CHAPTER 3

MATERIALS AND METHODS

3.1 Selection of sampling site:

The Siri cattle are generally found in the remotest hilly areas which are mostly inaccessible by vehicles of Darjeeling and Jalpaiguri District in West Bengal and Sikkim. The distribution of the particular cattle generally ranges between 26°42' to 28° 10' north latitude and between 89°4' to 88°58' east longitude.

Present study was conducted in the hilly areas of Malbazar block of Jalpaiguri district, Kalchini block of Alipurduar district, Gorubathan, Kalimpong–I and Kalimpong–II blocks of Kalimgpong district in West Bengal, and in the Ravong and Jorethang blocks of South Sikkim district in Sikkim (Fig-3.1). The areas under study were subdivided into four zones in such a way so that there was no chance of natural mixing or natural migration of animals from one zone to another zone. The selected zones were named as Zone–I (Kalchini block), Zone-II (Malbazar block), Zone-III (Gorubathan, Kalimpong–I and Kalimpong–II blocks) from West Bengal and Zone-IV (Ravong and Jorethang blocks) from Sikkim. Biological samples and data were collected from above stated zones during the period 2012 to 2014.

3.2 Materials:

3.2.1 Biological sample:

Total 930 Siri cattle were selected randomly and studied for colour pattern of different body parts (coat colour, colour of muzzle, eyelid, horn, hoof and switch) from different location in the four zones.

For study of physical, reproductive and productive characters of Siri cattle, total one hundred and sixty (160) animals from four zones (twenty adult males and twenty adult females from each zone of different distinct coat colours) of the study areas with average five to eight years of age were randomly selected. For study of birth weight of Siri calf, milk fat and solid non fat percentage in milk of Siri cattle, total twenty samples were considered from different zones for each character.

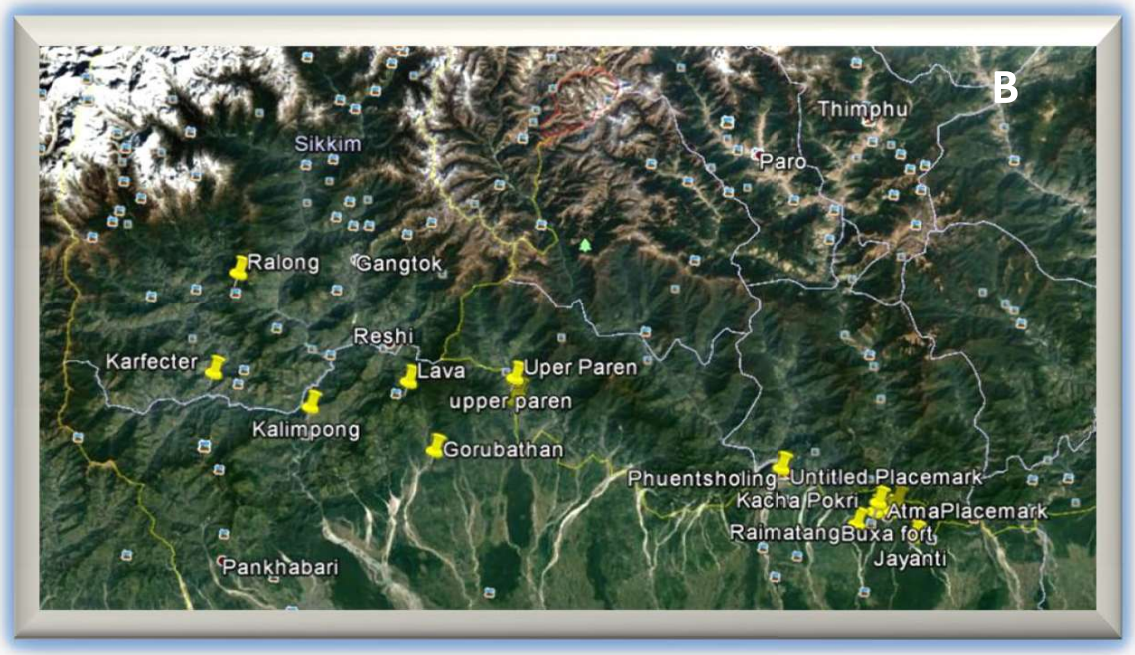

Fig- 3.1 Map showing the different locations of sample collection in West Bengal and Sikkim, India. A: Area of study is shown in pink. B: Sites of sample collection (shown in yellow).

For study of cytogenetic, male reproductive and productive characters, three adult Siri bulls were considered. Those three Siri bulls were provided by the Sikkim government to Frozen Semen Bull Station, Haringhata Farm, Nadia, under Paschim Banga Go-Sampad Bikash Sanstha for collection of semen and its cryopreservation. For molecular characterization study of Siri cattle, 34 blood samples were collected from different animals from four zones.

