Section A: Project Details - Consolidated Annual Progress Report

FINAL REPORT OF DBT-TWINING SCHEME
(01.04.2011 -16.03.2015)

A1. IDENTIFICATION OF SNPs IN LEPTIN GENE FOR SELECTION OF MITHUN (*Bos frontalis*) FOR HIGHER GROWTH TRAITS AND CHARACTERIZATION OF LEPTIN PROTEIN: C1-16

A2. DBT Sanction No. BT/01/NE/TBP/2010 dated 14/03/2011

Department of Animal Genetics and Breeding
National Research Centre on Mithun (ICAR)
Jharnapani, Medziphema, Nagaland – 797 106, INDIA
&
Department of Veterinary Microbiology
WEST BENGAL UNIVERSITY OF ANIMAL AND FISHERY SCIENCES
37, K.B. SARANI, KOLKATA- 700037

IDENTIFICATION OF SNPs IN LEPTIN GENE FOR SELECTION OF MITHUN (*Bos frontalis*) FOR HIGHER GROWTH TRAITS AND CHARACTERIZATION OF LEPTIN PROTEIN

CONSOLIDATED ANNUAL PROGRESS REPORT (1st April, 2011 -31st March, 2015)

A3. Name of the Principal Investigator :

1. Dr. Sabyasachi Mukherjee,
Sr. Scientist (AGB)
NRC Mithun (ICAR), Nagaland

2. Dr. S. N. Joardar
Reader, WBUAFS, Kolkata Centre

Name of Co-PI/Co-Investigator :

Dr. (Mrs) Anupama Mukherjee
Sr. Scientist (AGB)
NRC Mithun (ICAR), Nagaland

Name of the Junior Research Fellow :

1. Ms. Moonmoon Mech, M.Sc.
NRCM, Nagaland centre
2. Sk Sahanawaz Alam, M.Sc
WBUAFS, Kolkata centre

A4. Institute :

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National Research Centre on Mithun (ICAR), Nagaland
2. Department of Veterinary Microbiology,
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Department of Veterinary Microbiology,
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68 K.B.Sarani, Kolkata-700037, West Bengal.

A6. Total Cost of the Project: Rs.46.05 lakhs (NRCM, Nagaland : Rs.29.32 lakhs and WBUAFS, Kolkata - Rs. 16.73 lakhs)

A7. Duration: Three Years (14.03.2011- 31.03.2014) extension up to 14 September, 2014 vide e-mail order dated 25.03.2014.

A8. Approved Objectives of Project

- Genetic characterization of Leptin gene of Mithun.
- Identification of SNPs in Leptin genes in Mithun
- Association of Leptin polymorphism/SNPs with growth traits of Mithun.
- Isolation and characterization of leptin protein in Mithun.

A9. Specific Recommendations made by the Task Force (if any) -

The following Recommendations were made by Task Force held on 12 April 2013:

- **Progress report: Satisfactory**
- For association of Leptin SNPs with growth traits & weight of Mithun -
    - Animals subject to ideal Management practice to be taken up for study.
    - Mithun population from various environments to be studied. Within each group look for contrasting wt. animals viz. heavy and light and study their SNP profile.
    - Confirm SNPs by sequencing at least 5 times or Real time PCR.
- For serological characterization of leptin -
    - Check Mithun-leptin for cross reactivity with Anti cattle-leptin antibody.
- Contractual of skilled labour approved for 3rd year.
- **Rs. 40,000 allowed for refrigerator purchase, however, not sanctioned !**

- **Further, another recommendations were received on 01 October 2013 as below -**

- With reference to the proposal entitled “Identification of SNPs in leptin gene for selection of Mithun (*Bos frontalis*) for higher growth traits and characterization of leptin protein” for additional grant for sequencing work.
- In this regard we would like to inform you that the Expert Committee has recommended your proposal, in principle, **an additional grant of Rs. 8.00 lakhs was approved for the sequencing work.**

Section B: Scientific and Technical Progress

Consolidated Annual Progress Report (1st April, 2011 -31 March, 2015)

Period of reporting : 1/4/2011 To 31/03/2015

INTRODUCTION

Leptin is one of the most useful biomolecule to act as a marker for identifying the high performing individuals leading to better adaptability and productivity. Leptin has a pleiotropic effect on regulating appetite, energy metabolism, growth, reproduction, body composition and immunity.

Leptin, the 16-kDa molecular weight protein having 146 amino acids, is classified as cytokine due to its structural similarity with leptin receptor and glycoprotein (gp) 130, a member of IL-6 family. Leptin is a four helix cytokine and contains a single disulphide linkage (cys 96, cys 146) connecting the cluster of differentiation (CD) loop to the carboxyl terminus. Although report on mammalian leptin are available, information regarding leptin protein of mithun is scare.

Mithun (*Bos frontalis*) is a domesticated yet forest dwelling bovine species found in the north eastern hilly region of India. This animal has been referred to as the 'sacrificial ox' of the Naga people and mainly used for its meat during various social ceremonies. This animal has a very important place in the lives of tribal people of North East part of our country.

The present project has been continued to characterize this important gene in Mithun at the molecular level and to study its association with live weight and growth traits. This will also include identification of SNPs in the leptin gene of Mithun and characterization of leptin protein. Efforts will be made to identify SNPs in this gene and to find out their association with higher growth traits in Mithun. This will be very much helpful for selection of Mithuns with high growth rates for meat production. On the other

hand, this important leptin protein will also be isolated and characterized to study at the molecular level and to find out any difference from bovine leptin.

B1. Progress made against the Approved Objectives, Targets and Timelines during the Reporting Period

- **Work plan for entire course of study (0-48 months)**

NRC-M (ICAR), Nagaland

0-24 Months	• Notification and engagement of JRFs/SRFs and contractual workers • Selection of mithuns for experiment • Completion of codal formalities for procurement of chemicals, consumables, and equipments • Regular monitoring of mithuns for growth purpose • Initiation of experiments, blood sample collections • Isolation of DNA • PCR amplifications standardization • Single Sequencing of leptin gene fragments of Mithuns through outsourcing
30 Months	• Continuation of earlier studies • Analysis of sequence data and identification of SNPs • Study on the association of leptin polymorphism with growth traits of Mithuns • Statistical analysis • Annual evaluation by DBT experts committee • Recommendations for five time sequencing of PCR products for confirmation of SNPs and inclusion of more number of mithuns for the study
36 - 48 Months (under extension)	• Body weights taken for more than 100 mithuns belonging to various strains • Large scale PCR amplifications, gel elution of PCR products and outsourcing of sequencing job. • Five times sequencing of leptin gene fragments for confirmation of SNPs • Analysis of sequence data • Statistical analysis • Report writing.

Work Done during the entire period - NER Institute (NRCM, Nagaland)

- A group of 25 growing Mithuns calves belonging to Nagaland and Arunachal Pradesh strains were selected initially for this study. However, nine animals died during entire period of study due to various reasons.
- Procurement of consumables for the period was be taken up very late due to delay in availability of fund during the third year.
- Monitoring of body weight of experimental animals which are above one year in monthly interval.
- Body measures consisting of length, height and girth are recorded after every three months.
- PCR amplifications using primers for Leptin 422bp, and Leptin 588bp sequences of Mithuns.
- Screening of animals for Leptin polymorphism taking 422 bp PCR products.
- Screening of animals for Leptin polymorphism taking 588 bp PCR products by PCR-RFLP technique.
- Collection of animal sera and animal adipose tissue.
- Serum samples of all the experimental animals were sent for further analysis of protein.
- Sequencing of 24 mithuns in respect of 422 and 588 bp PCR amplicons.
- Apart from these, around 100 adult mithuns body measurements were recorded in terms of their birth weight, maturity weight, average daily weight gain (ADG), heart girth, body length, and height at weither.
- Five times sequencing of total 108 samples of leptin gene amplicons (422 bp and 588 bp) of mithuns were carried out. Consensus sequences were constructed from these raw sequences.
- These sequences were aligned and SNPs were identified using different bioinformatic software (MEGA4, Muscle, Bioedit, Megalign etc).
- The project gets six months extension from DBT and 3rd year grant was released very late during July, 2014.
- However, due to delay in large number of sequencing work and their analysis, the work was only completed recently.
- Statistical analysis using SAS software was carried out taking the SNP as one fixed effect along with year of birth, sex and strains of mithuns on growth traits like birth weight, adult weight and average daily wt gain (ADG).
- However, SNPs was having no significant effect on any of the growth traits studied so far.

Detailed Achievements against the Targets/Objectives

Objective - 1

- ***Genetic characterization of Leptin gene of Mithun.***

Achievements - Amplification and sequencing of leptin gene of mithun

Materials and methods used for the study

Glassware, plastic-wares and chemicals

All the glassware and plastic-wares used throughout the study were of neutral glass of Borosil and Tarsons make, respectively. The chemicals used for the experiments were of standard make namely Promega, Sigma Laboratories (USA) and Bangalore Genei. Primes for PCR amplification were also synthesized from IDT lab.

