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Genetic polymorphism and expression analysis of leptin gene in rabbit

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ABSTRACT

Leptin plays an important role in the regulation of feed intake, energy metabolism, growth and reproduction of rabbit. We used the polymerase chain reaction- single stranded confirmation polymorphism (PCR-SSCP) technique to screen for DNA polymorphisms of the leptin gene in 150 New Zealand White rabbits. In all the New Zealand White rabbits, we amplified different regions of the leptin gene. PCR-SSCP of all these fragments revealed monomorphic pattern. All the amplified fragments of leptin gene were monomorphic, which is in contradiction to many earlier reports of mammalian species. All the fragments were sequenced and compared with the available sequences of different species. The nucleotide sequence analysis indicated that the per cent similarity of leptin gene fragments of New Zealand White rabbit with rat, mouse, cattle and pig was 79.6–87.5%. At various places, the amino acids and protein were different in New Zealand White rabbit as compared to rat, mouse, cattle and pig. For relative quantification, total RNA was isolated from liver tissues collected at 2 different stages of growth i.e. pre-weaning and post-weaning. Our results indicate no significant differences in the mRNA expression of leptin between two different stages of growth.

Key words: DNA polymorphism, Expression analysis, Growth, Leptin gene, Rabbit

Growth is an important economic trait for rabbit farming, which has a vast potential to improve the socio-economic status of the rural and urban poor and to provide food security in terms of low cost animal protein. Leptin, a hormone secreted from adipose tissue, plays a vital role in regulation of haematopoiesis, angiogenesis, wound healing, immune inflammatory response, fetal development, growth and reproduction. Leptin genotypes have significant effect on growth traits in cattle (Dandapat *et al*. 2009). Leptin, a protein, plays an important role in the regulation of feed intake, energy metabolism, growth and reproduction of cattle (Ramsay and Cranwell 1999) and thus the leptin gene is a potential candidate gene for QTL studies. Many polymorphic studies and characterization of bovine leptin gene are available (Pomp *et al*. 1997, Lien *et al*. 1997, Wilkins and Davey, 1997, Konfortov *et al*. 1999, Haegeman *et al*. 2000, Buchanan *et al*. 2002, Dubey *et al.* 2012). To our knowledge there is no reported polymorphic study involving New Zealand white rabbit *(Oryctolagus cunniculus*). In a study about growth traits of rabbits, low to moderate heritability was reported (Dige *et al.* 2012). The objective of the present investigation was to study genetic variations and expression analysis in the leptin gene of New Zealand white rabbit and its association with growth traits.

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MATERIALS AND METHODS

Experimental animals for PCR-SSCP analysis: The present study was conducted on New Zealand White rabbits maintained at Laboratory Animal Resource, Indian Veterinary Research Institute, Izatnagar. New Zealand White rabbits (150) were selected and 1 ml of venous blood was collected from each animal under sterile conditions from jugular vein in vacutainer containing EDTA. Genomic DNA was isolated from the blood samples following standard phenol-chloroform extraction protocol. The purity of genomic DNA was assessed spectrophotometrically. Data were generated on growth as well as carcass traits.

Polymerase chain reaction: Amplification of 3 fragments (239 bp, 204 bp and 265 bp) spanning the promoter, 5' UTR, exon 1 and intron 1, exon 2 and 3'UTR regions of leptin gene was done using 2 sets of primers (Table 1). 25 µl of PCR reaction mixture was prepared using 10 pmoles of each primer, 2.5 mM of dNTPs, 1.5 mM $MgCl_2$, 1 X PCR buffer, 80–100 ng DNA template and 1 U Taq DNA polymerase. The optimization of thermal cycle conditions was done as

Table 1. Primer sequences used to amplify fragments of leptin gene

Gene/locus	Primer sequence (5' to 3')	Size of PCR product
Leptin (promoter)	TAAAGGCTCAGCTTTGGTGCAGAGG TGGAACACAGGACAGACAG	239 bp
Leptin (5' UTR and exon 1)	GGTGGTTCCTTCTGCCTTCA AGCCTCTGTACCGTGTGTGAG	204 bp
Leptin (exon 2 and 3' UTR)	AATAGCCAACGACCTGGAGAACCT GATTCTTTCCTCAGCGTGGTCCTT	265 bp

follows: 5 min at 95°C; followed by 30 cycles of 95°C for 1 min, 65°C for 1 min for 239 bp, 63°C for 1 min for 286 bp fragment, and 61.9°C for 1 min for 265 bp, followed by 72°C for 1 min extension; finally final extension of 10 min at 72°C.

