



Cloning and Characterization of NAD-dependent Deacetylase Sirtuin 2 Homolog from the Silkworm, *Bombyx mori*.

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Abstract

Sirtuin2 (Sirt2) is a kind of NAD⁺-dependent deacetylases ranging from bacteria to human and play an important role in many biological processes especially in lifespan. We performed genome analysis and protein prediction of Sirt2 of *B. mori* (BmSirt2). The cDNA sequence of BmSirt2 contains an ORF of 1164 bp encoding 387 amino acid residues with a

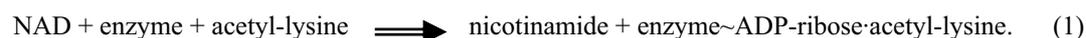
predicted molecular mass and isoelectric point of 43.37 kDa and 5.02, respectively. This protein shows high degrees of identity with other species. Phylogenetic relationship analysis showed that the BmSirt2 protein was in the same subgroup as the Sirt2 from invertebrate animals. RT-PCR analysis of gene expression in multiple tissues showed that Sirt2 gene was widely expressed in *B. mori*. BmSirt2 was successfully expressed in *E. coli* with a molecular mass of 48.0 kDa. The identification of the recombinant protein by MALDI-TOF-MS and western blotting showed this fusion protein was the correct one.

Keywords: *Bombyx mori*, Sirtuin2, Bioinformatics, RT-PCR, Prokaryotic expression, Mass spectrographic

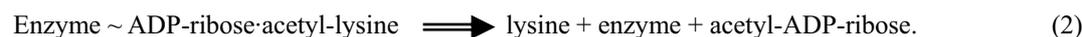
1. Introduction

Sirt2 enzymes, also known as sirtuins, or silence information regulator (Sir), comprise an ancient family of NAD⁺-dependent deacetylases conserved from bacteria to human and play a role in a wide variety of important biological processes, including development, heterochromatin formation, transcriptional silencing, DNA recombination and repair, genome stability, apoptosis, axonal protection, fat mobilization, metabolic regulation, and longevity (Avalos, Bever, & Wolberger, 2005, p.855; Hoff et al., 2006, p.1231). Sirtuins link aging, cancer, and diet and thus are potential molecular targets for the development of pharmaceuticals to treat human metabolic and neurological diseases and malignancy (Schuetz et al., 2007, p.377).

Sirt2 enzymes comprise a universally conserved family of NAD⁺-dependent deacetylases, which of the reaction can be thought of as occurring by a two-step mechanism. In the first step, two substrates, an acetylated protein and NAD, interact with the active site of the enzyme. The first reaction is reversible in the presence of excess nicotinamide:



In the second step the high-energy ADP-ribose intermediate reacts with the acetyl group on the ε amino group of the lysine:



(Landry & Sternglanz, 2003, p.34).

Sirtuins was first found in *S. accharomyces cereviae* and ranging from bacteria to humans. The hallmark of the family is a domain of approximately 260 amino acids that has a high degree of sequence similarity in all sirtuins. The family is divided into five classes (I-IV and U) on the basis of a phylogenetic analysis of 60 sirtuins from a wide array of organisms (Frye, 2000, p.793). Class I and class IV are further divided into three and two subgroups, respectively. The U-class sirtuins are found only in Gram-positive bacteria. The *S. cerevisiae* genome encodes five sirtuins, Sir2p and four additional proteins termed 'homologs of sir two' (Hst1p-Hst4p) (North & Verdin, 2004, p.224.1). The human genome encodes seven sirtuins, with representatives from classes I-IV (Frye, 2000, p.793).

In this paper, Sirt2 of *Bombyx mori* (BmSirt2) was cloned and analyzed with bioinformatics tools, including its genomic organization and the deduced amino acid sequence. The gene was confirmed by RT-PCR and prokaryotic expression. The deduced protein sequence was compared with that of some homologous protein from other species.

2. Materials and methods

2.1 Animals and RNA extraction

The silkworm, *B. mori* used in this study, was reared routinely on fresh mulberry leaves at 25 °C. Epidermis, midgut, fat body, silk gland, hemolymph, nerve knot, testis and ovary were dissected and washed in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ and 1.4 mM KH₂PO₄), frozen immediately in liquid nitrogen and stored at -80 °C until extraction of RNA. Total RNA was isolated from 30 mg each of *B. mori* larvae tissues using the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA). The extracted RNA was treated with RNase-free DNase (Promega, Madison, WI, USA), then was used to synthesize first strand cDNA using the SuperScript II™ reverse transcriptase (Promega) following the manufacturer's instructions (Wang et al., 2008, p.177).

