CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental animals

Data taken from 310 F_2 animals of a cross breed between Duroc and Pietrain pig (DUPI resource population, University of Bonn) (Figure 3.1) were used in this study.

All animals were kept and performance tested at the research farm Frankenforst, Institute of Animal Science, University of Bonn, Germany. The animals were exposed to uniform environmental conditions and were given an *ad libitum* diet containing 16% crude protein, 1% lysine, 0.6% (methionine and cystine) 0.6% threonine and 13 MJ metabolizable energy during the whole testing period. Immediately postmortem left femoral and humerus bones were dissected. Articular joints were dissected from both proximal and distal femoral and humerus and were kept at -20 for histopathological screening of osteochondrosis lesion. Moreover, bone mineral density, content and area were measured by DXA instrument and done by Lehr- und Versuchsgut Oberschle ißheimder Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München. The phenotype data used in this study are given in Table 3.1.

Table 3.1 Phenotype data number of animals, mean, minimum and maximum

Traits	Total				Male				Female			
	N	Mean	Min	Max	N	Mean	Min	Max	N	Mean	Min	Max
OC Score at shoulder joint (VLC)	278	1.7769	1.000	4.000	134	1.8134	1.000	4.000	144	1.7430	1.000	4.000
OC Score at elbow joint (VLK)	279	1.8207	1.000	4.000	134	1.8955	1.000	4.000	145	1.7517	1.000	4.000
OC Score at hip joint (HLC)	274	1.9817	1.000	4.000	128	2.0625	1.000	4.000	146	1.9109	1.000	4.000
OC Score at knee joint (HLK)	277	2.5884	1.000	4.000	136	2.7426	1.000	4.000	141	2.4397	1.000	4.000
Total OC Score	237	8.2109	4.000	13.00	114	8.5350	4.000	13.00	123	7.9105	4.000	13.00
Bone mineral density (BMD)	275	0.9586	0.688	1.2540	130	0.9525	0.7850	1.172	145	0.9640	0.688	1.254
Bone mineral content (BMC)	275	66.7186	45.526	87.3570	130	66.3776	45.526	83.292	145	67.0244	48.418	87.357
Bone mineral area (BMA)	275	69.6714	55.908	84.6360	130	69.7542	55.908	84.636	145	69.5972	57.355	83.290