Single strand conformation polymorphism analysis: SNPs were screened in these 3 fragments using SSCP technique (Orita *et al*. 1989). The 239 bp, 204 bp and 265 bp PCR products of leptin gene were resolved on 12% polyacrylamide gel. For visualization of bands, silver staining was carried out (Bassam *et al*. 1991).

DNA sequencing and sequence analysis: After the polymorphism analysis, the purified PCR products of different electrophoresis patterns were sequenced in both directions and these sequences were analyzed with the DNA star software. These sequences were submitted online in NCBI gene bank with GeneBank Accession No JX868865.

Expression profiling of leptin: Healthy New Zealand White rabbits at 2 different stages of growth i.e pre-weaning and post-weaning stage, were divided in to 2 groups (6 in each group). These groups were again subdivided into fast growing and slow growing rabbits in each group (3). The liver tissue (100 mg) was collected from the rabbits under sterile conditions after slaughter and carried to the laboratory on ice. The samples were stored at –70°C till further use.

Total RNA extraction and quality determination: The liver tissues were homogenised for isolation of total RNA and was isolated from liver tissue using TriZol, following standard protocol and was further used for cDNA synthesis. The RNA was dissolved in nuclease free water and the purity of RNA was verified by optical density (OD) absorption ratio OD 260 nm/OD 280 nm between 1.8 and 2.0 using nanodrop spectrophotometer. The quality and integrity of the total RNA was checked using denaturing agarose gel (1%) electrophoresis and visualization under UV light. Two intact bands of 28s, 18s with smearing indicated good quality and intactness of RNA.

RNA reverse transcription: Constant amount of 1µg of total RNA were reverse transcribed to cDNA using cDNA synthesis kit with following master mix: 20 µL reaction mixture contained 5 µg of total RNA, 0.5 µg of oligo dT primer (16–18 mer), 20 U of ribonuclease inhibitor, 1000 µM of dNTP mix, 10 mM of DTT, and 5 U of Mu MLV reverse transcriptase in 5X reverse transcriptase buffer. The reaction mixture was gently mixed and incubated at 37°C for 1 h. The reaction was stopped by heating the mixture at 70°C for 10 min and chilled on ice. The integrity of the cDNA was checked by PCR with GAPDH primers which amplified 200 bp GAPDH gene fragment.

Primers: Primers were designed for leptin gene of rabbit by the integrated DNA technologies (IDT) online software. The sequences of primers used were forward 5'-TCAGTGACATCTCACACACGCAGT-3'and reverse 5'-TCTGTTGGTAGATGGCCAACGTCT-3' and expected PCR product length was 128 bp.

Quantitative real time PCR: Quantitative real-time PCR was performed with a commercial kit. A master mix of following components was prepared, 8 µl nuclease free water, 0.5 µl forward primer (0.25µM), 0.5 µl reverse primer (0.25µM) and 10 µl SYBR mix. The master mix (19 µl) was added to the strip tubes and 1 µl of cDNA template was added. Following real time PCR protocol was employed for all investigated factors: initial denaturation at 95°C for 10 min, denaturation at 94°C for 30 sec, annealing at 60°C for leptin and GAPDH in for 30 sec, and extension at 72°C for 30 sec for 40 cycles and continuous fluorescence measurement, and a final cooling down to 40°C. After the run ended, cycle threshold (Ct) values and amplification plot for all determined factors were acquired by using the SYBR green (with dissociation curve) method. Real-time PCR efficiencies were determined by amplification of a standardized dilution series, and slopes were obtained. The specificity of desired products was documented using analysis of melting temperature, which is product specific and a high resolution gel electrophoresis to verify that transcripts were of exact molecular size. Relative expression of PCR product was subjected to statistical analysis by Livak method.

