work was to investigate the genetic basis of the phenotypic differences observed in two genetically diverse chicken strains by using a candidate gene approach. To this end, the chicken *IGF1* and *IGF2* genes were sequenced in several individuals from each strain to identify single nucleotide polymorphisms (SNP) that might be associated with growth and feeding traits. Moreover, an attempt was made to amplify and sequence the chicken leptin gene in order to isolate and characterize new genetic variants at this locus.

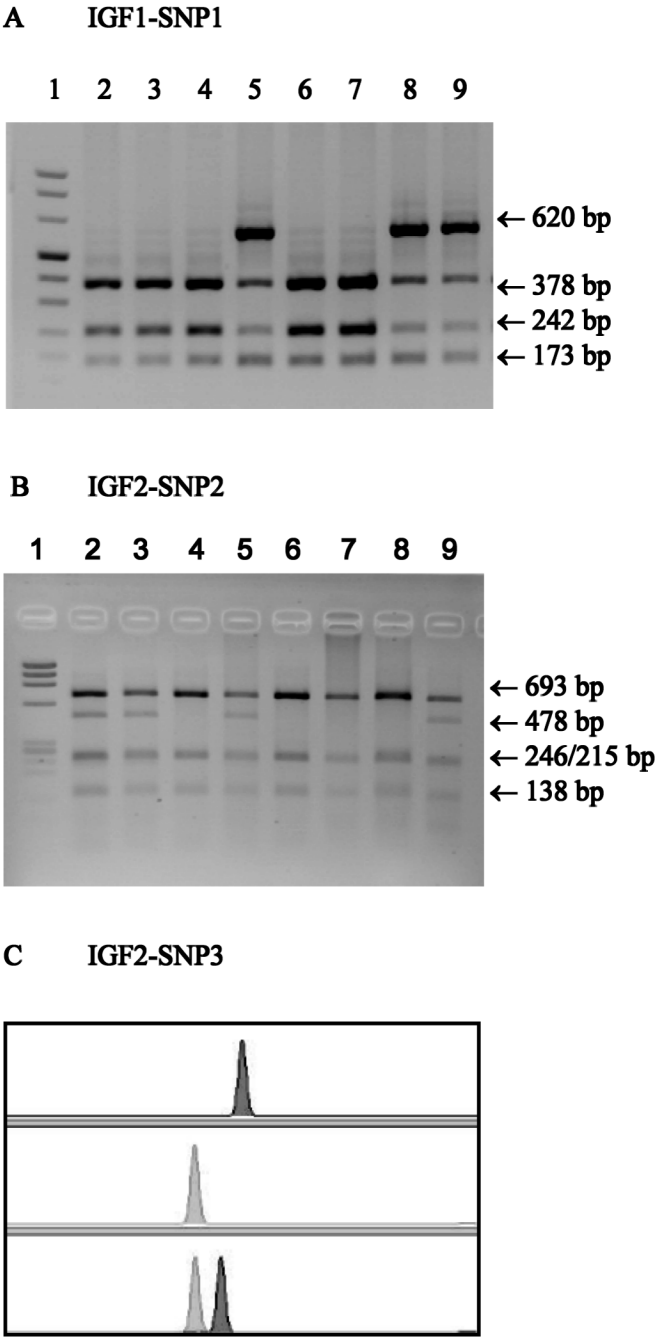

FIGURE 1. (A) Gel picture of two insulin-like growth factor (IGF) 1-single nucleotide polymorphism (SNP) 1 genotypes: lane 1, Marker VIII; lanes 2, 3, 4 , 6, 7, genotype AA; lanes 5, 8, 9, genotype AB; (B) Gel picture of two IGF2-SNP2 genotypes: lane 1, Phi X174 *Hae*III marker; lanes 4, 6, 7, 8, genotype AA; lanes 2, 3, 5, 9, genotype AB; (C) GeneScan 3.7 image of three IGF2-SNP3 genotypes: Homozygous 1/1 (upper lane), homozygous 2/2 (central lane) and heterozygous 1/2 (lower lane).

MATERIALS AND METHODS

Animal Material and Phenotypic Traits

Two genetically diverse maternal (MN) and paternal (PN) Black Penedesenca chicken strains bred at the Institute of Agro-Food Research and Technology (IRTA) Centre Mas Bové were used in the present study. These two strains were derived from a founder Black Penedesenca population (Francesch and Jordà, 1988). The MN strain had been selected for egg number at 39 wk of age and egg weight and egg shell color at 25 wk of age over 12 generations. Mean live BW was not modified noticeably during this selection procedure. The PN strain was obtained by crossing the same Black Penedesenca founder population with a Cornish chicken strain. Subsequently, this strain was selected over 12 generations for live BW and breast angle at 11 wk (see Francesch, 2002 for a review). At present, male live BW means for the MN and PN strains at 11 wk of age are 1.11 ± 0.01 kg and 2.22 ± 0.02 kg, respectively (Francesch, 2002).