2.2 Genome analysis for BmSirt2

A putative cDNA of BmSirt2 (AB194684) was obtained by the BLAST program using XP_001350011 from *Plasmodium falciparum* (Chakrabarty et al., 2008, p.140). The DNASTar software was used to find the open reading frame (ORF). TATA box and poly-A signal was predicted by Primer premier software. SIM4 (<http://pbil.univ-lyon1.fr/sim4.php>) was used to align the cDNA sequence with the genomic sequences to search potential introns. SilkMap of BGI (<http://silkworm.genomics.org.cn/>) was used to find the location of BmSirt2 on Chromosome.

2.3 Protein prediction and analysis

The deduced amino acid sequence was analyzed by the Expert Protein Analysis System (<http://www.expasy.org/>). Homologues were explored by BLASTP searching tool in the updated GenBank/EMBL databases. Multiple sequence

alignment was performed by Clustal W (<http://www.ebi.ac.uk/clustalw/>) and edited using GeneDoc software. Phylogenetic relationships were assessed and trees generated by the neighbour-joining method using MEGA 3.1 (<http://www.megasoftware.net>).

2.4 Cloning, sequencing and expression of *BmSirt2*

An ORF region of *BmSirt2* was amplified by PCR using the forward primer 5'-AGGATCCATGTCGGCAAATTCGCCT-3' and the reverse primer 5'-ACTCGAGTCATTCCGAGGCACTGGG-3' containing *Bam*HI and *Xho*I restriction sites (underlined) respectively. Actin A3 was amplified using the forward primer 5'-GCGCGGCTACTCGTTCACTACC-3' and the reverse primer 5'-GGATGTCCACGTCGCACTTCA-3'. PCR reaction was carried out for 35 amplification cycles (94°C/30 sec, 62°C/30 sec, 72°C/1 min).

The PCR product was ligated into pMD19-T (TaKaRa, Dalian) vector using T4 DNA ligase (TaKaRa, Dalian) and then transformed into *E. coli* (TG1 strain). After digestion with *Bam*HI and *Xho*I from the plasmid pMD19-T/*BmSirt2*, the purified DNA fragment was ligated into expression vector pET-30a (Novagen) and introduced into *E. coli* BL21 (DE3) by transformation. Sequencing was performed in Shanghai Sangon Bio-technology Corporation using the plasmid pMD19-T/*BmSirt2*.

The expression of recombinant *BmSirt2* tagged with histidine residues was induced overnight at 28°C by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM when the optical density of the culture at 600 nm reached 0.5. A 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to analyze the fusion protein.

2.5 Identification of *BmSirt2*

The band corresponding to our recombinant *BmSirt2* was excised as circular plugs 2-3 mm in diameter, and transferred to 1.5 ml Eppendorf tubes. Coomassie blue-stained gel pieces were first destained with 50 ul of 50% 50 mM NH₄HCO₃ and 50% acetonitrile for 10 min, followed by three washes with 50 ul of MilliQ water. The gel pieces were then dehydrated with 100% acetonitrile for 5 min, and dried in a SpeedVac (Thermo Sacant) for 30 minutes. The dried gel particles were rehydrated at 4°C for 30 min with 2.5 uL/well trypsin (sequencing grade; Promega, Madison, WI) in 50 mM NH₄HCO₃ (20 ug/mL), and then incubated at 37°C overnight. Peptide mixture (1 ul) was mixed with 1ul 10 mg/ml a-Cyano-4-hydroxycinnamic acid (Sigma) and pointed on the MTP Anchor Chip (Bruker) and analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) (ultraflex tof/tof, Bruker, Germany). The mass spectra were interpreted using the Mascot peptide mass fingerprint engine available on the web site (<http://www.matrixscience.com>) for protein identification. To identify the fusion protein further, the western blotting was performed using anti-His-tag antibody (TIANGEN, Beijing).

3. Results

3.1 The analysis of the nucleotide and amino acid sequences

The sequence of a 1586 bp *B. mori* cDNA was obtained from GenBank under accession no. AB194684, which contains an ORF of 1164 bp encoding 387 amino acid residues sequence with a predicted molecular mass and isoelectric point of 43.37 kDa and 5.02, respectively (Figure 1). The amino acid sequence had a conserved SIR2 domain, which contains 183 amino acid residues from the position 334 to 882. No TATA box was found but two poly-A signals existed, which showed as AATAAA (Figure 1). Blasting the cDNA to contigs of *B. mori* genome in GenBank revealed that contig19687, Ctg001400, contig176714, contig484962 and contig544692 having a high similarity. After analyzing these five contigs, we found that contig19687 contained Ctg001400, contig176714, contig484962 and contig544692. Using SIM4, seven exons were found in the relevant DNA sequence. The length of the exons was 34bp, 267bp, 207bp, 150bp, 193bp, 187bp and 126bp, respectively (Figure 2). Furthermore, we also found *BmSirt2* gene was located on Chromosome 25.

