Molecular cloning, characterization and evolutionary analysis of leptin gene in Chinese giant salamander, Andrias davidianus

Abstract: Leptin is an important hormone possessing diverse physiological roles in mammals and teleosts. However, it has been characterized only in a few amphibian species, and its evolutions are still under debate. Here, the full length of the leptin (Adlep) cDNA of Chinese giant salamander (Andrias davidianus), an early diverging amphibian species, is characterized and according to the results of the primary sequence analysis, tertiary structure reconstruction and phylogenetic analysis is confirmed to be an ortholog of mammalian leptin. An intron was identified between the coding exons of A. davidianus leptin, which indicated that the leptin is present in the salamander genome and contains a conserved gene structure in vertebrates. Adlep is widely distributed but expression levels vary among different tissues, with highest expression levels in the muscle. Additionally, the leptin receptor and other genes were mapped to three known leptin signaling pathways, suggesting that the leptin signaling pathways are present in A. davidianus. Phylogenetic topology of leptins are consistent with the generally accepted evolutionary relationships of vertebrates, and multiple leptin members found in teleosts seem to be obtained through a Cluopeocephala-specific gene duplication event. Our results will lay a foundation for further investigations into the physiological roles of leptin in A. davidianus.

Keywords: Leptin (Lep); tissue expression; evolution; leptin signaling pathways; Andrias davidianus

1 Introduction

Leptin, the protein product of the obese (ob or Lep) gene, was first cloned in ob/ob mice [1], and then was identified in human and other mammals (reviewed in [2]). Leptins also were identified in non-mammalians, including birds [3-5], reptiles [2], amphibians [6-8], and teleosts, the later generally possess duplicated leptins [8-15]. Leptin has been found to be responsible for the regulation of body weight and energy homeostasis [16,17], and it also is involved in regulating appetite, reproduction [18], the immune system [19], bone formation [20], angiogenesis [21], and stress response [22,23].

The primary amino acid sequences of leptins show low conservation among vertebrates. The identity of the leptin protein in Xenopus to that of pufferfish, human, and tiger salamander (Ambystoma tigrinum) is 13%, 35%, and 60%, respectively [6,7,9]. Although, positive selections of leptins are revealed in several mammal lineages, for example, pikas (Ochotona curzoniae), Cetacea and Pinnipedia, and heterothermic bats [24-26], the conserved gene structure (three exons separated by two introns) and secondary and tertiary structures of leptins were found from teleosts to mammals [2,3,6,11,27,28]. Phylogeny reconstruction of vertebrate leptins showed that most vertebrates form distinct clades with topology consistent with the generally accepted evolutionary relationships of vertebrates, and multiple leptin members found in teleosts seem to be obtained through a Cluopeocephala-specific gene duplication event. Our results will lay a foundation for further investigations into the physiological roles of leptin in A. davidianus.
effects in both mammals and non-mammalians [2,3,6,29,32-34]. For example, leptins possess roles in food intake and energy metabolism in mammals [35,36], growth and reproduction in birds [3], growth and development of the hind limb in X. laevis [6], and food intake and reproduction in teleosts [37,38]. Generally, more diverse physiological roles have been characterized in teleosts in contrast to those found in mammals [39]. Hence, the evolution of multiple functional leptins becomes an interesting question. However, due to the inconsistency of the phylogenetic analysis of leptins, especially for those teleostean homologs [2,29,40,41], identifying more leptins and characterizing their physiological roles in non-mammals, especially in amphibians, would help to address this question. To our knowledge, leptin had only been characterized in two amphibian species, in which they are involved in regulating food intake, growth rate, and bone and lung development [6,7,42,43].

The Chinese giant salamander (A. davidianus) belongs to the family Cryptobranchidae, which is thought to be a primitive group within the Caudata based on molecular and fossil evidence [44-46]. Moreover, a rapidly growing industry to farm this species has been developed throughout China during past two decades [47], which is mainly based on controlled artificial propagation [48] and ecological breeding methods [49]. Noticeably, significant weight differences (5- to 15- fold) have been found in one- and two-year-old artificial propagated populations (unpublished data), which led us to identify and characterize leptin in A. davidianus. Therefore, the objectives of the present study were: (1) to clone and analyze the characteristics of leptin genes in A. davidianus; (2) to examine the tissue distribution of the genes in A. davidianus; and (3) to investigate the evolution of the leptin gene in vertebrates.

