A novel *HADHA* gene differentially expressed in muscle and other tissues from black-boned *vs*. ordinary sheep

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The mRNA differential display technique was applied to investigate the differences in gene expression in the muscle tissue between black-boned and ordinary ("normal") sheep. The gene that was differentially expressed was identified through semi-quantitative RT-PCR, and its complete cDNA sequence was then obtained using the rapid amplification of cDNA ends (RACE) method. The nucleotide sequence of the gene was not found homologous with any sheep gene described so far. Sequence prediction analysis revealed that the open reading frame of the gene encodes a protein of 763 amino acids, highly homologous with the hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A (HADHA) of bovine (98%), pig (90%), sumatran orangutan (87%), human (87%), chimpanzee (87%), mouse (83%) and rat (82%) – so that it can be defined as sheep *HADHA* gene. This novel sheep gene was finally assigned to GeneID: 100192316. Phylogenetic tree analysis revealed that the sheep *HADHA* gene is genetically closely related to the *HADHA* gene of cattle. Tissue expression analysis indicated that the sheep *HADHA* is also differentially expressed in different tissues of black-boned sheep. The present study established the primary foundation for further research on the *HADHA* gene in sheep.

The mRNA differential display first described by Liang and Pardee [1992] is a fast, and efficient method for isolating and characterizing altered gene expression in different cell types. It was statistically shown that 80-120 primer combinations would be sufficient to cover all the transcript populations in the cell [Liang *et al*. 1993].

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This technique possesses the following advantages over other similar techniques: it is based on simple and established methods, more than two samples can be compared simultaneously and only a small amount of starting material is needed [Yamazaki and Saito 2002].

Black-boned sheep (*Ovis aries*) is a special population found in Nanping County of Yunnan Province, China. The animals possess dark coloured ("black") tissues such as black bone, skin, muscle, liver, kidney and black heart, compared to the reddish colouration in ordinary sheep. The colouration was shown to result from the presence of the excess of melanin [Deng *et al*. 2007, 2008]. Phenotypic variants are mainly determined by the genetic differences. Detecting the genetic differences between black-boned and ordinary sheep or finding out the differentially expressed genes which determine these phenotypic variants is important for sheep breeders.

The present study was carried out with the mRNA differential display technique to isolate the differentially expressed genes in the muscle tissue from black-boned *vs*.local ordinary sheep.

Material and methods

Samples collection

In 2007 two groups (populations) of purebred sheep were created: one of ordinary ("normal") and another of local black-boned sheep. Spleen, muscle, skin, kidney, lung, liver, heart, fat, and pancreas samples were collected from 120-day old animals slaughtered in 2008. For each group, the total RNA was extracted from three males and three females using the Total RNA extraction kit (GIBCO, USA). Before the first-strand cDNA synthesis, DNase I treatment of the total RNA was done.

Differential display

The differential display PCR amplification of each reverse transcription product was carried out with ten arbitrary primers and nine oligo (dT) primers as described earlier [Liu *et al*. 2004]. The PCR products were then separated in the 8% non-denaturing polyacrylamide gel and displayed using the silver stain as described by Pan *et al*. [2003] and Liu *et al*. [2004].

Semi-quantitative RT-PCR

RT-PCR for tissue expression profile analysis was performed according to Liu *et al*. [2005ab]. The housekeeping β-actin gene (Accession no. NM_001009784) as an internal control. The following control primers were used: 5'- CTGTCCCTGTACGCCTCTG-3' (forward primer 1) and 5'- GTGGTGGTGAAGCTGTAGCC-3' (reverse primer 1). The PCR product was 191-bp in length. The following expressed sequence tag (EST) or gene specific primers were used to perform the RT-PCR for identification and tissue expression profile analysis of mRNA3: 5'- CTCCGCTGCTCAGGTTAT-3' (forward primer 2) and 5'-CAGGCTTTGTGGACTTCT-3' (reverse primer 2). The PCR product

was 355-bp long. The 25-µl reaction mixture was: 2 µl cDNA (100-500 ng), 5 pmoles of each oligonucleotide primer (forward primer 1 and reverse primer1, forward primer 2 and reverse primer 2), 2.5 µl 2 mmol/l mixed dNTPs, 2.5 µl 10×Taq DNA polymerase buffer, 2.5 µl 25 mmol/l MgCl$_2$, 1.0 units of Taq DNA polymerase, and finally sterile water to reach the volume of 25 µl. The PCR programme initially started with a 94°C denaturation for 4min, followed by 25 cycles of 94°C/ 50s, 54°C /50s, 72°C /50s, then 72°C extension for 10 min, finally 4°C to terminate the reaction.

5'- and 3'-RACE

5'- and 3'-RACE were performed according to the instructions of BD SMART™ RACE cDNA Amplification Kit (BD SCIENCE, USA). The Gene-Specific Primers (GSPs) were:

5'-RACE GSP: 5'-TCCTCCTAAGCAGGATCCATTGATG-3' and

3'-RACE GSP:5'- GTGCCGTCCTTATCTCAACAAAGCC-3'.