3.2 Protein prediction and analysis

The deduced amino acid sequence shared 62% identity to *Apis mellifera*, 61% identity to *Tribolium castaneum* and *Nasonia vitripennis*, 60% identity to *Anopheles gambiae*, 59% identity to *Ciona intestinalis* and *Homo sapiens*, and 56% identity to *Mus musculus*. Multiple sequence alignment (Figure 3) showed that the deduced amino acid sequence of *Sirt2* from *B. mori* shared high level identity among these sequences in the conserved domain, SIR2 Domain. To investigate the evolutionary relationships between *Sirt2* of *B. mori* and other organisms, phylogenetic analysis was performed by neighbour-joining (NJ) method using MEGA 3.1 software (Kumar, Tamura, & Nei, 2004, pp.150-161). Phylogenetic relationship analysis was showed in Figure 4.

3.3 Cloning, sequencing and gene distribution

The DNA fragment was obtained through PCR (Figure 5A) and the expression plasmid pET-30a/*Sirt2* was successfully constructed. (Figure 5B). The result of sequencing showed that there were two different nucleotide acids, which was pointed in Figure 1. But this mutation was a kind of nonsense mutation and their amino acid residues didn't change.

Through semi-quantitative RT-PCR, BmSirt2 gene was detected in multiple tissues, including epidermis, midgut, fat body, silk gland, hemolymph, nerve knot, testis and ovary. This gene was expressed in all these tissues (Figure 6), which suggested that BmSirt2 was a very important and widely expressed gene in *B. mori*.

3.4 Expression of BmSirt2

BmSirt2 was successfully expressed in transformed *E. coli* BL21 with the pET-30a vector. After induction with IPTG over night at 28°C, the *E. coli* cells were collected from LB medium. SDS-PAGE analysis revealed that the recombination protein was overexpressed in a soluble form. Since the fusion protein had a His-tag at the N-terminus, its molecular weight was 48.0 kDa (Figure 7) which was higher than that of the predicted mass of 43.37 kDa.

3.5 Identification of BmSirt2

Two methods were utilized to identify the recombination protein. After identification by MALDI-TOF-MS, the peptide fragments of the recombination protein matched with the deduced amino acid sequence of Sirt2 from *B. mori* was showed in Figure 8 and Table 1. It indicated that the recombination protein we got was proved to be the BmSirt2 protein. Simultaneity, the western blotting using anti-His-tag antibody revealed that the fusion protein was the one we wanted to get.

4. Discussion

Silkworm (*B. mori*) is an important economic insect and is regarded as a model insect of Lepidoptera. Studies on structures and functions of certain related genes in *B. mori* have attracted more and more attention.

In this study, we cloned and analyzed the nucleotide sequences of BmSirt2 gene and further predicted its conserved domain. The gene includes an ORF of 1164 bp encoding 387 amino acid residues and the ORF contains seven exons. BmSirt2 gene locates on Chromosome 25 and is showed as a single copy gene. The deduced amino acid sequences show high identity to many other organisms, including from protista to mammals, especially among the SIR2 domain, which indicated that Sirt2 gene is conserved from invertebrates to mammals. Phylogenetic relationship analysis showed that the BmSirt2 protein was in the same subgroup as the Sirt2 from invertebrate animals, such as *Tribolium castaneum*, *Apis Mellifera*, *Aedes aegypti*, *Anopheles gambiae*, and *Drosophila melanogaster*.

BmSirt2 gene was widely expressed in *B. mori*, including epidermis, midgut, fat body, silk gland, hemolymph, nerve knot, testis and ovary. It indicated that BmSirt2 gene may play an important role in many biological processes in almost all the tissues in *B. mori*. BmSirt2 was successfully expressed and identified by MALDI-TOF-MS and western blotting. The results showed that the recombination protein we got was proved to be the BmSirt2 protein. BmSirt2 can be expressed in a soluble form in *E. coli* and its fusion protein can be purified using the Ni resin. Thus, further study like enzyme activity assay could be operated to indicate the function of BmSirt2.

