

**COMPARATIVE GENETIC STUDY ON
MITHUN (*BOS FRONTALIS*) AND THO-THO (*BOS INDICUS*)
CATTLE IN NAGALAND**

Thesis submitted to

NAGALAND UNIVERSITY

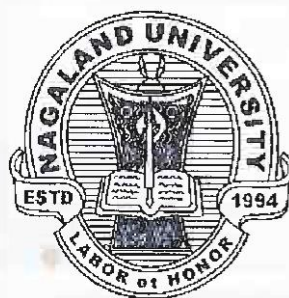
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DOCTOR OF PHILOSOPHY IN ZOOLOGY

By

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2015

DEDICATED TO MY BELOVED

DAUGHTERS

PUTICEN AND LENISA



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CERTIFICATE

This is to certify that the thesis entitled “Comparative genetic study on Mithun (*Bos frontalis*) and Tho-tho (*Bos indicus*) cattle of Nagaland” is a record of original research work done by **Mr. Imsusosang Longkumer**, under our personal guidance and supervision. He is a registered research scholar (*Regd. No- 376/2009*) of the Department.

The candidate has fulfilled all the requirements of Ph.D regulations of Nagaland University for the submission of thesis. The work is original and neither the thesis nor any part of it has been submitted elsewhere for the award of any degree or distinctions. The thesis is therefore, forwarded for adjudication and consideration for the award of degree of Doctor of Philosophy in Zoology under Nagaland University.

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I Mr. **Imsusosang Longkumer** hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University.

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A handwritten signature in black ink, appearing to read 'Imsusosang Longkumer'. The signature is fluid and cursive, with a large initial 'I' and 'L'.

(Imsusosang Longkumer)

ABBREVIATIONS

A	: Adenine (Purine base)
Ba(OH) ₂	: Barium Hydroxide
BrdU	: 5'-BromodeoxyUridine
bp	: Base pair
C	: Cytosine (Purine base)
C-bands	: Centromeric bands
CBG	: C-banding using Barium Hydroxide and Giemsa
D	: Genetic Distance
DNA	: Deoxy ribonucleic acid
FISH	: Fluorescent <i>in-situ</i> Hybridization
G	: Guanine (Pyrimidine base)
G-bands	: Giemsa bands
GDW	: Glass Distilled Water
gm	: Gram
HCl	: Hydrochloric acid
Hoechst-33258	: 2'-(4-Hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2-5-bi-1H-benzimidazole trihydrochloride
I	: Genetic Identity
lbs	: Pounds
ICAR	: Indian Council of Agricultural Research
ISCNDB	: International System for Chromosome Nomenclature of Domestic Bovids
Kb	: Kilo Base pair
L	: Liter
M	: Molarity of solution
ml	: Milliliter
mOsm	: Milli Osmolarity

N	: Normality of solution
nm	: Nano meter
OD	: Optical Density
PAGE	: Poly Acrylamide Gel Electrophoresis
PCR	: Polymerase Chain Reaction
Pg	: Pico gram
PHA	: Phytohaemagglutinin
pH	: Puisse-de-hydrogen
pMole	: Pico Mole
PWM	: Poke weed mitogen
RAPD	: Randomly Amplified Polymorphic DNA
R-band	: Reverse band
RBG	: R-banding by late Bromodeoxy Uridine incorporation and Giemsa staining
rpm	: Revolution per minute
SSC	: Sodium Saline Citrate
T _A	: Annealing Temperature
TBE	: Tris Borate EDTA
U.V.	: Ultra Violet
µg	: Micro gram
°C	: Degree centigrade

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M-1Kb DNA ladder, TT-Tho-tho cattle, NL-Nagaland Mithun, AR-Arunachal Mithun, MZ-Mizoram Mithun, MN- Manipur Mithun.

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M-1Kb DNA ladder, TT-Tho-tho cattle, NL-Nagaland Mithun, AR-Arunachal Mithun, MZ-Mizoram Mithun, MN- Manipur Mithun.

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PREFACE

PREFACE

The Mithun (*Bos frontalis*) is a heavily built semi domesticated bovine species found in north eastern hill region in four states of India. Mithun are classified into four strains based on their geographical habitat and phenotypic features as Arunachal, Manipur, Mizoram and Nagaland. Mithun is considered to be an animal of special significance by the people of North Eastern Hill Region, which is the home tract of this magnificent animal. It is also known to be a symbol of status prosperity and peace among the locals. This animal is largely dependent on the thick vegetation of the hilly terrains of north eastern states. Due to various biotic and abiotic factors viz. deforestation, disease outbreak, inbreeding and shifting cultivation, the population of this animal is dwindling, thereby causing serious concern for protection of this beautiful species.

Tho-tho (*Bos indicus*) cattle, which is claimed to be the native cattle breed of Nagaland is a short to medium stature black color animal available in different hilly pockets of Nagaland. This cattle is primarily used for meat and dung purposes. It also plays an important role in the socic-cultural life of the local tribal people.