Sequence analysis

The cDNA sequence prediction was conducted using GenScan software (http:// genes.mit.edu/GENSCAN.html). The protein prediction and 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 ClustalW software (http://www.ebi.ac.uk/ clustalw)

Results and discussion

Differential display of mRNA

From the mRNA differential display, one gene, named mRNA3, was found to be almost not expressed in the muscle of ordinary sheep, while strongly expressed in the muscle of black-boned sheep (Photo 1).
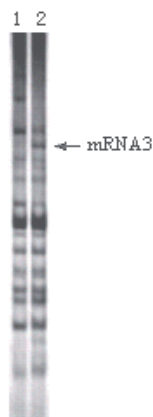

Photo 1. Results of the differential expression analysis of mRNA3. The arrow indicates the cDNA profile for the mRNA3 in a polyacrylamide gel of 8%, stained with silver nitrate; 1- ordinary sheep muscle; 2 - black-boned sheep muscle.

Semi-quantitative RT-PCR

The mRNA3 band was recovered from gel and used as a template for the re-amplification, which was performed with the corresponding oligo(dT) primer and the arbitrary primers used in the mRNA differential display. The resulting PCR product was 483 bp, being in accordance with the mRNA differential display. The purified PCR product was then cloned into the T-vector and the recombinant plasmid was sequenced. Semi-quantitative RT-PCR was then conducted using the EST specific primers. The results are presented in Photo 2.

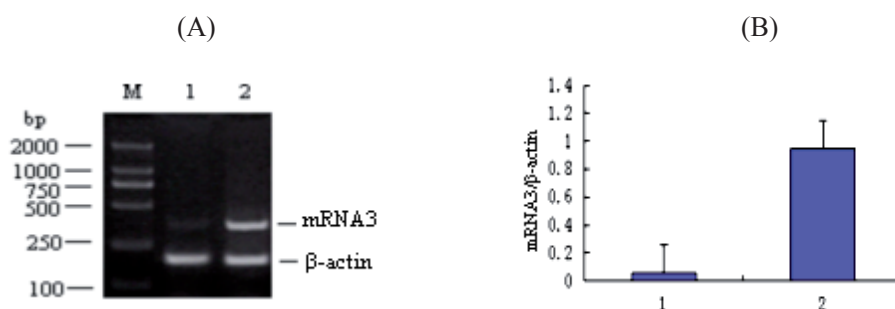(A)   (B)

Photo 2. Identification of mRNA3 (HADHA). (A): the semi-quantitative RT-PCR analysis of mRNA3 (HADHA) in the 1% agarose gel stained with ethidium bromide; (B): the bar graph of the mean ratio of mRNA3/β-actin. M – DL2000 marker, 1 – ordinary sheep muscle; 2 – black-boned sheep muscle**.** The mRNA3/ beta-actin ratios are the means of five semi-quantitative RT-PCRs using 100, 200, 300, 400 and 500 ng cDNA as templates. The signals of the PCR products were measured by BandScan software version 4.50 (http://www.Glyco.com).

Semi-quantitative RT-PCR results indicated that mRNA3 was highly expressed in the muscle of black-boned while weakly in the muscle of ordinary sheep. This also coincided with the result of mRNA differential display.

5'- and 3'-RACE

Through 5'-RACE, one PCR product of ~500-bp was obtained. The 3'-RACE product was ~2400-bp. Both products were then cloned to T-vector and sequenced. Taken together, a 2743-bp cDNA complete sequence was finally obtained.

Sequence analysis

The nucleotide sequence analysis using the BLAST software at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) revealed that the 2743-bp cDNA was not homologous with any of the known sheep genes and was, therefore, deposited into the GenBank database (accession number FJ211195). The sequence prediction was carried out using the GenScan software. An open reading frame encoding 763 amino acids was found in 2743-bp cDNA sequence. In the predicted results, probability of exon was 0.999 and poly-A signal was from 2699-bp to 2704-bp (consensus: AATAAA). Further