2 Materials and Methods

2.1 Sample collection

The individuals of A. davidianus used in this study were cultured in Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Science. Three one-year-old male salamanders were anesthetized according to the standards of the Chinese Council on Animal Care. Organ tissues, including kidney, spleen, lung, stomach, skin, muscle, intestine, gonad, heart, liver, brain, and pituitary gland, were collected and preserved in RNA Lock Stabilizer Reagent (E.Z.N.A.® RNA-Lock Reagent; Omega Bio-Tek Inc.) for RNA extraction.

2.2 Cloning the full-length cDNA and intronic DNA of Adlep

Total RNA of 12 tissues were isolated using TRIZOL (Takara, Dalian, China) based on the manufacturer’s protocol, and then tissues were treated with DNase I (THERMO SCIENTIFIC) to remove genomic DNA and used as templates for the following experiments. The first stand of cDNA was obtained by using SuperScript III transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. One annotated leptin gene sequence (m.112490) was found in the transcriptome of A. davidianus in our previous work [50]. Here, the internal region of leptin in A. davidianus was obtained by using one pair of primers designed according to the partial sequence of leptin in A. davidianus mentioned above (Table 1). Then, based on the obtained sequence, primers were designed (Table 1) and the 5’- and 3’- terminus were obtained by using 5’- and 3’- RACE System (Clontech, Palo Alto, CA, USA) according to the manufacturer’s recommendations. PCR amplification products were examined by electrophoresis using 1% agarose gel with the DL2000 marker (Takara, Dalian, China). The purified sequence fragments were cloned into a pMD-18 T vector and sequenced. All obtained sequence data were assembled using the LasergeneSeqMan program (DNAStar, Madison, WI, USA). Prediction of Open Reading Frames (ORF) were examined using the EditSeq program (DNASTAR). The sequence of the signal peptide was predicted using the program SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) [51]. The domains were predicted on the Pfam server (http://pfam.xfam.org/).

For the quantitative real-time PCR (qPCR) analysis, total RNA from 12 different tissues were extracted, and their first-strand cDNAs were synthesized as mentioned above. The qPCR was performed on a BIO-RAD Connect™ CFX using Power SYBR® Green PCR Master Mix. The specific primers for qPCR are listed in Table 1. The reaction contained: 10 µL of 2× PCR Master Mix, 1 µL of forward primer, 1 µL of reverse primer, 1 µL of cDNA template, and 7 µL of H2O. The PCR protocols were as follows: initial denaturation at 95°C for 2 min, 40 cycles of 95°C for 10 s, 55°C for 20 s, 72°C for 20 s. The melting curves were analyzed at 65–95°C after 40 cycles. Each PCR analysis was performed in triplicate. Relative expression analysis was normalized against the β-actin. The values were expressed as mean ± standard error of the mean (SEM). The expression difference was analyzed by one-way ANOVA with Tukey adjustment using GraphPad Prism Software (GraphPad Software Inc., San Diego, CA, USA). Significance was set at P < 0.05.
In order to obtain the genomic sequence of the leptin in *A. davidianus*, genomic DNA of muscle was isolated using the Blood & Cell Culture DNA Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s recommendations. Primers for PCR amplification of the leptin gene intron are listed in Table 1. The obtained PCR products were sequenced and then assembled by using DNASTAR. The intron was characterized by using sequence alignment of the obtained genomic region with the full length cDNA.

### 2.3 Tertiary structural analysis

The predicted amino acid sequence of Adlep gene was used as query to Blast search against the Protein Data Bank (PDB) to obtain the template protein based on sequence homology. The X-ray structure of the Crystal structure of the human obesity protein, leptin (Chain A) was the best mold found (PDBID: 1AX8) [1]. Thus, the human obesity protein leptin (Chain A) was used as template to analyze the structural model of the enzyme Adlep in *A. davidianus*.