Gene expression and statistical analysis: The moderate growth values were used as control for obtaining relative mRNA expression. GAPDH was used as housekeeping gene. The statistical significance of differences in mRNA expressions between the pre-weaned and post-weaned rabbits group for leptin was tested using the non-parametric Mann-Whitney statistics owing to the smaller sample size (6 in each group).

RESULTS AND DISCUSSION

PCR-SSCP analysis: Three fragments i.e. 239 bp (promoter region), 204 bp (spanning 5' UTR, exon 1 and intron 1) and 265 bp (spanning exon 2 and 3'UTR) of leptin gene were amplified using DNA samples of all the animals of rabbit included in the study with optimized PCR

programme and reaction conditions. SSCP patterns were detected for all the 3 fragments to find the single nucleotide polymorphism (SNPs). The DNA samples of all the rabbits for all 3 fragments revealed the similar type of SSCP pattern, which showed that these fragments of leptin gene are monomorphic in nature (Fig. 1). Consequently only one SSCP genotype (AA) and only one allele (A) were observed. Thus the allele (A) and genotype (AA) frequency were eventually one in these fragments. In order to better understand the genetic variations across breed and species of the leptin gene, all monomorphic SSCP patterns were sequenced. These sequences were submitted to NCBI GenBank Accession No JX868865. Comparison between the nucleotide sequences of New Zealand rabbit leptin gene with published sequence of rabbit (ENSOCUG00000002828) showed no differences.

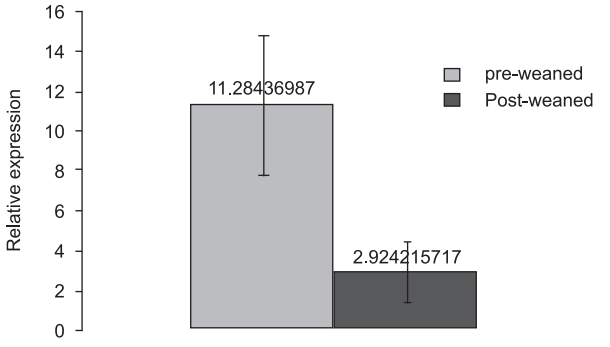

Fig. 1. Relative expression of leptin gene New Zealand White rabbit.

Association study: One of the important objectives of this study was to find an association of variation in leptin gene with the growth phenotype in the New Zealand White rabbit. The association of the growth phenotype with genotypes could not be established as the monomorphic pattern for the leptin gene was detected. The results of this

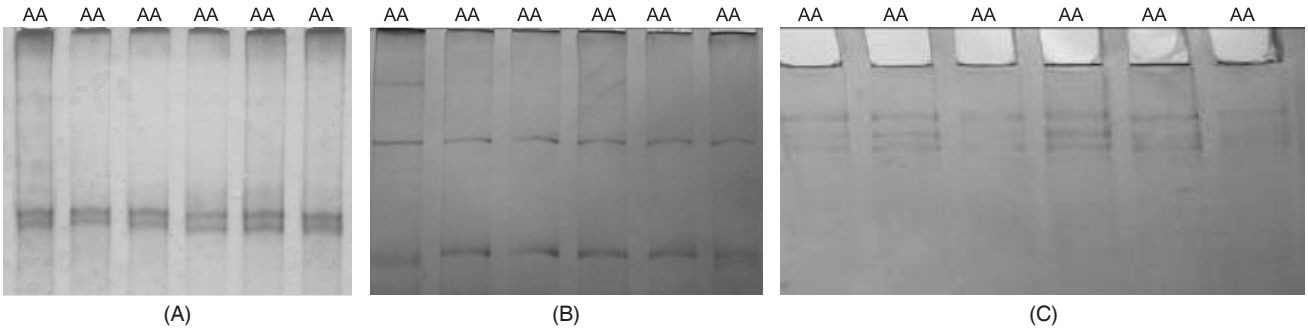

Fig. 2. SSCP genotypes of leptin gene in New Zealand White rabbit. (A) 204 bp fragment (B) 239 bp fragment (C) 265 bp fragment.