Isolation of genomic DNA of Mithun

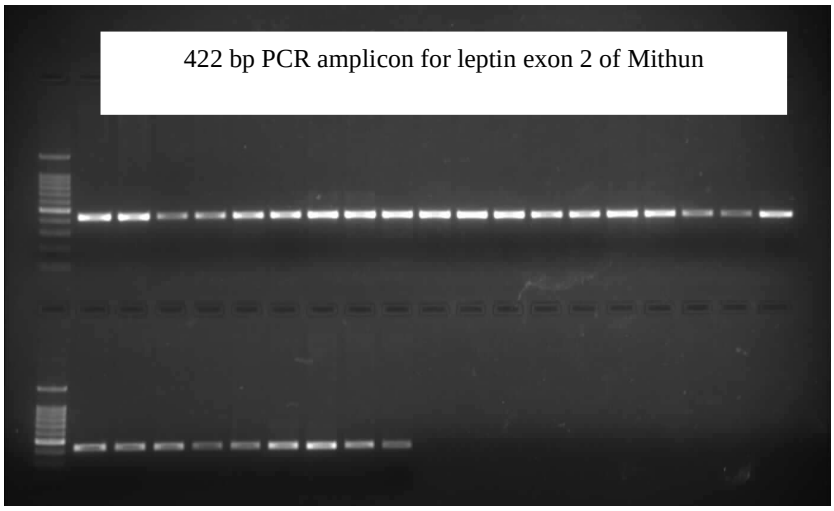
Blood samples were collected from Mithun (*Bos frontalis*) in heparinized vacuutainer tubes and genomic DNA was isolated from this whole blood using Promega Wizard DNA isolation kit as per manufacturer protocol. Quality and quantity was checked for each DNA samples and nanodrop rating (260/280) between 1.7-1.8 for each DNA was considered to be of good quality for further work.

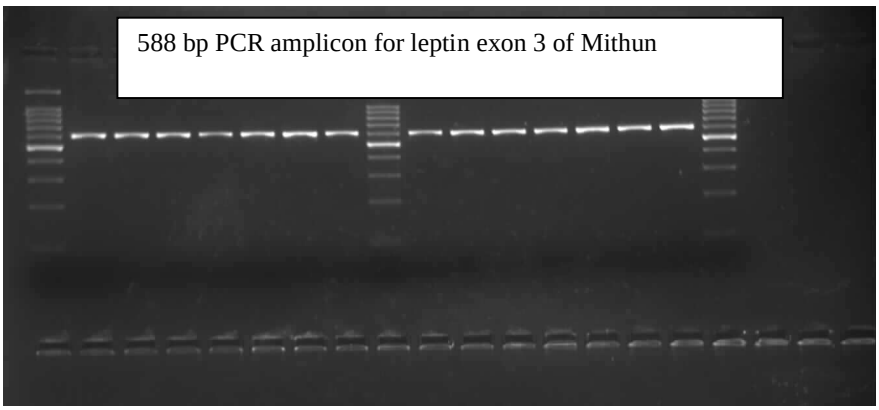
PCR amplification standardization

Initially two reported bovine leptin primers (Lagonigro et. al. 2003) were tried for PCR amplification of mithun leptin gene. However, these two primers could not amplify mithun leptin gene fragments. Subsequently, two primers were designed using DNASTAR software for amplification of exon 2 and exon 3 region of Mithun leptin gene based on Mithun leptin sequence available in the Genbank. These two primers successfully amplified the two exonic regions of Mithun to produce 422 bp and 588 bp PCR amplicons. One more primer pair (Pomp et al. 1997) was used to amplify a 1820 bp fragment of leptin gene of Mithun.

Table : Details of primers used for leptin gene (lep) Amplification

Sl No.	Primer sequences (5'- 3')	Annealing temperature (°C)	Product size	Length
1	F-5-CCCTCTCTCCCACTGAGCTC-3 R-5-GCCTATGTGGGCATCCTTTA-3	Not amplified	496 bp	20 20
2	F-5-GATTCCGCCGCACCTCTC-3 R-5-GGCTGTGCAGCCTTGCACAGG-3	Not amplified	467 bp	18 21
3	F-5-TGAAGACGTGGATGCGGGTGGTAA -3 R-5- AGGCAGGAGGCAAGGGAAGTGGAG -3	57.0^{0}C	422 bp Exon 2	24 24
4	F-5-GCTCTAGGGAAAGGCGAAGTC -3 R-5- TTTGGAAGAGCGGCTGAGAGGA -3	59.0^{0}C	588 bp Exon 3	21 22
5	F-5-GTCACCAGGATCAATGACAT-3 R-5-AGCCCAGGAATGAAGTCCAA-3 (Pomp et al., 1997)	55.0^{0}C	1820 bp	20 20





588 bp PCR amplicon for leptin exon 3 of Mithun

Collection of Animal sera

Blood samples were collected from Mithun (*Bos frontalis*) from Nagaland, sera were separated and kept in deep freezer (-20^0 C) till transportation to collaborating centre for further processing.

Collection of Adipose Tissue samples

Adipose Tissue samples of mithuns (Bos frontalis) were collected from Nagaland and Roing, Arunachal Pradesh and kept in freezing condition (- 80^{0}C) till processing of these samples.

Objective - 2

- ***Identification of SNPs in Leptin genes in Mithun***

Achievements - PCR amplification, PCR-RFLP, Sequencing for SNP identification

All the PCR products were gel eluted and sent for sequencing to Chromous Biotech Pvt Ltd, Bangalore through outsourcing of services. In the initial stages, only one time sequencing of PCR products was practiced. However, during the mid-term review of the project, Expert Committee suggested five times sequencing of all the PCR products for confirmation of SNPs identified.

Special requirement - Five times sequencing work

- Sequencing of 216 samples (each 108 PCR products samples) for 2 genes 5 times with double pass analysis.

Genes sequenced

- *Bos frontalis* Leptin gene exon 3: 588 bp

- *Bos frontalis* Leptin gene exon 2: 422 bp

Steps followed

- The PCR products were run on a 2% Agarose gel for sizing.
- The products were subjected to sequencing reactions 5 times bi-directional
- The data was aligned and SNP's were marked for all the samples.

Sequencing Reaction

The Sequencing mix Composition and PCR Conditions are as follows:

10µl Sequencing Reaction

- Big Dye Terminator Ready Reaction Mix : 4µl
- Template (100ng/ul) : 1µl
- Primer (10pmol/λ) : 2µl
- Milli Q Water : 3µl

PCR Conditions: (25 cycles)

Initial Denaturation	: 96°C for 5 min
Denaturation	: 96°C for 30 sec
Hybridization	: 50 °C for 30 sec
Elongation	: 60 °C for 1.30 min

Instrument and Chemistry used

Sequencing Machine	:	ABI 3130 Genetic Analyzer
Chemistry Cycle sequencing kit.	:	Big Dye Terminator version 3.1"
Polymer & Capillary Array	:	POP_7 polymer 50 cm Capillary Array.
Analysis protocol	:	BDTv3-KB-Denovo_v 5.2
Data Analysis Software	:	Seq Scape_ v 5.2
Reaction Plate	:	Applied Biosystem Micro Amp Optical 96-Well Reaction plate

- The PCR amplification of exon 3 and intron 3 of **leptin 588 bp** amplicon were carried out and fixed the annealing temperature at 59°C. The PCR-RFLP was done with 5U of NruI restriction enzyme at 37^0C for 1 hour. The RFLP product was run at 3%

agarose gel electrophoresis; however no cut were observed for leptin 588 bp in the present study as shown (figure).

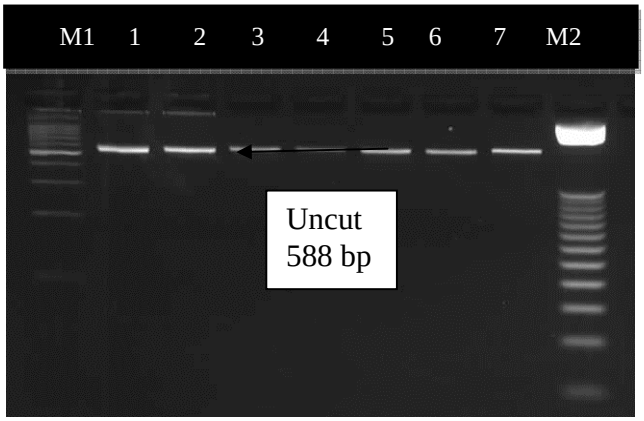


Figure. PCR-RFLP product of leptin 588 bp using restriction enzyme Nrul. M1 – 100 bp DNA marker, M2 – 25 bp DNA marker, 1-7 uncut sample by restriction enzyme.