BmSirt2 gene is a kind of silence information regulator, which also has the NAD⁺-dependent deacetylase activity. Recently, many researches found that Sirt2 is a strong candidate to regulate calorie restriction (CR) in many model organisms, such as yeast, nematodes, fruitflies and even mammals (Guarente, 2005, p.923). Guarente and Picard (2005, pp.473-480) reported that limiting calories resulted in activation of the Sir2-family of protein deacetylases, and that this activation accounted for the reduced rate of aging and increased life span observed in response to CR (Masoro, 2004, pp.591-594; Guarente & Picard, 2005, pp.473-480). Therefore, we used silkworms to study BmSirt2 gene. We thought it was so significant that it would be used to explain the mechanism of longevity which is the eternal topic in life science.

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Table 1. Results of NCBI Inr Database Search for the Identification of BmSirt2

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Sequence
34-45	1494.8590	1493.8517	1493.6933	0.1585	R.NMFRDLVDVDDVR.M
38-45	946.4945	945.4872	945.4404	0.0469	R.DLDVDDVR.M
52-68	1867.1019	1866.0947	1865.9411	0.1536	K.LGLFSPQDLEPAEPPEK.V
89-108	2004.2975	2003.2902	2003.1051	0.1851	K.KIITLSGAGISTSAGIPDFR.S
90-108	1876.19	1875.1884	1875.0102	0.1782	K.IITLSGAGISTSAGIPDFR.S
109-120	1386.8173	1385.8100	1385.6939	0.1161	R.SPETGLYHNLQK.Y
121-136	2012.2227	2011.2154	2011.0203	0.1951	K.YELPQPQAIFEINFFR.Q
137-147	1273.8038	1272.7966	1272.6867	0.1099	R.QNPKPFFTLAK.E Gln->pyro-Glu (N-term Q)
137-147	1290.8409	1289.8337	1289.7132	0.1205	R.QNPKPFFTLAK.E
148-164	2039.2980	2038.2907	2038.0676	0.2231	K.ELFPGSFKPTISHYFIR.L
175-185	1389.8080	1388.8007	1388.6684	0.1323	R.HYTQNIDTLER.G
194-210	2066.1602	2065.1529	2064.9146	0.2383	.LVEAHGTFYTSCLDCR.K
194-211	2194.2659	2193.2586	2193.0096	0.2490	K.LVEAHGTFYTSCLDCRK.E
211-219	1152.7128	1151.7055	1151.6226	0.0829	R.KEYPLEFVK.E
212-219	1024.6399	1023.6326	1023.5277	0.1049	K.EYPLEFVK.E
212-221	1309.8035	1308.7962	1308.6714	0.1248	K.EYPLEFVKER.I
297-307	1235.8621	1234.8548	1234.7510	0.1038	R.EKAGVRPPILR.I
299-307	978.7280	977.7207	977.6134	0.1073	K.AGVRPPILR.I
310-324	1655.9109	1654.9036	1654.7443	0.1593	R.GLMCGGLQLDEGSYR.D
310-328	2097.2471	2096.2398	2095.9779	0.2619	R.GLMCGGLQLDEGSYRDVAR.L
329-342	1623.7961	1622.7889	1622.6301	0.1588	R.LGDCDEGCQDLADR.L
343-350	945.5690	944.5618	944.4716	0.0902	R.LGWGDELRA

There listed the start and end position of the matched peptide, the observed molecular mass, expective Mr, calculative Mr, the delta value between expective Mr and calculative Mr and the matched sequences.