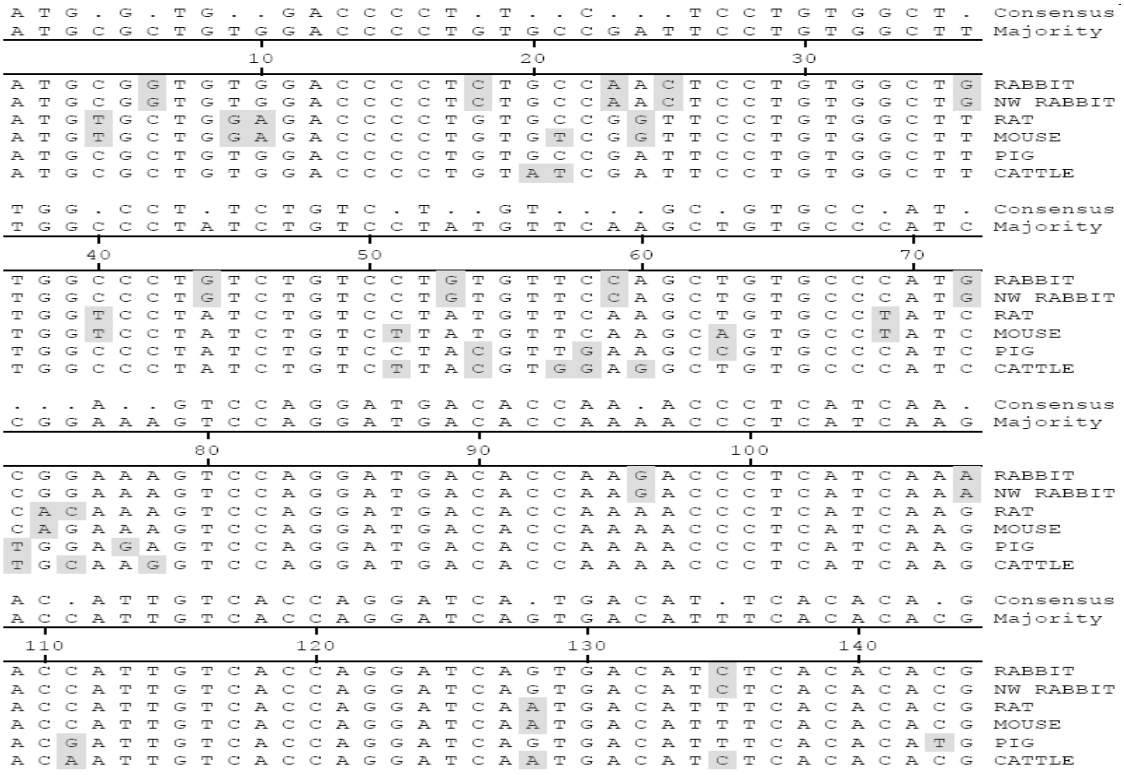

Fig. 3. Nucleotide sequence comparison of amplified 204 bp fragment of letin gene with different species.

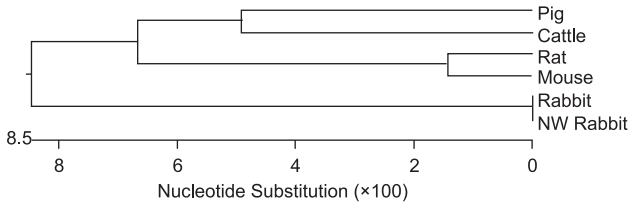

Fig. 4. Phylogenetic tree based on nucleotide sesuence comparison of 204 bp fragment of leptin gene.

study demonstrated that leptin locus in the New Zealand White rabbit is monomorphic.