- The PCR amplification of exon 2 and intron 2 of **leptin 422 bp** were carried according to Liefers *et al*. (2002) and the annealing temperature was fixed at 57°C. PCR-RFLP was carried out using ClaI restriction enzyme. The PCR product was digested with 5U ClaI at 37°C for 1 hour. The digested product was run at 3% agarose gel electrophoresis and a product with single cut with two bands (about 287 bp and 135 bp were observed in the present study as shown (figure).
- 145 animals were screened for leptin polymorphism using PCR-RFLP study taking 422 bp amplicons and all were found to be BB genotypes having two RFLP products (287 bp and 135 bp)

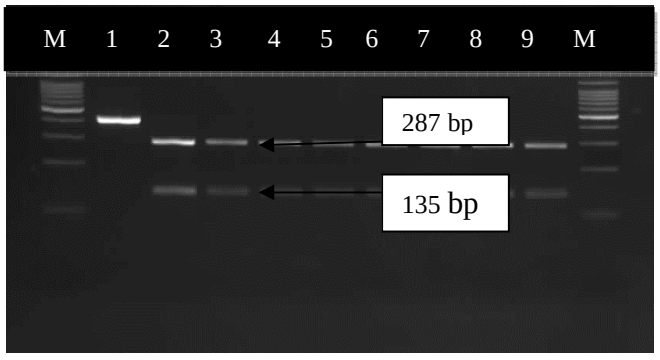


Figure. PCR-RFLP product of leptin 422 bp using restriction enzyme ClaI. M – 100 bp DNA marker, 1 – negative control uncut 422 bp, 2 – 9 digester samples with estimated band size of 272 bp and 150 bp.

- **Gene and genotype frequency in terms of PCR-RFLP (ClaI) of leptin 422 bp amplicon**

GENOTYPE	NO. OF ANIMALS
AA	0
AB	8
BB	137
TOTAL	145

n	ALLELIC FREQUENCY		GENOTYPIC FREQUENCY		
	A	**B**	**AA**	**AB**	**BB**
145	0.02	0.97	0	0.05	0.94

A 422 bp fragment of the leptin gene was amplified by PCR. Genotype analyses were performed using PCR-RFLP method. After digestion of 422 bp PCR products with R.E. ClaI, two genotypes were determined. The digested BB genotype gave two fragments of 287 and 135 bp whereas the AB genotype gave fragments of 422, 287 and 135 bp. On the basis of the Hardy-Weinberg equilibrium law, the expected frequencies of A and B alleles were in population of 145 mithuns 0.02 and 0.97 respectively. The observed frequencies of genotypes were 0 (n=0), 0.05 (n=8), 0.94 (n=137) for AA, AB and BB genotype respectively.

SNP identification in leptin gene of mithun

- A total of 17 SNPs have been identified in the 422 bp amplicons sequenced from a total 132 animals including 25 experimental mithuns. Out of these 17 SNPs, four are detected in coding region (exon 2 - 115-258 bp). These are as below -

Sl No.	SNP	Location	Frequency
1	A>G	26	32/132
2	C>T	52	30/132
3	G>A	54	2/132
4	C>T	59	32/132
5	T>C	131	32/132
6	A>T	191	2/132
7	A>C	191	30/132
8	G>A	229	32/132
9	G>A	310	23/132
10	T>C	314	115/132
11	C>T	322	115/132
12	C>T	361	32/132
13	C>T	371	85/132
14	C>T	382	30/132
15	T>A	383	2/132
16	T>G	383	30/132
17	G>A	387	32/132

- In another PCR leptin amplicon of 588 bp, nine SNPs have been identified sequenced from a total of 122 animals including 25 experimental mithuns. Out of these nine SNPs, four are detected in coding region (exon 3 - 87-446 bp). These are as below -

Sl No.	SNP	Location	Frequency
1	G>A	33	5/122
2	G>T	60	120/122
3	G>C	61	120/122
4	G>T	63	120/122
5	A>C	82	121/122
6	G>A	302	65/122
7	T>C	353	85/122
8	T>C	368	108/122
9	T>C	480	110/122

Objective - 3

- ***Association of Leptin polymorphism/SNPs with growth traits of Mithun.***

Achievements - Association study of SNPs with growth parameters of mithun

- For the association study, three growth parameters were taken (birth weight, maturity weight, average daily weight gain - ADG). While the genetic and non-genetic factors included sex and strain of the animals (four - Nagaland, Arunachal, Manipur and Mizoram), period of birth (clubbing various year of birth into three period) and SNP position (SNP26, 314, 322, 371).
- SAS statistical package was used taking PROC GLM (generalized least squares method) procedure taking total 132 mithuns in the analysis.

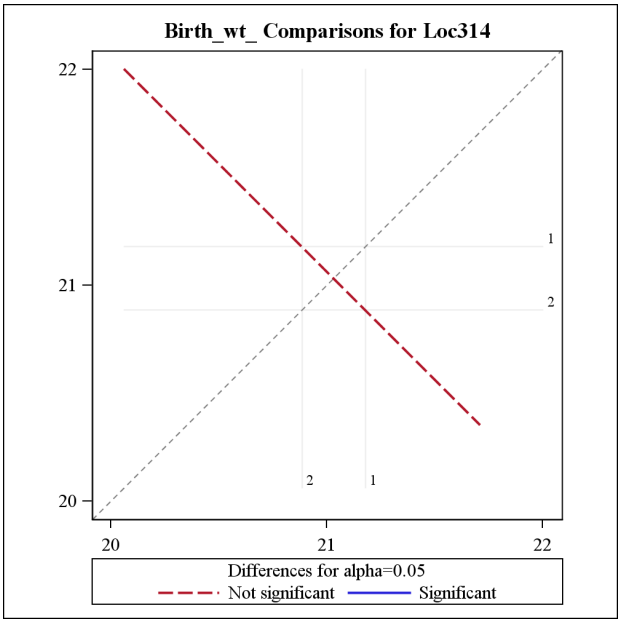
Class Level Information		
Class	**Levels**	**Values**
Sex	2	Female Male
Strain	4	1 2 3 4
Year_code	3	1 2 3
Loc314	2	1 2

Number of Observations Read	132
Number of Observations Used	130

- PROC GLM procedure - Association of dependent variable Birth_Wt with SNP Loc314 was found to be non-significant.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	33.2228161	4.7461166	0.94	0.4796
Error	122	617.0009531	5.0573849		
Corrected Total	129	650.2237692			

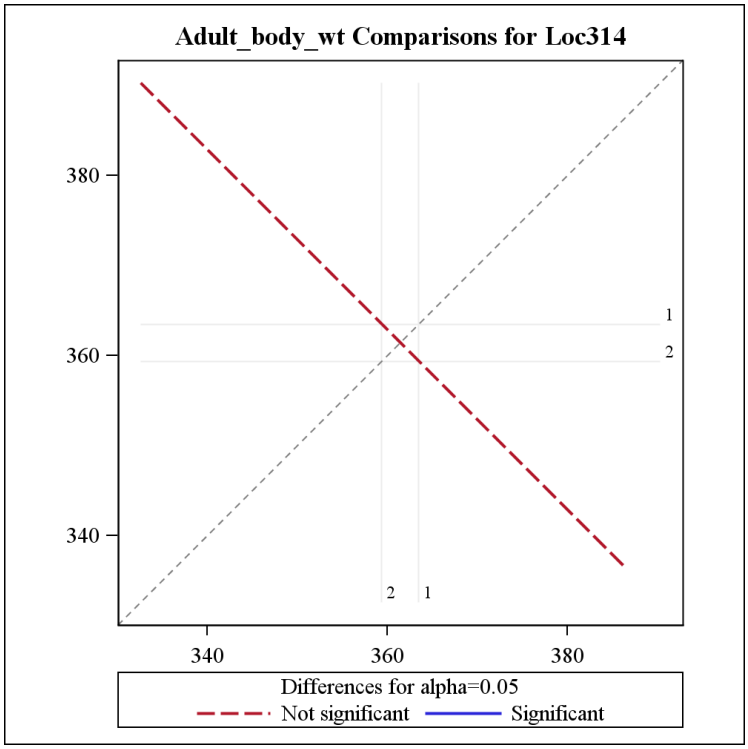
Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	5.62822161	5.62822161	1.11	0.2935
Strain	3	15.98704795	5.32901598	1.05	0.3715
Year_code	2	10.97642648	5.48821324	1.09	0.3411
Loc314	1	0.63112007	0.63112007	0.12	0.7245



- PROC GLM procedure - Association of dependent variable Adult Body_Wt with SNP Loc314 was found to be non-significant