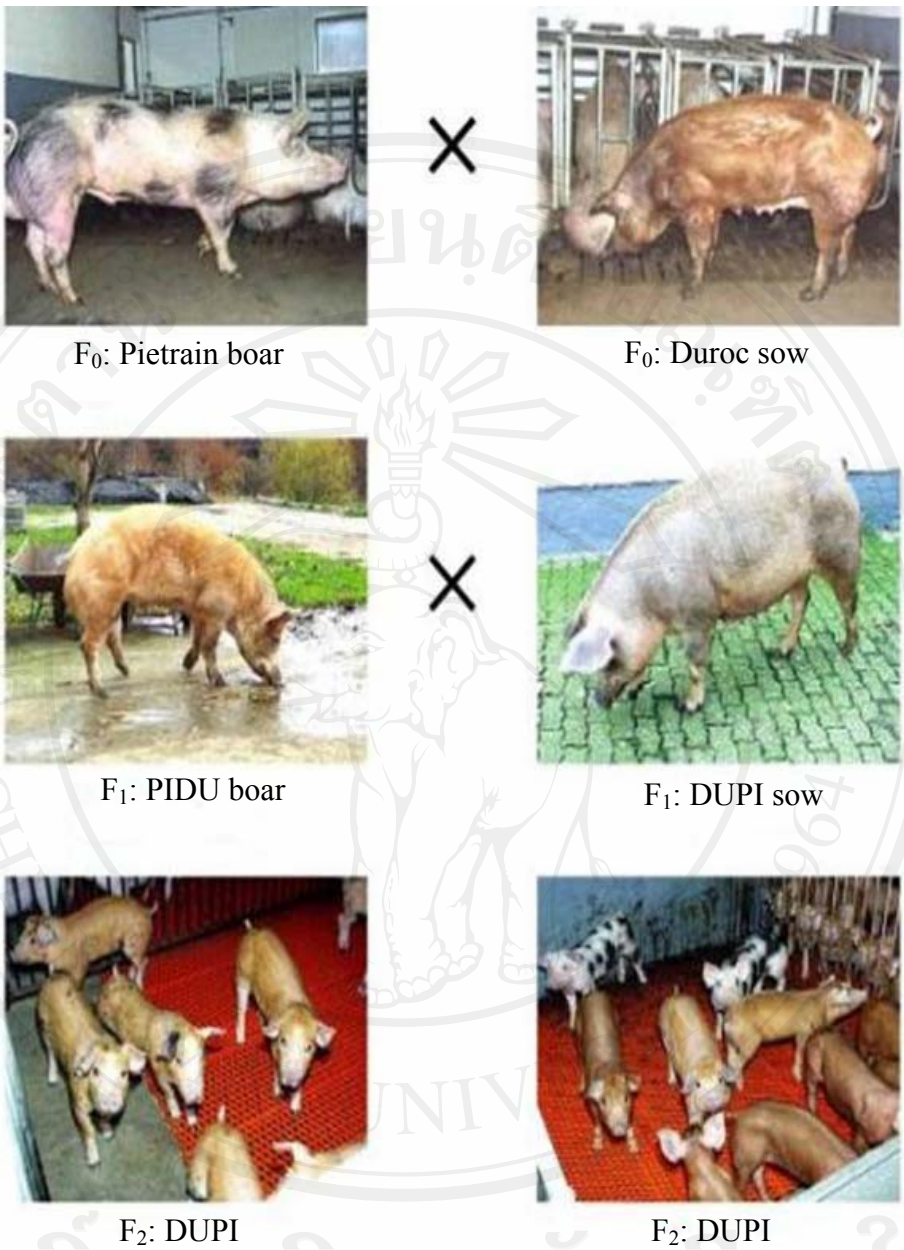

Figure 3.1 Structure of Duroc x Pietrain resource population

3.2 Chemicals and equipments

3.2.1 Chemicals and kits

1. Acetic acid (glacial) (Amersham Bioscience, Germany)
2. Agarose (ultra pure) (Merck, Germany)
3. Agar-Agar (Roth, Germany)
4. Ampicillin (Roth, Germany)

5. Boric acid (Roth, Germany)
6. Bromophenol blue (Sigma, USA)
7. dNTPs (Fermentas, USA)
8. E. coli XL1-blue (Promega Germany)
9. EDTA (Roth, Germany)
10. Ethidium bromide (Roth, Germany)
11. Exo-SAP-IT (USB Coporation, USA)
12. GenomeLab™ DNA Size Standard 80 (Beckman Coulter, USA)
13. GenomeLab™ DTCS Quick Start Kit (Beckman Coulter, USA)
14. GenomeLab™ SNP-Primer Extension Kit (Beckman Coulter, USA)
15. Glycerol (Roth, Germany)
16. Glycerin (Merck, Germany)
17. HCl (Roth, Germany)
18. iTaq^{TM}SYBR Green supermix with ROX (Bio-Rad, Germany)
19. Isopropanol (Roth, Germany)
20. KCl (Roth, Germany)
21. KH_2PO_4 (Roth, Germany)
22. KOH (Roth, Germany)
23. NaCl (Roth, Germany)
24. Proteinase K (Qiagen, Germany)
25. RNeasy Kit (Qiagen, Hilden, Germany)
26. Shrimp Alkaline Phospatase (SAP) (USB Corporation, USA)
27. Sodium dodecyl sulphate (SDS) (Roth, Germany)
28. Taq DNA polymerase (New England Biolabs Inc., USA)
29. Tetracycline (Roth, Germany)
30. Tris (hydroxymethyl) aminomethane (Roth, Germany)
31. TRI Reagent (Sigma-Aldrich, Munich, Germany)
32. Tween 20 (Roth, Germany)
33. X-Gal (Roth, Germany)
34. Yeast extract (Roth, Germany)
35. 10 X PCR reaction buffer (Sigma-Aldrich Chemie GmbH, Germany)

