Table 1 Frequencies of SNP alleles within the *CRP* locus of commercial breeds.

		Allele frequency			
		SNP1 (nt 788)		SNP2 (nt 1271)	
	No. of animals	*T*	*C*	*A*	*G*
German Landrace	23	0.93	0.07	0.04	0.96
Large White	25	0.28	0.72	0.70	0.30
Pietrain	31	0.47	0.53	0.35	0.65

primers was used to screen a panel of 118 hamster-porcine hybrid cell lines. Amplification results were submitted to the IMpRH database (http://imprh.toulouse.inra.fr). The most significantly linked marker (2pt analysis) is *SW286* on chromosome 4 (29 cR; LOD = 13.02).

For genetic mapping of *CRP*, two-point and multipoint procedures of the CRI-MAP package version 2.4 revealed linkage to *S0001* (proximal) and *S0214* (distal) with distances of 35.4 cM (recombination fraction = 0.27, lods = 13.64) and 16.2 cM (recombination fraction = 0.14, lods = 30.58). *Sw286* has been mapped 1 cM proximal of *S0214* (USDA-MARC). Between *S0001* and *CRP* we previously mapped *CRH*[4] (physically allocated to Sscr 4q13[5]) that is 16.1 cM proximal of *CRP* (recombination fraction = 0.13, lods = 30.28).

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Comparison of avian myostatin genes

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Source/description: Myostatin is a member of the transforming growth factor (TGF)-β superfamily and shares all the hallmark features associated with this superfamily.[1] In myostatin gene knock-out mice, the skeletal muscle mass is two to three times greater than that of wild-type mice because of a combination of hyperplasia and hypertrophy.[1] Recent studies have revealed that myostatin function is also correlated with adipose growth in mouse and chicken.[2–4] In order to study the structural and functional roles of myostatin in other avian species, we report here the sequences of myostatin cDNA from duck (*Anas platyrhynchos*), goose (*Anser anser*), pigeon (*Columba livia*) and quail (*Coturnix chinensi*), and 1.2 kb of 5′-genomic sequence from duck, goose and quail.

Primers: Based on mouse and chicken myostatin sequences (GenBank accession numbers AF019621 and AF019627, respectively), primers were designed (sense: 5′-GGAATTCCATATGCAAAAGCTAGCAGTCTATGTT-3′ and antisense 5′-CGCGGATCCCTCATGAGCACCACGATCTAC-3′) for cloning myostatin cDNA from duck, goose, pigeon and quail. Two primers (P84 5′-CTTCAGGTGTGTTCATAATGGAAAAG-3′; P85 5′-GCGATCTGCATGAACAGGTAAATATA-3′) for the avian myostatin 5′-region were designed based on the chicken myostatin sequence (GenBank accession number AF346599).

PCR conditions: Reverse transcription reactions were performed as follows: 1 μg total RNA from avian muscle was reversed transcribed by MMLV Reverse Transcriptase (RT; Clontech, Meadow Circle, Palo Alto, CA, USA) using oligo-dT as the primer. The RT products were subjected to polymerase chain reactions (PCR) (1 cycles of 94 °C for 4 min, 35 cycles of 94 °C for 40 s, 56 °C for 40 s, 72 °C for 40 s, and a final extension of 10 min at 72 °C) in a GeneAmp 9700 thermocycler (Applied Biosystem, Foster City, CA, USA). The PCR was conducted using 0.2 mM of each dNTP, 20 pmol of each primer, and 1 U *Taq* polymerase (Perkin-Elmer, Emeryville, CA, USA) in 1X PCR buffer in a 25 μl volume. The reaction system for amplifying avian myostatin promoter comprised 50 ng genomic DNA, 0.2 mM each dNTP, 0.5 μM each primer, 1.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 1 U *Taq* polymerase (TakaRa Bio Inc., Otsu, Shiga, Japan) in 25 μl. The PCR profile included an initial 5 min denaturation step at 94 °C followed by 35 cycles of 94 °C for 40 s, 55 °C for 40 s, 72 °C for 90 s, and a final extension of 72 °C for 10 min.

Evolutionary analysis: Myostatin protein sequences were aligned by CLUSTAL W[5] with manual adjustment. The nucleotide sequences were then aligned with the protein alignment as a guide. Phylogenetic analysis was conducted using MEGA2.[6] To eliminate the noise caused by saturation of synonymous nucleotide substitution, protein sequences were used to construct the phylogenetic tree using the p-distance. Reliability of the trees was evaluated by the bootstrap method[7] with 1000 replications. To examine the pattern of nucleotide substitution in this gene, the number of synonymous substitutions per synonymous site (dS) and the number of non-synonymous sub-

stitutions per non-synonymous site (dN) were estimated using the modified Nei-Gojobori method.[8] Furthermore, Yang's method[9] was used to detect positive selection for sites with the PAML computer package.