BLAST analysis of the protein encoded by mRNA3 revealed its high homology with hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A (HADHA) of seven mammalian species: bovine (AAI03308, 98%), sumatran orangutan (NP_001126017, 87%), human (P40939, 87%), pig (Q29554, 90%), chimpanzee (XP_515339, 87%), mouse (Q8BMS1, 83%) and rat (Q64428, 82%). Figure 1 shows the complete cDNA sequence of the gene and its encoded amino acids.

```
TTCCCGGGATGGGCTTTCCACTTTCGTCCGCTCCCACTCAAGATGGTGGCCTCTCGAGCAATTGGCAGCCTCAGCCGCTTT
                                         M   V   A   S   R   A   I   G   S   L   S   R   F

ACTGCCTCCAGGACGCTCCGCTGCTCAGGTTATATTTGCCGCAGTTTTACAAGGGCCTCTGCTCTGCTGACCAGAACCCAT
  T   A   S   R   T   L   R   C   S   G   Y   I   C   R   S   F   T   R   A   S   A   L   L   T   R   T   H

ATTAACTATGGAGTCAAAGGGGATGTGGCAGTTATTCGAATTAACTCACCCAATTCAAAGGTGAATACTCTAAGTCAAGAA
  I   N   Y   G   V   K   G   D   V   A   V   I   R   I   N   S   P   N   S   K   V   N   T   L   S   Q   E

CTGCATTCAGAGTTCATGGAAGTAATGAATGAAGTCTGGTCTAGCAGTCAGATCAGAAGTGCCGTCCTTATCTCAACAAAG
  L   H   S   E   F   M   E   V   M   N   E   V   W   S   S   S   Q   I   R   S   A   V   L   I   S   T   K

CCAGGCTGCTTCATTGCAGGTGCTGATCTGAACATGTTAAATGCTTGTACAACCTCTCAAGAAGTAACCCAAATATCACAA
  P   G   C   F   I   A   G   A   D   L   N   M   L   N   A   C   T   T   S   Q   E   V   T   Q   I   S   Q

GAAGCACAGAAAATGTTTGAGAAACTTGAGAAGTCCACAAAGCCTGTTGTGGCTGCCATCAATGGATCCTGCTTAGGAGGA
  E   A   Q   K   M   F   E   K   L   E   K   S   T   K   P   V   V   A   A   I   N   G   S   C   L   G   G

GGACTTGAGCTTGCCATTTCATGCCAATACAGAATAGCAACAAAAGATAAGAAAACAGTATTGGGTTCCCCTGAGGTCTTA
  G   L   E   L   A   I   S   C   Q   Y   R   I   A   T   K   D   K   K   T   V   L   G   S   P   E   V   L

CTGGGAATCTTACCAGGAGCAGGAGCCACACAAAGGCTGCCCAAGATGGTGGGTATACCTGCTGCTTTTGACATGATGCTG
  L   G   I   L   P   G   A   G   A   T   Q   R   L   P   K   M   V   G   I   P   A   A   F   D   M   M   L

ACTGGTAGAAGCATTCGTGCAGACAGAGCAAAGAAAATGGGACTGGTTGACCAACTGGTGGAACCACTGGGACCAGGA
  T   G   R   S   I   R   A   D   R   A   K   K   M   G   L   V   D   Q   L   V   E   P   L   G   P   G

GTAAAACCTCCAGAGGAACGGACAATTGAATACCTAGAAGAAGTTGCGATTACTTTTGCCAAAGGACTAGCTGATAAAAAG
  V   K   P   P   E   E   R   T   I   E   Y   L   E   E   V   A   I   T   F   A   K   G   L   A   D   K   K

ATCACTCCAAAGAGAGACAAGGGATTGGTGGAAAAAATGACGTCATACGCCTTGAGTATTCCATTTGTCAGGCAACAGATT
  I   T   P   K   R   D   K   G   L   V   E   K   M   T   S   Y   A   L   S   I   P   F   V   R   Q   Q   I

TACAAAAAGGTGGAAGAAAAAGTACAAAAGCAGACCAAAGGCCTTTATCCTGCACCCCTGAAAATAATTGATGTGGTGAAG
  Y   K   K   V   E   E   K   V   Q   K   Q   T   K   G   L   Y   P   A   P   L   K   I   I   D   V   V   K

ACTGGAATCGAGCAAGGGAGTAATGCTGGCTATCTCTCTGAATCTCAGAGATTTGGAGAGCTCGCAATGACCAAAGAATCA
  T   G   I   E   Q   G   S   N   A   G   Y   L   S   E   S   Q   R   F   G   E   L   A   M   T   K   E   S

AAAGCCTTGATGGGACTTTACCGTGGTCAAACCCAATGCAAGAAGAATAAATTTGGAGCTCCGCAGAAGGAGGTTAAGCAG
  K   A   L   M   G   L   Y   R   G   Q   T   Q   C   K   K   N   K   F   G   A   P   Q   K   E   V   K   Q

CTTGCTGTTCTTGGTGCAGGGCTGATGGGAGCCGGCATTGCTCAAGTCTCCGTGGATAAGGGCCTACAGACCATACTTAAG
  L   A   V   L   G   A   G   L   M   G   A   G   I   A   Q   V   S   V   D   K   G   L   Q   T   I   L   K

GACACTACACTGCCCGCGCTGGGTCGAGGACAGCAGCAGGTGTTCAAAGGACTGAATGATAAAGTGAAGAAGAAAACA
  D   T   T   L   P   A   L   G   R   G   Q   Q   Q   V   F   K   G   L   N   D   K   V   K   K   K   T