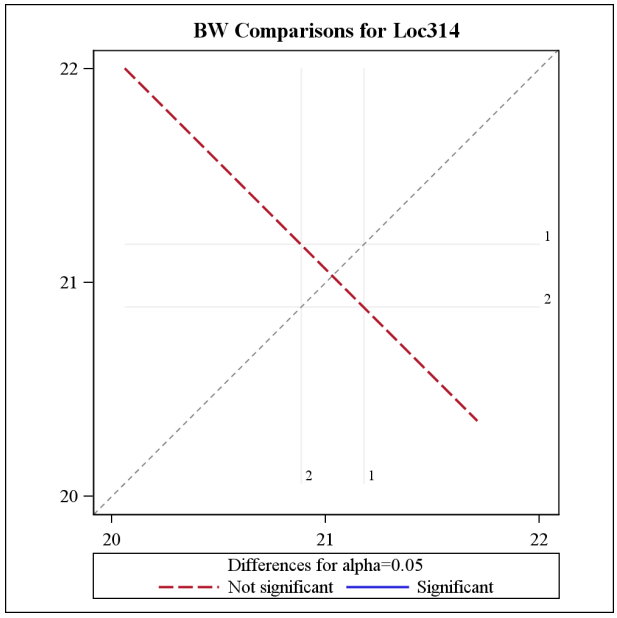
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	366232.142	52318.877	9.77	<.0001
Error	122	653422.406	5355.921		
Corrected Total	129	1019654.548			

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	122115.3594	122115.3594	22.80	<.0001
Strain	3	22042.6191	7347.5397	1.37	0.2546
Year_code	2	221950.2017	110975.1008	20.72	<.0001
Loc314	1	123.9616	123.9616	0.02	0.8793

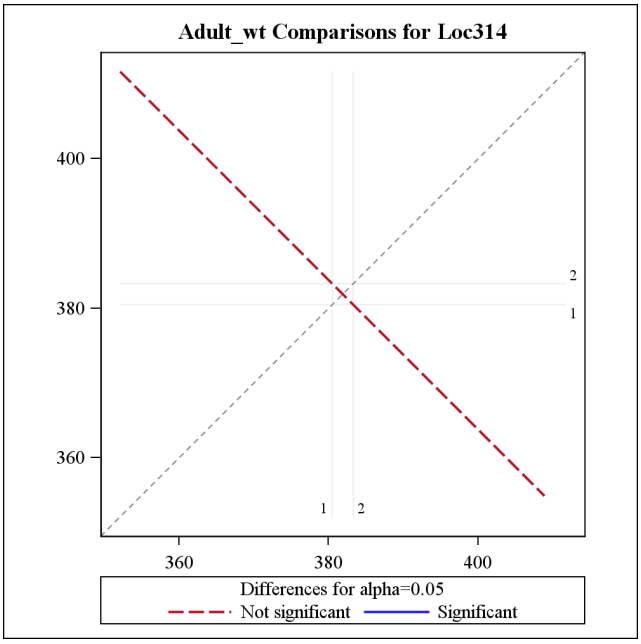


- PROC GLM procedure - Association of Dependent variable Birth wt (BW), Adult Body_Wt and ADG with SNP Loc314 was found to be not-significant.

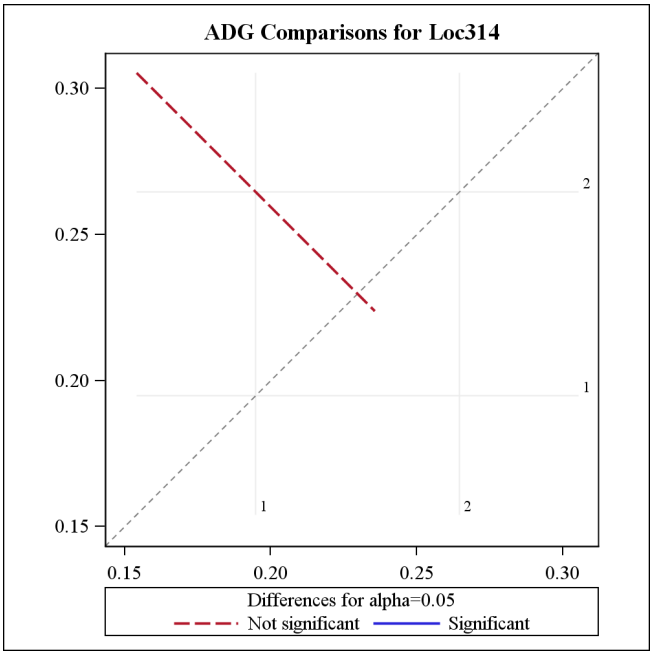
Loc314	BW LSMEAN	Standard Error	H0:LSMEAN=0	H0:LSMean1=LSMean2	
			Pr > |t|	t Value	Pr > |t|
1	21.1782381	0.2318848	<.0001	0.35	0.7245
2	20.8843068	0.8176334	<.0001		



Loc314	Adult_wt LSMEAN	Standard Error	H0:LSMEAN=0 Pr > |t|	H0:LSMean1=LSMean2 t Value	H0:LSMean1=LSMean2 Pr > |t|
1	380.463791	8.000562	<.0001	-0.10	0.9221
2	383.277180	28.210237	<.0001		



Loc314	ADG LSMEAN	Standard Error	H0:LSMEAN=0 Pr > |t|	H0:LSMean1=LSMean2 t Value	Pr > |t|
1	0.19480781	0.01147473	<.0001	-1.70	0.0924
2	0.26464204	0.04046026	<.0001		



- Similarly, association of dependent variables Birth weight, Adult Body_Wt and ADG were also found to be non significant with other SNPs found in leptin gene fragments (422 bp and 588 bp) in the analysis with PROC GLM of SAS.

B2. Summary and Conclusions of the Project

- A group of 25 Mithuns calves belonging to Nagaland and Arunachal Pradesh strains were selected initially for this study over. Monitoring of their body weight (growth), body measurements in regular intervals. DNA was also isolated from these animals.
- PCR amplification using primers for Leptin 422bp, and 588bp PCR products of Mithuns.
- Growth and body weight of around 23 experimental mithuns were recorded regularly. Apart from these, around 100 adult mithuns body measurements were recorded in terms of their birth weight, maturity weight, average daily weight gain (ADG), heart girth, body length, and height at weither.
- Sequencing of 168 samples of leptin gene amplicons (422 bp and 588 bp) of mithuns were carried out. Consensus sequences were constructed from these raw sequences.
- These sequences were aligned and SNPs were identified using different bioinformatic software (MEGA4, Muscle, Bioedit, Megalign etc).
- Statistical analysis with SAS v.9.3 was carried out for association study between various SNPs identified and growth parameters of mithun.
- No SNPs identified so far were found to be have significant correlation with any growth traits in mithun

B3. Connectivity of the partnering Institutes (Institute wise achievements to be given separately for each objectives)

- Institute wise achievements are already given separately

B4. Details of New Leads obtained, if any

- A number of SNPs have been identified in the leptin gene of mithuns and association of two SNPs (SNP Loc3014 & Loc322) was found to have positive and significant effect on growth rate of mthun (ADG). These may be important markers for selection of mithuns of growth traits. However, studies is continued in this aspects.

B5. Details of Publication and Patents, if any - Please see Annexure-V

Two Genbank submissions -

- GenBank: JX273647.1
- GenBank: JX273648.1

B6. Training undertaken by the NER PI and the recruited manpower at the Collaborating Institute

- The trips were made to the Collaborating Institute (WBUAFS, Kolkata) by the NER PI for transportation of samples, discussion related with the project etc.
- A number of field trips were also conducted in the native breeding tracts of mithuns in four NEH States (Nagaland, Arunachal Pradesh, Manipur and Mizoram) for field samples collections from time to time.

B7. Details of visits of the Collaborating Institute PI and the personnel's to NER

- One visit of the Collaborating Institute PI (WBUAFS) was made during the reported period.
- Other than this, there was regular interactions and visit of PI of NER Institute (ICAR: NRCM, Nagaland) was made to Collaborating Institute (WBUAFS, Kolkata) as and when necessary.