Phylogenetic analysis: Nucleotide sequences for the coding region of myostatin cDNA cloned from duck, goose, pigeon and quail have been deposited into GenBank (accession numbers AF440861, AF440862, AF440863, and AF440864, respectively). Each cDNA encodes a 375 amino acid polypeptide and each polypeptide contains a putative proteolytic processing site, which includes four disulphide bonds at the appropriate positions as observed in other TGF-family members. This high level of sequence conservation among all known myostatin orthologues suggests that the structure and function of each is conserved.

A phylogenetic tree for myostatin was constructed with the neighbor-joining method[10] using the sequences reported herein as well as previously reported sequences (Fig. 1). In this analysis, we divided the myostatin genes into three groups: mammals, birds and fish. The mean non-synonymous (amino acid replacement) distances (dN) and synonymous (silent) distances (dS)[11] were compared separately within each group and among different groups. We found that the mean dN for two different comparisons was significantly smaller than the mean dS in all three groups in comparisons within- and between-groups ($P < 0.01$). It is interesting that the mean dS in both the mammalian group and bird group were similar, but the mean dN in the bird group was significantly smaller than in mammals, suggesting that selection constraints in birds are stronger than those in mammals and that the function of myostatin has been conserved in birds. It is well known that different amino acids sites have different biological functions. Thus, we applied Yang's method[9] to test the evolutionary forces among sites, and the results were consistent with the pairwise comparisons.

Characterization of the avian myostatin gene 5′-region: We cloned 1.2 kb 5′-sequences of the myostatin gene from duck, goose and quail by direct PCR cloning (accession numbers AY329600, AY329601, and AY329602, respectively). Com-

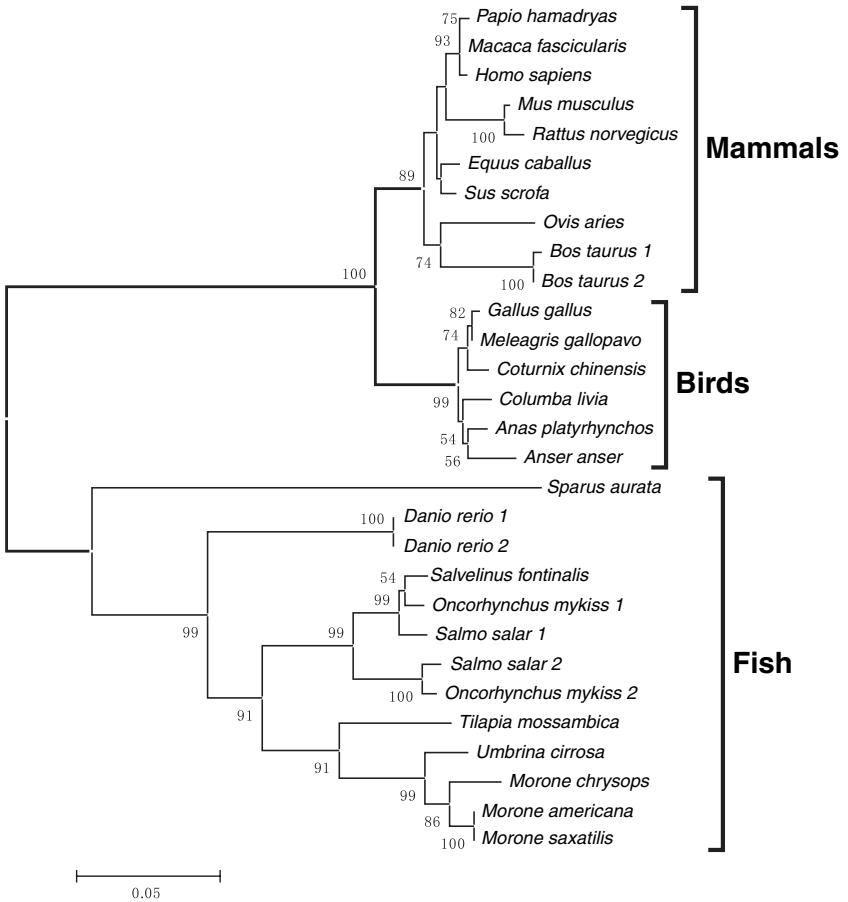

Figure 1 Neighbor-joining tree based on myostatin protein sequences. After the removal of signal peptides and gaps, a total of 337 amino acid were used in constructing the tree. Percentage bootstrap values (50) are shown on interior branches. Protein sequences are from GenBank (accession numbers: *Papio hamadryas* AF019619, *Macaca fascicularis* AY055750, *Homo sapiens* AF019627, *Mus musculus* AY204900, *Rattus norvegicus* AF019624, *Equus caballus* AB033541, *Sus scrofa* AY448008, *Ovis aries* AF019622, *Bos Taurus 1* AF019620, *Bos taurus 2* AY160688, *Gallus gallus* AY448007, *Meleagris gallopavo* AF019625, *Coturnix chinensis* AF440864, *Columba livia* AF440863, *Anas platyrhynchos* AF440861, *Anser anser* AF440862, *Sparus aurata* AF258448, *Danio rerio 1* AF019626, *Danio rerio 2* AY258034, *Salvelinus fontinalis* AF247650, *Oncorhynchus mykiss 1* AF273035, *Salmo salar 1* AJ297267, *Salmo salar 2* AJ344158, *Oncorhynchus mykiss 2* AF273036, *Tilapia mossambica* AF197193, *Umbrina cirrosa* AF316882, *Morone chrysops* AF197194, *Morone americana* AF290911 and *Morone saxatilis* AF290910).