Expression analysis: Total RNA was in good yield in all the samples. The bands of 28sRNA and 18sRNA reflected the high quality of extracted total RNA. Isolated RNA samples were free from the protein contamination as the OD 260: OD 280 values were more than 1.8. The concentrations of the RNA samples were in the range of 200–2000 ng/µl. The relative expressions of mRNA for leptin in liver tissues at pre-weaned and post-weaned stages of growth showed that leptin in pre -weaned rabbits were 4 fold higher as in post-weaned rabbits. However, these differences were statistically nonsignificant (Fig. 2).

Nucleotide sequencing and sequence comparison analysis: Nucleotide sequencing of amplified fragments of leptin gene of New Zealand White rabbit were done and submitted to the NCBI GenBank (Accession no. JX868865). Although Yang *et al*. (2008) had first reported the cDNA sequence of this gene but this is the first report of nucleotide sequencing of leptin gene of New Zealand White rabbit using genomic DNA. The lengths of the amplified products of three fragments of leptin gene were 239 bp (promoter region), 204 (spanning 5' UTR, exon1 and intron 1) and 265 bp (spanning exon 2 and 3'UTR) in length. The gene and genotype frequencies for all the amplified fragments of leptin gene were one as all fragments were found to be monomorphic.

The sequences of same exonic region of 2 fragment i.e. 204 bp and 265 bp of leptin gene of New Zealand White rabbit (Accession no. JX868865) were compared with published sequence of rabbit, rat, mouse, pig and cattle. In 204 bp fragment at 34 places, there were transitions and transversions amongst inter and intra species comparison of sequences (Fig. 3). There was a high degree of identity with published rabbit sequence (100%) and 85.4, 84, 86.8 and 84% with rat, mouse, pig and cattle sequences, respectively. Based on respective leptin gene sequences, the phylogenetic tree was constructed among rabbit, New Zealand White rabbit, rat, mouse, pig and cattle which showed their relative distance. It revealed that pig and cattle form a single monophyletic group. The species closest to these 2 species were rat and mouse. Rabbit and New Zealand White rabbit forms one cluster and were most distant to the pig and cattle (Fig. 4). The comparison of translated protein sequence of New Zealand white rabbit, rat, mouse, pig and cattle showed differences at 15 places by several amino acids (Fig. 5). At the amino acid level, highest identity (100%) was observed with rabbit, 75% with rat, mouse and 81.2% with pig and cattle.

In 265 bp fragment at 67 places, there were transitions and transversions amongst inter- and intra- species comparison of sequences (Fig. 6). These insertions/deletions could be utilized as a marker for species differentiation/ identification. There was a high degree of identity with published rabbit sequence (100%) and 79.6, 80.1, 87.5, 82.9% with rat, mouse, pig and cattle sequences,