3.2.2 Chemicals and reagents:

The entire chemicals used for this study of Siri cattle were molecular biology grade. The following chemicals were used for this study.

i. Ethylene diamine tetrachloro acetic acid (EDTA) (Sigma).
ii. Tris Base (Sigma).
iii. Sodium dodecyl sulfate (SDS) (Sigma).
iv. Sodium chloride (Sigma).
v. Ethidium bromide (EtBr) (Sigma).
vi. Tris saturated phenol (Sigma).
vii. Proteinase-K (Promega, USA).
viii. Glycerol (SRL, India).
ix. Chloroform (Sigma).
x. Absolute Ethanol (Bengal Chemical, Kolkata).
xi. Iso-amyl alcohol (Sigma).
xii. dNTPs Master mix (Fermentas).
xiii. *Taq* DNA polymerase (Fermentas).
xiv. Magnesium chloride (Bioline, U.K).
xv. PCR Buffer (Applied Biosystems USA).
xvi. Rose Bengal stain (Sigma).
xvii. Eosin stain (Sigma).
xviii. Nigrosin stain (Sigma).
xix. R.P.M.I1640 media (Hi-media).
xx. Lectin (Sigma).
xxi. Colchicine (Loba Chemicals).
xxii. Benzyl penicillin (Alembic).
xxiii. Streptomycin sulphate (Alembic).
xxiv. Bovine serum (collected).
xxv. Potassium chloride (Merck).

xxvi. Disodium hydrogen phosphate (Merck).
xxvii. Potassium dihydrogen phosphate (Merck).
xxviii. Sodium bicarbonate (Merck).
xxix. Methanol (Ranbaxy).
xxx. Glacial acetic acid (Merck).
xxxi. Giemsa stain (BDH).
xxxii. Bromophenol blue (Sigma).
xxxiii. Xylene cyanol (Himedia).
xxxiv. Sucrose (Sigma).
xxxv. Sodium hydroxide (Himedia).
xxxvi. Hydrochloric acid (Himedia).
xxxvii. Agarose (Sigma).
xxxviii. BDTv3.1 (Applied Biosystems).
xxxix. Sequencing buffer (Applied Biosystems).
xl. HiDI Formamide (Applied Biosystems).
xli. Sodium acetate (Thermo Fisher Scientific)
xlii. DNA marker (NEB, Biobharti)

3.2.3 Working reagent solution preparation:

(i) Tris (hydroxymethyl) aminomethane-HCl (1M, pH-8.00).

Tris base	1.211 gm
Nuclease free water	10 ml

At first 1.211 gm Tris base was dissolved in 7 ml of nuclease free water; initial pH was measured and adjusted to 8 by adding concentrated HCl. Once pH became stable at 8, rest amount of nuclease free water was added to make the final volume to 10 ml. Then the prepared solution was autoclaved at 15 lb for 15 min and stored for subsequent use at room temperature.

(ii) Ethylene diamine tetra-acetic acid (0.5M, pH-8.00).

EDTA	1.862 gm
Nuclease free water	10 ml

At first 1.862 gm EDTA was added first in 7 ml of nuclease free water and initial pH was measured and adjusted to 8 by adding NaOH. Once pH became stable at 8, rest amount of nuclease free water was added to make the final volume to 10 ml. Then solution was autoclaved at 15 lb for 15 min and stored for subsequent use at room temperature.

(iii) Sodium chloride (5M).

NaCl	2.422 gm
Nuclease free water	10 ml

2.422 gm NaCl was mixed in 10 ml of nuclease free water and then autoclaved for15 min at 15 lb and stored at room temperature for future use.

(iv) Sodium dodecyl sulfate (20%).

SDS	2 gm
Nuclease free water	10 ml

Initially 2gm SDS powder was dissolved properly into 7 ml of nuclease free water and then added water up to 10ml. This was heated to about 68°C and stored at 37°C.

(v) Ethidium bromide or EtBr-(10 mg/ml).

Ethidium bromide	10 mg
Nuclease free water	1ml

After thorough mixing, the solution was taken in a container which was wrapped with aluminium foil and kept at room temperature.

(vi) Proteinase K (20 mg/ml).

20 mg lyophilized powder of Proteinase K was dissolved in 1ml sterile nuclease free water. This solution was divided into small aliquots and store at -20°C for subsequent use.

(vii) Chloroform-Isoamyl alcohol (24:1).

One part of isoamyl alcohol was added with 24 parts of chloroform and mixed properly and kept at 4°C.

(viii) Alcohol - 70%.

Absolute Ethanol	70ml
Double distilled H_2O	30ml

(ix) Phenol: Chloroform: Isoamyl alcohol (25:24:1), pH 7.8-8.0.

Tris-saturated phenol (pH 7.8-8.0), chloroform and isoamyl alcohol were mixed properly in the ratio of 25:24:1. Some amount of 100 mM Tris-HCl was given for layering above this mixture and stored in air tight dark bottle at 4°C for period of up to 1 month because phenol was readily oxidized in the presence of air and light, and oxidized phenol (such as quinones) damaged the DNA by breaking down of phosphodiester bonds or cause cross-linking of RNA and DNA.

(x) Bromophenol blue.

Bromophenol blue	25mg
Xylene cyanol FF	25mg
Sucrose	4g
Double distilled H_2O	10ml

All the above mentioned materials were mixed properly and stored at 4^0C temperature for future use.

(xi) Lectin (0.125mg/ml).

One milligram Lectin was dissolved in 8 ml double distilled water for making working solution.

(xii) Colchicine (0.04mg/ml).

One milligram Colchicine was dissolved in 25 ml of double distilled water for making working solution.

(xiii) Benzyl penicillin (10000IU/ml).

Benzyl penicillin (500000 IU) was dissolved in 5 ml double distilled water (D.D.W) for making stock solution. Then 1 ml of stock solution was added to 9 ml of D.D.W for making working solution.