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CTAACATCATTTGAAAGGGATGCCATGTTCAGCAACTTGATCGGGCAGCTTGATTACCGGGGTTTTGAAAAGGCCGACATG
  L T S F E R D A M F S N L I G Q L D Y R G F E K A D M
GTGATTGAAGCCGTTTTTGAGGACCTCAGCCTTAAGCACAGAGTGCTGAAGGAGACAGAAGCGGTGATTCCAGATCACTGT
  V I E A V F E D L S L K H R V L K E T E A V I P D H C
GTCTTTGCCAGCAACACATCTGCTCTTCCAATCGGTGAGATTGCTACTGTCAGTAAAAGACCTGAGAAGGTGATCGGCATG
  V F A S N T S A L P I G E I A T V S K R P E K V I G M
CACTACTTCTCTCCCGTGGACAAGATGCAGCTGCTGGAGATTATCACAACTGAGAAGACTTCGAAGGACACAACTGCCTCT
  H Y F S P V D K M Q L L E I I T T E K T S K D T T A S
GCTGTAGATGTCGGCCTCAGACAAGGGAAGGTCATCATTGTGGTTAAGGATGGACCTGGCTTCTATACCACCAGGTGTCTC
  A V D V G L R Q G K V I I V V K D G P G F Y T T R C L
ACACCCATGATGTCTGAAGTCATCAGAATTCTCCAGGAAGGAGTTGGCCCTAAGAAGCTGGACTCCCTGACCACGAGCTTC
  T P M M S E V I R I L Q E G V G P K K L D S L T T S F
GGCTTCCCTGTGGGTGCCGCTACACTGGTGGATGAAGTTGGTGTGGATGTCGCAAAACACGTGGCAGAAAATCTCGGCAAA
  G F P V G A A T L V D E V G V D V A K H V A E N L G K
GAGTTTGGGGAGCGGTTTGCAGGTGGAAGCCTGGAAATGCTGCAGCAGATGATATCCAAGGGCTTCCTGGGTCGCAAGTCT
  E F G E R F A G G S L E M L Q Q M I S K G F L G R K S
GGGAAAGGCTTTTACATCTACCAGGAGGGTGTGAAGAATAAGAATTTGAATTCTGACATGGACAGTATTTTGGCAAGTCTG
  G K G F Y I Y Q E G V K N K N L N S D M D S I L A S L
AAGATGCCTTCTCAGCCTGACGTCTCCTCAGATGAAGACATCCAGTACCGCCTAGTGACCAGGTTTGTGAATGAAGCCATC
  K M P S Q P D V S S D E D I Q Y R L V T R F V N E A I
CTGTGCCTGCAGGAAGGGGTCTTGGCCACGCCCACAGAGGGAGACATCGGTGCGGTCTTTGGGCTTGGCTTCCCCCCGTGT
  L C L Q E G V L A T P T E G D I G A V F G L G F P P C
CTTGGAGGGCCCTTCCACTTTGTGGATCTGTACGGGGCACAGAAAATAGTGGACCGGCTCCGGAAGTACGAGGCTGTCTAT
  L G G P F H F V D L Y G A Q K I V D R L R K Y E A V Y
GGAAAACAGTTCACCCCGTGTCAGCTGCTTGTGGACCATGCCAACAACCCTAACAAGAAGTTCTACCAATGAGCAGGCCCT
  G K Q F T P C Q L L V D H A N N P N K K F Y Q *
GTCCCCTGCCCACTCGCCCACTAACACCAACCATGGCAGCACTGTTTCTCAACAGAGTGGCATCTAGATTATCAGAGTGAC
CAGAAGGAAGACAAACTCTGGCACCGGGTATGTGCCTTGATCAAAGTGCCTTTAGCTAGGACATCTCTCCCTCCCAGCTAGG
TCGGGCTGTGATGTAGTTTGTACTTCCTGTTGGAAGGTGGAACCCTCTGTATGCACCAACTTATAAGCCCTGAGGCCTAGGGT
GGCAGTGTTTCTGGAGCCATCCTTGTTGCCTGCTCCTCCGAGAGCCAGTCGTGGTCACTGGTGGCCAGTGGTGGTGAGGAC
AAGTTTGCACTCAGCCAGATACTTAACAATAAAAATCAAACTTTTTGTCGTAAAAAAAAAAAAAAAAAAAAAAA

Fig. 1. The complete cDNA sequence and amino acid sequence of the protein encoded by mRNA3 (GenBank accession number: FJ211195). ATG – start codon (double underlined); TGA – stop codon indicated by the asterisk.

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Sheep               MVASRAIGSLSRFTASRTLRCSGYICRSFTRASALLTRTHINYGVKGDVAVIRINSPNSK
Bovine              MVASRAIGSLSRFTASRTLRCSGYICRSFTRSSALLTRTHINYGVRGDVAVIRINSPNSK
Pig                 MVASRAIGSLRRFTASQTLRCPGYICRNFTRSSALLTRTHINYGVKGDVAVIRINSPNSK
Human               MVACRAIGILSRFSAFRILRSRGYICRNFTGSSALLTRTHINYGVKGDVAVVRINSPNSK
Chimpanzee          MVACRAIGILSRFSAFRILRSRGYICRNFTGSSALLTRTHINYGVKGDVAVVRINSPNSK
Sumatran_orangutan  MVACRAIGILSRFSAFRILRSRGYICRNFTGSSALLTRTHINYGVKGDVAVVRINSPNSK
Mouse               MVASRAIGSLSRFSAFRILRSRGCICRSFTTSSALLTRTHINYGVKGDVAVIRINSPNSK
Rat                 MVASRAIGSLSRFSAFRILRSRGCICHSFTTSSALLSRTHINYGVKGDVAVIRINSPNSK
                    ***.**** * **:* : **. * **:.** :****:********:*****:********

Sheep               VNTLSQELHSEFMEVMNEVWSSSQIRSAVLISTKPGCFIAGADLNMLNACTTSQEVTQIS
Bovine              VNTLSQELHSEFMEVMNEVWSSSQIRSAVLISTKPGCFIAGADLNMLNSCTTSQEVTQIS
Pig                 VNTLGQELHSEFIEVMNEVWSSSQIRSAVLISSKPGCFIAGADINMLSACTTSQEVTQIS
Human               VNTLSKELHSEFSEVMNEIWASDQIRSAVLISSKPGCFIAGADINMLAACKTLQEVTQLS