Two hundred eighty-seven male chicks (122 PN and 165 MN) were used in the current experiment. Chickens were

divided into four lots and reared in pens. At 7 wk of age, 60 chicken from each strain were randomly selected and transferred to individual cages. Chickens had free access to water and were given ad libitum access to a starter diet (3,100 kcal ME/kg and 230 g/kg CP) until 7 wk of age and a grower diet (3,100 kcal ME/kg and 210 g/kg CP) from the 7th wk until slaughter. Live BW was recorded weekly until 20 wk of age. Pen and individual feed intake were recorded weekly from 1 to 7 wk and from 7 to 20 wk of age, respectively. Each cage was 0.5 × 0.5 × 0.8 m (long × wide × height), and it had a nipple drinker and one feeder (0.4 m long). Each feeder was weighted and filled weekly. Feed intake was measured as the difference between the initial and the final weight of the feeder. Animal weight was also recorded the same day and weekly using an electronic scale (0.1 g of precision). In this way, it was possible to obtain cumulated feed intake for each bird at each weight. The derivative of weight as a function of cumulative intake gives feed efficiency (FE) at cumulative intakes. The cumulative intake at different ages was obtained from the mean curve of intake as a function of age for each strain. Alternatively, the derivative of cumulative intake as a function of weight gives feed conversion ratio at different ages (weights). Plasma IGF1 concentrations were measured in duplicate at 73 d with the Multispecies IGF1 ELISA kit using an antibody which displayed 70% relative binding potency to chicken IGF1[2]. The IGF-binding proteins were separated, according to Bowsher et al. (1991), and the assay was standardized with recombinant chicken IGF1[3]. In the current study, the assay had a repeatability of 0.93.

DNA and RNA Extraction and cDNA Synthesis

Genomic DNA was isolated from 120 blood samples (60 MN and 60 PN) obtained by intracardiac puncture. Five microliter blood samples were mixed with 250 μL of HNC buffer (10 m*M* Tris, 400 m*M* NaCl, and 2 m*M* EDTA) plus 50 μL SDS (5%) and 5 μL of proteinase K (10 mg/mL). This mixture was incubated at 56°C for 3 h and 150 μL NaCl (5 *M*) were added to the lysate. Subsequently, the lysate was centrifuged at 13,000 rpm for 15 min. The supernatant was transferred to a fresh 1.5-mL eppendorf tube. Genomic DNA was ethanol precipitated, washed with ethanol 70%, and resuspended in 500 μL of sterile water.

Total RNA was isolated from six individuals (3 PN and 3 MN). Liver and adipose tissue samples were frozen in liquid nitrogen, pulverized with a mortar and a pestle, and homogenized with an Ultra Turrax T8 homogenizer.[4] The RNA was extracted with the Trizol reagent[5] according to the instructions of the manufacturer, resuspended in diethyl pyrocarbonate treated water, and quantitated by spectrophotometry. Total RNA was reverse transcribed to cDNA by using the kit ThermoScript RT-PCR System.[6] The RT reaction contained 1 to 3 μg of total RNA in a final 20-μL volume.

TABLE 1. Genotype frequencies of the the insulin-like growth factor 1 gene single nucleotide polymorphism 1 (IGF1-SNP1), IGF1-SNP4, IGF2-SNP2, and IGF2-SNP3 in the PN and MN chicken strains[1]

Genotype	PN strain	MN strain	Significance[2]
IGF1-SNP1			
AA	0.49	0.63	NS
AB	0.38	0.37	NS
BB	0.13	0.00	NS
IGF1-SNP4			
+/+	0.63	1.00	***
+/−	0.26	0.00	***
−/−	0.11	0.00	***
IGF2-SNP2			
AA	0.51	0.50	NS
AB	0.44	0.43	NS
BB	0.05	0.07	NS
IGF2-SNP3			
1/1	0.89	0.42	***
1/2	0.11	0.48	***
2/2	0.00	0.10	***

[1]PN = Black Penedesenca paternal chicken strain; MN = Black Penedesenca maternal chicken strain.

[2]Significance of a Chi-square test comparing genotypic frequencies of the PN and MN strains.