3.2.2 Equipments

1. ABI Prism® 7000 thermal cycler (Foster City, CA, USA)
2. CEQ™ 8000 genetic analysis system (Beckman Coulter, Fullerton, CA, USA)
3. Centrifuge Hermle (Hermle Z 233MK, Wehingen)
4. Centrifuge Hermle (Hermle Z 323K, Wehingen)
5. Electrophoresis for agarose gel (BioRad, Germany)
6. Incubator (Memmert)
7. Millipore ®Milli Q system (Millipore Corporation, USA)
8. pH Meter (Kohermann)
9. Power supply PAC 3000 (BioRad, Germany)
10. PCR thermal cycler Bio-Rad I cycler (Bio-Rad, USA)
11. PCR thermal cycler (PTC-100™) (MJ Research, Inc. U.S.A)
12. Spectrophotometer Ultrospec™ 2100 (Amersham Bioscience, Freiburg, Germany)
13. UV Transilluminator (Uvi-tec) (Uni Equip, Martinsried, Germany)

3.2.4 Used software

1. BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/)
2. DNA alignment (http://searchlauncher.bcm.tmc.edu/)
3. FBAT statistic analysis(http://www.biostat.harvard.edu/~fbat/fbat.htm)
4. Multiple sequence alignment (http://pbil.ibcp.fr/htm/index.php)
5. NCBI (http://www.ncbi.nlm.nih.gov/)
6. Primer 3(http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)
7. SAS package version 9.1 (SAS Institute Inc., Cary, NC)

3.3 Methods

3.3.1 Identification of nucleotide sequence and polymorphisms in porcine *MGP* gene

3.3.1.1 DNA isolation

Hundred mg of sample tissue were cut into small pieces and placed in a 1.5 ml tube, after that 700 µl of digestion buffer, 70 µl of 10% SDS and 18 µl of proteinase K were added. The mixture was incubated at 55^{o}C in shaker water bath for overnight. Completely digested tissue resulted in a viscous homogeneous solution. To extract DNA, 700 µl of phenol-chloroform was added and gently mixed by several inversions until an emulsion formed. The mixture was separated into 3 phases after centrifugation at 10,000 rpm for 10 min, a lower phenol-chloroform phase, an interphase of precipitated protein and an upper phase containing DNA. The aqueous phase was transferred to another 2 ml tube followed by an addition of 700 µl chloroform and the centrifugation at 10,000 rpm for 10 min. For DNA precipitation, the DNA phase was transferred to a fresh tube and mixed with 700 µl of isopropanol and 70 µl of sodium acetate. After centrifuging at 10,000 rpm for 5 min, a DNA pellet was collected and the supernatant was removed. The pellet was washed with 200 µl of 70% ethanol for the removal of excess salt. In the final step, the pellet was dissolved in 200-500 µl of 1x TA buffer. DNA concentration and integrity was evaluated by a spectrophotometer. The working solution of DNA was prepared by diluting stock DNA in 1x TA buffer to the concentration 50 ng/µl. Stock DNA solution was stored at -20^{o}C and the working solution was kept at 4^{o}C.

3.3.1.2 Polymerase chain reaction (PCR)

Primers (Table 3.2) were designed from porcine *MGP* mRNA (GenBank Accession no. AF525316.1). PCR amplification was performed in 25 µl reaction volume in 1X PCR buffer, containing 100 ng genomic DNA, 0.2 µM of each primer, 50 µM of each dNTP and 0.5 U of Taq polymerase. The PCR reaction was carried out in a PCR thermocycler and the thermal cycling program with the following PCR cycling conditions.