Chimpanzee          VNTLSKELHSEFSEVMNEIWASDQIRSAVLISSKPGCFIAGADINMLAACKTLQEVTQLS
Sumatran_orangutan  VNTLSKELHSEFSEVMNEIWASDQIRSAVLISSKPGCFIAGADINMLAACKTLQEVTQLS
Mouse               VNTLNKEVQSEFIEVMNEIWANDQIRSAVLISSKPGCFVAGADINMLSSCTTPQEATRIS
Rat                 VNTLNKEVQSEFVEVMNEIWANDQIRSAVLISSKPGCFVAGADINMLASCTTPQEAARIS
                    ****.:*::*** *****:*:..*********:*****:****:*** :*.* **.::: *

Sheep               QEAQKMFEKLEKSTKPVVAAINGSCLGGGLELAISCQYRIATKDKKTVLGSPEVLLGILP
Bovine              QEAQKMFEKLEKSTKPVVAAINGSCLGGGLELAISCQYRIATKDKKTVLGSPEVLLGILP
Pig                 QEAQRTFEKLEKSTKPIVAAINGTCLGGGLELAISCQYRIATKDKKTVLGAPEVLLGILP
Human               QEAQRIVEKLEKSTKPIVAAINGSCLGGGLEVAISCQYRIATKDRKTVLGTPEVLLGALP
Chimpanzee          QEAQRIVEKLEKSTKPIVAAINGSCLGGGLEVAISCQYRIATKDRKTVLGTPEVLLGALP
Sumatran_orangutan  QEAQRIVEKLEKSTKPIVAAINGSCLGGGLELAISCQYRIATKDRKTVLGTPEVLLGALP
Mouse               QEGQRMFEKLEKSPKPVVAAISGSCLGGGLELAIACQYRIATKDRKTVLGVPEVLLGILP
Rat                 QEGQKMFEKLEKSPKPVVAAISGSCLGGGLELAIACQYRIATKDRKTVLGVPEVLLGILP
                    **.*: .******.**:****.*:********:**:*********:***** ****** **

Sheep               GAGATQRLPKMVGIPAAFDMMLTGRSIRADRAKKMGLVDQLVEPLGPGVKPPEERTIEYL
Bovine              GAGATQRLPKMVGIPAAFDMMLTGRGIRADRAKKMGLVDQLVEPLGPGVKPPEERTIEYL
Pig                 GAGGTQRLPKMVGVPAAFDMMLTGRGIRADKAKKMGLVDQLVEPLGPGLKPPEERTIEYL
Human               GAGGTQRLPKMVGVPAALDMMLTGRSIRADRAKKMGLVDQLVEPLGPGLKPPEERTIEYL
Chimpanzee          GAGGTQRLPKMVGVPAALDMMLTGRSIRADRAKKMGLVDQLVEPLGPGLKPPEERTIEYL
Sumatran_orangutan  GAGGTQRLPKMVGVPAALDMMLTGRSIRADRAKKMGLVDQLVEPLGPGLKPPEERTMEYL
Mouse               GAGGTQRLPKMVGVPAAFDMMLTGRNIRADRAKKMGLVDQLVEPLGPGIKSPEERTIEYL
Rat                 GAGGTQRLPKMVGVPAAFDMMLTGRNIRADRAKKMGLVDQLVDPLGPGIKSPEERTIEYL
                    ***.*********:***:*******.****:************:*****:*.*****:***

Sheep               EEVAITFAKGLADKKITPKRDKGLVEKMTSYALSIPFVRQQIYKKVEEKVQKQTKGLYPA
Bovine              EEVAITFAKGLADKKITPKRDKGLVEKLTSYALSIPFVRQQIYKKVEEKVRKQTKGLYPA
Pig                 EEVAVTFAKGLADKKISPKRDKGLVEKLTSYAMSIPFVRQQIYKKVEEKVRKQTKGLYPA
Human               EEVAITFAKGLADKKISPKRDKGLVEKLTAYAMTIPFVRQQVYKKVEEKVRKQTKGLYPA
Chimpanzee          EEVAITFAKGLADKKISPKRDKGLVEKLTAYAMTIPFVRQQVYKKVEEKVRKQTKGLYPA
Sumatran_orangutan  EEVAITFAKGLADKKISPKRDKGLVEKLTAYAMTIPFVRQQVYKKVEEKVRKQTKGLYPA
Mouse               EEVAVNFAKGLADRKVSAKQSKGLVEKLTTYAMTVPFVRQQYKTVEEKVKKQTKGLYPA
Rat                 EEVAVNFAKGLADRKVSAKQSKGLMEKLTSYAMTIPFVRQQVYKTVEEKVKKQTKGLYPA
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Sheep               PLKIIDVVKTGIEQGSNAGYLSESQRFGELAMTKESKALMGLYRGQTQCKKNKFGAPQKE
Bovine              PLKIIDVVKTGIEQGSNAGYLSESQTFGELAMTKESKALMGLYRGQTQCKKNKFGAPQKE
Pig                 PLKIIDVVKTGIEQGSDAGYLSESQKFGELAMTKESKALMGLYRGQTLCKKNKFGAPQKE
Human               PLKIIDVVKTGIEQGSDAGYLCESQKFGELVMTKESKALMGLYHGQVLCKKNKFGAPQKD
Chimpanzee          PLKIIDVVKTGIEQGSDAGYLSESQKFGELVMTKESKALMGLYHGQVLCKKNKFGAPQKD
Sumatran_orangutan  PLKIIDVVKTGIEQGSDAGYLSESQKFGELVMTKESKALMGLYHGQVLCKKNKFGAPQKD
Mouse               PLKIIDAVKAGLEQGSDAGYLAESQKFGELALTKESKALMGLYNGQVLCKKNKFGAPQKN
Rat                 PLKIIDAVKTGLEQGNDAGYLAESEKFGELALTKESKALMGLYNGQVLCKKNKFGAPQKT
                    ******.**:*:***.:****.**: ****.:*************.**. ***********

Sheep               VKQLAVLGAGLMGAGIAQVSVDKGLQTILKDTTLPALGRGQQQVFKGLNDKVKKKTLTSF
Bovine              VKQLAVLGAGLMGAGIAQVSVDKGLQTILKDTTLPALGRGQQQVFKGLNDKVKKKTLTSF
Pig                 VKHLAILGAGLMGAGIAQVSVDKHLKTILKDASLPALGRGQQQVFKGLNDKVRKKALTSF