(xiv) Streptomycin sulphate (10mg/ml).

One gram of Streptomycin sulphate was dissolved in 10 ml of D.D.W to prepare stock solution. Then 1 ml of stock solution was added to 9ml of D.D.W for preparation of working solution.

(xv) KC1 (0.075 M) as hypotonic solution:

KCl (0.56 gm) was dissolved in 100ml of D.D.W to prepare 0.075 (M) KCl and stored in room temperature.

(xvi) Sodium bicarbonate (4.4%).

Sodium bicarbonate	4.4 gm
Distilled water	100ml

(xvii) Methanol: Glacial acetic acid (3:1) as a fixative solution.

Three parts of methanol were added to 1 part of glacial acetic acid for fixative solution preparation.

3.2.4 Working buffer preparation.

(i) Lysis buffer (pH 8.0).

1M TrisHCl	1ml
5M NaCl	1ml
0.5M EDTA	2ml
20% SDS	10ml
DDW	86ml

All the above mentioned materials were mixed properly and autoclaved at 15 lb for 15 min and stored at room temperature.

(ii) Tris-EDTA salt buffer (TES).

1M Tris HCl	500 µl
0.5M EDTA	100 µl
NaCl	0.292g
DDW	49.4ml

All the above mentioned materials were mixed properly and autoclaved at 15 lb for 15 min and stored at room temperature.

(iii) TAE (Tris-acetate-EDTA) buffer (50X).

Tris base	24.2g
Glacial acetic acid	5.71ml
0.5M EDTA	10ml
DDW	84.29ml

All the above mentioned materials were mixed properly. To make 1X TAE, 20 ml of stock was diluted with 980 ml of double distilled water.

(iv) Sorenson's 0.1M phosphate buffer pH-6.8.

(A) Disodium hydrogen phosphate (1.419 gm/100ml).

Disodium hydrogen phosphate (Na_2HPO_4)	1.4198 gm
Distilled water	100ml

(B) Potassium dihydrogen phosphate (1.3609 gm/100ml).

Potassium dihydrogen phosphate (KH_2PO_4)	1.3609 gm
Distilled water	100ml

Thirty-three milliliters of solution 'A' was mixed with 17 ml of Solution 'B' to obtained 50 ml Sorenson's phosphate buffer of pH 6.8.

3.2.5 Kit:

Gel extraction kit (Qiagen), was used for elution of specific amplicon from the gel for generation of sequence information.

3.3 Methods:

3.3.1Field data collection and morphometric measurements for phenotypic characterization:

The study-sites were selected based on the availability of pure Siri cattle. The reliability of other available data and cooperation on the part of farmers were also important. Part of the survey was done based on question and answer sessions conducted with cattle owners for this purpose. The prescribed format of National Bureau of Animal Genetics Resources (NBAGR), Karnal was used for evaluation of breed under field condition. The questionnaire was prepared with the objective to acquire knowledge about the animal on a scientific basis, as well as to understand the socio-economic status of the animal owners. For the purpose of field data collection, co-operations were taken from local veterinary doctors and key persons (e.g. Panchayet members, group leaders and/or other elderly respected persons) in the study areas. The data regarding management practices, reproductive performances of males and females and production performances of female Siri cattle were taken from the field through questions and answers from the cattle owners as well as from the persons engaged with animal husbandry practices. Morphometric measurements of adult Siri males and females were taken as per prescribed format of National Bureau of Animal Genetics Resources (NBAGR), Karnal (Appendix-1).

3.3.2 Semen collection, quality and quantity assessment of collected semen:

Semen of three Siri bulls was collected twice in a week with the help of artificial vagina from each bull. Altogether 43 collections of semen were considered. For male productive performance study, age of 1st semen donation, semen quality, quantity and different fertility tests were measured. The colour, volume and pH of neat semen as well as concentration of sperm were assessed.

Semen, after collection was stored in graduated glass test tube. Volume of the semen was measured by observing the amount of semen in the graduated test tube. Colour of the semen was estimated by simply observing the neat semen in the fine glass test tube. Semen pH was measured with the help of pH meter (Merck). The concentration of the neat semen was estimated by spectrophotometer (IMV, France).

3.3.3 Microscopic evaluation of semen:

Microscopic evaluation of semen provides significant information about the quality of semen such as mass motility, initial motility, vitality and sperm morphology. The test involves handling of living cells which are extremely sensitive to extraneous influences.

3.3.3.1 Mass motility:

Motility estimation of semen is a subjective test. Mass motility was examined by placing a small drop of neat semen on a warm glass slide (without placing cover slip) and observing under phase contract microscope at 10X. The edge of the drop provided the best estimate of the mass motility.

3.3.3.2 Initial motility:

A small drop of neat semen was taken on a clean warm glass slide. A cover slip was placed on the drop and lightly pressed for even spreading of the drop. The motility was measured at 37°C in 20x magnification under a phase contrast microscope with a warm stage facility.

Motility can only be assessed by viewing the forward motion of individual sperm cells. It is the percentage of rapidly moving, forwardly progressive individual sperm cells per field (moving at greater than two body lengths per second) but not those that are swimming backwards, in a circle or moving slowly. Approximate percentage of progressively motile spermatozoa was observed in different fields to arrive at an accurate assessment.