Initiate denature	94°C 5 min	
Denature	94°C 30 sec	35 cycles
Annealing	60-65°C 30 sec	
Extension	72°C 1 min	
Final extension	72°C 5 min	

Five microliters of PCR product were electrophoresis in 1.5% (w/v) agarose gels in 1X TAE containing ethidium bromide.

Table 3.2 Primer sequences used for amplification of *MGP* gene

Primer name	**Primer sequence 5' ⇒ 3'**	**Annealing temperature**
MGP F1	AGACCCTGAGAGCAACCTCA	60.0
MGP R2	TTCATAGGATTCCAAGCTCTCA	
MGP In1F	CCAGGGAGAGGACTGTCTCA	60.0
MGP R2	TTCATAGGATTCCAAGCTCTCA	
MGP W-F1	CTGGAGCCAGTGGTTTCTGT	65.0
MGP W-R1	TCTGTGTGGTTGATGTGGTG	
MGP F2	GCCACAGCAGAGATGGAGA	60.0
MGP R1	CCATGGCACTTTCATTCCTT	
MGP W-F1	CTGGAGCCAGTGGTTTCTGT	60.0
MGP Ex2R	GCTTTCGCTCTCCATCTCTG	
MGP F2	GCCACAGCAGAGATGGAGA	60.0
MGP R3	AAATAACGATTGTAGGCGGCA	

3.3.1.3 Purification of PCR products

ExoSAP-IT™ was used for fast and efficient purification of PCR products for sequencing. ExoSAP-IT consists of two hydrolytic enzymes, exonuclease I and Shrimp Alkaline Phosphates (SAP), in a specially formulated buffer for the removal

of unwanted primers and dNTPs from a PCR product mixture with no interference in sequencing. For purification of PCR product, a mixture of 1 µl of ExoSAP-IT was added to 5 µl of PCR product and incubated at 37°C for 30 min followed by inactivation step at 80°C for 15 min.

3.3.1.4 Ligation

The PCR fragments were ligated with the pGEM®-T vector (Promega). The ligation reaction was performed in 5µl reaction volume containing 2.5 µl ligation buffers, 0.5 µl vectors, 0.5 µl T4 DNA ligase (3 U/µl) and 1.5 µl DNA templates. The reaction was overnight incubated at 4 °C or 20 °C for 2 h.

3.3.1.5 Transformation

For cloning of PCR fragments, 3 µl of the ligation reaction was co-incubated with 60 to 80 µl of DH5α *E. coli* competent cells (Stratagen) for 25 min on ice. The mixture was heat shocked by putting it into a 42 °C water bath for 60 second and immediately transferred on ice for 2 min. SOC medium (750 ml) was added to the bacteria solution and shaken at 120 rpm at 37 °C for 90 min. Each bacterial suspension was plated on two ampicillin containing LB-agar plates. The medium was containing 20 µl X-gal and an IPTG solution was overnight incubated at 37 °C. Colonies were differentiated by the activity of β-galactosidase as white and blue for the presence of inserted DNA fragment. Due to the activation of LacZ gene on the vector, colonies containing the insert target DNA appeared as white colonies and those with active LacZ gene without insert DNA formed blue colonies. To identify and screen insert target DNA fragment, two white colonies were picked up from each plate and suspended in 30 µl 1X PCR buffer (Sigma). One blue colony was picked up as a control standard to differentiate the presence of the target insert by comparing the length of amplified DNA fragments from white and blue colonies. M13 primer was used for amplified and PCR products were subject to sequencing.

