CDNA CLONING, SEQUENCE IDENTIFICATION AND TISSUE EXPRESSION DISTRIBUTION OF THREE NOVEL GENES: DFP, ITM2B AND PQLC1 FROM BLACK-BONED SHEEP (OVIS ARIES)

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ABSTRACT
The complete coding sequences of three of Black-boned sheep (Ovis aries) genes DFP, ITM2B and PQLC1 were amplified using the reverse transcriptase polymerase chain reaction (RT-PCR) according to the conserved sequence information of the cattle or other mammals and known highly homologous sheep ESTs. Black-boned sheep DFP gene encodes a protein of 213 amino acids which contains the conserved putative DNA/pantothenate metabolism flavoprotein domain and has high homology with the DFP proteins of four species: cattle (99%), dog (91%), human (88%) and zebrafish (53%). Black-boned sheep ITM2B gene encodes a protein of 266 amino acids that contains the conserved putative BRICHOS domain and has high similarity with the ITM2B proteins of five species: cattle (98%), human (95%), mouse (94%), rat (94%) and pig (94%). Black-boned sheep PQLC1 gene encodes a protein of 253 amino acids that contains the conserved putative PQ-loop repeat domain and has high homology with the PQLC1 proteins of four species: cattle (99%), human (91%), mouse (90%) and rat (84%). The phylogenetic tree analysis demonstrated that Black-boned sheep’s DFP, ITM2B and PQLC1 proteins have a close relationship with cattle. The tissue expression analysis indicated that Black-boned sheep DFP, ITM2B and PQLC1 genes were expressed in a range of tissues including leg muscle, kidney, skin, longissimus dorsi muscle, spleen, heart and liver. Our experiment is the first to provide the primary foundation for further insight into these three sheep genes.

Keywords: Black-boned sheep, DFP, ITM2B and PQLC1, tissue expression analysis

Introduction
Biogenesis of coenzyme A (CoA) from pantothenic acid is a necessary universal pathway from prokaryotes to eukaryotes. Phosphopantothenoylcysteine synthase/decarboxylase (DFP), one of the last enzymes in this pathway, metabolizes phosphopantothenate to phosphopantothenoylcysteine (6). DFP used ATP for the activation of substrate in the ligation reaction four times more efficiently than CTP. Drosophila phosphopantothenoylcysteine synthetase is required for tissue morphogenesis during oogenesis (2).

Amyloid precursor proteins are processed by beta-secretase and gamma-secretase to synthesize beta-amyloid peptides which lead to the characteristic plaques of Alzheimer disease (17). The integral membrane protein 2B (ITM2B) gene encodes a transmembrane protein which is processed at the C-terminus by furin or furin-like proteases to synthesize a small secreted peptide which hinders the deposition of beta-amyloid (18). Mutations which lead to the extension of the C-terminal end of the encoded protein, thereby increasing the size of the secreted peptide, are correlated with two neurodegenerative diseases in terms of familial Danish dementia and familial British dementia (1, 11).

Members of the proline and glutamine loop repeat containing 1 (PQLC1) family are all membrane bound proteins possessing a pair of repeats each spanning two transmembrane helices connected by a loop. The proline and glutamine (PQ) motif found on loop 2 is critical for the localisation of cystinosin to lysosomes (3). However, the PQ motif appears not to be a general lysosome-targeting motif. It possesses a more general function practically involving a glutamine residue (19).

Surprisingly, we reported the discovery of Black-boned sheep (Ovis aries) in a population of sheep found in Nanping County of Yunnan Province, China (7, 8, 9, 14). These sheep had dark coloured (black) tissues, compared to the reddish coloration of normal sheep (Ovis aries), and the coloration was shown to be due to the presence of excessive melanin, as in the silky fowl. The trait for dark coloration in sheep has been found to be inherited in cross-breeding studies (16).

The preceding description of the functions of DFP, ITM2B and PQLC1 proteins that are encoded by the DFP, ITM2B and PQLC1 genes, the association of the genes with growth, health, cell morphology, melanogenesis regulation and other important functions that are highly related or potentially related to melanocyte and melanosome distributions in Black-boned sheep, justifies the cloning of these genes in Black-boned sheep which are natural and rare mammal model for pigment cell research.

In the current experiment, we have cloned the coding sequences of Black-boned sheep DFP, ITM2B and PQLC1.
genes based on the conserved sequence information of cattle or other mammals and highly homologous sheep ESTs sequence information. We have also conducted sequence analysis of established nucleotide sequences, some necessary function analyses, and finally examined the expression of these genes in a range of Black-boned sheep tissues. The information provides a foundation for further research on these three sheep genes.

Materials and Methods

Sample collection, RNA extraction and first-strand cDNA synthesis
Samples of leg muscle, kidney, skin, longissimus dorsi muscle, spleen, heart and liver were collected from six adult Black-boned sheep (Ovis aries). Total RNA extraction and first-strand cDNA synthesis were conducted according to previous studies (8, 20). Moreover, a pooled cDNA from the above mentioned seven tissues was conducted.