3.3.3.3 Live and dead sperm estimation:

To assess sperm vitality, as soon as possible after collection, the semen samples were prepared for staining. Before staining and evaluation of semen sample, thorough mixing of fresh neat semen sample of bull is important before pipetting aliquots. One drop of fresh neat Siri bull semen sample was mixed with two drops of 1% Eosin Stain and then wait for 30 seconds. After that, three drops of 3% Nigrosin Stain was added to the previous mixture and gently swirled to mix. A smear was made on microscopic slides with uniform distribution and allowed to air-dry. Then the prepared slides were noticed under microscope in 100X. For estimation of live and dead sperms, 200 sperms were counted. Viable sperm showed white/faint pink heads. On the other hand non-viable sperm took red/dark pink colour in heads part with dark background.

3.3.3.4 Sperm morphology assessment:

For evaluation of the morphology of the sperm of fresh semen, Rose Bengal dye was used. First of all a thin smear was prepared on a clean glass slide with one drop of fresh neat Siri bull semen and then prepare slide was dried. Then one drop of Rose Bengal dye was added on the smear and dried at room temperature for a period of 5 minutes. Later on, differential count of 200 sperm under microscope (100X magnification) was done and the numbers of normal and abnormal sperm were determined in percentage. The deviation from normal structure and contour of head, neck and tail of sperm were considered as abnormal.

3.3.4 Cytogenetic study:

The present cytogenetic study in Siri cattle were carried out by using the whole blood lymphocyte culture technique as mentioned by Oh et al. (2011) with some modification.

3.3.4.1 Preparation of lymphocyte culture media:

The composition of working tissue culture medium for whole blood lymphocyte culture is as follows:

Medium R.P.M.I1640	100 ml
Lectin (Poke weed mitogen)	2.0 ml (0.25 mg)
Benzyl penicillin	2ml (20000IU)
Streptomycin sulphate	2 ml (20 mg).
Bovine Serum	25ml

The all chemicals were dissolved completely through gentle swirling of the flask with in bio-safety cabinet. Sterile sodium bicarbonate (4.4%) solution was added into the medium drop by drop till the pH of the medium reached 7.2, as judged by light pink colour of the medium. Additionally, pH of cell culture medium was also tested with pH meter. After that the prepared culture medium was filtered with the help of syringe filter having 0.2µ pore size. After filtration, 4.5 ml cell culture medium was transfer to each culture vials with capacity of 30 ml in laminar flow. The vials were stored at -20°C till needed. Generally these were used within 2-3 weeks.

3.3.4.2 Collection of blood samples and transportation to the laboratory:

Blood samples (3 ml) for culture were collected aseptically from the jugular vein of each selected Siri cattle. After washing with 90% alcohol, the jugular vein was punctured and the blood was collected in heparinised tubes. The vials were gently shaken for a few seconds to prevent clotting of blood. The collected samples were brought to the laboratory in double jacketed ice container (at 4°C) after giving proper identification mark and stored in refrigerator at 4°C. An aliquot of 0.5 ml of blood was used for setting up of cultures.

3.3.4.3 Establishing the culture:

Strict aseptic conditions were maintained for establishment of culture. The entire process was conducted within a bio-safety cabinet. The culture vials containing frozen medium were first thawed and thereafter blood (0.5ml) which was drawn in glass syringe was inoculated into the culture vial containing 4.5 ml medium. For each blood sample, double sets of culture were established. The culture vials were then labeled appropriately and incubated at 38°C (±1°C) for 72 hours.

3.3.4.4 Harvesting of cultures:

An aliquot of 0.1 ml of colchicine solution was added to each culture vial 45 minutes prior to harvesting of culture. The required quantity of fixative solution was prepared and kept in readiness in the refrigerator after thorough mixing. Hypotonic solution (0.075M KCl) was also prepared and kept in readiness inside the water bath (37°C). At the end of 45 minutes the contents of the culture vials were transferred into 15 ml centrifuge tubes. Centrifugation was done for 15 minutes at 2000 r.p.m. Most of the supernatant was then discarded leaving only one or two drops above the settled cells. The pellet was resuspended by gently tapping the tube with the finger and 8-10 ml of warm hypotonic solution was added into each tube. The cell pellet was mixed properly with the help of a Pasteur pipette and was kept for 10 minutes at 37°C in water bath. Subsequently 2 ml of chilled fixative was added in each tube to stop further action of the hypotonic solution and mixed with the help of Pasteur pipette. Then centrifugation was done at 1000 r.p.m. for 10 minutes after which the supernatant was discarded with the help of Pasteur pipette. The cell pellet of lymphocyte at the bottom of the tube was resuspended again with 5 ml of fixative solution and centrifuged it for 10 minutes at 1000 r,p.m. The entire process was repeated three to four times to obtain a clean whitish pellet in the centrifuge tube. After final centrifugation, the supernatant was discarded leaving 0.5 to 1.0 ml above cell pellet. The cell pellet was then mixed gently with the remaining supernatant by a Pasteur pipette to make it a cellular suspension.

3.3.4.5 Preparation of slides:

After thorough mixing, a small quantity of cell suspension was taken from centrifuge tube into a long nozzled Pasteur pipette. A clean grease free slide was moistened by blowing with air from mouth and 2-3 drops of cell suspension was dropped on the slide from the Pasteur pipette from a height of about 3 feet. The cellular material was spread in the form of expanding rings. The slides were then allowed to dry in air after appropriate labeling. After preparing a few slides (4-6), the remaining cells in culture were transferred into clean siliconised tubes and stored at -20°C for future use.