3.3.1.6 Comparative sequencing

Polymorphisms in the porcine MGP gene were detected by a direct comparative sequencing. Prior to the sequencing procedure, PCR with specific primer was performed and 5 µl of the PCR product was checked on 2% agarose gel. A sharp band visualized under UV/transilluminator showed primer specifity and the PCR products were ready to be cleaned up. Sequencing was done by SEQ 8000 Genetic Analysis System (Beckman coulter) using CEQ Dye Terminator Cycle sequencing (DTCS) Quick start kit. The sequencing PCR reaction was done according to the recommendation of the company with minor modification as follows: To clean PCR product subject to sequencing PCR, 2 µl of either forward or reverse primer, 4 µl DTCS master mix (DNA polymerase, pyrophosphates, buffer, dNTPs, and dye terminators) and 8 µl of Millipore H_2O were added. The sequencing PCR was done for 30 cycles at 96°C for 20 sec, 50°C for 20 sec and 60°C for 4 min, followed by holding at 4°C. Precipitation and holding the samples into CEQ sample plate was performed according to the instruction in the kit protocol. Briefly, 2 µl of 3M Sodium Acetate (pH 5.2), 2 µl of 100 mM EDTA (pH 8.0) and 1 µl of glycogen (20 mg / ml) was prepared. To each of the labeled tubes, 5 µl of the stop solution and 60 µl cold 99% ethanol was added, mixed thoroughly and immediately centrifuged at 18,000 rpm at 4°C for 20 min. The supernatant was removed and the pellet was rinsed twice with 200 µl cold 70 % ethanol. For each rinse, a centrifugation was immediately applied at 18,000 rpm at 4°C for 5 min. After that, the supernatant was removed and the pellet was air dried (or vacuum dried) for 10 min. Finally, the pellet was resuspended with 40 µl of sample loading solution (SLS) (Beckman Coulter) and subject to sequencing. Samples were transferred to a CEQ sample plate and overlaid with mineral oil at the mean time, the separation buffer was prepared in another plate and both plates were loaded into the instrument and started the desired method in the CEQ™ 8000 Genetic Analysis System.

The completed sequence of the fragment was utilized to search for homologous sequences in GenBank data base using BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence similarities were considered to be significant when identity percentage was ≥ 90%. Pool DNA form 8 individuals was

subject to sequence to screen for polymorphisms and if polymorphisms were detected, DNA form individual animals were sequence.

3.3.2 Isolation of RNA and gene expression analysis

3.3.2.1 RNA isolation

Articular cartilage tissue samples of six OC and six healthy pigs were used for RNA isolation. 100 mg of samples were powdered by mortar and pestle, 1 ml of TRI® reagent was added and homogenized. The homogenized samples were incubated for 5 min at room temperature. Then 0.2 ml of chloroform was added to homogenized samples, mixed thoroughly by shaking and incubated for 15 min at 15 – 30 °C. Samples were centrifuged at 12,000 rpm for 15 min at 2 to 8 °C. The RNA remains exclusively in the upper aqueous phase which was transferred to a fresh tube. The RNA in the aqueous phase was precipitated by adding 0.5 ml of isopropyl alcohol. The samples were incubated at room at room temperature for 10 min and centrifuged at 12,000 rpm for 10 min at 2 to 8 °C. The supernatant was removed and the RNA pellet was washed once with 1 ml of 75 % ethanol. After centrifugation and removal of supernatant, the RNA pellet was air dried and dissolved in 50 µl RNase-free water and stored at –80 °C for further use. The RNA was treated with DNase to remove residual DNA. The DNA digestion was performed by mixing the following components in a 0.5 ml RNase free tube: 25 µl RNA, 5 µl 10X buffer, 2 µl RQ1 RNase-Free DNase, 1 µl RNase inhibitor (40 U/µl), and 7 µl RNase-free water and incubated at 37 °C for 1 hour. After incubation, the RNA was purified by RNeasy Mini Kit (Qiagen). To assess the quality of RNA, 2 µl RNA sample was electrophoresed in duplication on 1.2% agarose gel. RNA was checked for any DNA contamination by performing PCR with GADPH housekeeping gene. The purified RNA was used to synthesize cDNA.