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1      TGTC AATATTGGACAAAGGTTTCGAGACGCAATTTTGTATTTTTAOGTAAATGTCGGCA
                                         M S A
61     AATTCGCCTCCCGAAAAAGTGGGGTGCATGAAACCGTGGATGAGTCACGCGAGAATGTC
      N S P P G K S G G H E V D E S P Q N N V
121    CCGCCAACAACCTCGATGGAAAGCCTCAGAAATATGTTTCGGATCTCGACGTGGATGAC
      P P T T S M E S L R N M F R D L D V D D
181    GTTCGAATGTACTTGGCCTTAAAACTCGGACTCTTCAGCCCTCAGGATCTTGAACCGCA
      V R M Y L A L K L G L F S P Q D L E P A
241    GAACCACCCGAAAAAGTTTTGGATGAAGTCAGCCTTATGGGTATTGTCAGGTGGATCAAG
      E P P E K V L D E V S L M G I V R W I K
301    AGOGATAGGTGCAAGAAGATCATAAACACTTTCAGGAOCTGGTATTCTAOCCTGCTGCGA
      S D R C K K I I T L S G A G I S T S A G
361    ATTCCAGACTTCCGTAGTCCCGAAACAGGATTATATCATAACTTACAGAAGTAOCGAACTA
      I P D F R S P E T G L Y H N L Q K Y E L
421    CCTCAACCACAGCGATATTCGAAATAAATTTCTTCAGGCAAAATOCCAAACCTTTCTTC
      P Q P Q A I F E I N F F R Q N P K P F F
481    ACATTAGCAAAGGAATTATTTCCAGGAAGCTTTAAGCCTACAATTTACATTATTTTATA
      T L A K E L F P G S F K P T I S H Y F I
541    AGACTTCTACATGAGAAAGGGCTCTTATTACGTCATTATACACAAAACATTGACACATTA
      R L L H E K G L L L R H Y T Q N I D T L
601    GAGCGAGGAGCCCGCATTCCCGAAGAAAAATTAGTCCGAAACACACGGAAOCTTCTACACA
      E R G A G I P E E K L V E A H G T F Y T
661    TCACATTGCTGGATTGCCGCAAGGAATACCCACTGGAGTTTGTCAAAGAAAGGATATTT
      S H C L D C R K E Y P L E F V K E R I F
721    GCTGACCAGATOCCAATCTGCACATGAATGCCCTGGTGTGTGAAGCCTGACATTGTGTTC
      A D Q I P I C T E C P G V V K P D I V F
781    TTOGGTGAAGTCTGCCGAGAGGTTCCAGATGTGTCTAGAGGAGGACTTCAAGCAATGT
      F G E S L P E R F Q M C L E E D F K Q C
841    GACATGCTTATTATTATGGGATCTTCACTTGAAGTGAACCATTTGCATCATTATAGAT
      D M L I I M G S S L E V Q P F A S L I D
901    ATGGTCCCAGATTGGTCTCCTCGACTTCTGATAAACCGTGAGAAGCCCGCGTGGAGCCA
      M V P D S C P R L L I N R E K A G V R P
961    CCTATATTGAGAATAAGAGGATTAATGTGTGGAGGTTTACAGTTAGACGAAGGTTCCCTAT
      P I L R I R G L M C G G L Q L D E G S Y
1021   AGAGATGTGGCGAGGCTAGGAGATTGTGATGAAGGCTGCCAGGACTTGGCAGACAGACTG
      R D V A R L G D C D E G C Q D L A D R L
1081   GGATGGGGGTTGAACCTCCGACATTAGTAGCCGTGAACATGAAOCTTGGACCAAGAA
      G W G D E L R A L V A R E H E R L D Q E
1141   CTTTGTACTGCGTCTOCCCATGCOCCGTACTGATAOCTTCAGAAGCAAACGCGGGAOCC
      L L T A S P H A P V L I P S E A N A G P
1201   AGTGCCTCGGAAATGATCAAAACAATTAACAATTAGATTAACCTCGAATTTAATTGTACC
      S A S E
1261   TCGGATGACCTGAATGAAGOGAAGAAAAGCCATGATGTGATTCAAATAAAAAATATTGGAA
1321   AATTAATAAAAAATTTACAGATATGTAAATCGAACTACAAGCACCTTCTTGTCTATTTTAA
1381   TAATAATTTAAAAAGGTACTTAGTAAGAATACTCAATTTAATATTCGCTCTATTTTCAT
1441   GCGGTTAATACAGATAAAAATTCCTCTGGGTCCTCCGTTTCTATTGATTTCAAAGAAGT
1501   ACGCAAACAATATACTCATAAAATTTTGTATATAGCAACATTCAATTATTTATTGGTAAA
1561   CTGAAAACCACTTCAAGTAOCTAATT

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Figure 1. The cDNA sequence and deduced amino acid sequence of BmSirt2. The start codon (ATG) and stop codon (TGA) are boxed. Two poly-A signals are underlined. In amino acid sequences, SIR2 domain is framed. Two nonsense mutation found in the sequencing are shaded.

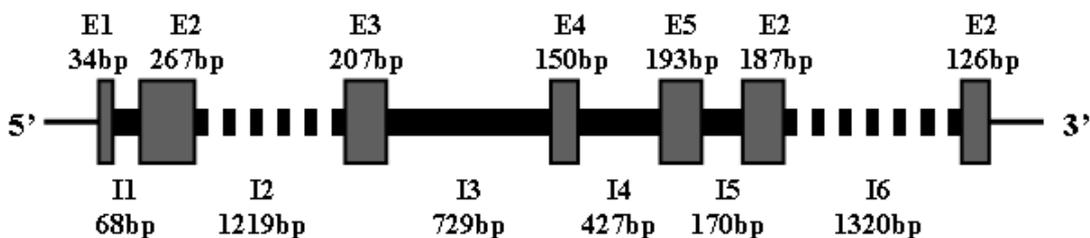


Figure 2. The gene structure of BmSirt2. Seven exons (E) are indicated as large open boxes with the length in nucleotides just above the box. Introns (I) are thick lines with lengths in nucleotides listed below the lines.