Table 1 Sequence identify among myostatin gene promoter regions.

	Chicken	Quail	Duck	Goose	Human	Bovine	Murine
Chicken	100.00	78.06	61.70	68.89	30.96	25.22	14.51
Quail		100.00	58.13	65.42	33.35	46.20	8.30
Duck			100.00	94.82	41.90	44.13	16.34
Goose				100.00	18.36	47.00	14.33
Human					100.00	69.53	43.10
Bovine						100.00	31.76
Murine							100.00

parison of these sequences with the chicken myostatin gene 5′-sequence showed a high degree of identity (Table 1). The number and location of the E-boxes identified in the chicken and quail myostatin promoter region are well conserved, which is consistent with the high sequence similarity in these promoter regions (78%). Comparison of the bird myostatin gene promoters with mammal (human, mouse and bovine) sequences revealed a lower level of similarity (Table 1). Our analysis demonstrated that the myostatin promoter sequences and regulatory elements among birds and mammals are less conserved. These data may explain the diversity and complexity of regulation of myostatin gene expression among different species during development.

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Supplementary material: The following material is available from: http://www.blackwellpublishing.com/products/journals/suppmat/AGE/AGE1194/AGE1194sm.htm

Figure S1 Nucleotide sequences of the coding region of myostatin cDNA from Duck (*Anas platyrhynchos*), Goose (*Anser anser*), Pigeon (*Columba livia*) and Quail (*Coturnix chirensi*).

Figure S2 Alignment of the deduced amino acid sequences for the myostatin proteins.

Figure S3 Comparison of E-box locations in avian myostatin gene promoter regions.

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Radiation hybrid mapping of four genes (*MYBPC1*, *LUM*, *ZRF1* and *ATP2B4*) expressed in embryo skeleton muscle to pig chromosomes 5 and 9

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Source/description: MYBPC1 encodes the slow type isoform 1 of myosin-binding protein C, slow type.[1] LUM is a keratan sulphate proteoglycan found in connective tissue including the interstitial collageneous matrices of skeletal muscle.[2] *ZRF1* encodes zuotin-related factor 1 also known as M-phase phosphoprotein 11.[3,4] ATP2B4 (ATPase, Ca++ transporting, plasma membrane 4) is one of a family of ion transport ATPase that plays an important role in intracellular calcium homeostasis.[5] The cDNA clones encoding pig *MYBPC1*, *LUM*, *ZRF1* and *ATP2B4* were identified in a cDNA library constructed from pig embryonic skeletal muscle mRNA in our laboratory.

The identity of these cDNAs was determined by sequence analysis and BLAST searches of the GenBank non-redundant (nr) sequence database (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The degree of sequence identity between the pig cDNA sequences and the putative human homologues was as follows: *MYBPC1* (AY654607, NM_002465) 92%; *LUM* (AY654606, NM_002345) 90%; *ZRF1* (AY654608, XM_168590) 93%; *ATP2B4* (AY654605, NM_001684) 79%.

Primer designation, PCR condition and sequencing: Primers were designed from the pig cDNA sequences in order to amplify the corresponding genomic sequences. The amplified products were obtained in 20 μl reaction volume consisting of 50 ng of porcine genomic DNA, 1X polymerase chain reaction (PCR) buffer, 0.3 μM of each primer, 75 μM of each dNTPs, 2.0 mM $MgCl_2$ and 2 U *Taq* DNA polymerase (Promega, Madison, WI, USA). The PCR conditions were as follows: 95 °C for 5 min and 31 cycles of 94 °C for 30 s, 58–60 °C (Table 1) for 30 s, and 72 °C for 20 s, followed by a further 5-min extension at 72 °C. The PCR products were then examined by electrophoresis through 1.5% agarose gel with 1X TAE buffer. The gels were stained with ethidium bromide and photographed. The PCR products were purified with Wizard prep PCR purification system (Promega) and sequenced. The primer sequences, annealing temperature and expected product sizes are shown in Table 1.The identities of the PCR products amplified from genomic DNA were confirmed by sequence analysis and new primers designed for mapping *MYBPC1* (Table 1).

Chromosomal location: The four genes (*MYBPC*, *LUM*, *ZRF1*, *ATP2B4*) were mapped using a whole genome porcine radi-