***$P \leq 0.0001$.

Amplification of the Chicken Leptin Gene

Four differents primer pairs were used to amplify conserved regions of the chicken leptin gene. Primer sequences were GALLEP2-FW, 5′-TGC TGG AGA CCC CTG TGT CGA-3′; GALLEP2-REV, 5′-TGT GTG AAA TGT CAT TGA TCC TGG TG-3′; GALLEP3-FW, 5′-GTA TCC GCC AAG CAG AGG GTC A-3′; GALLEP3-REV, 5′-CAG CAT TCC GGG CTA ATA TCC AAC-3′; LEPGALL-FW, 5′-TGG CAA TCT ACC AAC AGA TCC T-3′; LEPGALL-REV, AGA GGG AGG CTT CCA GGA CG-3′; CHICKLEP-FW, 5′-SYT TRT CCA AGA TGG ACC AGA C-3′, and CHICKLEP-REV, 5′ CYT CAR RGC CAC CAC CTC-3′. Primer pairs GALLEP2 and 3 were derived from the available chicken leptin sequences. Primer pairs LEPGALL and CHICKLEP are consensus primers derived from an alignment of mammalian and avian leptin sequences. Genomic DNA (GALLEP2 and 3 PCR) or cDNA synthesized from liver and adipose tissue RNA (LEPGALL and CHICKLEP PCR) were used as templates in the amplification reaction. All these primer pairs were assayed at different annealing temperatures (51.4°C, 55°C, 60.6°C, 65.2°C, and 67.6°C) and magnesium chloride concentrations (1 m*M*, 1.5 m*M*, and 2.5 m*M*) in a gradient thermocycler PTC-200.[7] The thermal profile included 34 cycles of 94°C for 1 min, annealing step for 1 min, and 72°C for 1 min. The PCR reaction contained 100 μM of each deoxyribonucleotide (dNTP), 0.5 μM of each primer, 50 ng of genomic DNA or 1.5 μL of RT reaction, and 0.25 U of *Taq* DNA polymerase[8] in a final volume of 12.5 μL.

[2]Immunological & Biochemical Testsystems GmbH, Reutlingen, Germany.
[3]Gro Pep Pty Ltd., North Adelaide, Australia.
[4]IKA-WERKE GmbH, Staufen, Germany.
[5]Gibco BRL, Life Technologies S.A., Barcelona, Spain.
[6]Invitrogen S.A., Barcelona, Spain.

TABLE 2. PN and MN[1] strain means for body weight, average daily gain, cumulated feed intake, and feed efficiency at 44, 73, and 107 d of age

Variable	PN strain	MN strain	SED[2]	Significance
Body weight, g				
44 d	1,429	660	66	***
73 d	2,747	1,321	159	***
107 d	3,856	2,015	345	***
Average daily gain, g/d				
44 d	44.4	21.4	1.1	***
73 d	41.3	24.4	2.3	***
107 d	18.5	13.0	6.3	NS
Cumulated feed intake, g				
44 d	3,338	1,711	20	***
73 d	6,974	3,989	47	***
107 d	11,994	7,591	108	***
Feed efficiency, g/g				
44 d	0.39	0.31	0.005	***
73 d	0.28	0.25	0.006	***
107 d	0.16	0.13	0.011	***

[1]PN = Black Penedesenca paternal chicken strain; MN = Black Penedesenca maternal chicken strain.

[2]SED = Standard error of the difference.

***$P \leq 0.0001$.

Amplification and Sequencing of the Chicken IGF1 and IGF2 Genes

One fragment of 655 bp corresponding to the chicken *IGF1* cDNA and one of 593 bp of the *IGF2* cDNA were targeted. The PCR conditions were 1.5 m*M* $MgCl_2$, 100 μM of each dNTP, 0.5 μM of each primer, 3 μL of cDNA, and 0.5 U of *Taq* DNA polymerase[8] in a final 25-μL volume. Primers for cDNA amplification were IGF1-CHK-FW, 5′-CAG AGC AGA TAG AGC CTG CG-3′; IGF1-CHK-REV, 5′- TCT GCA GAT GGC ACA TTC AT-3′; IGF2-CHK-FW, 5′-GCC AGG CAG ATA CTG CTG CTA-3′, and IGF2-CHK-REV, 5′-TCC CCA GGA GAT CAC AAA TC-3′. The thermal profile was 94°C for 2 min followed by 34 cycles of 94°C for 1 min, 65°C for 1 min (*IGF1*) or 63°C for 1 min (IGF2), and 72°C for 3 min. In addition, two genomic sequences were amplified: one 793-bp fragment, corresponding to the 5′ end of the chicken *IGF1* gene, and one 1,496-bp fragment including exon 2, intron 2, and most of exon 3 of the chicken *IGF2* gene. Primer sequences were IGF1-FW, 5′-GCT GGG CTA CTT GAG TTA CTA C-3′; IGF1-REV, 5′-TTG CGC AGG CTC TAT CTG CTC-3′; IGF2-FW, 5′-ACA GGT AGA CCA GTG GGA CG-3′; and IGF2-REV, 5′-CTA GTG TTG GCA CTG GGG ATG-3′. In both cases, the amplification reactions contained 1.5 m*M* $MgCl_2$, 100 μM of each dNTP, 0.5 μM of each primer, 200 ng of genomic DNA, and 1.25 U of *Taq* DNA polymerase[8] in a final 50-μL volume. The thermal profile was 94°C for 2 min, followed by 32 cycles of 94°C for 1 min, 65°C for 2 min, and 72°C for 3 min. Amplified products were sequenced forward and reverse by using the BigDye Terminator Cycle Sequencing kit.[9] Sequencing reactions were precipitated, resuspended in 25 μL of formamide, denatured at 95°C and analyzed in an ABI 310 capillary electrophoresis device.[9] Sequences were aligned with the Multalin program (Corpet, 1988).