Cytogenetics applied to domestic animals is a useful biotechnology for the genetic improvement of livestock. Indeed, it can be used to select reproducers free of chromosome abnormalities that are responsible for abnormal body conformation, low fertility or sterility (Iannuzzi, 2007). Mithun and Tho-tho cattle are less explored cytogenetically and it is very essential to identify and understand the nature of chromosome before starting any biotechnological interventions including genome analysis in this species, as karyotype features allow individual species to be distinguished. It is well known fact, that the cytogenetic variation leads to evolutionary divergence across the species and it is therefore important to study the karyotype and morphological parameters not only for scientific breeding, but also for planning conservation and propagation strategies.

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Chromosome banding techniques have proved to be useful in pairing of chromosomes and thus making karyotype more accurate. Any variation in chromosome number and /or structure may result in unbalanced karyotype. Thus the detection of chromosomal abnormalities in domestic animals will prevent the rapid spread of chromosomal defects in subsequent generations through natural and/or advanced reproductive tools. One of the prime objective of the present study was to construct the karyotype with all the details of chromosome morphometric parameters for complete chromosomal characterization of Mithun and Tho-tho cattle.

Genetic diversity is the basis for present day diversified living systems and future genetic improvement needs. This diversity should be properly utilized, improved and conserved. Conservation and improvement strategies ought to be based on proper genetic characterization in association with phenotypic characterization. The present work was carried out to ^{assess} assist the genetic variation between Mithun and Tho-tho cattle using molecular marker (RAPD). Genetic diversity study was done ^{using} based on PCR technique.

CHAPTER I

**CYTOGENETIC ANALYSIS OF MITHUN AND
THO-THO CATTLE**

INTRODUCTION:

Mithun

The Mithun (*Bos frontalis*) is a heavily built semi domesticated bovine species found in north eastern hill region in four states of India (Figure 1). Mithun are classified into four strains based on their geographical habitat and phenotypic features as Arunachal, Manipur, Mizoram and Nagaland (Figure 2). They prefer to roam about in the forest for browsing on shrubs and seldom on forage grasses. The most conspicuous feature of the Mithun is a prominent dorsal ridge (Buchholtz, 1989). The other distinguishing features are dewlap, white stockings on all four legs, grey spot on the forehead and broad prominent frontal bone (from where the name *Bos frontalis* hails). In morphological characteristics, the Mithun appears somewhat similar to that of Gaur, also known as Indian bison (a wild variety), but are distinctly smaller in size. Moreover, the mithun is a far more amenable and docile animal, unlike the gaur which could not be tamed. At higher elevations, Mithun territories are also shared by Yak (*Poephagus grunniens*), while at lower altitudes domestic cattle (*Bos indicus*) and Mithun co-habit. According to the latest census the population of Mithun in India is 2,98,264 (Livestock survey GOI, 2012). The taxonomy indicated that this unique bovine species belongs to genus *Bos* similar to other large ruminants. The zoological place of Mithun is given below:-

Taxonomy of Mithun

Kingdom	:	Animalia
Phylum	:	Chordata
Class	:	Mammalia
Order	:	Artiodactyla
Family	:	Bovidae
Subfamily	:	Bovinae
Genus	:	<i>Bos</i>
Species	:	<i>frontalis</i>

Mithun "Ceremonial Ox" is considered to be sacred animal by the Mithun rearing society. It is commonly used as a meat animal; however the slaughter is restricted only during

ceremonial or religious functions. In the old Naga tradition system, the Mithun was considered to be the best gift for the state official and dignitaries, it was considered as an act of honor. The picture of the head of the Mithun is official logo of the state government of Nagaland.

Mithun a unique bovine species, is less explored cytogenetically and it is very essential to identify and understand the nature of chromosome before starting any biotechnological interventions including genome analysis in this species, as karyotype features allow individual species to be distinguished. It is well known fact, that the cytogenetic variation leads to evolutionary divergence across the species and it is therefore important to study the karyotype and morphological parameters not only for scientific breeding of Mithun, but also for planning conservation and propagation strategies.

The population of Mithun is not very high and the rate of growth is also very slow in this state, in such situation risk of demographic extinction and reduction in the population size is always there. With these available genetics resources and genetic diversity, one of the prime objectives of the present study was to construct the karyotype of Mithun with all the detail chromosome morphometric parameters for complete chromosomal characterization of Mithun.

Tho-tho Cattle

Tho-tho cattle is short to medium stature, black color animal available in different hilly pockets of Nagaland, especially in Kohima and Phek districts. Body is mostly covered with short and glossy hairs. Coat color is either pure black or black with white spot; long and fine neck, horns are blackish and curved upward with pointed tips. Udder is small and hump is prominent in this animal. Although, the Tho-tho is the popular name, but is also known by the different other names with location specific tribal names Ashucho, Ameshi, Sheapi, Chokru, Tseso. These cattle are primarily used for meat purposes (Figure 3). However, the evidences are there, that these cattle are also used as milk and draught purposes. They also play an important role in the socio-cultural life of the local tribal people and are considered as the ceremonial animal of various social rituals. The

total population of Tho-Tho cattle has been estimated about 2,07,220 (Livestock census, GOI 2007). The taxonomic position of these cattle is similar to indigenous cattle and is given below:-

Taxonomy of Tho-tho cattle

Kingdom	:	Animalia
Phylum	:	Chordata
Class	:	Mammalia
Order	:	Artiodactyla
Family	:	Bovidae
Subfamily	:	Bovinae
Genus	:	<i>Bos</i>
Species	:	<i>indicus</i>

Cytogenetics is the study of chromosome number, structure, function and behavior in relation to gene inheritance, organization and expression. Chromosomes are complex structures located in the cell nucleus; they are composed of DNA, histone and non-histone ~~and~~ polysaccharides. They are basically the "packages" that contain the DNA. To collect cells with their chromosomes in this condensed state they are exposed to a mitotic inhibitor which blocks formation of the spindle and arrests cell division at the metaphase stage. Chromosomes are the nuclear components of special organization, individuality and function. They are capable of self reproduction and play a vital role in heredity, mutation, variation and evolutionary development of the species.