Human               VKHLAILGAGLMGAGIAQVSVDKGLKTILKDATLTALDRGQQQVFKGLNDKVKKKALTSF
Chimpanzee          VKHLAILGAGLMGAGIAQVSVDKGLKTILKDATLTALDRGQQQVFKGLNDKVKKKALTSF
Sumatran_orangutan  VKHLAILGAGLMGAGIAQVSVDKGLKTILKDATLTALDRGQQQVFKGLNDKVKKKALTSF
Mouse               VQQLAILGAGLMGAGIAQVSVDKGLKTLLKDTTVTGLGRGQQQVFKGLNDKVKKKALTSF
Rat                 VQQLAILGAGLMGAGIAQVSVDKGLKTLLKDTTVTGLGRGQQQVFKGLNDKVKKKALTSF
                    *::**:************* *:*:***::..*.****************:**:****

Sheep               ERDAMFSNLIGQLDYRGFEKADMVIEAVFEDLSLKHRVLKETEAVIPDHCVFASNTSALP
Bovine              ERDAMFSNLIGQLDYRGFEKADMVIEAVFEDLNLKHRVLKEAEAVIPDHCVFASNTSALP
Pig                 ERDSLFSNLTGQLDYQGFEKADMVIEAVFEELSLKHKVLKEVEAVIPDHCVFASNTSALP
Human               ERDSIFSNLTGQLDYQGFEKADMVIEAVFEDLSLKHRVLKEVEAVIPDHCIFASNTSALP
Chimpanzee          ERDSIFSNLTGQLDYQGFEKADMVIEAVFEDLSLKHRVLKEVEAVIPDHCIFASNTSALP
Sumatran_orangutan  ERDSIFSNLTGQLDYQGFEKADMVIEAVFEDLSLKHRVLKEVEAVIPDHCIFASNTSALP
Mouse               ERDSIFSNLIGQLDYKGFEKADMVIEAVFEDLGVKHKVLKEVESVTPEHCIFASNTSALP
Rat                 ERDSIFSNLIGQLDYKGFEKADMVIEAVFEDLAVKHKVLKEVESVTPEHCIFASNTSALP
                    ***:.**** *****:*****************:* :**:****.*:* *:**:*******

Sheep               IGEIATVSKRPEKVIGMHYFSPVDKMQLLEIITTEKTSKDTTASAVDVGLRQGKVIIVVK
Bovine              IGEIATVSKRPEKVIGMHYFSPVDKMQLLEIITTEKTSKDTTASAVDVGLRQGKVIIVVK
Pig                 ISEIAAVSKRPEKVIGMHYFSPVDKMQLLEIITTEKTSKDSTASAVEVGLKQGKVIIVVK
Human               ISEIAAVSKRPEKVIGMHYFSPVDKMQLLEIITTEKTSKDTSASAVAVGLKQGKVIIVVK
Chimpanzee          ISEIAAVSKRPEKVIGMHYFSPVDKMQLLEIITTEKTSKDTSASAVAVGLKQGKVIIVVK
Sumatran_orangutan  ISEIAAVSKRPEKVIGMHYFSPVDKMQLLEIITTEKTSKDTSASAVAVGLKQGKVIIVVK
Mouse               INQIAAVSKRPEKVIGMHYFSPVDKMQLLEIITTDKTSKDTTASAVAVGLKQGKVIIVVK
Rat                 INQIAAVSQRPEKVIGMHYFSPVDKMQLLEIITTDKTSKDTTASAVAVGLKQGKVIIVVK
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Sheep               DGPGFYTTRCLTPMMSEVIRILQEGVGPKKLDSLTTSFGFPVGAATLVDEVGVDVAKHVA
Bovine              DGPGFYTTRCLTPMMSEVIRILQEGVGPKKLDSLTTSFGFPVGAATLVDEVGVDVAKHVA
Pig                 DGPGFYTTRCLAPMMSEVLRILQEGVGPKKLDSLTTSFGFPVGAATLMDEVGMDVAKHVA
Human               DGPGFYTTRCLAPMMSEVIRILQEGVDPKKLDSLTTSFGFPVGAATLVDEVGVDVAKHVA
Chimpanzee          DGPGFYTTRCLAPMMSEVIRILQEGVDPKKLDSLTTSFGFPVGAATLVDEVGVDVAKHVA
Sumatran_orangutan  DGPGFYTTRCLAPMMSEVIRILQEGVDPKKLDSLTTSFGFPVGAATLVDEVGVDVAKHVA
Mouse               DGPGFYTTRCLAPMMSEVMRILQEGVDPKKLDALTTGFGFPVGAATLADEVGVDVAQHVA
Rat                 DGPGFYTTRCLAPMMSEVIRILQEGVDPKKLDALTTGFGFPVGAATLADEVGIDVAQHVA
                    ***********:******.*******.*****:***.*********** ****:***:***

Sheep               ENLGKEFGERFAGGSLEMLQQMISKGFLGRKSGKGFYIYQEGVKNKNLNSDMDSILASLK
Bovine              ENLGKEFGERFAGGSLEMLQQMISKGFLGRKSGKGFYIYQEGVKNKNLNSDMDSILASLK
Pig                 ENLGKIFGERFAGGNLDVLRQMISKGFLGRKSGKGFYVYQEGVKNRNVNSDTESILASLK
Human               EDLGKVFGERFGGGNPELLTQMVSKGFLGRKSGKGFYIYQEGVKRKDLNSDMDSILASLK
Chimpanzee          EDLGKVFGERFGGGNPELLTQMVSKGFLGRKSGKGFYIYQEGVKRKDLNSDMDSILASLK
Sumatran_orangutan  EDLGKVFGERFGGGNPELLTQMVSKGFLGRKSGKGFYIYQEGVKRKDLNSDMDSILASLK
Mouse               EDLGKAFGERFGGGSVELLKQMVSKGFLGRKSGKGFYIYQEGSKNKSLNSEMDNILANLR
Rat                 EDLGKAFGERFGGGSVELLKLMVSKGFLGRKSGKGFYIYQSGSKNKNLNSEIDNILVNLR
                    *:*** *****.**. ::*  *:************:**.* *.::**: :.**..*:

Sheep               MPSQPDVSSDEDIQYRLVTRFVNEAILCLQEGVLATPTEGDIGAVFGLGFPPCLGGPFHF