The template and target sequences were aligned using the align2d script available in comparative protein modeling program MODELLER9v14 [52-54]. After alignment, a bundle of 20 models from random generation of the starting structure was calculated, and the best one was selected according to the lowest molpdf and DOPE score implemented within Modeller [52-54]. The selected best model was further subjected to loop refinement in Modeller. To gain better relaxation and a more correct arrangement of the atoms, the best model after loop refinement was further subjected to energy minimization by GROMOS96 force field provided by Swiss-Pdb Viewer V4.1.0 [55] using the steepest descent method of 200 steps. The predicted model also was subjected to energy minimization using the steepest descent technique to check for non-compatible contacts within the protein. Computations were carried out in vacuo with the GROSOMS96 43B1 parameters set, implemented through Swiss-pdb Viewer [56] with default settings. The obtained model was then validated using Ramachandran plot with the PROCHECK program [57] (a Windows version prepared by Bernhard Rupp, http://www.ruppweb.org/ftp_warning.html) and the overall quality factor with Errat on the Structural Analysis and Verification Server (http://nihserver.mbi.ucla.edu/SAVES/).

**Table 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Primer Names</th>
<th>5'-3' sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal region 1</td>
<td>Adlep_f:</td>
<td>GGAAAGCCAGGGAACCCAA</td>
</tr>
<tr>
<td></td>
<td>Adlep_r:</td>
<td>TGCTCCAGATGTTTGACAGATTTAT</td>
</tr>
<tr>
<td>5'RACE</td>
<td>Adlep5'-1:</td>
<td>ATCTCCAGGGTGCTCTCAT</td>
</tr>
<tr>
<td></td>
<td>Adlep5'-2:</td>
<td>TGCTCTCTGGGATAGAAGTC</td>
</tr>
<tr>
<td></td>
<td>Adlep5'-3:</td>
<td>AAGGAACTGGGAGGGGTG</td>
</tr>
<tr>
<td>3'RACE</td>
<td>Adlep 3'-1:</td>
<td>AGGAGTACGCCAAGTCCCCCATACA</td>
</tr>
<tr>
<td></td>
<td>Adlep 3'-2:</td>
<td>CCATAACATGCTAAACATCCG</td>
</tr>
<tr>
<td>qPCR</td>
<td>Adlep-F2</td>
<td>AGAACCTCCGAAGCTTCTTC</td>
</tr>
<tr>
<td></td>
<td>Adlep-R2</td>
<td>CCCCCAAGCCAAACCGAGAAA</td>
</tr>
<tr>
<td>Control</td>
<td>β-actin-F1</td>
<td>CCCCCAAGCCAAACCGAGAAA</td>
</tr>
<tr>
<td></td>
<td>β-actin-R1</td>
<td>GACACCATCACAGAGTCCA</td>
</tr>
<tr>
<td>Intron-cloning</td>
<td>F1:</td>
<td>AAATGCTGCTACACCCTGGT</td>
</tr>
<tr>
<td></td>
<td>R1:</td>
<td>CAGGGGTGGTCTGTAGTAGC</td>
</tr>
<tr>
<td></td>
<td>F2:</td>
<td>GCGACAGTACAGGACAC</td>
</tr>
<tr>
<td></td>
<td>R2:</td>
<td>ATCTCCAGGGTGCTCTCAT</td>
</tr>
<tr>
<td></td>
<td>F3:</td>
<td>TGGACTCTGTGATGAGAC</td>
</tr>
<tr>
<td></td>
<td>R3:</td>
<td>AGCTCAAGGTCCTGCTGTC</td>
</tr>
<tr>
<td></td>
<td>F4:</td>
<td>CTGCTGCGCTGACGAC</td>
</tr>
<tr>
<td></td>
<td>R4:</td>
<td>TCCTCAGCAGCTGTAAGC</td>
</tr>
</tbody>
</table>
2.4 Phylogeny analysis