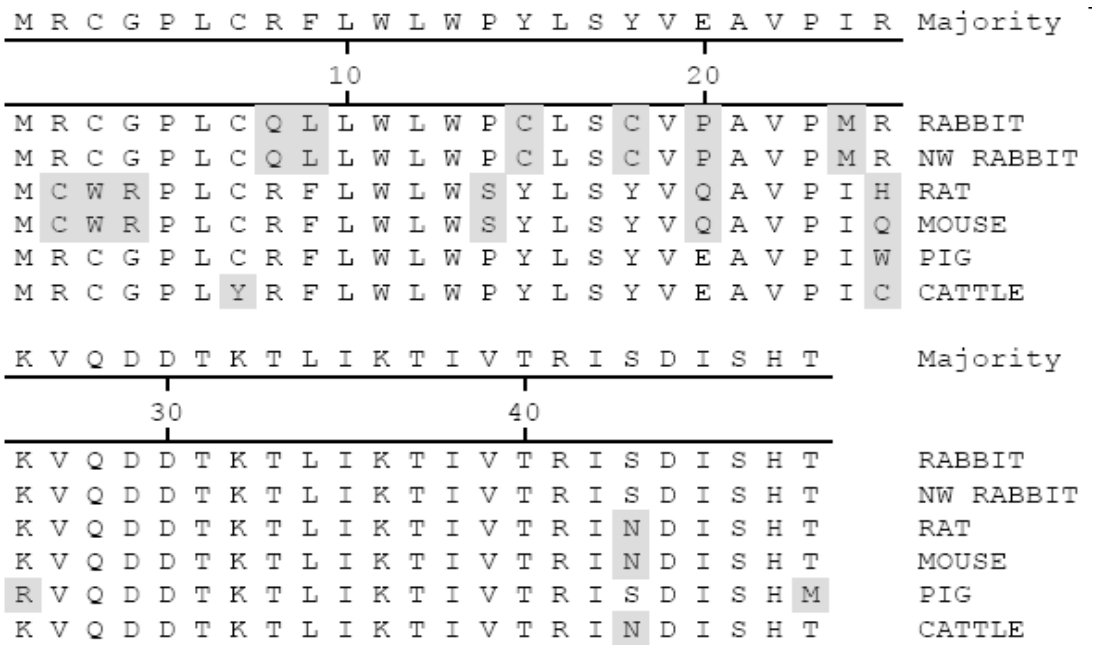

Fig. 5. Amino acid sequence comparison of amplified 204 bp fragment of letin gene with different species.