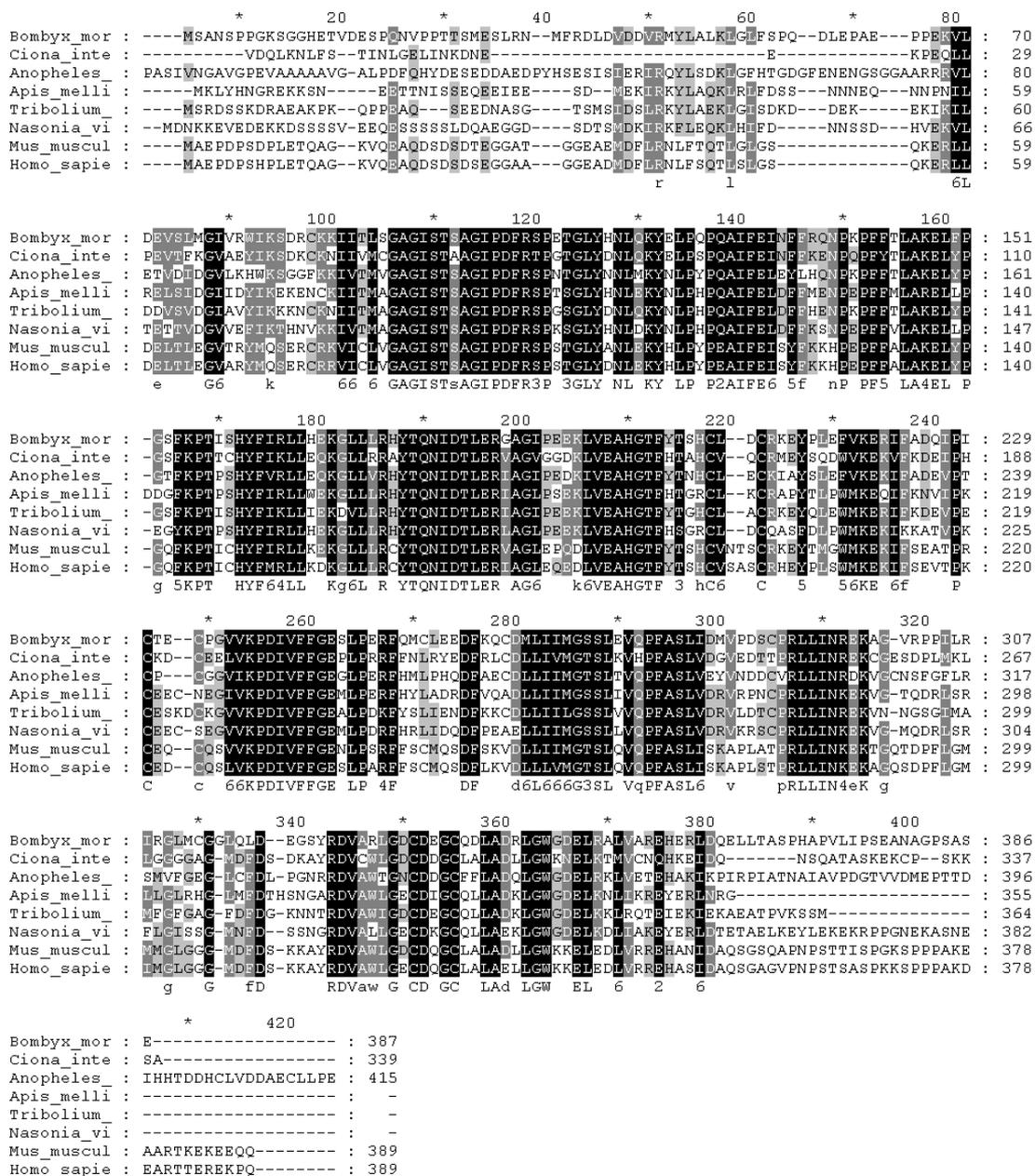


Figure 3. Multiple alignments of the amino acid sequences of Sirt2 proteins from *B. mori*, *Ciona intestinalis*, *Anopheles gambiae*, *Apis mellifera*, *Tribolium castaneum*, *Nasonia vitripennis*, *Mus musculus* and *Homo sapiens*. Invariant residues are highlighted in black.