Typing of the Chicken IGF1 and IGF2 Genes

The IGF1-SNP1 was typed by digesting 5 μL of the 793-bp PCR product with 5 U of *Hinf*I[10] at 37°C overnight. Restriction patterns were visualized by electrophoresing the digestion product in a 3% agarose gel stained with ethidium bromide. The IGF1-SNP4 was typed according to Nagaraja (2000). The typing of the *IGF2* gene involved the analysis of two polymorphisms (IGF2-SNP2 and IGF2-SNP3). One genomic fragment of 1,146 bp encompassing exons 2 and 3 was amplified by using primers GALIGF2-4-5′, 5′-CCA GTG GGA CGA AAT AAC AGG AGG A-3′ and GALIGF2-5-3′, 5′-TTC CTG GGG GCC GGT CGC TTC A-3′. The thermal profile was 94°C for 2 min followed by 34 cycles of 94°C for 1 min, 67°C for 2 min, and 72°C for 3 min. The IGF2-SNP2 polymorphism was detected by digesting 5 μL of the 1,146-bp PCR product with 10 U of the *Hsp 92* II enzyme[11] at 37°C overnight. Genotyping of IGF2-SNP3 was performed by using the SnaPshot ddNTP Primer Extension kit[9] according to the manufacturer instructions. The amplification primers were IGF2-FW and IGF2-REV. The sequence of the extension primer was SNAP, 5′- GGA AAC CAT TGG TGG GGG AG-3′.

Statistical Analysis

Polynomial models with random regression coefficients were used to describe the repeated measurement data. Individual performances for BW and feed intake were analyzed as a third degree polynomial on age. The degree of the polynomial model was chosen according to the re-

[7]MJ Research, Ecogen, S.R.L., Barcelona, Spain.
[8]Ecogen S.R.L, Barcelona, Spain.
[9]Applied Biosystems, Foster City, CA.
[10]Roche Diagnostics S.L, Barcelona, Spain.
[11]Promega, Innogenetics Diagnóstica y Terapéutica S.A., Barcelona, Spain.

TABLE 3. Association between the insulin-like growth factor 1 gene single nucleotide polymorphism 1 (IGF1-SNP1) genotypes and body weight, average daily gain, cumulated feed intake, and feed efficiency at 44, 73, and 107 d in the PN and MN chicken strains[1]

Variable	PN strain (mean ± SE)		MN strain (mean ± SE)
	AA-AB	Dominance effect[2]	AA-AB
Body weight, g			
44 d	23.8 ± 29.9	–20.7 ± 29.6	31.2 ± 29.9
73 d	–37.9 ± 59.8	81.1 ± 58.6	77.0 ± 60.3
107 d	–232.5 ± 131.2	325.4 ± 128.7*	90.9 ± 131.2
Average daily gain, g/d			
44 d	–0.7 ± 1.3	1.7 ± 1.3	3.2 ± 2.6
73 d	–3.7 ± 2.1	5.3 ± 2.0**	5.9 ± 5.3
107 d	–7.9 ± 3.7*	9.0 ± 3.6*	9.0 ± 8.5
Cumulated feed intake, g			
44 d	15.73 ± 27.8	–19.1 ± 27.7	17.7 ± 27.4
73 d	–3.5 ± 61.0	34.6 ± 60.0	27.1 ± 61.0
107 d	–54.5 ± 139.2	163.2 ± 136.5	33.2 ± 140.5
Feed efficiency, g/g			
44 d	–0.003 ± 0.009	0.007 ± 0.009	0.023 ± 0.011*
73 d	–0.030 ± 0.015*	0.037 ± 0.014*	0.014 ± 0.009
107 d	–0.068 ± 0.030*	0.080 ± 0.030**	0.001 ± 0.016

[1]PN = Black Penedesenca paternal chicken strain; MN = Black Penedesenca maternal chicken strain.

[2]Dominance effects were estimated as the difference between the heterozygote and the mean of the two homozygotes. Consequently, dominance effects were not calculated in the MN strain since the BB genotype was missing.

*$P \leq 0.05$.