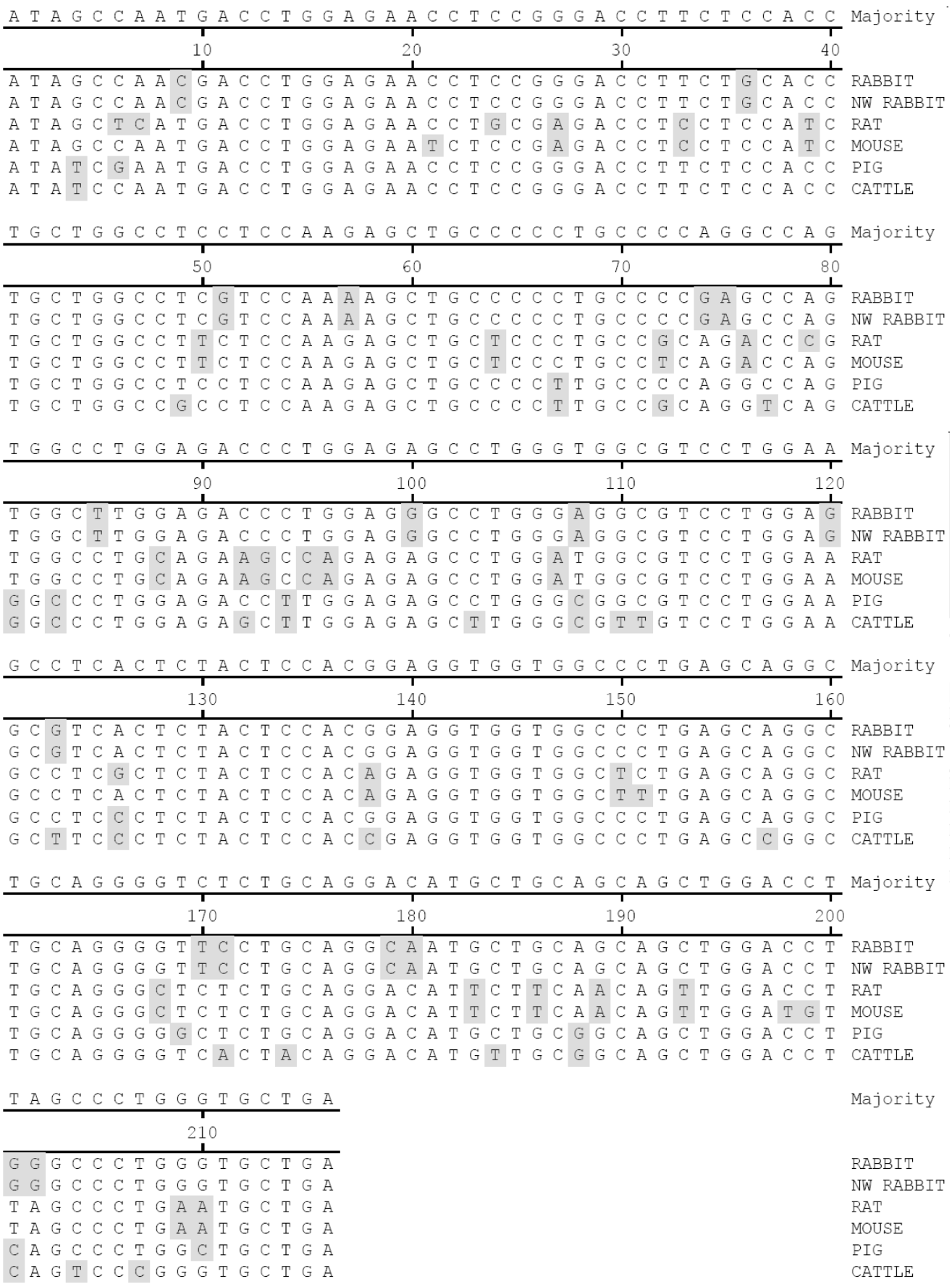

Fig. 6. Nucleotide sequence comparison of amplified 265 bp fragment of leptin gene with different species.

respectively. Based on respective leptin gene sequences the phylogenetic tree was constructed among rabbit, New Zealand White rabbit, rat, mouse, pig and cattle, which revealed that, rabbit and New Zealand White rabbit forms one cluster and was found to be most distant to the rat and mouse. The species closest to these two species were pig and cattle. Rat and mouse were found to form a single monophyletic group (Fig. 7). The translated protein sequence of New Zealand White rabbit is different from the rabbit, rat, mouse, pig and cattle at twenty one places by several amino acids (Fig. 8). At the amino acid level, highest identity (100%) was observed with rabbit and 77.5, 78.9, 87.3 and 81.7% with rat, mouse, pig and cattle, respectively.

While no SNPs were identified in this study, it is possible that other SNPs in remaining intronic and 3' untranslated regions of the leptin gene may be correlated with growth or carcass traits. A number of SNPs have been reported in the leptin gene coding region (Buchanan *et al*. 2002, Vallinoto *et al*. 2004, Lagonigro *et al*. 2003), intronic region (Jane de *et al*. 2009) and in the promoter region (Leifers *et al*. 2005, Nkrumah *et al*. 2005). Several of these polymorphisms have been associated, or have had tendency towards association, with diverse quality traits; e.g. increased fat deposition in beef bulls (Buchanan *et al*. 2002), greater feed intake (Lagonigro *et al*. 2003), fat and lean yield and tenderness in beef cattle (Schenkel *et al*. 2005), quality and yield grade in beef cattle (Kononoff *et al*. 2005), and feed intake and average daily gain in beef cattle (Nkrumah *et al*. 2005), protein yield and lactose yield in Holstein Friesian cows (Liefers *et al*. 2002), feed intake in cattle (Lagonigro *et al*. 2003), body weight up to one year of age, milk production and age at first calving in crossbred cattle (Choudhary 2004), 305–days milk yield and reproductive performance in Iranian Holstein cows (Heravi Moussavi *et al*. 2006), growth traits, first lactation and second lactation milk yield and average daily milk yield in cattle (Dandapat *et al*. 2009) and slaughter age, bacon weight, Boston shoulder weight, midline backfat thickness, midline backfat thickness at last rib, and rib weight in pig (Jane de *et al*. 2009). However, few authors reported no polymorphisms and these findings are consistent with our finding e.g. leptin gene of buffalo (Kumar *et al*. 2003), bovine exon 2 of leptin gene (Vallinoto *et al*. 2004), and bovine leptin gene in indicine cattle (Choudhary 2004). The results of this study demonstrated that leptin locus in the New Zealand White rabbit is monomorphic. Moreover, relative expression analysis of leptin also indicated that there were no significant differences between pre-weaned and post-weaned stages of growth in New Zealand White rabbit. This could be due to vital role of leptin in growing stages of rabbit.