Under the microscope, chromosomes appear as thin, thread-like structures. They have a short arm and long arm separated by a primary constriction called the centromere. The short arm is designated as *p* and the long arm as *q*. The centromere is the location of spindle attachment and is an integral part of the chromosome. It is essential for the normal movement and segregation of chromosomes during cell division. Sub-metacentric chromosomes have short and long arms of unequal length with the centromere more

towards one end. Acrocentric chromosomes have a centromere very **near** to one **end** and have very small short arms.

A karyotype is the observed characteristics i.e. number, type and shape of the chromosomes of species. In normal diploid organisms, autosomal chromosomes are present in two identical copies. Karyotypes are prepared to study chromosomal aberrations, and other macroscopically visible differences among individuals. In order, to observe the chromosome and to study their size and morphology cell division is arrested at the metaphase stage. Any abnormality related to number or shape and/or structure of chromosome can be studied by various banding techniques. The chromosomes are arranged and displayed as an idiogram: in pairs, ordered by size and position of centromere for chromosomes of the same size.

Cytogenetic analysis was done for karyotype construction, estimate of relative length, arm ratio, centromeric index, number of bands and idiogram of the chromosome. Relative length (RL) of the chromosome is the length of an individual chromosome to the total length of all chromosomes. Arm Ratio is the ratio of chromosomal arm i.e. length of long arm to the length of short arm. Centromeric index is the measurement of short arm to the chromosome length and is done only for metacentric and sub-metacentric chromosome. Number of bands is the total number of bands shown in the arms of chromosome. Idiogram is the diagrammatic representation of the chromosome. The above cytogenetic parameters estimate the accuracy in the structure, shape, size and the position of the centromere which enable to distinguish the type and position of chromosome, making the karyotype easy. These parameters were used not only for the construction of karyotype and idiogram, but also required for morphology description of the chromosome.

Chromosome abnormalities can be very complex and are two basic types: numerical and structural. Both types can occur simultaneously. Numerical abnormalities involve the loss and/or gain of a whole chromosome or chromosomes and can include both autosomes and sex chromosomes. Generally chromosome loss has a greater effect on an individual than does chromosome gain although these can also have severe consequences. Cells

which have lost a chromosome are monosomy for that chromosome while those with an extra chromosome show trisomy for the chromosome involved. Structural abnormalities involve changes in the structure of one or more chromosomes.

Not listed in
References

REVIEW OF LITERATURE:

A chromosome is an organized structure of DNA and protein found in nucleus. They are capable of self-reproduction and play a vital role in heredity, mutation, variation and evolutionary development of the species. Chromosomes were first observed in plant cells by Karl Wilhelm von Nägeli in 1842. Their behavior in animal cells was described by Walther Flemming, the discoverer of mitosis, in 1882. In a series of experiments beginning in the mid 1880s, Theodor Boveri demonstrated that the chromosomes are the vectors of heredity, although the term chromosome was coined by Waldeyer (1888). Chromosome studies have attracted the attention of research worker since Roux (1883), pioneer in foretelling the involvement of chromosome in the mechanism of inheritance. The study of the chromosome was continued by Benden and Boveri (1887) and they have reported that the number of each species were constant. With the advent of twentieth century Sutton and Boveri (1902) proved and confirm that chromosomes were the physical structures acting as messengers in heredity.

Cytogenetics deals with the study of the structure and function of the cell, especially the chromosomes. In 1953, Hsu and Pomerat showed that hypotonic treatment before fixation would result in a better separation of individual chromosomes, thus making cytogenetic analysis more precise in terms of chromosome number and morphology. Tizo and Levan (1956) cultured somatic cells from fibroblasts of human embryos and counted the human chromosome number as 46, including the sex chromosomes, this was confirmed by Ford and Hamerton (1956) while working with the testicular material in the same year. Moorehead et al. (1960) described a simple method of culturing lymphocytes from human blood. Observations of heredity defects, had led cytogenetists to look for possible association between abnormalities of chromosome and respective phenotypes, in both human (e.g. Turner's syndrome, Klinefelter's syndrome, hermaphroditism etc.) and in various livestock species (e.g. hermaphroditism, testicular hypoplasia, intersexuality etc.) for both reproductive and economically important traits.