**$P \leq 0.01$.

sults of a likelihood ratio test. Let Y_{id} denote the observation of animal i at age d (in days). The model was then:

$$Y_{id} = \sum_{j=0}^{j=3} (b_{strain \times genotype,j} + a_{i,j})d^j + e_{id} \quad [1]$$

where $b_{strain \times genotype,j}$ is the fixed effect of strain by genotype interaction associated to the degree j of the polynomial; $a_{i,j}$, is the random effect of animal i associated to the degree j of the polynomial; and $e_{i,d}$ the residual term. Additive genetic values are multivariate normally distributed $N(0,A \otimes V_a)$, in which A is the numerator relationship matrix and V_a is the variance-covariance matrix of the additive effects associated to the degrees of the polynomial ($V_a = V(a_{i0}, a_{i1}, a_{i2}, a_{i3})'$). A two-generation pedigree was considered to calculate A. Animal effects and residuals were assumed to be mutually independent. Thus, variation between individuals is explained in terms of $a_{i,j}$, whereas variation within individuals is done in terms of e_{id}. A univariate mixed model, equivalent to model [1] but doing j = 0, was used to analyze plasma IGF1 concentrations at 73 d. The first derivative of equation [1] for BW, gives the growth rate curves. When BW is modeled substituting d in model (1) by the cumulated feed intake, the first derivative gives FE as a function of feed intake.

All variance-covariance matrices have been estimated by restricted maximum likelihood using the EM algorithm, as applied in the REMLF90 programs (Misztal, 1999). Iterations were performed until the criterion of convergence was less than 1×10^{-7}. The estimated fixed effects and predicted random effects were obtained regarding the estimated variance-covariance parameters as the true parameters. Henderson's mixed model equations were solved by direct inversion of the coefficient matrix. Differences between genotypes (i.e., AA and AB for IGF1-SNP1 marker in strain PN) for trait k (being k a trait with repeated measurements) at age d (H_{kd}) were estimated as follows:

$$H_{kd} = \sum_{j=0}^{j=3} (b_{genotype = AA, strain = PN,j} - b_{genotype = AB, strain = PN, j)}d^j \quad [2]$$

Within strain estimates of the difference between the most abundant homozygote (AA, +/+ or 1/1) and the heterozygote were obtained for all four markers. The dominance effect, estimated using equation [2] as the difference between the heterozygote and the mean of homozygotes, only was calculated when the two homozygotes were available in a strain. Estimation of the standard error of genotype means and H_{kd} were calculated using the (co)variances of the estimated genotype effects. These values were obtained from the inverse coefficient matrix from the associated mixed-model equations. Differences between genotypes within strain were tested using the *t*-test. The Bonferroni adjustment within strain and trait was also performed to correct for multiple tests. Accordingly, the critical *P*-value of 0.05 (suggestive) was corrected to an experiment-wide error level of 0.01 (significant). Strain means and their standard errors were also obtained from equation [2] but only using strain as a fixed effect. The proc FREQ procedure of the SAS software (SAS Institute, 1992) was used to perform chi-square tests aimed to identify the existence of significant differences between strains with regard to genotypic frequencies and significant associations between the allelic segregation of the *IGF1* markers (IGF1-SNP1 and IGF1-SNP4) and between the allelic segregation of the *IGF2* markers (IGF2-SNP2 and IGF2-SNP3).

TABLE 4. Association between the insulin-like growth factor 1 gene single nucleotide polymorphism 4 (IGF1-SNP4) genotypes and body weight, average daily gain, cumulated feed intake, and feed efficiency at 44, 73, and 107 d in the PN chicken strain[1]

	PN strain	
Variable	+/+ – +/– (mean ± SE)	Dominance effect[2]
Body weight, g		
44 d	–25.9 ± 39.3	–28.7 ± 42.3
73 d	17.7 ± 79.2	33.6 ± 84.7
107 d	257.8 ± 223.5	399.5 ± 241.5
Average daily gain, g/d		
44 d	–0.1 ± 1.7	–0.2 ± 1.8
73 d	3.5 ± 2.1	5.2 ± 3.4
107 d	11.18 ± 8.1	17.4 ± 8.9*
Cumulated feed intake, g		
44 d	–17.4 ± 60.2	–8.15 ± 64.5
73 d	12.8 ± 113.1	40.6 ± 120.7
107 d	90.5 ± 193.7	154.1 ± 206.5
Feed efficiency, g/g		
44 d	–0.017 ± 0.023	–0.031 ± 0.015*
73 d	–0.018 ± 0.026	–0.031 ± 0.020
107 d	–0.017 ± 0.029	–0.030 ± 0.027

[1]PN = Black Penedesenca paternal chicken strain. Genotypic differences were not calculated in the Black Penedesenca maternal chicken (MN) strain since all the individuals were +/+.