Isolation of the DFP, ITM2B and PQLC1 genes
The sequences published on GenBank for DFP for human (Accession no. NM_024664), cattle (Accession No. NM_001102228) and their highly homologous sheep eSts sequences: DY500759, DY521039 were used to design a primer pair to amplify the complete coding sequence of DFP by using Primer Premium 5.0 software. Similarly, other two primer pairs for sheep ITM2B and PQLC1 genes were designed based on the conserved coding sequences from cattle and their highly homologous sheep ESTs sequences.

RT-PCR was performed for Black-boned sheep DFP, ITM2B and PQLC1 genes using the above synthesized cDNAs. The RT-PCR was performed in 25 μl reaction volume as follows: 2.0 μl cDNA, 2.0 μl 10 mM mixed dNTPs, 2.5 μl 10×Taq DNA polymerase buffer, 0.8 μl 10 pm forward primer, 0.8 μl 10 pm reverse primer, 0.4 μl Taq DNA polymerase (5 U/μl), and 16.5 μl sterile water. The primer sequences and their annealing temperatures for RT-PCR were shown in Table 1. PCR products for Black-boned sheep DFP, ITM2B and PQLC1 genes were cloned into PMD18-t vector and bidirectionally sequenced.

Semi-quantitative RT-PCR
Semi-quantitative RT-PCR was performed as previously described elsewhere (5, 10, 15). To eliminate the effect of cDNA concentration, the RT-PCR was repeated five times using 1, 2, 3, 4 and 5 μl of cDNA as template. The housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was selected as an internal control. The used control primers were: 5’-AACGTGAGCCGCACAGCTCA-3’ (GAPDH 5’ primer) and 5’-TCATAGTCCTCCAGCAT-3’ (GAPDH 3’ primer). To ensure that no false positive PCR fragments were generated from pseudogenes in the contaminating genomic DNA, GAPDH primers were derived from different exons in the same gene. PCR primer combinations were tested using ovine genomic DNA as a negative control and an approximately 364 bp PCR fragment was amplified when cDNA was contaminated by genomic DNA. The primers for amplification of Black-boned sheep DFP, ITM2B and PQLC1 genes which were used to perform the semi-quantitative RT-PCR for tissue expression profile analysis were the same as the above described primers for isolation RT-PCR. The PCR reactions were optimized for a number of cycles to ensure product intensity within the linear phase of amplification.

Bioinformatic analysis
The cDNA sequence prediction was conducted using GenScan software (http://genes.mit.edu/GENSCAN.html). The protein prediction and alignment and phylogenetic analysis were performed using the Conserved Domain Architecture Retrieval Tool of BLAST at the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST) and the Clustal W software (http://www.ebi.ac.uk/clustalw). Secondary structures of deduced amino acid sequences were predicted by SOPMA (http://npsa-pbil.ibcp.fr/).

Results and Discussion

RT-PCR result for Black-boned sheep DFP, ITM2B and PQLC1 genes
Through RT-PCR with pooled cDNA for Black-boned sheep DFP, ITM2B and PQLC1 genes, the resulting PCR products were 642, 801 and 762 bp, respectively (Fig. 1).

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
<th>Ta/°C</th>
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<tbody>
<tr>
<td>DFP</td>
<td>Forward 5’-ATG GCC GCC GTG GAC CTG-3’</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-TCA GAT CTC TGG AAG AGA AGC-3’</td>
<td></td>
</tr>
<tr>
<td>ITM2B</td>
<td>Forward 5’-ATG GTG AAG GTG ACG TTC-3’</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-TCA AGA GCA AAT TAA AGT TTC-3’</td>
<td></td>
</tr>
<tr>
<td>PQLC1</td>
<td>Forward 5’-ATG GAA GCG GAA GGC TTG-3’</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-TCA GAG GGC CTT GGC ACT-3’</td>
<td></td>
</tr>
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TABLE 1
Sequence analysis

The cDNA nucleotide sequence analysis using the BLAST software at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) showed that all of the isolated in this study genes (these three) were not homologous to any of the known sheep genes. They were then deposited into the GenBank database (Accession Nos: EU583471, EU797607 and EU583473). The sequences prediction were carried out using the GenScan software and the results showed that the 642, 801 and 762 bp cDNA sequences represent three single genes which encoded 213, 266 and 253 amino acids. The theoretical isoelectric point (pI) and molecular weight (Mw) of the three putative proteins of the sheep genes were also computed using the Compute pl/Mw Tool (http://www.expasy.org/tools/pi_tool.html). The pI of sheep DFP, ITM2B and PQLC1 were 6.85, 5.14 and 8.28. The Mw of the three putative proteins were 22606.86, 30305.95 and 28433.50.

These putative proteins were also blasted using the Conserved Domain Architecture Retrieval Tool of Blast at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) and their conserved domains were identified as DFP, BRICHOS and PQ-loop, respectively (Fig. 2).