3.3.4.6 Staining of prepared slides:

Dried slides were stained by Giemsa stain. An aliquot of 5ml of Sorenson's 0.1M Phosphate buffer (pH-6.8) and 90 ml of DDW were taken into a staining jar. Then 5 ml of stock Giemsa stain (BDH) was added drop by drop with constant swirling to avoid precipitation. The slides were stained for 20 to 30 minutes, and rinsed thoroughly in distilled water. The extra moisture on slides was removed by absorbing in the folds of a filter paper. Slides were observed under microscope using 10X, then in 40X and finally in 100X. Four slides from each Siri bull were taken and minimum 5 metaphase spreads from each prepared stain slide were examined to determine the chromosome number, morphology and abnormalities, if any. In case of any suspected abnormalities, 50 metaphase spreads in each animal were examined. Metaphase spread from each slide were selected at random and photographed for documentation, karyotype and further study.

For study of cytogenetic parameters like Karyotype, relative length, centromeric index, arm ratio and ideogram preparation, total 60 karyotypes (20 from each male) were considered.

3.3.4.7 Preparation of karyotypes and measurements:

Preparation of karyotypes of chromosomes was done by LUCIA Karyotyping software. The centromeric index, relative length and arm ratio of chromosomes of Siri cattle were calculated as follows.

Centromeric index = (short arm length/ total length of chromosome) X 100.

Relative length measurement of chromosomes = (Absolute length of each pair /total length of all chromosome) X100.

Arm ratio= Long arm length/short arm length.

3.3.5 Statistical methodology:

The statistical methods used in the present study include percentage analysis, calculation of arithmetic mean and standard error. Chi-squared test was done to determine differences in percent frequencies of physical traits. One way analysis of variance was done to determine differences among the means by SPSS 16.0®.

3.3.6 Molecular characterization:

3.3.6.1 Isolation of genomic DNA:

Blood samples were collected from jugular vein of Siri cattle under aseptic condition in EDTA Vacutaine tube (BD) and stored at 4°C. For isolation of genomic DNA, phenol-chlorofrom-isoamyl method (Sambrook and Russell 1989) was followed with slight modification. Blood sample of 50-100 µl was taken in sterile 1.5 ml micro-centrifuge tube. Then TES buffer was added to it and volume was adjusted to 500µl. An aliquot of 50 µl of 10% SDS, followed by 5-10 µl of 20mg/ml of Proteinase-K were added to the above mixture and incubated at 56°C for 1-2 hours. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed thoroughly for few minutes. The sample was centrifuged for 10 minutes at 12000 r.p.m.. The upper phase was transferred to new 1.5 ml micro-centrifuge tube. Then equal volume of chloroform: isoamyl alcohol (24:1) was mixed with transferred solution in the micro-centrifuge tube and then centrifuged the tube for 10 minutes at 12000 r.p.m. After centrifugation, the upper portion of aqueous layer in the micro-centrifuge tube was transferred to another fresh sterilized 1.5 ml micro-centrifuge tube. After that the chilled absolute ethanol amounting double volume of transferred aqueous solution was added in the micro-centrifuge tube. The above sample was stored overnight at -20° C for precipitation. It was then centrifuged at 10,000 r.p.m. for 10 minutes. After that the supernatant in micro-centrifuge tube was thrown out without disturbing the pellet at bottom. After adding of 500 µl of 70% ethanol, the micro-centrifuge tube was then spin at 7000 r.p.m for 10 minutes. Then the supernatant in micro-centrifuge tube was thrown out and the pellet at bottom was air dried under laminar air flow. The pellet was mixed with 30 µl nuclease free water and stored either in -20° C for regular use or in -70^{0}C for long preservation.

3.3.6.2 DNA quantification and its purity:

Initially, the purity of DNA was evaluated by determining the OD_{260}/OD_{280} value. The optical density (OD) of isolated DNA samples from different individual Siri cattle was measured at two different wavelengths i.e. 260 nm and 280 nm. The absorbance value at 260 nm relates to the concentration of

nucleic acid in the sample whereas the value at 280 nm indicates the amount of protein in the sample. OD_{260}/OD_{280} value in the range of 1.8-2.0 indicates a pure preparation of DNA. Standard value of 1 O.D. of double-stranded (ds) DNA sample at 260nm corresponds to 50 µg/ml of dsDNA. For quantification, the purified DNA sample (1 µl) was diluted in nuclease free water (dilution factor = 50) and the absorbance was recorded in a UV-spectrophotometer (Biophotometer, Eppendorf).

3.3.6.3 Agarose gel electrophoresis:

The DNA samples isolated from different individual cattle were passed through agarose gel electrophoresis in a submarine horizontal electrophoresis apparatus (BioRad, USA) for checking its purity. Agarose gel (0.8%) was prepared by boiling agarose (Invitrogen) in an appropriate volume of 1X TAE buffer. Ethidium bromide (10 mg/ml) was added to the agarose solution after cooling to about 50-55^{0}C, so that a final concentration of 0.5 µg/ml was achieved and mixed properly. After cooling the agarose solution mixture was poured into the gel casting tray very slowly and allowed it to solidify. After solidification, the gel was transferred to the electrophoresis tank in such a manner that the gel block was completely submerged in 1X TAE running buffer. The DNA sample (4 µL) was mixed with 1 µL 6X loading dye (Fermentas) and loaded into wells. Electrophoresis was done at 50 volt and mobility was monitored by the migration of dye. After sufficient migration (2-3 h), the gel was placed on an ultraviolet trans-illuminator to visualize DNA and photograph was taken (Gel-DOC, BioRad).