Bovine              MPSQPDVSSDEDIQYRLVTRFVNEAILCLQEGVLATPTEGDIGAVFGLGFPPCLGGPFHF
Pig                 IPSRPDISSDEDIQYRLVTRFVNEAVLCLQEGILATPAEGDIGAVFGLGFPPCLGGPFRF
Human               LPPKSEVSSDEDIQFRLVTRFVNEAVMCLQEGILATPAEGDIGAVFGLGFPPCLGGPFRF
Chimpanzee          LPPKSEVSSDEDIQFRLVTRFVNEAVMCLQEGILATPAEGDIGAVFGLGFPPCLGGPFRF
Sumatran_orangutan  LPPKSEVSSDEDIQFRLVTRFVNEAVMCLQEGILATPAEGDIGAVFGLGFPPCLGGPFRF
Mouse               LPAKPEVSSDEDVQYRVITRFVNEAVLCLQEGILATPAEGDIGAVFGLGFPPCLGGPFRF
Rat                 LPAKPEVSSDEDIQYRVITRFVNEAVLCLQEGILATPEEGDIGAVFGLGFPPCLGGPFRF
                    :*.:.::*****.*:.*:;********.:*****.**** ********************:*

Sheep               VDLYGAQKIVDRLRKYEAVYGKQFTPCQLLVDHANNPNKKFYQ
Bovine              VDLYGAQKIVDRLRKYEAAYGKQFTPCQLLVDHANNPNKKFYQ
Pig                 VDLYGAQKVVDRLRKYEAIYGKQFTPCQLLIDHASSPNKKFYQ
Human               VDLYGAQKIVDRLKKYEAAYGKQFTPCQLLADHANSPNKKFYQ
Chimpanzee          VDLYGAQKIVDRLKKYEAAYGKQFTPCQLLADHANSPNKKFYQ
Sumatran_orangutan  VDLYGAQKTVDRLKKYEAAYGKQSTPCQLLADHANSPNKKFYQ
Mouse               VDLYGAQKVVDRLRKYESAYGTQFTPCQLLDHANNSSKKFYQ
Rat                 VDLYGAQKVVDRLRKYESAYGTQFTPCQLLRDLANNSSKKFYQ
                    ******** ****:***: **.* ****** * *....***:*
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Fig. 2. The alignment of the protein encoded by sheep mRNA3 and HADHA proteins of mammals.

The alignment of the proteins encoded by mRNA3 from sheep and seven other HATHA mammalian proteins is shown in Figure 2.

From the sequencing and structural results described, the gene in question could be defined as the sheep *HADHA* gene. The novel sheep gene was finally assigned to GeneID: 100192316. Based on the results of the alignment of HADHA of thirty different species, a phylogenetic tree was constructed using the ClustalW software (http://www.ebi.ac.uk/clustalw) – Figure 3. The tree analysis revealed that the sheep *HADHA* is genetically closer to the bovine *HADHA* than to those of remaining seven species.
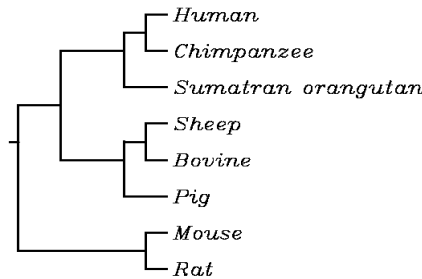

Fig. 3. The phylogenetic tree for *HADHA* gene of sheep, bovine, pig, sumatran orangutan, human, chimpanzee, mouse and rat.

Tissue expression profile

The RT-PCR analysis of the tissue expression profile was carried out using the cDNAs from different tissues of one adult black-boned sheep as the templates (Photo

3). The result indicates that the sheep *HADHA* gene is strongly expressed in liver, moderately in fat, muscle and spleen, weakly in skin, and hardly expressed in kidney, heart, pancreas and lung.
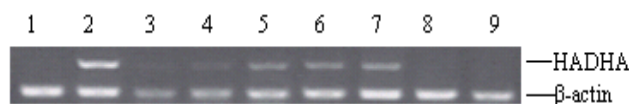

Photo 3. Tissue expression profile analysis of the sheep *HADHA* gene (1% agarose gel stained with ethidium bromide). The beta-actin expression was the internal control. 1 – kidney; 2 – liver; 3 – heart; 4 – skin, 5 – spleen, 6 – muscle, 7– fat, 8 – lung; 9 – pancreas.

HADHA gene encodes the alpha subunit of the mitochondrial trifunctional protein, which catalyzes the last three steps of mitochondrial beta-oxidation of long-chain fatty acids. The mitochondrial membrane-bound heterocomplex is composed of four alpha and four beta subunits, with the subunit alfa catalyzing the 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities. Mutations in that gene result in human trifunctional protein deficiency or LCHAD deficiency. The genes of the alpha and beta subunits of the mitochondrial trifunctional protein are located adjacent to each other in the human genome in a head-to-head orientation [Spiekerkoetter *et al* 2003, Olpin *et al*. 2005, Choi *et al*. 2007]. Up to date, the sheep *HADHA* has not been reported.