The full-length Adlep gene sequence was used as a query to search against the NCBI Refseq protein database to obtain leptin homologs in other species, and then they were extracted along with corresponding nucleotide sequences. Amino-acid sequences were aligned using MUSCLE (version 3.8.31) [58] and ambiguous regions were removed manually. Phylogenetic analyses were conducted using Bayesian inference and Maximum-Likelihood (ML) methods. Bayesian inference was carried out in MrBAYES v3.2.2 [59] using the Metropolis-coupled Markov chain Monte Carlo (MCMC) algorithm, with four incrementally heated Markov chains, sampled every 1,000 generations with the temperature set to 0.5. Amino acid site substitution rate heterogeneity was corrected with an invariable and eight Γ- distributed substitution rate categories and the Whelan and Goldman (WAG) model for amino acid substitutions, abbreviated herein as WAG+I+8G. Two separate runs were performed to confirm the convergence of the chains. The average standard deviation of split frequencies and the potential scale reduction factor convergence diagnostic was used to assess the convergence of the two runs. Trees below the observed stationarity level were discarded, resulting in a ‘burnin’ that comprising of 25% of the posterior distribution of trees. The 50% majority-rule consensus tree was determined to calculate the posterior probabilities for each node. The ML tree was inferred with FastTree 2.1.3 [60], with default Jones-Taylor-Thornton (JTT) amino acid substitution matrix and the “CAT” approximation model to account for rate across sites; parameters recommended by the authors were used to improve the effectiveness of the tree search at a slight cost of increased running time (flags: -spr 4 -mlacc 2 -slownni) as described by Beiko et al. [61].

2.5 Mining the leptin signaling pathway in A. davidianus

Leptin (http://www.kegg.jp/dbget-bin/www_bget?K05424) mediates its effects by binding to its receptor (leptin receptor or LEPR), which activates the following signaling pathways within the cell, such as the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (ko04630), AMP-activated protein kinase (AMPK) signaling pathway (ko04152), adipocytokine signaling pathway (ko04920). In order to investigate whether the leptin signaling pathways are present in A. davidianus, our previously assembled transcriptome was subjected to analysis against the Kyoto Encyclopedia of Genes and Genomes (KEGG) to assign their functions to the known biological pathways.

Ethical approval: The research related to animals use has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

3 Results

3.1 Cloning and sequence analysis of the leptin in A. davidianus

One full length coding sequences (CDS) of leptin named as Adlep was obtained in A. davidianus and deposited into GenBank under the accession number KX241573. The one leptin sequence segment found in the transcriptomic sequences of A. davidianus in our previous work was confirmed to be the internal region of the Adlep. The obtained Adlep is composed of a 169 bp 5’UTR sequence, a 1,232 bp 3’ UTR sequence, and a 510 bp CDS sequence encoding a protein of 169 amino acids with a calculated molecular mass of 19.33 kDa and an estimated isoelectric point of 5.88. Typical polyadenylation signal (AATAAA) and poly (A) stretch signal are found (Fig. 1). The predicted Adlep protein sequence contains a signal peptide composed of 21 amino acid N-terminal residues (Fig. 1). One leptin domain (PF02024) was characterized using the Pfam server.

Using four intron primer pairs, the genomic sequence of Adlep gene was obtained and sequenced to be 1,415 bp (Fig. 2). Sequence alignment revealed that two exons were present in the genomic sequence of the Adlep gene, which corresponds to exon 2 and 3 in other vertebrates. Additionally, one intron with 906 bp was found in the genomic sequence of Adlep gene (Fig. 2). The intron of the Adlep gene started as GT and ended as AG (Fig. 1), and its length was shorter than that of the second intron in other tetrapods while longer than that of teleosts (Fig. 2).

Two conserved cysteine residues in positions from 119 aa to 169 aa forming a disulfide bond, which is revealed to be present in all the leptin sequences of tetrapods, including Adlep (Fig. 1 & Fig. 3). Furthermore, the most highly conserved regions among tetrapod leptins also were present (Fig. 3). The identity between the 1AX8 protein sequence in human and Adlep protein sequence in A. davidianus is 35.62%, which is sufficient to use the 1AX8 protein model as a template to yield a reliable model. One model (Adleptin.B99990017.pdb) of the twenty models
Fig. 1. Nucleotide and deduced amino acid sequences of the Adlep in A. davidianus. The intron sequence is shown in lowercase and exon sequence in uppercase. Start codon (ATG) and putative stop codon (TGA) are shown in red. The predicted signal peptides are shown in bold. The termination codon is marked with an asterisk. Two cysteine residues are marked in circle. The GenBank accession number is KX241573.
Molecular cloning, characterization and evolutionary analysis of leptin gene in Chinese giant salamander

Fig. 2. Leptin gene structure of *A. davidianus*. Exons are shown in dark shading, introns are shown as lines with length marked above, and undetermined genomic regions are shown with a dashed line.