3.3.6.4 PCR based amplification of COI gene and D-loop of mtDNA:

Selection of Primer: Following primers (Sigma Aldrich) were used for amplification of the D-loop (Kshetrimayum & Ghosh 2013) and *COI* (Kshetrimayum et al. 2013) region:

Farm animal D-loop (Fadl) primer:

Fadl F: 5'- CTCATCTAGGCATTTTCAGTGCC -3'

Fadl R: 3'-CACCATCAGCACCCAAAGCTGA -5'

Economic Animal *COI* (Eco) primer:

Eco F: 5'-CTCTGTCCTTAGATTTACAGTC-3'

Eco R: 3'-CGTGTATCGACGTCTATTCC- 5'

PCR settings:

Each 50 µl PCR mixture contains:

10X PCR buffer :	5µl
dNTPs Mastermix (10 mM) :	5µl
Forward primer (20 pmole/µl :	1µl
Reverse primer (20 pmole/µl) :	1µl
High fidelity DNA polymerase (5 Unit/µl) :	1µl
Genomic DNA (100-200 ng) :	1µl
Nuclease free water:	upto 50 µl

PCR cycling condition for COI gene amplification:

For amplification of COI gene of mtDNA, PCR reaction was done using the following program: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 30 seconds, extension at 72°C for 2 min and a final extension at 72°C for 5 min on a thermal cycler (Applied Biosystems, Inc. USA).

PCR cycling condition for D-loop amplification:

For amplification of mtDNA D-loop, PCR reaction was done using the following program: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 30 seconds, extension at 72°C for 2 min and then final extension at 72°C for 5 min through thermal cycler (Applied Biosystems, Inc. USA).

Detection of PCR amplicon in agarose gel:

Aliquots of 10µl of DNA products from PCR amplification were loaded in 0.8-1.2 % agarose gel mixed with ethidium bromide (0.5µg/ml) and electrophoresed in 1X TAE buffer along with 100bp ladder. The resolved amplicons were noticed under UV transilluminator and documented through Gel-DOC (BioRad).

3.3.6.5 Purification of PCR products:

The PCR-amplified products were electrophoresed through 1% low melting agarose gel containing ethidium bromide (10 mg/ml). The single distinct uniform band visible under UV-transilluminator was excised and purified using QIA quick^{R} Gel extraction kit (QIAGEN, USA) following manufacturer's instructions. The procedure is as follows:

The part of gel containing DNA fragment was excised with a clean, sharp scalpel blade and transferred the gel slice to a 1.5ml or 2.0ml centrifuge tube. Then 650µl Gel solubilizer was added. The tube containing gel was incubated at 50°C for ten minutes in a water bath until the sliced gel had completely dissolved. After that the 50µl of binding optimizer was added to the sample and then mixed properly by pipetting and vortexing for several time. The sample (750µl) was transferred to spin column 'A' placed in a 2ml collection tube and centrifuged at 10,000 x g (12,000r.p.m.) for one minute. The filtrate at the bottom of the collection tube was thrown out and then the spin column was placed back in the collection tube. Residual solution was poured in the spin column and centrifuged it again at 12,000r.p.m. Wash buffer 'A' (700µl) was added and centrifuged at 10,000 x g (12,000r.p.m.) for one minute. After centrifugation the filtrate in the bottom of collection tube was again discarded and then the spin column was placed back in the collection tube. Then the spin column was centrifuged for two minutes at maximum speed to remove all traces of ethanol and then the collection tube was discarded. The spin column A was placed into a 1.5ml elution tube and 30-50µl elution buffer was added directly to the spin column membrane. Then it was incubated at room temperature for one minute and centrifuged at 10,000 x g (12,000r.p.m.) for one minute to elute the DNA. The purified DNA was stored at -20°C.

3.3.6.6 Sequencing of PCR amplicons:

The amplified products for D-loop and COI gene were purified and then sequenced bi-directionally using automated DNA Sequencer (ABI 3500 Genetic Analyzer; Applied Biosystem, Inc. USA). The sequencing reaction was done using 1 µl of BDTv3.1 (Applied Biosystems), 1.5 µl of 5x Sequencing buffer; a final immersion of 0.5 pmol of each of the primers was maintained in separated reaction, template DNA 70ng/µL. The Chain

termination reaction was carried out at 25 rounds of one minute at 94°C, 5 Sec at 50°C and four minutes at 60°C as per manufacturer's protocol. Prior to run in the 3500 Genetic Analyzer, the fragments were purified by Sodium Acetate/EDTA/Ethanol method. The Sodium Acetate 3M, pH-5.2 and 125mM EDTA were added maintaining 1/10th of the total volume of the product followed by the addition of double the volume of absolute ethanol and consecutive wash with 70% ethanol. The sample was dried properly and resuspended in Hi-DI Formamide (Applied Biosystems) prior to loading in the capillary.