Technical developments in cytogenetics

Phytohaemagglutinin (PHA) was discovered as mitogen by Nowell (1960) while using it to separate leucocytes from whole blood due to its powerful erythroagglutination

property. PHA, extracted from red kidney bean (*Phaseolus vulgaris*) was found to stimulate the T-lymphocytes. Later on, pokeweed mitogen (PWM), a lectin from the roots of *Phytolacca americana* was discovered by Frances *et al.* (1964). PWM stimulated both T- cells and B- cells. PHA and PWM mitogens have been used widely to stimulate multiplication of buffalo lymphocytes in *in vitro* cultures (Rathnasabhapathy and Ganesh, 1980; Thiagarajan, 1987; Ramesha, 1988). However, PHA was found to agglutinate buffalo blood cells and consequently resulted in poor cultures compared to PWM (Bongso and Hilmi, 1982; Thiagarajan, 1987; Ramesha and Hegde, 1992). Joshi *et al.* (1999b) used both PHA and PWM, both of which yielded good results, but recorded PWM to be superior over PHA.

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Hypotonic treatment was accidentally discovered by Hsu (1952), who observed that chromosomes could be clearly visualized in cells treated with hypotonic solution instead of isotonic solution. Hypotonic treatment causes the swelling of lymphocytes that enables better separation of chromosomes and better chromosomal spreads. A hypotonic potassium chloride solution was first recommended by Hungerford (1965). Most of the workers used 0.075 M potassium chloride as standard hypotonic solution. However, the duration of hypotonic treatment varied from eight to 30 minutes in different laboratories.

A synthetic medium for tissue culture was introduced by Fisher (1948), where he used plasma as a basal medium and systematically replaced it with a mixture of amino acids, which were biologically active nutrients. Morgan *et al.* (1950) prepared a completely defined medium that was capable of maintaining chick embryo cells alive for a few weeks, subsequently called as the TC- 199 medium which was used widely in animal cell culture laboratories.

Serum was accepted as an essential component of the TC medium for induction and maintenance of growth of lymphocytes. Human inactivated serum (Gupta and Raychaudhuri, 1978); autologous plasma (Rathnasabhapathy and Ganesh, 1980), foetal calf serum (Bongso and Hilmi, 1982) and adult cattle serum (Goswami and Balakrishnan, 1983) had been incorporated in the tissue culture media at various levels to culture

buffalo lymphocytes. However, Eldridge (1982) avoided the addition of serum or plasma from exogenous source. Likewise, Joshi and Govindaiah (1999) also avoided supplementing culture medium with exogenous serum/plasma and did not observe any adverse affect on the growth of the lymphocytes.

Standardization of chromosome nomenclature

The chromosome nomenclature of domestic animals was standardized during First International Conference on Domestic Animals held at University of Reading, England, in 1976 (Ford *et al.*, 1980). It was recommended to follow strictly the systems developed for the description of human banded chromosomes. The chromosomes were distinguished by the following structural features viz., a) the length of each chromosome relative to the total length of the normal autosomes and X containing haploid set, b) the ratio of length of longer arm relative to length of its short arm and c) the centromeric index expressed as the ratio of the length of shorter arm to the total length of the chromosome. The species discussed during the conference were cattle, sheep, goats, pigs, horses, cats and rabbits.

Not
Ref.

Chromosomal morphometric parameters

Relative length

The lymphocytes in culture respond to colchicine treatment depending on the stage of cell cycle. Consequently, the chromosomes tend to vary in length from cell to cell due to condensation process. Even among the members of a pair of homologous chromosomes variations were recorded (Ford, 1973). Therefore, the concept of relative length at chromosomes was introduced as a parameter to compare the chromosome length reported in different karyotypes.

Centromeric index

The centromeric index is the ratio of short arm of the chromosome to its total length and is expressed in percentage. The centromeric index value indicates the position of centromere in the chromosome which enables to classify the chromosomes according to the morphology. Alteration in this index implies variation in the concerned chromosome

pair and is important criteria in locating the chromosomal aberrations that may lead to large phenotypic variations.

Chromosomal studies in cattle

The normal karyotype of cattle (*Bos taurus* and *Bos indicus*) comprises of $2n=60$ chromosomes of which fifty-eight are acrocentric autosomes and two sex-chromosomes (Melander, 1959; Sasaki and Makino, 1962; Gustavsson, 1969). The X - Chromosomes in both the species are ~~of~~ sub-metacentric (Sasaki and Makino, 1962). The Y-chromosome of the exotic (*Bos taurus*) male is metacentric and that Zebu (*Bos indicus*) acrocentric (Kieffer and Kartwright, 1968).

Not in References

The cytogenetic studies by Gupta *et al* (1974) revealed that the Y-Chromosome in humped cattle (i.e. Red Sindhi and Sahiwal) was acrocentric. It has been proposed that difference between Y chromosomes of *Bos taurus* and *Bos indicus* is due to a 'Y' to autosome balance translocation (Rendel, 1980). Potter and Upton (1979) described the Y in a Jersey bull as metacentric rather than sub-metacentric. Nevertheless, there is yet no overview ⁵¹ for the morphology of the Y chromosome in cattle; unlike the situation in human studies where Y polymorphisms are expected between races or individuals (Makino, 1975). Yadav *et al.* (1984) reported only sub-metacentric 'Y' chromosomes obtained from crossing exotic males and zebu females on F_1 crossbred males.

In 2004, Ahmad *et al.* carried out study of chromosomal analysis for 200 breeding bulls of different breeds of cattle (Jersey, Holstein Friesian, Sahiwal and Cross-bred) and Nili-Ravi buffalo, maintained at Semen Production Unit, Qadirabad and Livestock Experiment Station, Bhunikey (Pattoki) ~~was carried out~~. Micro method was adopted for leukocyte culture and chromosomes were trapped at metaphase stage.