The results reported here indicate that the *HADHA* gene was differentially expressed in the muscle tissues from black-boned *vs*. ordinary sheep. Muscles of black-boned sheep contain more melanin compared to the ordinary sheep muscles. The melanin content in muscle tissue in two populations of sheep in question shows the trend of black-boned sheep – high, ordinary sheep – low. It is interesting that the expression of *HADHA* gene in the sheep muscle also shows the trend of black-boned sheep-high, ordinary sheep-low. As we know, phenotypic variants are mainly determined by the gene expression differences. May the sheep *HADHA* gene expression be associated with the melanin metabolic process in muscle tissue? The question remains open.

This study also showed that the *HADHA* gene was differentially expressed also in other sheep tissues. Would this also be associated with the melanin metabolic process of these tissues?

In this investigation the cDNA sequence of the sheep *HADHA* gene was established and the gene was found to be differentially expressed between the black-boned and ordinary sheep. However, the association between the differential expression of the gene and the melanin metabolism is still uncertain. So, to further understand the function of the novel gene, more research is needed based on the primary results presented in this paper.

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Identyfikacja nowego genu owcy – *HADHA* – i jego zróżnicowanej ekspresji w tkance mięśniowej owiec z syndromem „czarnych kości" (*black-boned sheep*) i owiec pospolitych (*ordinary sheep*)

S t r e s z c z e n i e

Owce z syndromem „czarnych kości" (*black-boned sheep),* którego przyczyną są przypuszczalne mutacje w genach tyrozynazy i melanokortyny charakteryzują się nadmiernym odkładaniem pigmentu – melaniny – i ciemnym zabarwieniem kości, mięśni, a nawet osocza krwi. W prezentowanej pracy zastosowano metodę różnicowego namnażania (*differential display*) i półilościową metodę RT-PCR (*reverse transcription PCR)* do poszukiwania genów ulegających zróżnicowanej ekspresji w tkance mięśniowej owiec *black-boned* i owiec pospolitych („normalnych"). Zidentyfikowano jeden taki gen o zróżnicowanej ekspresji i określono pełną sekwencję jego cDNA za pomocą metody szybkiej amplifikacji końców cDNA (*rapid amplification of cDNA ends* – RACE). Stwierdzono brak podobieństwa tego cDNA do sekwencji któregokolwiek ze znanych genów owcy. Analiza komputerowa *(sequence prediction analysis)* wykazała, że odkryty gen zawiera otwartą ramkę odczytu (*open reading frame* – ORF) kodującą białko o 763 aminokwasach (aa) homologiczne z dehydrogenazą hydroksyacylo koenzymu A/3-ketoacylo-koenzymu A (HADHA) siedmiu następujących gatunków: bydło (identyczność sekwencji aa 98%), świnia (90%), orangutan sumatrzański (87%), człowiek (87%), szympans (87%), mysz (83%) i szczur (82%) i w związku z tym zostało określone jako owczy gen *HADHA*. Gen ten został zarejestrowany w GenBank pod numerem FJ211195. Analiza filogenetyczna wykazała, że owczy gen *HADHA* jest blisko "spokrewniony" z genem *HADHA* bydła. Poza różnicami w ekspresji genu HADHA między owcami *blak-boned a* owcami pospolitymi, różnice w poziomie ekspresji tego genu stwierdzono także między różnymi tkankami owiec *black-boned*.

Uzyskane wyniki mogą być podstawą dalszych, bardziej szczegółowych badań nad owczym genem *HADHA*.