3.3.6.7 Sequence analysis using Bioinformatics tools:

The raw sequences generated after sequencing was annotated and brought in proper format in order to make the sequences applicable for phylogenetic analysis. The techniques and procedures followed for the annotation and analysis of the sequences are detailed below.

3.3.6.7.1 Interpretation of sequencing chromatogram:

Automated DNA sequencers generated a four-colour chromatogram showing the results of the sequencing run, as well as a computer program's best guess at interpreting that data - a text file of sequence data. That computer program, however, does make mistakes and thus the interpretation of the primary data was manually double-checked. Predictable errors occurred near the beginning and again at the end of sequencing run. The sequence chromatogram was viewed using software SeqScanner Version 1.0 (Applied Biosystems Inc., CA, USA). The software was used in this study to display, edit, trim, print, generate and export reports for sequencing sample files from Applied Biosystems Genetic analyzer instrument.

3.3.6.7.2 Sequence Scanner:

Initially a new folder was created which contain sequencing results (.ab1 files). The imported file (ctrl + click to get alternative files or shift + click to get multiple files once) were highlighted and afterwards 'Add selected trace' option was clicked. In the Trace Manager tab, highlighted file was clicked. Under File Tasks on the left hand side, 'Open Traces' option was selected. This would open the trace window at the bottom. 'Raw' tab option was selected to view the actual fluorescence then 'Annotation' tab was selected to

view the signal strength along with other details of the data. After that the 'Sequence' tab was selected to view the results in text format. In the Reports Tab, report type option~~ed~~ was clicked to view information about all the sequencing files. To check the sequence data, the file was double clicked to open the trace window at the bottom. To print the file on a single page, highlighted file in the Trace Manager tab was clicked and then 'File' 'Print Setup' was clicked. The parameters (Panels per page to 6 and Points per page to at least 1800) were arranged. To visualise the quality bars on the graph, 'Print QV information' was selected and then 'Apply' option was clicked. Then 'Print preview' option was clicked to make sure the whole trace file would be printed on one page and 'Print' option was clicked to print.

3.3.6.7.3 Reverse Complement (http://www.bioinformatics.org/sms2/rev_comp.html):

This software program is one of the essential Sequence Manipulation Suite that transfers a DNA sequence into its reverse-complement counterpart. Here the DNA sequences were converted to reverse-complement for future analysis.

3.3.6.7.4 BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html):

BioEdit is a mouse-driven, sequence alignment, sequence analysis and easy-to-use editor program. BioEdit is a software program that can manage most simple sequence, alignment editing and manipulation functions that researchers have been done on a daily basis and also for few basic sequences analyses (Hall 1999).

3.3.6.7.5 Open Reading Frame (ORF) Finder:

The ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) is a graphical analysis tool which finds all open reading frames of a selectable minimum size in a user's sequence or in a sequence already in the database. This tool identifies all open reading frames using the standard or alternative genetic codes. The deduced amino acid sequence can be saved in various formats. The ORF Finder is very much useful tool in getting ready for complete and accurate submission of sequences. This tool is also tied together with the Sequin sequence submission software. The program returns the range of

each ORF, along with its protein translation. ORF Finder supports the entire IUPAC alphabet and several genetic codes.

3.3.6.7.6 Alignment of the sequences with ClustalX:

ClustalX2 (Thompson et al. 2002) was used for multiple alignments of the sequences. Here a input of fasta formatted sequences set has been taken and automated procedure of entire progressive alignment are carried out. The sequences were aligned in pairs in order to generate a distance matrix that can be used to make a simple initial tree of the sequence. ClustalX2 is freely available and can be downloaded from EMBL/EBI file server (ftp://ftp.ebi.ac.uk/pub/software/) or from ICGEB in Strasbourg, France (ftp://ftp-igbmc.ustrasbg.fr /pub/ClustalW). In individual case, ClustalX2 presented a graphical user interface having alignments with colourful display. ClustalX2 performed the progressive alignment and created an output guide tree file and an output alignment file in the default Clustal format. ClustalX2 indicated the degree of conservation at the bottom of the aligned sequences, which was used to evaluate a given alignment.

3.3.6.7.7. Procedure for sequence analysis:

For each sample, two chromatograms were obtained. Each chromatogram represents sequence of each strand of both the strands of DNA. Ends of the noisy sequences were trimmed. To compare the sequences from the two chromatograms, BLASTN software program (Altschul et al. 1990) was used and the two fragment sequences showing 100% alignment with no gap or indels (insertion/deletions) were chosen. In case of any discrepancy, both the sequences were reviewed and quality value of the sequence was considered to determine the most likely nucleotide using SeqScanner Version 1.0 (Applied Biosystems Inc., CA, USA). Here all selected specimen sequence fragments were aligned using software, ClustalX (Thompson et al. 2002) and subjected to BLASTN searches at the National Centre for Biotechnology Information to see whether the developed sequences align with database mitochondrial COI gene and D-loop without having any indels. The sequences of *COI* were translated using the online software ORF finder (http://www.ncbi.nlm.nih.gov/ gorf/gorf.html) and aligned through BLASTP (Altschul et al., 1990) to further check matching of the array of

amino acid in the developed sequences in comparison to mitochondrial *COI* barcode sequences.

3.3.6.7.8 Sequence submission:

All the analyzed sequences were deposited in GenBank through the Banklt sequence submission tool (http://www.ncbi.nlm.nih.gov/ WebSub/?tool= genbank) of GenBank and received valid accession numbers. The sequences were also submitted in BOLD following BOLD sequence submission protocol (BOLD management paper) and received valid ID's.