✓ Balaji *et al.* (2006) characterized the purebred of Deoni cattle revealing the diploid chromosome number to be 60 and the chromosome architecture of Deoni cattle was proved to be similar to other breeds of Zebu cattle.

The chromosome number and structure of Umblachery cattle breed was characterized by Kumarasamy (2008) and described that the diploid chromosome number in Umblachery cattle was $2n=60$ comprising of 29 pairs of autosomes and one pair of sex chromosomes. All autosomes are acrocentric, the X- chromosome is sub-metacentric and Y chromosome is acrocentric.

Cytogenetics studies of Thai's native cattle (*Bos indicus*) by Sitthisak Jantararat *et al.* (2009) revealed that the diploid chromosomes number was $2n = 60$, the fundamental numbers (NF) being 61 in male and 62 in female. The autosomes were with 18 large, 20 medium and 20 small acrocentric chromosomes. The X chromosome was a large sub-metacentric and the Y was the smallest acrocentric.

Tho-tho cattle

The period of domestication of the Tho-tho cattle breed is not clear. However, the local people know this particular animal since last 500 years (according to traditional oral history). Tho-tho cattle is having significant distinct phenotype as compared to the other breeds of cattle due to geographical boundaries and environmental uniqueness. Since the traits are heritable in nature it needs to be evaluated cytogenetically as well as at molecular level. Therefore, the present study was focused on the chromosome morphology and cytogenetic analysis in this breed. The information generated will be helpful for providing the basis for comparative analysis with the other breeds of *Bos indicus*. Since, there is no report of chromosomal study on this breed of cattle till date, this cytogenetic study will be useful to generate a base for further detail analysis using molecular technique and also in culling the animals with chromosomal abnormalities, if any, which might affect the productive and reproductive performance and overall herd economic loss.

Mithun

The cytogenetic study on Mithun has been previously reported (Winter, 1984; Gupta, 1995). However, no detail information on chromosome morphometric parameters of Mithun is available in present scenario. Therefore, the present study was taken up to carry out the detail cytogenetic analysis that will provide clearer and standard karyotype with accurate arrangement and pairing of chromosomes. The information generated will play an important role in genetic conservation and breeding of Mithun.

The diploid chromosome number in Mithun, $2n=58$, was first reported by Winter *et al.* (1984), however no detail studies on the chromosome morphology were described. Later on Gupta *et al.* (1995) described the morphology of Mithun chromosome that the first pair of autosome is of sub-metacentric while remaining 27 pairs of autosomes are acrocentric. X chromosomes are sub-metacentric and similar to cattle. The Y chromosome is small metacentric like *Bos taurus*. However, after this none of the reports were published on detailed chromosome morphology, banding and cytogenetic analysis in Mithun.

MATERIALS AND METHODS:

Experimental area

The research lab and the experimental farm ^{was} is located at National Research Centre on Mithun, ICAR, Nagaland at 25° 45' 43" N latitude and 93° 53' 04" E longitude at an altitude of 310 m above the sea level. The climate broadly represents sub humid tropical climate zone with high relative humidity, moderate temperature with medium to high rainfall. The mean temperatures ranges from 21°C to 32°C during summer and rarely goes below 8°C in winter due to high atmospheric humidity. The average rainfall varies between 2000-2500 mm, starting from April till September while the period from October to March remains comparatively dry.

Materials

The material, i.e. blood sample, ^{of} of the present study were collected from 115 Mithun including both male and female and 28 Tho-tho cattle of both sexes kept at the station farm of National Research Centre on Mithun and the field samples were also collected from Visvema, Kidema, Kigwema and Merima under Kohima district. The composition of the chemical reagents and preparations, buffers, equipments and lab₃ wares are given in the appendix.

Methods

Cleaning and Rinsing

1. Extensive and careful preparation of glassware was a key to successful lymphocyte culture technique.
2. All glassware to be used were kept in chromic acid solution overnight, washed thoroughly first with running tap water and then rinsed with GDW and finally dried in hot air oven at 100°C for 24 hours.
3. Screw caps were soaked in mild detergent fluid overnight and washed in running tap water. They were later rinsed in distilled water and air-dried.

Sterilization

1. Glassware and rubber wares used for preparation of culture medium were sterilized in autoclave under 15 lb pressures for 15 minutes.
2. Ethanol (70%) was used to sterilize the hands during culturing operation and collection of blood samples.
3. The following materials were autoclaved usually by serial arrangements.
 - About 20 to 30 culture vials of 30 ml capacity with plastic cap and rubber linings.
 - About 8 to 10 pipettes of 10 ml capacity.
 - Two conical flask of 250 ml capacity.

Preparation of Culture media

RPMI 1640/Ham'sF-10	82 ml
Fetal calf serum	15 ml
Streptomycin	01 ml
Penicillin	01 ml
Pokeweed Mitogen	01 ml

The pH of the media was checked and adjusted to 7.2

Sample collection

The samples were collected from Mithun and Tho-tho cattle kept at the station farm of National Research Centre on Mithun and the field samples were also collected from Visvema, Kidema, Kigwema and Mkrima under Kohima district. The blood samples were collected from all age groups including breedable animals maintaining aseptic condition during the process of sample collection. The blood was collected following the guidelines suggested by animal ethics committee of the institute. The blood was aseptically taken out from jugular vein by using vaccutainer tube. This creates a negative pressure and the animal does not feel pain while taking out the blood. After the blood was collected in tubes it was stored in icebox and proper care was taken while handling like ^{avoid} shaking, jerk. The blood samples were transported to the laboratory as soon as possible for culture.