[2]Dominance effects were estimated as the difference between the heterozygote and the mean of the two homozygotes. Dominance effects were not calculated in the MN strain since all the individuals were +/+.

*$P \leq 0.05$.

RESULTS AND DISCUSSION

Amplification of the Chicken Leptin Gene

Amplification of any region of the chicken leptin gene was unsuccessful in spite of the fact that four different primer combinations were used in a range of magnesium chloride concentrations and annealing temperatures. Even when highly conserved primers complementary to the published chicken sequences and to a broad diversity of mammalian leptin sequences were used, amplification was always abolished or unspecific. The successful amplification of the *IGF1* and *IGF2* cDNA clearly demonstrates that technical procedures and chemical reagents used were not the cause of this problem. Pitel et al. (2000) reported that they were unable to amplify any region of the chicken leptin gene when using a panel of primers derived from the published chicken sequences. Surprisingly, these primers amplified murine and human leptin targets. Friedman-Einat et al. (1999) found similar problems and suggested that the chicken leptin sequences available in the Genbank might be artifactual. Phylogenetic analysis of these sequences, which are unexpectedly highly similar to rodent leptin sequences, gives support to this hypothesis (Dunn

TABLE 5. Association between insulin-like growth factor 2 gene single nucleotide polymorphism 2 (IGF2-SNP2) genotypes and body weight, average daily gain, cumulated feed intake, and feed efficiency at 44, 73, and 107 d in the PN and MN chicken strains[1]

	PN strain (mean ± SE)		MN strain (mean ± SE)	
Variable	AA-AB	Dominance effect[2]	AA-AB	Dominance effect[2]
Body weight				
44 d	26.4 ± 30.0	–9.1 ± 37.0	8.2 ± 29.6	–12.6 ± 33.3
73 d	–108 ± 58.7	–13.3 ± 71.3	–11.7 ± 57.9	–30.6 ± 64.3
107 d	–22.6 ± 179.7	81.7 ± 219.5	–24.0 ± 176.2	–56.3 ± 194.4
Average daily gain, g/d				
44 d	–0.8 ± 1.2	–0.7 ± 1.5	–0.5 ± 1.2	–0.6 ± 1.3
73 d	–1.3 ± 2.5	0.8 ± 3.1	–0.7 ± 2.5	–0.7 ± 2.7
107 d	1.2 ± 6.8	5.3 ± 8.5	0.2 ± 6.6	–0.8 ± 7.3
Cumulated feed intake, g				
44 d	27.3 ± 26.8	–43.6 ± 34.1	–11.2 ± 26.4	–18.4 ± 30.4
73 d	26.1 ± 59.9	–106.5 ± 73.3	–22.0 ± 58.6	–20.9 ± 65.8
107 d	1.5 ± 138.1	–203.7 ± 167.3	–36.6 ± 135.6	–9.8 ± 150.5
Feed efficiency, g/g				
44 d	0.008 ± 0.015	0.002 ± 0.010	–0.004 ± 0.010	–0.007 ± 0.011
73 d	0.003 ± 0.009	–0.001 ± 0.018	0.0002 ± 0.008	–0.009 ± 0.009
107 d	0.009 ± 0.016	–0.006 ± 0.037	0.006 ± 0.016	–0.012 ± 0.017

[1]PN = Black Penedesenca paternal chicken strain; MN = Black Penedesenca maternal chicken strain.

[2]Dominance effects were estimated as the difference between the heterozygote and the mean of the two homozygotes.

TABLE 6. Association between insulin-like growth factor 2 gene single nucleotide polymorphism 3 (IGF2-SNP3) genotypes and body weight, average daily gain, cumulated feed intake, and feed efficiency at 44, 73, and 107 d in the PN and MN chicken strains[1]

	PN strain (mean ± SE)	MN strain (mean ± SE)	
Variable	1/1–1/2	1/1–1/2	Dominance effect[2]
Body weight, g			
44 d	–6.7 ± 41.8	8.2 ± 29.3	–2.7 ± 30.1
73 d	89.1 ± 81.4	39.8 ± 57.7	–5.6 ± 58.5
107 d	252 ± 197	60.2 ± 140.0	–8.7 ± 140.9
Average daily gain, g/d			
44 d	2.2 ± 1.7	0.9 ± 1.2	–0.1 ± 1.2
73 d	4.2 ± 3.0	1.1 ± 2.1	–0.1 ± 2.1
107 d	5.1 ± 6.3	–0.1 ± 4.4	–0.1 ± 4.4
Cumulated feed intake, g			
44 d	78.7 ± 80.2	19.4 ± 55.2	17.6 ± 58.2
73 d	145.3 ± 151.3	47.3 ± 105.1	19.1 ± 109.5
107 d	230.2 ± 284.4	91.7 ± 198.9	8.1 ± 205.5
Feed efficiency, g/g			
44 d	0.006 ± 0.012	0.010 ± 0.010	–0.001 ± 0.010
73 d	0.006 ± 0.020	0.006 ± 0.008	–0.001 ± 0.009
107 d	0.006 ± 0.067	–0.001 ± 0.016	–0.001 ± 0.016

[1]PN = Black Penedesenca paternal chicken strain; MN = Black Penedesenca maternal chicken strain.