Section C: Details of Grant Utilization

C1. Equipment acquired or placed order with actual cost

Sl No.	Name of equipment procured	Cost (Rs.)
1	Deep freezer (- 80^0C) – NSW/Eppendorf	478970
2	Power back up system (5 kv Inverter with 8 batteries)	121000
	Total	**599970**

Details of Expenditure – Please see the table below

C2. Details of Expenditure – Please see the table below

Consolidated statement of Expenditure											
DBT project entitled "Identification SNP's in Leptin gene for selection of Mithuns" (*Bos Frontalis)*											
Reciept (Rs)						**Expenditure (Rs)**					**Balance**
Name of Head	**1st Year**	**2nd Year**	**3rd Year**	**4th Year**	**Total**	**1st Year**	**2nd Year**	**3rd Year**	**4th Year**	**Total**	
Non Recurring											
Equipment	600000				600000	599970				599970	30
Recurring											0
Manpower	247000	200000		298000	745000	200355	227800	200400	25226	653781	91219
Consumables	400000	400000		300000	1100000	400000	398220		299529	1097749	2251
Travel	50000	50000		50000	150000	49908	50025		50067	150000	0
Contigency	50000	50000		50000	150000	49976	32052		49063	131091	18909
Overhead Charges	100000	50000		50000	200000	99947	69925		49956	219828	-19828
Sequencing				800000	800000				800000	800000	0
Non Recurring + Recurring	1447000	750000		1548000	3745000	1400156	778022	200400	1273841	3652419	***92581**

***Refund amount sent back to DBT vide Demand Draft No 277188 dated 23.03.2016**

Annexure – II

Collaborating Centre (WBUAFS, Kolkata)

IDENTIFICATION OF SNPs IN LEPTIN GENE FOR SELECTION OF MITHUN (*Bos frontalis*) FOR HIGHER GROWTH TRAITS AND CHARACTERIZATION OF LEPTIN PROTEIN

CONSOLIDATED ANNUAL PROGRESS REPORT
OF DBT-TWINING SCHEME

(Including Extension Period)

(01.04.2011 -31.03.2015)

Detailed Achievements against the Targets/Objectives

Objective - 4

- ***Isolation and characterization of leptin protein in Mithun.***

1. INTRODUCTION

2. Work plan for entire period (0-36 month + Extension period)

WBUAFS, Kolkata centre

Period of study	Achievable targets
0-24 months	• Collection of adipose tissue of goat as mithun adipose tissue was not readily available • Standardization of various purification techniques • Collection of mithun adipose tissue from the field samples
25-30 Month	• Purification of leptin protein by column chromatographic techniques • Serological characterization of leptin by Western blot.
31-36 Month + Extension period	• Characterization of leptin by mass spectroscopy (MALDI-TOF).

3. Work done during the third year including extension period

Collaborating Centre (WBUAFS, Kolkata)

3.1. Materials and Methods

3.1. 1. Materials

3.1.1.1 Glassware, plastic-wares and chemicals

All the glassware and plastic-wares used throughout the study were of neutral glass of Borosil and Tarsons make, respectively. The chemicals used for the experiments were of standard make viz. Sigma Laboratories (USA), Genetix/Fermentus, ProScience, Bangalore Genei and HiMedia.

3.1.2. Methods

3.1.2.1. Collection of Animal tissue

Adipose tissue samples were collected from Mithun (*Bos frontalis*) from different localities of Nagaland. Tissues were kept in deep freezer (-20^0 C) until use.

3.1.2.2. Ultrasonication of adipose tissue and protein estimation

Adipose tissue samples were ultrasonicate with the help of ultra sound sonicator (Hysel, Japan) in Phosphate Buffer Solution (pH -7.4). The samples were centrifuged and concentrated using sucrose. The total protein concentration was determined using Lowry's method (1951).

3.1.2.3. Protein extraction by Chemical method

Total adipose tissue and mature adipocytes were thawed in 0.4 ml of cold Urea/thiourea buffer (7M urea, 2M thiourea, 4% CHAPS, 45mM Tris, pH 7.4, 60mM DTT) and complete protease inhibitors (one tablet/20 ml, Roche, Barcelona, Spain) supplemented with 0.1 mM NaCl. Cells were mechanically disrupted and briefly sonicated. Samples were adjusted to 900µl with lysis buffer (20 mM Tris, pH 7.4; 100 mM Nacl; 1% Triton and protease inhibitors) and incubated for 15 min at 35^{0}C. After cooling on ice (10 min),100µl of 0.1M Tris, pH 7, and 50 mM $MgCl_2$ were added to the homogenate, which were then incubated with DNase-1 (30 U Sigma) on ice (10 min).

The homogenate was centrifuged (15 min, 10000 X g, 4^0C) and the aqueous phase between the upper lipid phase and the lower cellular debris phase was collected. Finally the extract was separated by chloroform/methanol precipitation. The protein estimation was done by Bradford assay for protein quantification.

3.1.2.4. Preparation of hyperimmune serum

Hyperimmune serum, against the affinity purified adipocyte antigen of Mithun, was raised in rabbit as per Mishra *et al.* (1997) with some modifications that encompass increased doses. Briefly, two New Zealand White (NZW) male rabbits weighing 1200 g were injected intramuscularly with 5 doses of affinity purified antigen, mixed with equal volume of Freund's adjuvant (Sigma, USA) at 10 days interval with increased subsequent doses ranging from 600 µg to 1200 µg per injection. First dose was given with Freund's complete adjuvant (FCA) and subsequent 4 doses with Freund's incomplete adjuvant (IFA). One rabbit of same breed, sex and weight was also maintained without immunization to collect normal serum.

3.1.2.5. Agar gel precipitation test (AGPT)

The specificity and the titre of the antiserum were determined using AGPT following Ouchterlony (1953).

3.1.2.6. Affinity column chromatography

Leptin protein was purified by affinity chromatography column chromatography using polyclonal antibody raised in the laboratory (Wilchek et al. 1984). Briefly, the sample protein was charged on the column and the unbound fraction was eluted with wash buffer. In next step, bound fraction was eluted with the help elution buffer. Fractions under each region were pooled and dialyzed against distilled water at 4°C. Proteins were concentrated by sucrose, sterilized through a membrane filter (0.22 µ), and stored at -20°C and estimated by Lowry's method (Lowry et al. 1951).

3.1.2.7. SDS-PAGE

Crude and affinity purified leptin proteins were analyzed by sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) as per Laemmli (1970) using 12.5% polyacrylamide gel in a vertical mini slab gel electrophoretic apparatus (Attao, Japan).

The samples were mixed with sample buffer in a proportion of 1:1 and subsequently the solution was heated at 100^0C for 3 min. The amount of protein applied was 50µg per track. Proteins were run at 18 mA for 150 min. The bands were visualized by staining with monochromatic silver staining (Shevchenko et al., 1996). Standard molecular weight marker (PMW-M, Genei, India) was run parallel along with sample proteins to determine the relative molecular weights of the polypeptides.

3.1.2.8. Western blot analysis

Affinity purified leptin protein fraction was separated on a 12.5% SDS-PAGE, and electro transferred onto NCM membrane on a semi dry blotting unit (Atto, Japan) as per Towbin *et al.* (1979) with some modifications. The blotted proteins were subjected to immunoblot analysis with anti- leptin mithun polyclonal antibodies and anti-goat horse radish peroxidase antibodies (Genei, India). Standard molecular weight marker (Fermentas) was run parallel along with sample proteins to determine the relative molecular weights of the polypeptides.

3.1.2.9. MALDI-TOF Mass Spectrometry

The molecular mass of affinity purified fraction was determined by Voyager DE Pro^{TM} mass spectrometer equipped with 337 nm N_2 laser (Applied Biosystems, USA) as per Mandal et al. (2009). Purified protein (2 µl) was mixed with MALDI matrix, synapinic acid (8 µl), then 2 µl of mixture solution was spotted onto the MALDI 100 well stainless steel sample plate and allowed to air dry (for 5 hr) prior to the MALDI analysis. The spectra were recorded in the positive ion linear mode in accelerating voltage 20 kV. Peptide mono-isotopic mass was obtained in linear mode with external calibration. It was performed using calibration mixture 1 (Applied Biosystems, USA) having Arg1-Bradykinin (*m*/*z,* 904.468), angiotensin I (*m*/*z,* 1296.685), Glu1-fibrinopeptide B (*m*/*z,* 1570.677) and ACTH (18-39) (*m*/*z,* 2465.199). Reproducibility of the spectrum was checked 5 times from separately spotted samples.

In-Solution tryptic digestion

Affinity purified fractions were tryptic digested for identification of peptides through MALDI TOF/ TOF MS following the method of OmPraba et al, (2010) with some modifications. Lyophilized dried fractions were initially dissolved in 50 mM ammonium bicarbonate (pH 8.0) solution. Then 20 µL of peptide solution (0.26 $\mu g.\mu L^{-1}$) was taken in a tube followed by reduction with 1 µL of 0.1M DTT at 56°C for 30 min. Afterward, alkylation of the solution was done with 1 µL of 0.3 M iodoacetamide at room temperature for 15 min in the dark. After incubation the solution was subjected to digestion with trypsin (1µL of 1 $mg.mL^{-1}$) at 37°C for overnight. One microliter of digested sample solution was spotted directly onto a MALDI target plate and1 µL of CHCA matrix solution was applied on the sample spot and allowed to air dry.

MS/MS analysis and database search

MALDI TOF/TOF MS (Applied Biosystems, CA, USA; model 4800) with (Nd: YAG) laser was used to analyze tryptic digested peptide samples. MS/MS spectral data were acquired in positive ion reflector mode with a mass range of 850-3000 Da after an average of 3000 laser shots. Maximum 20 most intense ion signals were selected with signal-to-noise ratio above 30 as precursors for MS/MS acquisition and externally calibration was made using fragment ion masses observed in the MS/MS spectrum of angiotensin I. Applied Biosystems GPS Explorer^{TM} (v3.6) software coupled with MASCOT (Matrix Science, London, UK) was used to search the protein databases using MS/MS spectral data for protein ID.