3.3.2.2 cDNA synthesis

The total RNA was used to synthesize first strand cDNA. The reaction was performed in a nuclease-free microcentrifuge tube, 1 µl oligo dT (11)N (500 µg/ml), 1 ng to 5 ng total RNA, 1 µl 10 mM dNTP mix and 12 µl sterile water was added in 0.2 ml PCR tube. The mixture was heated at 70 °C for 10 min and immediately chilled on ice. The contents of the tube were collected in bottom by brief centrifugation and 4 µl 5 X first-strand Buffer (Gibco BRL, Karlsruhe Germany) and 2 µl 0.1 M DTT was added. The reaction was gently mixed and incubated at 40 °C for 2 min. One microlitre (200 units) of Superscript™ II RNase H reverse transcriptase was added and further incubated at 42 °C for 90 min. The reaction was inactivated by heating at 70 °C for 15 min. The cDNA was diluted 1:4 in RNase free water and kept at -20°C for subsequent analysis.

Following the relative standard curve method, the quantitative real-time gene expression analysis was performed using an ABI PRISM® 7000 sequence detection systems thermo controller. SYBR® Green (Bio-Rad) was used as a double-strand DNA-specific fluorescent dye. Analyses were made using the ABI PRISM® 7000 SDS- CTS 1.0 software and results were analyzed by SAS. Quantification compares the point of dye fluorescence of an established standard curve with known molecules copy number against the unknown sample. To prepare an absolute standard curve, the double stranded DNA amplicon standard method was applied (Bustin 2000, Bustin et al. 2005). The method involves subcloning of the PCR amplicon within the T7-SP6 RNA polymerase promoters of the pGEM®-T (Promega) plasmid vector. The target C_T was compared directly with the calibrator C_T and was recorded as containing either high or low amplicon, which is relative to the mRNA. 2 genes GADPH and TBP were used as internal control.

3.3.3 Screening for polymorphisms in candidate genes

To identify polymorphisms of the porcine *TGF-β1, MMP3, COL2A1* and *COL10A1* genes, primers for PCR amplification were designed based on published sequence from GenBank (http://www.ncbi.nlm.nih.gov/). Genomic DNA from F1 resource population (Doruc x Pietrain) was used as DNA templates for PCR

amplification. PCR reactions were performed using the standard PCR protocol. The sequences of primers and annealing temperatures were shown in Table 3.3. The DNA sequences of pig were analyzed with software BLAST (Altschul *et al*., 1997) or software Multiple Sequence Alignment to find out the polymorphisms within these sequences.

3.3.4 Genotyping by multiplex reaction

3.3.4.1 Multiplex PCR

The primers for multiplex PCR were contained the SNPs site that shown in table 3.4. Multiplex PCR was performed in a total volume of 25 µl solution containing 50 ng genomic DNA, 0.2 µmol/l of each primer, 50µM of each dNTPs, 0.5 U of Taq polymerase and 1.5 mM $MgCl_2$ in 10X PCR buffer. In these experiments was used the following PCR cycling conditions:

Initiate denature	94°C 5 min	
Denature	94°C 30 sec	35 cycles
Annealing	60°C 1 min	
Extension	72°C 1 min	
Final extension	72°C 5 min	

DNA amplification for individuals, studied in present work, was done as described in single PCR cycling reactions. All PCR-products were checked by electrophoresis on 2% agarose gels. The PCR products have to be further processed to remove all the excess primer and dNTP before proceeding with the SNP reaction.