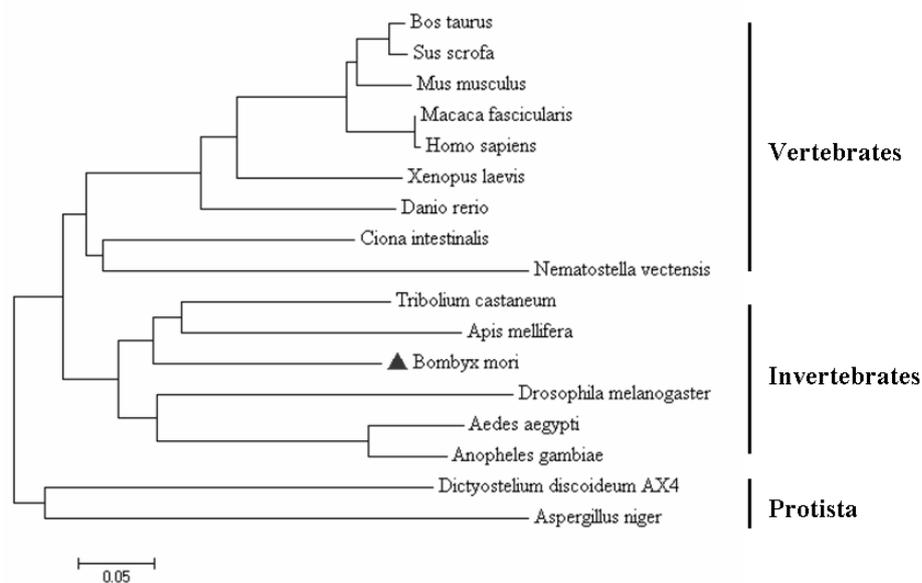


Figure 4. Neighbour-joining phylogram showing the relationships between Sirt2 from *B. mori* and other organisms. The tree distances were generated according to the ClustalW algorithm, and the tree was constructed using MEGA 3.1.

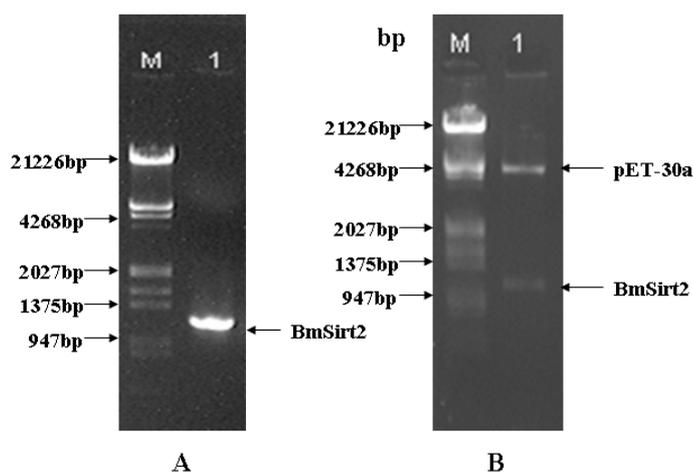


Figure 5. (A) The result of PCR for BmSirt2. Lane M, DNA molecular mass maker; Lane 1, PCR product. (B) Identification of the recombinant plasmid pET-30a/BmSirt2 by digestion by *Bam*HI and *Xho*I. Lane M, DNA molecular mass maker; Lane 1, digested pET-30a/BmSirt2

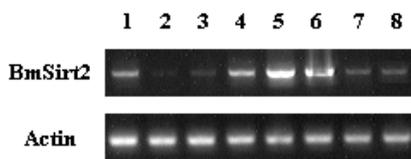


Figure 6. Distribution of BmSirt2 gene in multiple tissues. 1. epidermis; 2. midgut; 3. fat body; 4. silk gland; 5. hemolymph; 6. nerve knot; 7. testis; 8. ovary. *B. mori* Actin A3 gene was used as the control to show the normalization of the amount of templates in PCR reactions.

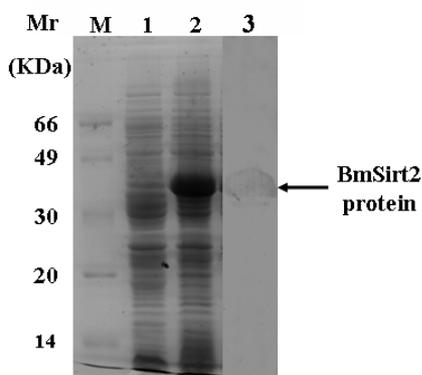


Figure 7. Expression of BmSirt2 in *E. coli*. BL21 cells. Cell extracts were separated by SDS-PAGE and stained with Coomassie brilliant blue. Lane M: protein molecular weight markers. Lane 1: proteins from BL21 transformed with pET-30a plasmid. Lanes 2: proteins from BL21 cells transformed with plasmid containing BmSirt2 gene. Lane 3: the western blotting of fusion protein using anti-His-tag antibody.