3.1.2.10. Enzyme linked immunosorbent assay (ELISA)

Indirect Enzyme linked immunosorbent assay (iELISA) was performed in a 96 well plate (NEST, China) as per Mishra et al. (1997) with slight modification in blocking buffer composition. Briefly, Affinity purified mithun antigen was coated @ 2 µg/well for overnight at 4ºC. The non-specific sites were blocked using 4% skimmed milk powder and 2% gelatin in phosphate buffered saline. After thorough washing 4 times by phosphate buffered saline (PBS)-Tween-20, diluted (1:200 in PBS) anti-leptin rabbit serum (100 µl) was dispensed keeping normal serum as control. Anti-rabbit horse radish peroxidase conjugate (HRPO,Sigma, USA) and O-phenylene diamine dihydrochloride substrate was used for colour development.

Dip-stick ELISA was performed with the affinity purified fraction following the procedure of Jiahao et al. (1997) with some modifications in substrate buffer composition. Coating of three sticks was done by affinity purified protein @ 2µg/srip. After blocking and subsequent washing of the sticks, anti-leptin antibody and normal serum were added in the three sticks, respectively. In the next step, after proper washing, anti-rabbit HRPO conjugate (Genei, India) was added to the sticks. The colour development step was carried out by dipping the sticks into substrate solution (40 ml H_2O_2, 0.025 gm diaminobenzydine in 10 ml Tris HCl, pH 7.5).

3.1.2.11. CIRCULAR DICHROISM (C.D)

Circular dichroism (CD) spectra were recorded using samples at 1% wt and pH 7 with a path length of 0.001 cm. Spectra were recorded at room temperature from 200 to 300 nm, with a 0.2 nm data pitch and a scan rate of 50 nm min^{-1} by Jasco-810 spectropolarimeter. Milli degrees of rotation were converted to molar residual ellipticity (MRE).

3.2. Results

3.2.1. Leptin protein estimation

The concentration of the crude protein from adipose tissue was estimated to be 8.2 mg/ml and affinity purified leptin protein concentration was found to be 0.16 mg/ml

3.2.2. Agar gel precipitation test

The specificity between crude mithun antigen and anti-mithun rabbit serum was indicated by clear band between the wells. The titre of the hyperimmune serum was found to be 4 (results not shown).

3.2.3. SDS-PAGE

When the crude protein obtained from adipose tissue was subjected to SDS–PAGE analysis, 31 (thirty one) polypeptides in the molecular weight range 14- to 100 kDa were obtained (Fig. 1). The affinity bound fraction yielded only three polypeptides of which one (16 kDa) was major and two (4- and 54 kDa) were minor. The unbound fraction contained several polypeptides in the molecular weight ranging from 3.5- to 100 kDa.

3.2.4. Western blot analysis

When Western blot analysis was performed with the affinity purified protein using anti-leptin antibodies, a band corresponding to 16 kDa was observed indicating the presence of 16 kDa leptin protein in the affinity purified fraction (Fig. 2).

3.2.6. MALDI-TOF mass Spectrometry

The affinity purified protein fraction when subjected to MALDI-TOF mass spectrometry to determine the molecular mass more precisely, it revealed as 17214.26 Da (Fig. 3). Further, we have confirmed the identity of the purified protein as leptin using Matrix science database analysis after MS/MS analysis of tryptic digested product. The sequence coverage was 67% as shown in Fig.4.

3.2.7. ELISA

Reactivity of affinity purified mithun leptin antigens with hyperimmune serum in iELISA is expressed in O.D. (at 492 nm) and is shown in Fig. 5. It indicated that the sero-reactivity of αand it was true for all the serum dilutions used (1:100 through 1: 3200).

Affinity purified mithun leptin antigen was further used in dip-stick ELISA to detect its viability to be used for diagnostic purpose in future. The difference in intensity of developed colour between hyperimmune and normal serum is shown in Fig. 6. Out of the two dip-sticks used, the first stick showed seroreactivity.

3.2.8. Circular Dichroism

Circular dichroism is a sensitive method to the stereoisometry of amino acids constituting the peptide backbone.This result shows that purified protein is very similar to leptin protein. This protein had 4α helix and 2 β sheet peptide which is identical with human leptin protein (Fig. 7).

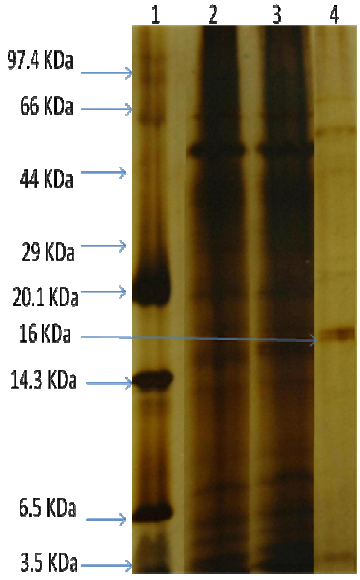


Fig.1. Polypeptide profile of crude and affinity fractionated proteins of adiposities obtained from Mithun as assessed by SDS-PAGE

1- Standard molecular weight marker
2- Crude adiposite protein
3- Affinity purified unbound protein
4- Affinity purified bound protein

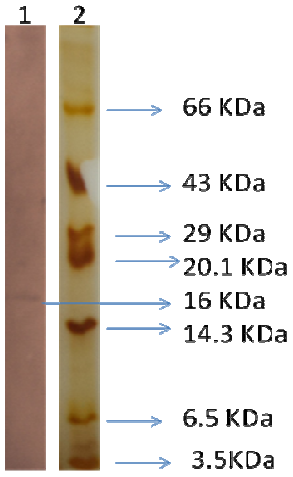


Fig. 2. Western blot analysis of affinity purified bound protein obtained from adiposities of Mithun.
Lane 1-Affinity purified bound protein fraction
Lane 2-Standard Molecular weight marker range (3.5 KDa to 205 KDa)

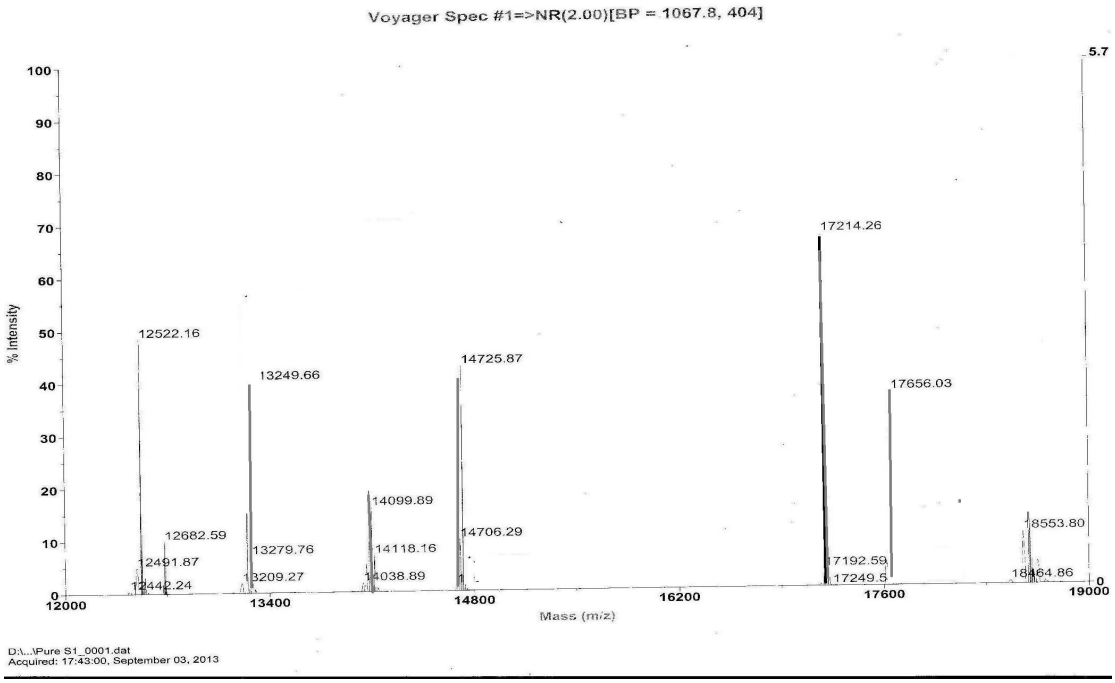


Fig. 3. MALDI-TOF Mass spectrometry of Purified Mithun leptin protein

1	MR**CGPLCRFL**	**WLWPYLSCVE**	**AVPIR**K**VQDD**	**TK**TLIKTIVA	RINDISHTQS
51	VSSKQR**VAGL**	**DFIPGLQPVL**	**SLSR**MDQTLA	IYQQILNSLH	SR**NVVQISND**
101	**LENLRDLLHL**	**LASSKSCPLP**	**R**AR**GLETFES**	**LGGVLEASLY**	**STEVVALNRL**
151	**QAALQDMLR**R	LDLSPGC			