3.3.6.7.9 GenBank DNA barcode sequences retrieved:

In addition to the developed sequences, DNA barcode sequences representing same and related taxa of the bovine species under study were acquired from GenBank as replicate data for evaluating the taxonomic status of our target species and detailed in Appendix-2.

3.3.6.7.10 Codon usages and nucleotide composition analysis:

For codon usages and nucleotide composition analysis, different software and computerized program were used.

A. MEGA

AT and GC skews of the sequences were calculated using MEGA software (Tamura et al. 2013). MEGA uses Graphic user interface for the insilico nucleotide as well as codon analysis. It can be employed for various statistical analyses. The tool performs nucleotide analysis for the query sequences and presents the results in spreadsheets which can be further utilized for statistical analysis.

Steps:

This program was started using the MEGA. The Input file was opened using "Open" button or by select "File -> Open"(Ctrl +O). The ".txt" file was selected that contains the DNA sequence for analysis. The Dialogue box showed the end of calculation. The "gird" showed the generated results. The result file was saved using the Save option on dialogue box (Ctrl + S).

B. Codon Usage (http://www.bioinformatics.org/sms2/codon_usage.html):

Codon usage of the sequences was calculated using Codon Usage program. It is one of the important programs of Sequence Manipulation Suite. It accepts one or more DNA sequences and returns the number and frequency of each codon type. Assessment of whether a sequence shows a preference for particular synonymous codons, can also be done. The said program also judges against the frequencies of codons that responsible for the same amino acid (synonymous codons).

3.3.6.7.11 Phylogenetic analysis:

All the generated sequence data of the *COI* and D-loop region of Siri cattle was subjected to phylogenetic analysis along with the available databases. Data were retrieved in two categories, those of Indian origin and those from rest of the world. For species identification through generated *COI* sequence similarity search was done in two public databases, viz., BOLD and GenBank and matches were conducted with retrieved sequences (Appendix2). The phylogenetic studies based on mtDNA D-loop were carried out using the molecular evolutionary genetic analysis (MEGA6) software in accordance with the Kimura 2- Parameter (K2P) model. DNA Barcoding sequences and D-loop were analyzed by using the phylogenetic tree reconstruction methods, such as Neighbor-joining (NJ) and Maximum likelihood (ML) method.

a) Neighbour-Joining (NJ) method: This method was developed by Saitou and Nei (1987) and subsequently also modified by Studier and Keppler (1988) to estimate the minimum evolution tree. The Kimura model is an extension of the Jukes and Cantor (JC) basic model (Saitou and Nei 1987, Studier and Keppler 1988). This type of model of analysis differentiates between two types of substitutions: i) **Transitions:** Here a pyrimidine is substituted by another pyrimidine (C<-->T) or a purine is substituted by another purine (A<-->G) and ii**) Transversions**: Here a purine is replaced by a pyrimidine or vice versa (A or G <--> C or T). The model assumes that the rate of transversions is dissimilar from the rate of transitions. For the species-level analysis, the Kimura-2-Parameter (K2P) model is used to compute nucleotide sequence divergences. This method is the best metric method when distances are low in DNA barcode sequence.

b) **Maximum likelihood (ML) method:** Maximum likelihood (ML) method was first described by Felsenstein (1973, 1981) for deducing evolutionary trees using discrete characters (such as nucleotide sequences) of extant species. In evolutionary perspective, the maximum likelihood tree is a significantly better representation of the true tree. It is important to find out the variance of the difference between log likelihood of different tree topologies (Yang 1993).

MEGA6 (http://www.megasoftware.net/): Phylogenetic and molecular evolutionary analysis were conducted using *COI* barcode and D-loop sequences using MEGA verrsion6 (Tamura et al. 2013) software to estimate the genetic distance (pairwise, over all mean, within group mean, between group mean and net between group mean) between the species, with in the species and to estimate the nucleotide and amino acid compositions.

Molecular phylogenetic analysis was done in four stages. First, the sequences were selected for analysis from available online records. Multiple sequence alignment of nucleic acid sequences was done using ClustalX2 analysis. Subsequently, the tree building was done through above mentioned method through MEGA6.

Time Tree construction: Time tree provide information about the evolutionary timescale of life. Following models are generally used for this purpose.

a) **Node time** – Here find the divergence time of higher taxa or two species.
b) **Time line** – It locate evolutionary branches from the viewpoint of a single species.

Advancement in Bayesian phylogenetic inference (Rannala and Yang 1996) has culminated in the field of phylogenetic inference in which both phylogenetic relationships and at the same time the times of divergence are estimated (Drummond et al. 2006). This evaluation is supported by molecular clocks (Thorne et al. 1998) which resolve nonclock-like evolution with an original time-tree in which on an axis of time the common ancestors are placed. To calculate results on an absolute time scale it is necessary to either supply information on the rate of molecular evolution or alternatively calibrate a subset of internal nodes with a calibration density (Thorne et al. 1998).

Haplotype analysis: A haplotype is a group of genes in an organism that are inherited together from a single parent (Cox et al. 2000). Haplotypes provide powerful required information about migration and population relationships (Jorde 2000). For haplotype study the nucleotide sequences analysis was done by DnaSP 4.10.9 software (Rozas 2009).