Table 3.3 The primer sequence used for SNPs screening in candidate genes

Primer name	Forward primer sequence (5' ⇒ 3')	Reverse primer sequence (5' ⇒ 3')	Anealing temperature (°C)	Accession number
TGFB1 F1/R1	CTACTCATCCATCTGAGTG	GAAGCAGTAGTTGGTATCCA	59	AF461808-9
TGFB1 In6 F/R	GTGCGGCAGCTCTACATTGACT	TTGCGGCCCACGTAGTACAC	59	AF461808
MMP3 F1/R1	GACGGGAAAGCTGGATTCTA	CCAGGTGCATAGGCATGAG	64	AF201725
MMP3 F2/R2	GGCCACCTCTTCCTTCAAGT	AACCCAGGGTGTGGATACTTC	64	AF069641
COL2A1 F1/R1	CAAAGATGGCGAGACAGGTG	CAGATGGCCCAGGAGCAC	65	AF201724
COL2A1 F2/R2	AAGGTGGGAAACCAGGTGAT	AGGAGCTCCAGCTTCACCAG	64	AF201724
COL10A1 F1/R1	TACTGCAGGACAGCTCTGGA	GAAACACACAAACTGTCATTGGA	61	AF222861
COL10A1 F2/R2	TTTCAGTCTACACCAAAGATGTGA	AACAACACACTAGTTAATGGAACAGA	61	AF222861
COL10A1 F2/R2	CCAACCAGGGAGTAACAGGA	TTTATACAGGCCCACCCAAG	60	AF222861
COL10A1 F4/R4	TTCAGCCTACCTCCATATGCAT	CAACAGCACTACGACCC	57	AF222861

Table 3.4 The primer sequence for multiplex PCR and genotyping

Primer name	Primer sequence 5' ⇒ 3'	Fragment (bp)	SNP site
MGP F2/R1	Fw: GCCACAGCAGAGATGGAGA Rw: CCATGGCACTTTCATTCCTT	1161	SNP1
MGP W-F1/W-R1	Fw: CTGGAGCCAGTGGTTTCTGT Rw: TCTGTGTGGTTGATGTGGTG	1088	SNP2, SNP8
MMP3 F1/R1	Fw: GACGGGAAAGCTGGATTCTA Rw: CCAGGTGCATAGGCATGAG	434	SNP3
COL2A1 F1/R1	Fw: CAAAGATGGCGAGACAGGTG Rw: CAGATGGCCCAGGAGCAC	397	SNP4, SNP5
TGFB1 F1/R1	Fw: CTACTCATCCATCTGAGTG Rw: GAAGCAGTAGTTGGTATCCA	218	SNP6
COL10A1 F4/R4	Fw: TTCAGCCTACCTCCATATGCAT Rw: CAACAGCACTACGACCC	400	SNP7
TGFB1 In6 F/R	Fw: GTGCGGCAGCTCTACATTGACT Rw: TTGCGGCCCACGTAGTACAC	1083	SNP9, SNP10

3.3.4.2 SNP primer design

SNP primers (Table 3.5) were designed using the same considerations as mentioned of PCR primer. SNP primers can sometimes be difficult to design using the ideal parameters because the locus is fixed. There are only two choices in designing the primers – either on the forward strand or the reverse strand. Length of primers starting at 20 nt. The Tm range should be around 60-75°C. For the multiplex reactions, there should be at least 5 nt between each primer, a poly A tail should be added to separate the different SNPs. The poly A tail is added to the 5' end of the primer. In a panel with more than 7 primers, the interrogation primers should be spaced out by 6 nts.

Table 3.5 SNP primers used in multiplex genotyping reaction

SNPs symbol	SNP (5'→3')	SNP location	Primer sequence	Primer size (bp)	SNP locus tag (bp)	SNP name
MGP:C1124T	G/A	Intron1	3'-GGCTGGGCAGCTGGTTCAGCT-5'	21	18	SNP8
MGP:C1185A	C/A	Intron1	5'-(A)4 GCCTGTTTTCCAGACCCTTTGTTC-3'	28	24	SNP2
MGP:C3817T	C/T	Exon4	5'-(A)9 GCTATGCCATGGTTTATGGATACAA-3'	34	31	SNP1
MMP3:C158T	C/T	Intron2	3'-(A)12 TCCTGTGATAGTAATTAATAAAATCTCA-5'	40	36	SNP3
COL2A:C81T	C/T	Intron29	5'-(A)25 CTGTCCAGGTGGCCAACGGGC-3'	46	41	SNP4
COL2A1:G156A	G/A	Intron29	5'-(A)35 TGTTGATCTGGGATGCCTGAGCC-3'	58	55	SNP5
COL10A1:G103A	G/A	Exon1	5'-(A)44 CAGCTGGAGCCATACCTGGTC-3'	65	61	SNP7
TGFβ1:A797G	C/T	Exon5	3'-(A)31 GCTGTGCAGGTGCTGGGCCCT-5'	52	48	SNP6
TGFβ1-B	G/A	Intron6	5'-(A)52 GGCGGGGATGGGGGAAGCAG-3'	72	69	SNP9
TGFβ1-C	C/T	Intron6	5'-(A)59 CGACCCTGACCCCGTCCACC-3'	79	75	SNP10