Fig. 3. Multiple amino acid sequence alignment of leptins from the mouse (Accession No. P41160), rat (P50595), cow (P50595), pig (Q29406), dog (Q02270), human (P41159), fat-tailed dunnart (*Sminthopsis crassicaudata*, a marsupial) (Q9XSW9), *Xenopus laevis* (AY884210), tiger salamander (*Ambystoma tigrinum*) (AY683941), Japanese fire belly newt (*Cynops pyrrhogaster*) (comp395199_c0_seq1), and Chinese giant salamander (*A. davidianus*) (KX241573). Alignment was performed by Clustal X 1.83 with default parameters. Note that all amphibian leptins comprise of 169 amino acids and all mammalian sequences comprises of 167 amino acids. Two conserved cysteine residues (positions 119 and 169 of the salamander sequence) forming a lasso knot are indicated by arrowheads. The mature peptide begins at position 22 of all these tetrapod sequences as indicated by a vertical line. Boxed regions are the most highly conserved sequences among tetrapod leptins.
generated by Modeller9v14 was selected based on the lowest DOPE score (-15826.54688 KJ/mol), and then it was further subjected to loop refinement with loop.py. The best model was selected and subjected to energy minimization. The Procheck analysis showed 90.2% amino acids in core, 9.1% amino acids in allow, 0.0% in gener, and 0.8% in disallowed in the Ramachandran plot, and the Errat analysis showed that the overall quality factor of the model rose to 86.466 from the original 77.206. These results supported the reliability of the modelled Adlep protein in *A. davidianus*. Superposition of the α-carbon skeleton of the template human leptin models (1AX8) and the theoretical model of Adlep showed that the root mean square deviation (rms deviation) is 0.51 Å, indicating a remarkable similarity of the structural conformation between the Adlep theoretical model with the previous experimental model, and that Adlep does not have major conformational differences with the initial model. The predicted structure of Adlep in *A. davidianus* showed a tertiary structure of a bundle of four main helices (Fig. 4).

3.2 Expression of Adlep in *A. davidianus* tissues

The distribution of salamander leptin expression was studied by qPCR on mRNA isolated from a variety of tissues in three one-year-old male individuals. As shown in Figure 5, expression levels differed significantly among different tissues, with high expression in muscle, moderate expression in skin, and weak expression in other tissues (i.e. heart, pituitary, liver, intestine, lung, spleen, kidney, stomach, and gonad). The tissue expression profiles show light difference from our preliminary expression analysis using one female individual (Supplementary Fig. 1).
3.3 Phylogenetic analysis of leptin

Both the Bayesian inference and Maximum Likelihood (ML) trees showed similar topologies, therefore, we chose to display the Bayesian tree as a representative with the support values of ML tree also on the tree. Phylogenetic reconstruction revealed that the topologies of vertebrate leptins are consistent with the general understanding of the evolution of vertebrates, corresponding to clades of mammals, birds, reptiles, amphibians and fishes, and that Adlep is recovered from the amphibian clade consisting of homologs of other amphibians (Fig. 6). Except for the basal position of leptins in *Latimeria chalumnae*, *Lepisosteus oculatus*, and *Anguilla anguilla*, all other leptins of Teleostei were recovered into two high-supporting clades, corresponding to leptin A and leptin B respectively (Fig. 6 and Supplementary Fig. 2).

![Phylogenetic tree of 78 vertebrate leptins](image)

*Fig. 6.* Phylogenetic tree of 78 vertebrate leptins, which was reconstructed using MrBayes 3.2.2 with 202 aligned amino acid sites. The GenInfo Identifier (GI) number of each sequence is given after species name (*A. davidianus*: KX241573; *A. tigrinum*: AAY68394.1; *C. pyrrhogaster*: comp395199_c0_seq1). Numbers at the nodes correspond to the bootstrap value of the ML tree (on the right of slashes) and the Bayesian posterior probabilities more than 0.50 (on the left of slashes). Scale bar, substitutions per position.
3.4 Leptin signaling pathways in A. davidianus

Through the KEGG annotation of our transcriptome, a total of 577 unigenes are assigned to 184 KEGG orthology (KO) identifiers, and the leptin receptor and other genes belong to subsequent signaling pathways were found. The three leptin signaling pathways were reconstructed as shown in Figure 7 (and Supplementary Fig. 2).