Fig. 4. Amino acid sequences of leptin from Mithun (*Bos frontalis*)
Matched peptides shown in *bold red* with human leptin protein sequence
Sequence coverage was highlighted with red colour. Obtained mass data (MH^+) of the matched sequences from leptin after tryptic digestion

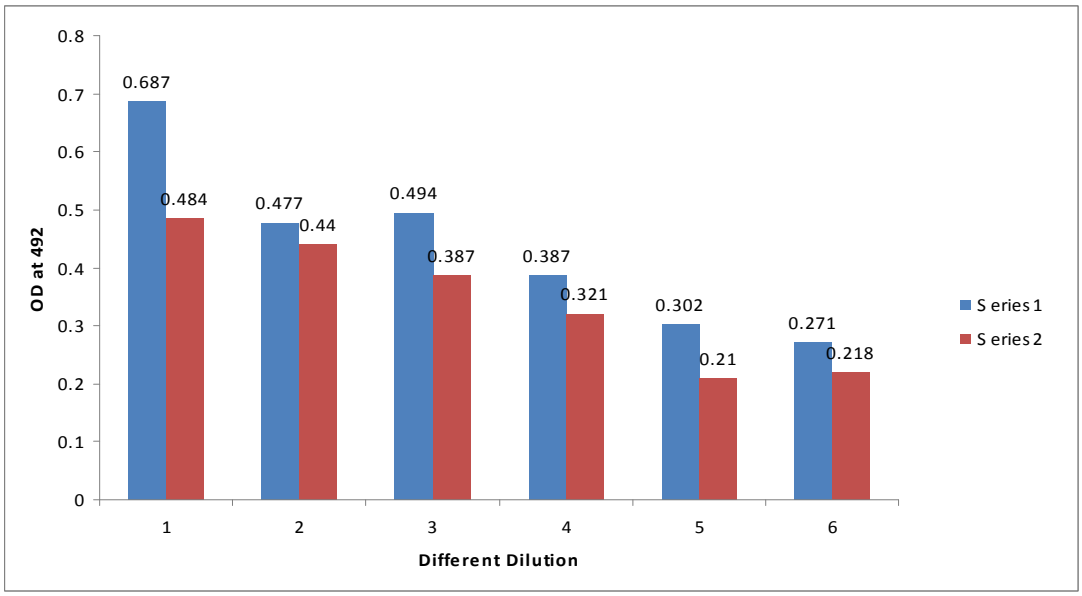


Fig. 5. Sero-reactivity of Mithun leptin protein antigens with hyperimmune and control rabbit sera as assessed by ELISA
Series 1: hyperimmune serum. Series 2: normal (control) serum.

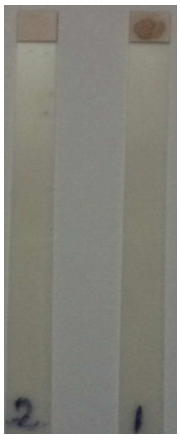


Fig. 6. Sero-reactivity of affinity purified bound protein with known antiserum and control serum as assessed by Dipstick ELISA
Dip-stick 1: Anti-mithun leptin antibody (hyperimmune serum)
Dip-stick 2: Negative control (normal serum)

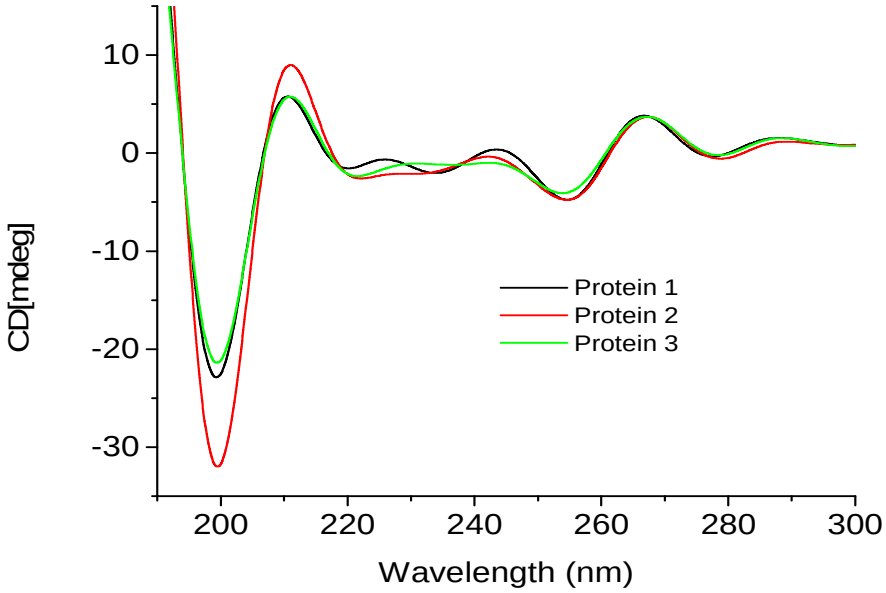


Fig.7. Circular Dichroism of Mithun leptin

Protein 1, 2, 3 are affinity purified mithun protein (leptin) samples

4. Discussion

Leptin is a protein hormone synthesized and secreted primarily by adipocytes of mammals in response to increased energy storage in adipose tissue (Zhang et al., 1994; Halaas et al., 1995). White adipose tissue is the key site of leptin production in mammals (Cinti et al., 1997). In the present study, mithun adipocyte tissue was considered for leptin protein isolation. After chemical treatment followed by ultra-sonication, crude proteins were obtained from adipocytes. In the present work, affinity purification of the crude protein was done using affinity column using polyclonal antibody. The affinity purified protein revealed a major band of molecular mass 16 kDa along with two minor bands of 4- and 54 kDa as assessed by SDS-PAGE. It showed that the affinity chromatographed fraction was a semi-purified preparation having a major protein of molecular mass 16 kDa. The present work corroborates with the previous work of Cohen and co-workers where the molecular mass of affinity purified recombinant human leptin protein was estimated as approximately 16000 Da by SDS-PAGE while the molecular mass of endogenous human leptin was reported as

16,026±9 Da by MALDI mass technique (Cohen et al., 1996). The molecular weight of leptin protein was reported earlier within the range of 16-18 kDa (Takahashi et al., 2002, Taniguchi et al., 2002). Recently, the molecular mass of native leptin from goat was found to be of 16 kDa by SDS-PAGE (unpublished observation). Subsequently, western blot analysis revealed that mithun adipocytes contained the leptin (16 kDa) protein both in affinity chromatographed bound fraction having seroreactive property. Earlier, western blot analysis with leptin-specific antibody detected a protein with a molecular mass of approximately 15–16 kDa in dunlin liver and adipose tissue (Kochan et al., 2006). The actual molecular mass of leptin protein of mithun was carried out using mass spectroscopic analysis and the molecular weight obtained was 17214.26 Da. In a previous study, gel pieces containing the leptin protein (16-kDa) of giant panda were excised and their peptide mass fingerprints were obtained and analyzed by MALDI TOF-TOF-MS. The mass of the mature protein without the signal peptides was detected as 16.8 kDa (Xu et al., 2010). Recently in our laboratory, MALDI-TOF mass spectroscopy was used to determine actual molecular mass of native goat leptin. It was found as 15948.72 Da (Alam et al., 2014). Circular Dichroism study revealed that the purified mithun protein samples contain 4 α-helix and 2 β-pleated sheet structure which is identical with human leptin protein. The sero-reactivity of affinity purified fraction was confirmed by plate and dipstick ELISA with the help of anti-mithun leptin antibodies. Moreover, this test (ELISA) revealed its potentiality in detecting leptin protein in clinical (suspected/unknown) samples and/or concentration thus explored the possibility of replacing other prevalent serodiagnostic assay viz. radioimmunoassay.

In short, it may be concluded that column chromatography may be used to purify native leptin from mithun (*Bos frontalis*). Moreover, imithun leptin possesses a molecular mass of 17214.26 Da and also sero-reactive property that might be exploited while preparing of sero-diagnostic tool(s) for detecting leptin in clinical samples and/or measuring leptin concentration in blood that might be needed to select specific animals(s) for selective breeding purpose.

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References

Ahima, R.S., Filer, J.S. (2000). Leptin and the neuroendocrinology of fasting. Annual Reviews of Physiology **62**: 413-437.

Alam, S., Joardar., SN and Panigrahi, AK. (2014). Immunobiochemical Characterization of Native Leptin from Goat (Capra hircus): Serodiagnostic Potentiality Revealed. Advance Animal and Veterinary Science **2(2**): 86 – 90

Cinti, S., Frederich, R.C., Zingaretti, M.C., De Matteis, R., Flier, J.S., Lowell, B.B. (1997). Immunohistochemical localization of leptin uncoupling protein in white and brown tissue. Endocrinology **138**: 797–804.