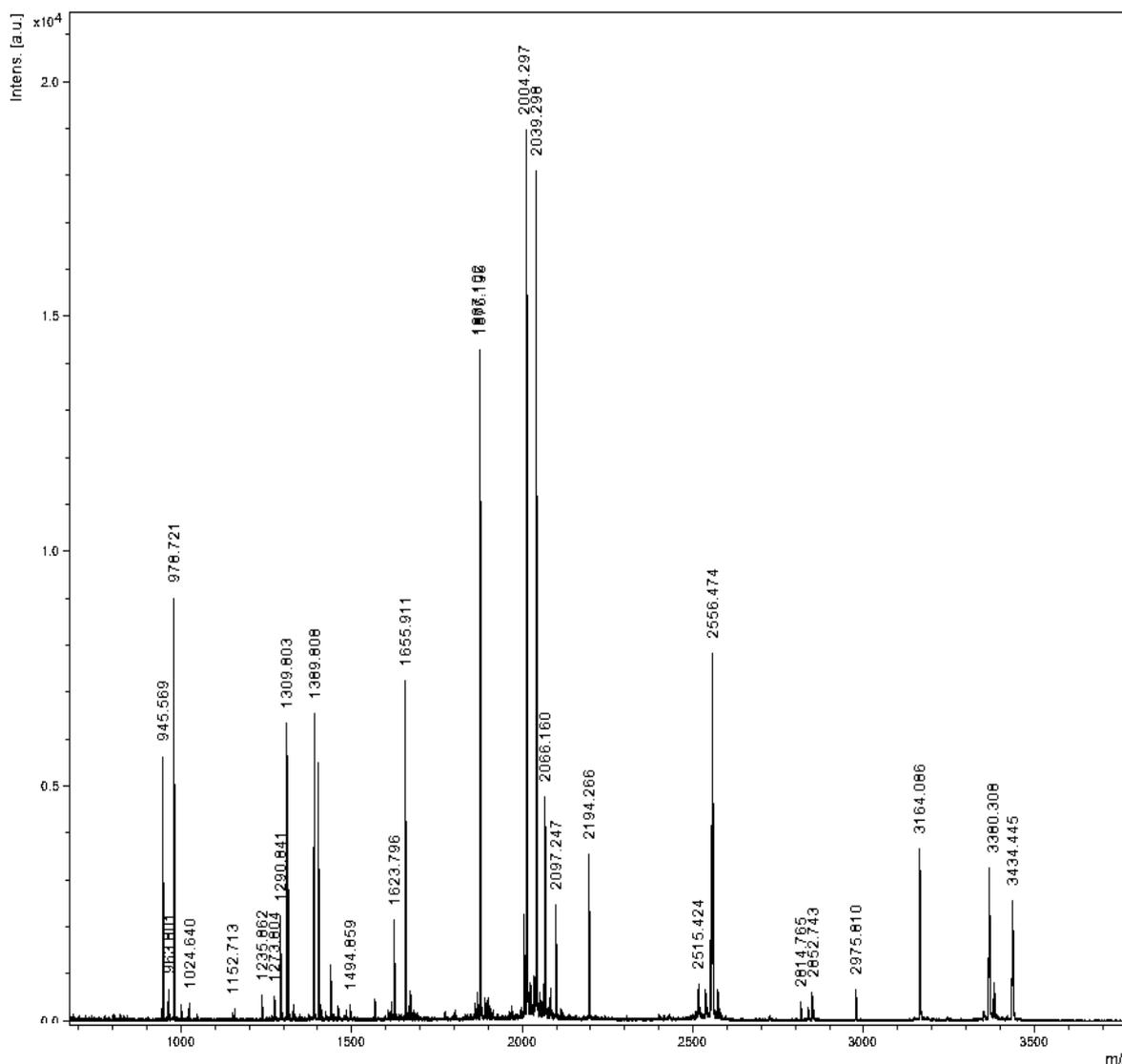


Figure 8. Peptide mass fingerprints (PMF) for BmSirt2. MALDI-TOF-MS spectrometry was done with in-gel-digested peptides of the recombination protein.