Setting up lymphocyte Culture

Whole blood lymphocyte culture technique was applied according to Yadav and Balakrishnan (1984) with little needed modification.

1. Whole blood culture were established either in freshly prepared medium or stored medium (-20°C).
2. If the culture medium was in frozen state, then the culture vials were kept in incubator ($37.5^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$) and brought the medium to room temperature.
3. The blood sample collected in vacutainer tubes were given a mild shaking by inverting the tubes a few times and spraying with 70% alcohol kept in the laminar air flow.
4. With the help of sterile syringes, 0.5 ml blood was taken out from the vacutainer tubes and transferred to the culture vials containing complete culture medium.
5. The content are given a gentle swirl for quick mixing.
6. The cap and the mouth of the culture vials were flamed over the spirit lamp before making it airtight to reduce the change of bacterial contamination.
7. Identification marks like date, animal number etc. were written on the label tag and pasted to each culture vials and also recorded in the laboratory notebook.
8. The culture vials were transferred to an incubator at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for a period of 65-72 hours.
9. The cultures were given a gentle swirl twice a day for mixing the growing cells into the media.

Harvesting of lymphocyte culture

The following procedure was adopted to harvest the chromosomes in metaphase stage.

1. Two drops of colchicine solution ($0.5\mu\text{g/ml}$) were added to 6 ml of media at about 68 hours (range 65-72 hours) to arrest the dividing cells at early metaphase and to have longer chromosome, respectively. The culture vials were gently mixed and incubated for 20 minutes.
2. The culture bottles were gently swirled to dislodge as many cells as possible. The contents of the culture vials were then transferred to 15 ml centrifuge tubes and subjected to centrifugation at 1500 rpm for 10 minutes.

3. The supernatant was discarded carefully with the help of Pasteur pipette leaving the cell suspension in the bottom of the centrifuge tubes in the form of pellets.
4. The pellets in each tube were treated with 10 ml of prewarmed hypotonic solution (0.075 M KCl) having the osmolarity of about 150 mOsm.
5. The cells pellets were resuspended by gentle pipetting using pasture pipette and the tubes were incubated at 37°C for 40 minutes.
6. To stop the action of hypotonic solution after incubation period 1ml of chilled fixative (methanol: acetic acid in the ratio of 3:1) was added to each centrifuge tubes and mixed gently.
7. Subsequently, centrifugation was done for 10 minutes at 1500 rpm, after which the supernatant was discarded and pellets were resuspended in 5 ml chilled fixative.
8. Steps (6) and (7) were repeated until a clear supernatant suspension and white cells button at the bottom of the tube were obtained.
9. Finally, cells were resuspended in 0.5 to 1.0 ml of freshly prepared fixative depending upon the size of cell button for the preparation of slides.

Cleaning of Slides

1. The slides (Himedia of 1mm thickness) were dipped overnight in chromic acid solution, thoroughly rinsed in tap water and then with GDW.
2. These were stored in a plastic bottle containing 5% HCl and absolute methanol and kept in the refrigerator (4°C).
3. Before use, slides were taken out, allowed to dry and cleaned thoroughly with a clean muslin cloth.

Preparation of Slides

Flame drying method for conventional giemsa staining

1. A clean polished slide was held with the help of forcep at an angle of about 30°.
2. The slide was moistened by blowing air from the mouth.
3. About 3-4 drops of cell suspension were dropped on this slide from a height of about 2 feet with the help of Pasteur pipette.
4. The slides were moved side wise to allow even flow of the cells on the slide.

5. The slides were touched at the edge to the flame of a spirit lamp.
6. This burnt the methanol on the slide and a mild jerk was given to remove the left over acetic acid.
7. Slides were kept in the incubator for hours before staining.

Conventional staining with Giemsa

1. One ml of giemsa solution and 49 ml of distilled water were thoroughly mixed in a coplin jar. The top layer of the solution was cleaned with blotting paper to remove the greasy layer.
2. The required slides were kept in the coplin jar.
3. The slides were allowed to stand in the staining solution for 30 minutes.
4. Subsequently, the slides were rinsed thoroughly with GDW and dried in folds of filter paper.
5. The slides were then transferred to the incubator and kept for a few hours before screening of slides for metaphase spread.

Preservation of the cell suspension

After preparation of sufficient numbers of slides, the remaining cells were resuspended in three ml of fixative. These centrifuge tubes were sealed with paraffin and stored in the fridge. Whenever required, frozen cells were thawed, washed with fresh chilled fixative and used for slide preparation.

Cytogenetic analysis

Screening of slides

The stained slides were systematically screened under low power objective (10X) to select suitable metaphase spreads considering the following criteria.

- i. Minimum chromosome overlapping.
- ii. Chromosome of sufficient length.
- iii. Chromosome with clear staining/banding.

The spreads were photographed under oil immersion objective to give a final magnification of 1000X. A minimum of 20 spreads from each animal were screened and photographed.