Cohen, S.L., Halass, J.L., Friedman, J.M., Chait, B.T., Bennett, L., Chang, D., Hecht, R., Collins, F. (1996). Human leptin characterization. Nature **382**: 15-20.

DiMarchi, R.D., Hermeling, R.N., Hoffmann, J.A. (1998). Anti-obesity proteins. US Patent. (Eilly & Lily co) 5,7,19,266.

Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Friedma, J.M., (1995). Weight reducing effects of the plasma protein encoded by the obese gene. Science **269**: 543-546.

Jiahao, M,. Xueqin, L., Zhenyin, W., Zhenguo, Y., Wendong, W. (1997). Rapid detection of motile Aeromonas species in fish by Dot ELISA. Journal of Dalian Fisheries College **12**: 72-78.

Peinado, J.R., Jimenez-Gomez, Y., Pulido, M., Ortega-Bellido, M., Diaz-Lopez, C., Padillo, J.F., Lopez-Miranda, J., Vazquez-Martı´nez, R.,Malagon, M. (2010). The stromal-vascular fraction of adipose tissue contributes to major differences between subcutaneous and visceral fat depots. Proteomics **10**: 3356–3366.

Kochan, Z., Karbowska, J., Meissner, W. (2006). Leptin is synthesized in the liver and adipose tissue of the dunlin (Calidris alpina). General and Comparative Endocrinology **148**: 336–339.

Laemmli, U.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriphage T_4. Nature **227**: 680-685.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J. (1951). Protein measurement with Folin-phenol reagent. Journal of Biological Chemistry **193**: 265-275.

Mandal, S.M., Dey, S., Mandal, M., Neto, S.M., Franco, O.L. (2009). Identification and structural insights of three novel antimicrobial peptides isolated from green coconut water. Peptides **30**: 633-637.

McGregor, G.P., Desaga, J.F., Ehlenz, K., Fischer, A., Heese, F., Hegele, A., Lammer, C., Peiser, C., Lang, R.E. (1996). Radio immunological measurement of leptin in plasma of obese and diabetic human subjects. Endocrinology **137**: 1501-1504

Mishra, S.S., Sekhar, M.S. (1997). ELISA and Dot immunoassay for detection of Vibrio spp. in tiger shrimp Penaeus monodon. Indian Journal of Fisheries **44**: 369-76

Ouchterlony, O. (1953). Antigen-antibody reactions in gels. IV. Types of reactions in coordinated systems of diffusion. Acta Pathologica et Microbiologica Scandinavica .**32:** 231-40.

Qinggang Xu., Xinming, Z., Ning, H., Jianxin, S., Rong, H., Zhihe, Z., Hongwu, B., Muyuan, Z. (2010). Characterization and Expression of *Ailuropoda melanoleuca* Leptin (*ob* gene). Zoological Science **27**: 41–46.

Shevchenko, A., Wilm, M., Vorm, O., Mann, M. (1996). Mass spectrometric sequencing Of proteins from silver-stained polyacrylamide gels. Analytical Chemistry **68**: 850–858.

Takahashi, N., Patel, H.R., Qi, Y., Dushay, J., Ahima, R.S.(2002). Divergent effects of leptin in mice susceptible or resistant to obesity. Hormone Metabolism Research **34:** 691–697.

Taniguchi, Y., Itoh, T., Yamada, T., Sasaki, Y.(2002). Genomic structure and promoter analysis of the bovine leptin gene. IUBMB Life **53(2)**: 131-135.

Towbin, H., Staehelin, T., Gorden, J.(1979). Electrophoretic transfer of proteins from Polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of National Academy of Sciences USA **76:** 4350–4354

Buchanan, F. C., Fitzsimmons, C. J., Van. Kessel, A.G., Thue, T. D., Dianne., Winkelman-Sim, C., and Schmutz, S. M. (2002). Association of a missense mutation in the bovine leptin gene with carcass fat content and leptin mRNA levels. *Gen. Selec. Evol* **34**: 105-116.

Buchanan, F. C.,Van Kessel, A.G., Waldner, C., Christensen, D.A., Laarveld, B. and Schmutz, S.M.(2003). Hot Topic: An Association between a Leptin Single Nucleotide Polymorphism and Milk and Protein Yield**.** *J. Dairy Sci*. **86**: 3164–3166.

Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L. and Friedma, J.M. (1995). Weight-reducing effects of the plasma protein encoded by the obese gene**.** *Science* **269**: 543-546.

Joardar, S.N. and Ram, G.C. (1999). Effect of fractionated antigens of *Mycobacterium bovis* AN5 on blood mononuclear cells of cattle. *Indian J. Ani. Sci*. **69**: 7-11.

Liefers, S.C., TePas, M.F., Veerkamp, R.F. and Vanderlende, T. (2002). Associations between Leptin Gene Polymorphisms and Production, Live Weight, Energy Balance, Feed Intake, and Fertility in Holstein Heifers. *J. Dairy Sci*. **85**: 1633–1638.

Liefers, S.C., TePas, M.F., Veerkamp, R.F., Delavaud, C., Chilliard, Y. and Vanderlende, T. (2003). Leptin Concentrations in Relation to Energy Balance, Milk Yield, Intake, Live Weight, and Estrus in Dairy Cows**.** *J. Dairy Sci***. 86**: 799–807.

Lowry, H., Rose brough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin-phenol reagent. *J. Biol. Chem*. **193**: 265-275.

Taniguchi, Y., Itoh, T., Yamada,T. and Sasaki,Y. (2002). Genomic structure and promoter analysis of the bovine leptin gene. *IUBMB Life* **53(2)**: 131-135.

Towbin, H, Staechelin and T, Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheets - Procedure and some applications. *Proc. Natl. Acad. Sci., USA* **70**: 4350-4354.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopoid, L.and Friedman, J.M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature* **372**: 425-432.

Annexure-V

Publications

DBT Twinning Programme

"Identification of SNPs in leptin gene for selection of Mithun..."

Research papers

1. Alam, S., Joardar, S.N. and Panigrahi, A. (2014). Immunobiochemical characterization of native leptin from goat (*Capra hircus*): serodiagnostic potentiality revealed. *Advances in Veterinary and Animal Sciences*. Vol. 2 (2): 86-90. **Global-based Impact Factor-0.547**

   IVC Index (Copernicus International Value)-5.8

2. Alam, S.S, Joardar, S.N., Panigrahi, A.K. Abraham, T.J. Mukherjee, S. and Mukherjee, A. (2014). Immunobiochemical characteristics of purified native leptin protein from Indian major carp, rohu (*Labeo rohita* Ham.). *Open Journal of Immunology* Vol. 4: 139-147. **Global-based Impact Factor-0.89**

3. Joardar, S.N., Mukherjee, S., Alam, S.S. and Mukherjee, A. (2014). Immunobiochemical characteristics of leptin protein from mithun (*Bos frontalis*) reveals sero-diagnostic potentiality. (Manuscript submitted)

Abstract presented in Symposia/Conferences

1. Joardar, S.N., Mukherjee, S, Alam, S.S and. Mukherjee, A. (2014). Immunobiochemical characterization of leptin protein from Mithun (*Bos frontalis*) reveals sero-diagnostic potentiality. International symposium and XXVIII convention of IAVMI, org. by IAVMI at DUVASU campus, on 30th Oct.-1st November, 2012 at Mathura, U.P.
2. Joardar, S.N., Mukherjee, S and Alam, S.S. (2014). Immunobiochemical Characteristics of Animal Leptin. UGC-sponsored National level Seminar. Org. by Department of Microbiology, Ramkrishna Mission Vidyamandira on 20-21 November, 2014 at Belur Math, Howrah, WB, India.

3. Alam, S.S., Joardar, S.N. and Panigrahi, A.K. (2013). Isolation and characterization of leptin protein from black Bengal goat. 100th Indian Science Congress. Org. by ISCA on 3-7 January, 2013 at University of Calcutta, Kolkata, WB.

4. Alam, S.S., Joardar, S.N. and Panigrahi, A.K. (2013). Immunobiochemical characterization of leptin protein from Indian major carp-*Labeo rohita*. 20th WB State Science and Technology Congress. Org. by DST, Govt. of WB, on 28 January – 2nd March, 2013 at Bengal Engineering and Science University, Shibpur, Howrah, WB, India.

5. Joardar, S.N., Alam, Sk. S. and Panigrahi, A.K. (2012). Immunobiochemical characterization of leptin protein of Black Bengal goat. 19th WB State Science and Technology Congress. Org. by DST, Govt. of WB, on 1st – 2nd March, 2012 at Saha Institute of Nuclear Physics, Salt Lake, Kolkata, WB, India.