4 Discussion

Leptins have been characterized and demonstrated to possess pleiotropic roles in many vertebrates over the past two decades [62]. Interestingly, leptins have been shown to be involved in early development [63] and innate immune response [64], and to be associated with growth rate [65-69] in different vertebrates. The JAK/STAT pathway is found to be conserved between frogs and mammals [70], suggesting conservation of the leptin signaling mechanism across vertebrate groups. More diverse physiological roles have been characterized in teleosts than those found in mammals [39]. However, further studies are needed to clarify the conservation and evolution of the physiological roles of leptin in diverse vertebrate groups. Here, one full length cDNA of Adlep was obtained in A. davidianus (Fig. 1). The gene organization of Adlep in A. davidianus is similar to that of human, X. laevis and Takifugu [6,9,28], which suggested that the leptin present in the salamander genome though the length of the second intron is between those of other tetrapods and those of teleosts (Fig. 2). Further homolog searching, domain and tertiary structure analysis, and phylogenetic analysis showed

![Image of the reconstructed Adipocytokine signaling pathway (ko04920) in A. davidianus](image-url)
that the identified Adlep is the ortholog of mammalian leptins. Furthermore, the characterization of two cysteine residues and conserved regions and determination of the 3D structure suggests that Adlep could bind to the leptin receptor and trigger the subsequent signaling pathways. Interestingly, we found broad tissue expression of Adlep in muscle, skin, heart, testis, pituitary, liver, kidney, spleen, and stomach, and the muscle and skin showed the highest expression levels (Fig. 5), which is similar in *X. laevis*, *A. tigrinum* and teleosts [2,6,7,15] and different from the restricted expression in mammals [16,71,72]. Noticeably, the expression patterns of Adlep in these three male individuals also differed from that of our preliminary work with one female individual (one-year-old), with high expression in skin, kidney, stomach, liver, and ovary (Supplementary Fig. 1). The expression level variation among individuals might be explained by different nutritional state among individuals or caused by seasonal variation as the samples in these two different expression analyses were collected at different times. This phenomenon also has been reported in *A. tigrinum* [7]. Altogether, the wide tissue distribution of Adlep seems different from the relatively restricted expression distribution of mammalian leptins. The different expression pattern of leptins among different vertebrate groups remains to be clarified in future. The presence of leptin receptor and the subsequent leptin signaling pathways (i.e. JAK/STAT, AMPK, and adipocytokine) in *A. davidianus* suggests that the conservation of the leptin signaling pathway could be traced back to the ancestor of Tetrapoda, and that Adlep might be correlated with homeostasis, energy status, and lipid regulating. Hence it would be interesting to investigate the potential physiological roles of Adlep in future studies, such as identification and association analyses of polymorphisms in Adlep with growth traits among different individuals in farmed populations of *A. davidianus* because significant growth rate differences among different individuals of different ages have been found.

The evolution of leptin is still under debate considering the presence of multiple copies in teleost fish [2,62,73]. Here, the overall topology of the leptins in vertebrates is consistent with the general accepted evolutionary relationships of vertebrates (Fig. 5). Noticeably, two leptins in *Anolis carolinensis* are clustered together as reported previously [2], indicating that they are obtained through species specific gene duplication. Moreover, leptins in *Lepisosteus oculatus* and *Anguilla anguilla* are branched first, and then leptins of other teleosts are generally recovered into two subclades, leptin A and leptin B, which are generally consistent with another research reported previously [41], indicating that the multiple leptins in teleost are obtained through gene duplication that occurred in the ancestor of Clupeocephala. Given the conserved tertiary structures from teleosts to mammals [2,34], the diverse physiological roles of leptins in different vertebrates characterized to date [74], and the common intracellular signaling pathways presented in all Tetrapoda as suggested by this research, the phylogenetic topology of vertebrate leptins obtained in this research suggest that it would be very interesting to detect whether there are different regulation mechanisms for pleiotropic roles or expressions of leptins throughout evolution.

**Acknowledgement:** This work was supported by the Central Public-interest Scientific Institution Basel Research Fund, CAFS (NO. 2016JBF0305). The authors also would like to thank anonymous reviewers who gave valuable suggestion that has helped to improve the quality of the manuscript.

**Conflict of interest:** Authors state no conflict of interest.

**Reference**