3.3.4.3 Multiplex SNP primer reaction

The SNP determination assay was developed using the CEQ SNP primer extension kit (part no. 390280; Beckman Coulter) and a Beckman Coulter CEQ8000 genetic analyzer. The SNP primer extension utilizes the annealing of a regular synthesized DNA primer one base short of the SNP site to DNA template. In the presence of dye-labeled ddNTP, DNA polymerase was added the complementary dye terminator to the 3' end of DNA primer corresponding to SNP site. A SNP-primer extension premix was prepared by mixing 210 µl each of 10X buffer, ddUTP, ddGTP, ddCTP, and ddATP and 105 µl of polymerase. Multiplex SNP primer extension was performed in a total volume of 20 µl was shown below the table 3.6. The extension reaction was carried out using the following thermal cycling conditions:

Initiate denature	96°C 1 min	
Denature	96°C 20 sec	28 cycles
Annealing	64°C 10 sec	
Extension	72°C 30 sec	
Hold on at	4°C	

Following thermal cycling, the extended product was treated with shrimp alkaline phosphatase (SAP) to remove unincorporated dideoxynucleoside triphosphates. In a separate tube, 1.5 U SAP and 2 µl deionized water were mixed with 1.5 µl of the primer extension reaction mix. This mix was then incubated at 37°C for 60 min, followed by 80°C for 15 min in a thermocycler.

Table 3.6 SNP primer extension components using CEQ SNP-Primer Extension Kit

Components	Volume (µl)
SNP-Primer Extension Premix	11.0
SNP primer(s) 1.0 µM	1.0
Template(s) 100 nM	1.0
Water	7.0
Total	**20.0**

3.3.4.4 SNP scorings

In order to detect extended products on the CEQ8000 genetic analyzer, an aliquot of the cleaned reaction mixture was diluted 1:10 in deionized water. A 0.5 µl aliquot of this dilution was added to 39 µl of Beckman SLS and 0.1 µl of CEQ DNA Size Standard Kit 80 in Beckman 96-well plates. The mixes were covered with a drop of mineral oil and immediately processed using the Beckman standard run method SNP-1 (denaturation at 90°C; injection time, 30 s; injection voltage, 2 kV; run temperature, 50°C; run current, 6.0 kV; run time, 16 min). SNP locus tags were defined on the basis of the midpoint of migration variability seen during optimization, and SNPs were called automatically using default SNP analysis parameters in the Beckman software (slope threshold, 10; relative peak height threshold, 10; dye mobility calibration, SNP version 1).

3.4 Statistical model and association analysis between the markers and osteochondrosis trait

The statistical model used in this study was:

$$Y_{ijk} = \mu + sex_i + family_j + slaughter\ weight_k + e_{ijk}$$

The model used for association analysis included fixed effects which showed significant effects on the traits in the variance analysis. Different linear models were applied to the data to test for any effects on the traits (SAS). Slaughter weights were included as fix effect. Then, association of SNP was tested for the derived residuals from the linear model with different bone-related traits using the family-based

association test (FBAT) (Horvath *et al.*, 2001). The FBAT statistics is based on a linear combination of offspring genotypes and traits; it is calculated under the null hypothesis of no association and no linkage, conditioning on traits and on parental genotypes. The quantitative analyses were done under the condition of an additive model (Horvarth *et al*., 2001). Significant results were determined by *P*–value less than or equal to 0.05 for the disease allele.