[2]Dominance effects were estimated as the difference between the heterozygote and the mean of the two homozygotes. Consequently, dominance effects were not calculated in the PN strain because the 2/2 genotype was missing.

et al., 2001). Moreover, a search of the Biotechnology and Biological Sciences Research Council chicken expressed sequence tag database (http://www.chick.umist.ac.uk) and the University of Delaware Chickest database (http://www.chickset.udel.edu) did not yield any sequence matching the ones described by Taouis et al. (1998) and Ashwell et al. (1999). The elucidation of the genomic structure of the chicken leptin gene and the optimization of one PCR-based protocol yielding consistent results will be essential to solve this controversy and find out why the reported avian sequences are so difficult to amplify.

Identification of Polymorphisms in the Chicken IGF1 and IGF2 Genes

Most of the coding sequence of the *IGF1* and *IGF2* genes and the 5′end of the IGF1 gene have been amplified successfully by using either cDNA or genomic DNA samples. Sequence alignment of the chicken *IGF* sequences revealed the existence of three SNP. One SNP (IGF1-SNP1) was located in the 5′UTR of the *IGF1* gene, near a putative TATA box, and consisted of one A→C substitution (position 570 of the sequence with accession number M74176). This mutation was associated with a *Hinf*I RFLP. The sequence of the 5′end also contained one monomorphic *Hinf*I site (position 191 bp of the sequence with accession number M74176). Digestion of the PCR product yielded two restriction patterns named as A (378/242/173 bp) and B (620/173 bp) (Figure 1A). Moreover, two SNP were found in the *IGF2* sequence. One of them was a neutral substitution C → T at exon 3 (IGF2-SNP2) and could be detected with the *Hsp*92 II restriction enzyme (position 315 of the cDNA sequence with accession number AY267181 and position 943 of the genomic DNA sequence with accession number S82962). Sequence analysis revealed the existence of four additional *Hsp*92 II sites located at intron 2. Digestion of the PCR product yielded two restriction patterns named as A (246/39/138/30/693 bp) and B (246/39/138/30/478/215 bp) (Figure 1B). The second SNP (IGF2-SNP3) was a G → A substitution at intron 2 (position 248 of the sequence with accession number S82962) that could be typed by using primer extension analysis (Figure 1C).

Associations Between Polymorphisms at the Chicken IGF1 and IGF2 Genes and Growth Traits

Three novel SNP (IGF1-SNP1, IGF2-SNP2 and IGF2-SNP3) and the SNP previously described by Nagaraja et al. (2000) (IGF1-SNP4) were typed in 60 individuals from each strain. Genotype frequencies between strains were analyzed by using a chi-square test (Table 1). Significant differences were found between the PN and MN strains for IGF1-SNP4 and IGF2-SNP3 ($P \leq 0.0001$) but not for IGF1-SNP1 or IGF2-SNP2 (Table 1). An association between the allelic segregation of the IGF1-SNP1 and IGF1-SNP4 markers was detected ($P \leq 0.0001$). Mean phenotypic records for the MN and PN strains are presented in Table 2. The results of the association analysis for IGF1-SNP1, IGF1-SNP4, IGF2-SNP2, and IGF2-SNP3 are given in Tables 3, 4, 5, 6, and 7. They are expressed as the difference between the heterozygous and one of the homozygous genotypes. No significant associations were found between the four analyzed polymorphisms and growth and feeding traits. However, suggestive associations ($P \leq 0.05$) were found among IGF1-SNP1 and average daily gain at 107 d and FE at 44, 73, and 107 d (Table 3). These associations were not simultaneously found in both strains and the difference of the genotypic means was negative or positive depending on the strain considered. Significant and sug-

TABLE 7. Association between plasma insulin-like growth factor (IGF) 1 concentration (ng/mL ± SE) at 73 d of age and IGF1-single nucleotide polymorphism (SNP) 1, IGF1-SNP4, IGF2-SNP2, and IGF2-SNP3 genotypes in the the PN and MN chicken strains[1]