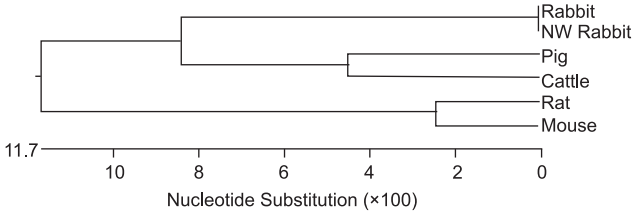

Fig. 7. Phylogenetic tree based on nucleotide sequence comparison of 265 bp fragment of leptin gene.

It is concluded that no SNPs were found for the leptin gene studied in New Zealand White rabbit population. PCR-SSCP studies indicated monomorphic pattern for leptin gene in New Zealand White rabbit. Although this is the first study in rabbit species, the results were consistent as well as showed disparity with some previous studies in other livestock populations. The nucleotide sequence analysis indicated that the percent similarity of leptin gene fragments of New Zealand White rabbit with rat, mouse, cattle and pig was 79.6–87.5%. The amino acids of leptin protein were different at various places in New Zealand White rabbit as compared to rat, mouse, cattle and pig. The amino acid sequence comparison indicated that the similarity of leptin protein of New Zealand White rabbit with rat, mouse, cattle

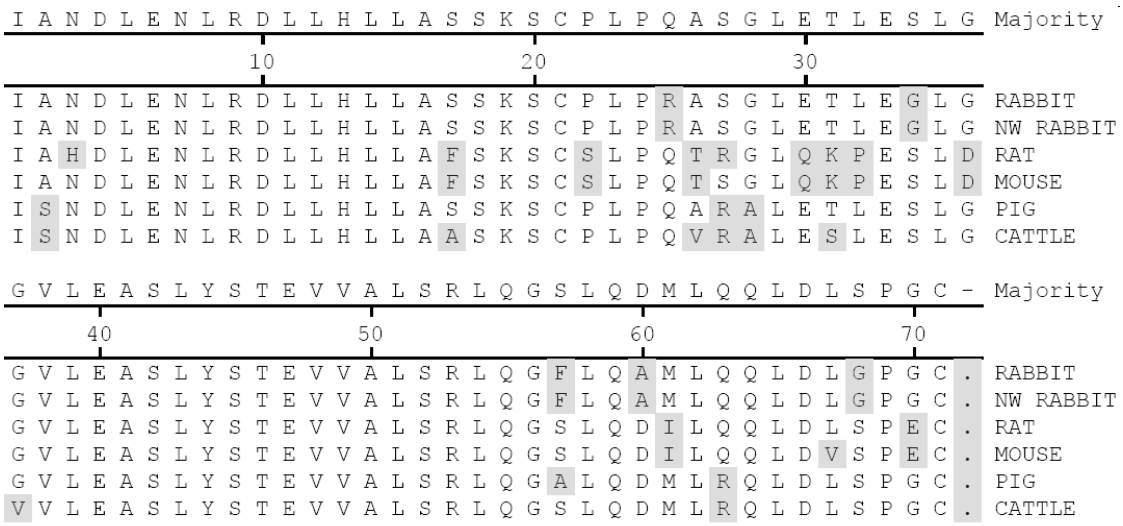

Fig. 8. Amino acid sequence comparison of amplified 265 bp fragment of leptin gene with different species.

and pig was 75–87.3%. The result also revealed that there were no significant differences in the relative expression of leptin at pre-weaning and post-weaning stages of growth. However our study tested only small number of animals in population and further investigation is required in large random mating population to identify the alleles associated with growth traits in rabbit.

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