Photomicrography

The good metaphase spreads from the slide were selected and photographs were taken. The measurements of chromosomes were done for identification and pairing of homologous chromosomes using ImagePro 6.0 software (Nikon) and Cytovision 7.2 (Leica, Microsystem).

Identification and classification of chromosome

Homologous pairs of chromosomes were identified and karyotype was made based on size and banding pattern of chromosomes.

Chromosome Morphology

Karyotypes established from the photomicrographs taken of several good metaphases were used for the measurement of length of chromosomes (Ford *et al.* 1980). These chromosomes were measured with Image Pro 6.0 at 20 micron. The following linear parameters of chromosomes were determined from their mean lengths by the quotients.

Not in Ref.

Relative length (RL)

$$RL = \frac{\text{Length of individual chromosome}}{\text{Total length of genome including sex chromosomes}} \times 100$$

Centromeric index (CI)

$$CI = \frac{\text{Short arm length}}{\text{Total length of its chromosome}} \times 100$$

Arm ratio (AR)

$$AR = \text{Long arm/Short arm}$$

OBSERVATION:

Before actually starting the experiment and setting up cultures from blood samples of experimental animals, the recommended experimental protocol was standardized according to the laboratory and the present experimental conditions. The standardization was done for various factors as follows:-

Standardization of media

At first, few trials were done to find out the differences between two media available in the laboratory viz. RPMI-1640 and Ham's F-10. These two media were considered as best and had given the optimum and consistent result of lymphocyte culture technique in the laboratory, where the present study was carried out. In both the medium the amount of blood used for setting up culture was 20 drops, with a hypodermic needle (gauge no. 20). The colchicines treatment was given for 20 minutes and the cells were allowed to swell up in hypotonic solution (0.075 M KCl) for 30 minutes. The metaphase spread obtained through this trials contained few good spread, many condensed spread and many blast cell in case of RPMI-1640. However, Ham's F-10 gave a better result with good metaphase spread and less blast cell. The morphology and the spread area of the chromosomes were good.

Standardization of hypotonic solution and treatment

The time and concentration of hypotonic solution treatment was a key to obtaining good and well spread metaphase chromosome. At first, the standard concentration of hypotonic solution i.e 0.075 M KCl was unchanged, but the duration of the treatment was varied. The timing of the treatment were kept as 15 mins, 20 mins, 25 mins 30 mins, 35 mins and 40 mins. Best result was obtained in 30 minutes in case of Tho-tho cattle. However these durations do not gave good metaphase spread chromosome in Mithun. Therefore second trial was made with change in the concentration of the hypotonic solution. A trial with a concentration of 0.056 M KCl (4.2gm/l) as hypotonic solution was set for Mithun with the incubation time variation as mention above. The incubation time of 40 minutes at 37.5°C gave the best metaphase spread chromosome in Mithun.

The result obtained in these trials were used as standard in all the subsequent cultures on the experimental blood samples i.e. Ham's F-10 was used as culture medium with pH adjusted to 7.4, followed by hypotonic treatment of 0.075 M KCl for 30 minutes in case of Tho-tho cattle and 0.056 M KCl for 40 minutes in Mithun.

Cytogenetic studies of Mithun (*Bos frontalis*)

Chromosomal profile studies in Mithun were carried out by karyotyping, cytogenetic analysis.

The collection of the blood sample was done using sodium heparinized vacutainer tube with sterile needle. The Mithun were controlled in a travis for the collection of the sample. The adult Mithun above three years of age especially the males were very difficult to handle as they are very aggressive in nature. The sample collection in the field was done by adapting the casting method.

The blood samples were cultured by lymphocyte culture technique in Ham's F-10 (w/L Glutamine) supplemented with foetal calf serum along with antibiotics and mitogen. The culture was incubated for 72 hours for normal morphological studies and 68 hours for chromosomal banding studies.

Lymphocyte culture harvesting

Colcemide was added 15 minutes before the end of the culturing. The hypotonic treatment was given for 40 minutes and washed with fixative till clear cell pellet were obtained. At least five slides were prepared by air dry method and stained with 2% giemsa stain.

Mithun chromosome number and Morphology

The slides with good metaphase spreads were selected for screening. A total of 80 to 100 spreads were screened and out of them best metaphase spreads from each individual

Mithun were photographed with Nikon 90i microscope. The photographs were used for chromosomal numerals as well as for any aberrations. The diploid chromosome number in Mithun was found to be 58 ($2n=58$), which include 28 pairs of autosomes and one pair of sex chromosomes. The normal karyotype of Mithun (Figure 5) shows, the first pair of autosome is the largest and sub-metacentric chromosome and the rest autosome were acrocentric. Among the sex chromosome the X-chromosome was sub-metacentric and Y-chromosome was the smallest metacentric chromosome.

Cytogenetic parameters of Mithun chromosome

In our present study, cytogenetic analysis was carried out for both male and female Mithun based on the morphometric measurement such as relative length, centromeric index, arm ratio and morphological index were generated. In addition, mean chromosome length for species, average arm ratio, morphological index, range between the longest and the shortest chromosome were calculated. Chromosome morphology was described using nomenclature proposed by Gurra (1986). The measurements of chromosomes were done for identification and pairing of homologous chromosomes using ImagePro 6.0 software (Nikon) and Cytovision 7.2 (Leica Microsystem).