Genotype	PN strain	MN strain
IGF1-SNP1		
AA	130.8 ± 13.46	153.7 ± 11.87
AB	106.6 ± 14.06	165.5 ± 14.61
BB	112.2 ± 23.41	—
IGF1-SNP4		
+/+	140.0 ± 15.26	167.7 ± 12.68
+/−	116.2 ± 21.41	—
−/−	110.7 ± 34.95	—
IGF2-SNP2		
AA	117.3 ± 12.98	162.8 ± 12.82
AB	121.0 ± 13.76	162.2 ± 13.68
BB	109.7 ± 37.68	104.2 ± 31.35
IGF2-SNP3		
1/1	119.1 ± 10.24	142.6 ± 14.01
1/2	114.3 ± 26.92	168.0 ± 12.54
2/2	—	172.2 ± 25.98

[1]PN = Black Penedesenca paternal chicken strain; MN = Black Penedesenca maternal chicken strain.

gestive dominant effects were detected in the PN strain when comparing the productive performance of heterozygous and homozygous IGF1-SNP1 genotypes for several parameters, including BW at 107 d ($P \leq 0.05$), average daily gain at 73 d ($P \leq 0.01$) and 107 d ($P \leq 0.05$), and FE at 73 d ($P \leq 0.05$) and 107 d ($P \leq 0.01$) (Table 3). Similarly, suggestive dominant effects ($P \leq 0.05$) were detected in the PN strain for the IGF1-SNP4 and average daily gain at 107 d and FE at 44 d. Previously, Nagaraja et al. (2000) described the existence of significant dominance effects for the IGF1-SNP4 genotype with regard to egg and egg shell weight. The absence of BB (IGF1-SNP1), −/− (IGF1-SNP4), and 2/2 (IGF2-SNP3) individuals did not allow the estimation of the within strain dominance effects for IGF1-SNP1 (MN strain), IGF1-SNP4 (MN strain), and for IGF2-SNP3 (PN strain). This feature, combined with the low frequency of BB chickens (IGF2-SNP2) for both strains, made it difficult to separate the additive and dominant effects and restricted the comparisons to the difference in performance between the most abundant homozygote (AA, +/+, or 1/1) and the heterozygote. No significant association was detected between growth and feeding traits for IGF2-SNP2 and IGF2-SNP3 (Tables 5 and 6). Moreover, plasma IGF1 levels at 73 d did not show any association with *IGF1* or *IGF2* genotypes (Table 7).

The IGF1-SNP1 marker is located in the promoter region of the *IGF1* gene, so the existence of suggestive associations among IGF1-SNP1 and growth and FE might be interpreted in the light of differences in the transcriptional rate of both alleles. In fact, the analysis of the promoter sequence with the TFSEARCH program[12] revealed that the substitution A → C involved the supression of one potential CdxA transcription factor binding site. At present, it is unknown if the loss of this site has any functional significance, since no significant relationship between IGF1-SNP1 genotypes and IGF1 plasma levels was found. Likely, the associations observed among IGF1-SNP1 and average daily gain and FE might have been produced by linkage disequilibrium between these polymorphisms and another mutation located in the *IGF1* locus or another linked gene directly involved in the regulation of these two phenotypic traits. This interpretation is more consistent with the current data because it may explain why no associations were found between IGF1-SNP1 genotypes and growth or intake traits simultaneously in both strains. Additional evidence gives strong support to the linkage hypothesis. In spite of the fact that both strains have been originated from the same Black Penedesenca population, the PN strain was crossed with Cornish chicken before the first generation of selection, thus, it is a mixture of two different genetic backgrounds. Moreover, the MN and PN strains have been selected for different breeding objectives, a feature that may favor the establishment of different allelic combinations positively correlated with the traits under selection. No significant or suggestive associations were found between IGF1-SNP4, which is located 7 kb upstream the *IGF1* promoter (Nagaraja et al. 2000), and growth and feeding traits. These results are consistent with the association analysis reported by Nagaraja et al. (2000), since they did not find significant associations between this SNP and BW or feed consumption.

Although the present results are not conclusive, the existence of suggestive associations between growth and consumption related traits with IGF1-SNP1 might be relevant. In this way, several reports indicate that IGF1 is more intimately linked to posthatch development than IGF2. For instance, Tomas et al. (1998) infused human recombinant IGF1 and IGF2 into three different strains of chickens (fat, lean, and control) and demonstrated that IGF1, but not IGF2, significantly increased growth rate and FE. Similarly, Beccavin et al. (2001) have shown that hepatic IGF1 mRNA levels are different in two chicken strains divergently selected for high or low growth rate, being higher in the high-growth strain. Other authors have reported that IGF1 is mainly involved in fat deposition and lipid metabolism (Huybrechts et al., 1992; Tixier-Boichard et al., 1992; Spencer et al., 1995). The search of mutations in the *IGF1* and *IGF2* coding and regulatory sequences will play an essential role for elucidating the molecular basis of these associations.

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