Further BLAST analysis revealed that Black-boned sheep DFP had high homology with the DFP proteins of four species: cattle (99%), dog (91%), human (88%) and zebrafish (53%). Black-boned sheep ITM2B had high homology with the ITM2B proteins of five species: cattle (98%), human (95%), mouse (94%), rat (94%) and pig (94%). Black-boned sheep PQLC1 had high similarity with the PQLC1 proteins of four species: cattle (99%), human (91%), rat (84%) and mouse (90%). The DFP, ITM2B and PQLC1 have common conserved domains with highly homologous proteins from other mammals.

The prediction of secondary structure by SOPMA (4) indicated that the deduced DFP contained 97 α-helices, 22 extended strands and 94 random coils. Furthermore, the deduced ITM2B consisted of 95 α-helices, 56 extended strands, 10 beta turns and 105 random coils compared to PQLC1 having 85 α-helices, 62 extended strands and 106 random coils (Fig. 3).

Gene expression profiles

Gene expression profile analysis was carried out and results showed that Black-boned sheep DFP gene was moderately
expressed in spleen, *longissimus dorsi* muscle and leg muscle, and weakly expressed in kidney. There was almost no expression in heart, liver and skin. Black-boned sheep *ITM2B* gene was middle-expressed in both liver and kidney. There was almost no expression in heart, spleen, leg muscle, *longissimus dorsi* muscle and skin. Moreover Black-boned sheep *PQLC1* gene was over-expressed in leg muscle, hardly expressed in heart, liver, spleen, kidney, *longissimus dorsi* muscle and skin (Fig. 5).

![Fig. 5. Tissue expression distribution of Black-boned sheep DFP, ITM2B and PQLC1 genes](image)


Comparative genomics determines the relationship of genome structure and function among different biological species or strains. Researchers have learned a great deal about the function of human genes by examining their counterparts in completed reference sequences of the human and mouse genomes and some results have shown that virtually all (99%) of the protein-coding genes in humans align with homologs in mouse, and over 80% are clear 1:1 orthologs (12, 13, 23). This extensive conservation in protein-coding regions implies that the same sequences could be expected in different animals such as cattle, human, rat and mouse (21, 22). This provides us with a useful method to identify the functional regions of different genes for sheep according to the conserved sequence information for the mouse, human, cattle or other animals and to predict what those functions are.

With the development of modern bioinformatics, many specific databases such as NCBI sheep EST database are established along with different convenient analysis tools and these make it much easier to search the sheep EST sequences which are highly homologous to some coding sequences of mouse, human or other animals. This implies that when we can clone the functional regions of different genes for sheep based on the conserved sequence information of the mouse, cattle or other animals we can also refer to this highly homologous EST sequence information.

In this experiment, the complete coding sequences of Black-boned sheep *DFP*, *ITM2B* and *PQLC1* genes were isolated based on the sequence information of the mouse, cattle or other animals and some reference sheep ESTs. Sequence identification further confirmed that the comparative genomics method is a useful tool for cloning unknown genes especially for the conserved coding region of genes for sheep. From our results we know that sheep DFP, ITM2B and PQLC1 are highly homologous with DFP, ITM2B and PQLC1 of cattle or other animals; and sheep DFP, ITM2B and PQLC1 also have the same domains with their corresponding highly homologous proteins from cattle or other animals. This implies that sheep DFP, ITM2B and PQLC1 will have similar functions as DFP, ITM2B and PQLC1 of cattle or other animals. We also find that sheep DFP, ITM2B and PQLC1 do not show complete identity to cattle or other animals. This implies that they will have some differences in functions to those of cattle or other animals.

The phylogenetic tree analysis revealed that the sheep DFP, ITM2B and PQLC1 proteins have a close genetic relationship with these of other species. This implies that different genes have different evolutionary model even though they are in one individual or in one species. But we could still find that these sheep proteins have a closer relationship with these of other animals. This supported the methods used in this experiment to clone the sheep encoding regions based on the information for conserved encoding regions from other animals.

In our experiment, we not only cloned the complete coding sequence of Black-boned sheep *DFP*, *ITM2B* and *PQLC1* genes but also conducted the sequence analysis and tissue expression profiles analysis. From the tissue profile analysis it could be seen that these genes were obviously differentially expressed in different tissues. The suitable explanation for this is that at the same time these biological activities, associated with the functions of these genes, were diversely presented in different tissues.

**Conclusions**

In summary, this is the first report for cloning of ovine *DFP*, *ITM2B* and *PQLC1* genes and conducting of the necessary functional analysis and tissue expression profiles. This information provides the primary foundation for further insight into these three sheep genes.

**Acknowledgements**

The authors Yuai Yang and Yongke Sun contributed equally to this work and should be considered first authors.

The study was supported by the Natural Science Foundation, Key Project of Yunnan Province (2006C0005Z), the National Nature Science Foundation of China (30760177 and 30860042), the Candidates of the Young and Middle Aged Academic and Technical Talents of Yunnan Province (2008PY039) and the Scientific Research Foundation of the State Human Resource Ministry and the Education Ministry for Returned Chinese Scholars, China (to Dr. WD Deng).

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