^{ed.}
The detail analysis of cytogenetic parameters are described below:

Relative length

The relative length (%) of the first pair was the largest chromosome that is sub-metacentric with 6.322 ± 0.191 and 6.131 ± 0.562 in male and female, respectively. The relative length of the smallest chromosome (chromosome number 28) was 1.705 ± 0.040 in male and 1.666 ± 0.146 in female. In sex chromosome, the X-chromosome was 5.284 ± 0.153 for male and 5.276 ± 0.282 for female. The percent relative length of Y-chromosome was 2.283 ± 0.190 . The detail relative length of the entire chromosome for male and female Mithun is shown in Table 1. The idiogram was prepared based on the mean value for both male and female (Figure 6).

Centromeric index

The centromeric index was calculated only on bi-armed chromosome i.e. first pair sub-metacentric autosome and sex chromosomes. The average centromeric index of chromosome number 1 in male and female Mithun was about 28.378 ± 2.261 and 28.126 ± 0.940 respectively. In sex chromosome the centromeric index for female was $X = 30.130 \pm 1.757$ and in male $X = 29.489 \pm 1.040$ and $Y = 45.53 \pm 1.904$. Since the value of centromeric index was in the range of 26-45 in case of X chromosome therefore it is categorized as sub-metacentric chromosome. However, in Y chromosome the value is little above 45% so it is considered as metacentric chromosome. The graphical representation of centromeric index of bi armed chromosome in Mithun is shown in Figure 7.

Arm ratio

The arm ratio was calculated in sub-metacentric chromosome i.e. chromosome 1 and sex chromosome. The value of the arm ratio for the first pair of chromosome was 2.269 ± 0.756 and 2.290 ± 0.930 in male and female, respectively. In female, the arm ratio for the sex chromosome was 2.290 ± 0.930 and in male X chromosome was 2.390 ± 0.903 , Y chromosome was 1.180 ± 0.482 . The graphical representation of arm ratio of bi armed chromosome in Mithun is shown in Figure 7.

Cytogenetic studies of Tho-tho (*Bos indicus*) cattle

The collection of the blood sample was done using sodium heparinized vacutainer tube with sterile needle. The samples were collected from 17 male and 11 female, out of which 5 male samples were collected from the station farm of National Research Centre on Mithun and the rest from the field i.e. Visverna, Kidema, Merima and kigwema all under Kohima district. The sample collection in the field was done by adapting the casting method since it was difficult to setup the traxis in field due to geographical conditions. The blood collection was done according to the guidelines suggested by animal ethics committee of the institute.

The blood samples were cultured by lymphocyte culture technique in Ham's F-10 (w/L Glutamine) supplemented with foetal calf serum along with antibiotics and mitogen. The culture was done for 72 hours for normal morphological studies and 68 hours for chromosomal banding studies.

Lymphocyte culture harvesting

Colcemide was added 15 minutes before the end of the culturing. The hypotonic treatment was given for 30 minutes and washed with fixative till clear cell pellets were obtained. At least five slides were prepared by air dry method and stained with 2% giemsa stain.

Tho-tho cattle chromosome number and Morphology

The diploid chromosome number in Tho-tho cattle was observed to be $2n=60$ in all complete metaphase examined. The normal karyotype (Figure 8) shows that all the autosomes are acrocentric chromosome and in sex chromosome, X chromosome was submetacentric chromosome and Y chromosome was the smallest acrocentric chromosome.

Cytogenetic parameters of Tho-tho cattle chromosome:

Relative length

Cytogenetic analysis was done for both male and female Tho-tho cattle and found that the mean relative length in autosome ranged from $5.48 \pm 0.107 \%$ to $1.79 \pm 0.105 \%$ in male and $5.31 \pm 0.148 \%$ to $1.86 \pm 0.055 \%$ in female. In Tho-tho cattle the largest chromosome in the genome was chromosome number 1. The detail relative length of all the chromosome for male and female Mithun is shown in Table 2.

Centromeric index

The morphology of the chromosome was ascertained by estimating the parameters like centromeric index and arm ratio on bi-armed X chromosomes. The average centromeric index in Tho-tho cattle was found to be $36.45 \pm 0.31 \%$ in male and $31.06 \pm 0.55 \%$ in female.

Based on the value of the cytogenetic analysis an ideogram was prepared as shown in Figure 9. This cytogenetics parameter of the present study for the Tho-tho breed of cattle are in agreement with those previously reported for the *Bos indicus*, thus confirming that the karyotype profile of investigated animals did not differ from the standard karyotype of the bovine species. However, the present study on chromosomes of Tho-tho cattle will be a platform for further comparative studies with other breed of same species by ~~Amoleentay~~ cytogenetics and molecular techniques. The graphical representation of centromeric index of bi armed chromosome in Tho-tho is shown in Figure 10.

Arm ratio

The arm ratio is calculated in sub-metacentric chromosome i.e. chromosome 1 and sex chromosome. The arm ratio was 2.60 ± 0.09 and 2.23 ± 0.06 for male and female Tho-tho cattle, respectively (Figure 10). Referring to the value of the cytogenetic analysis an ideogram was prepared.



Figure 1. Mithun Male and Female with Calf

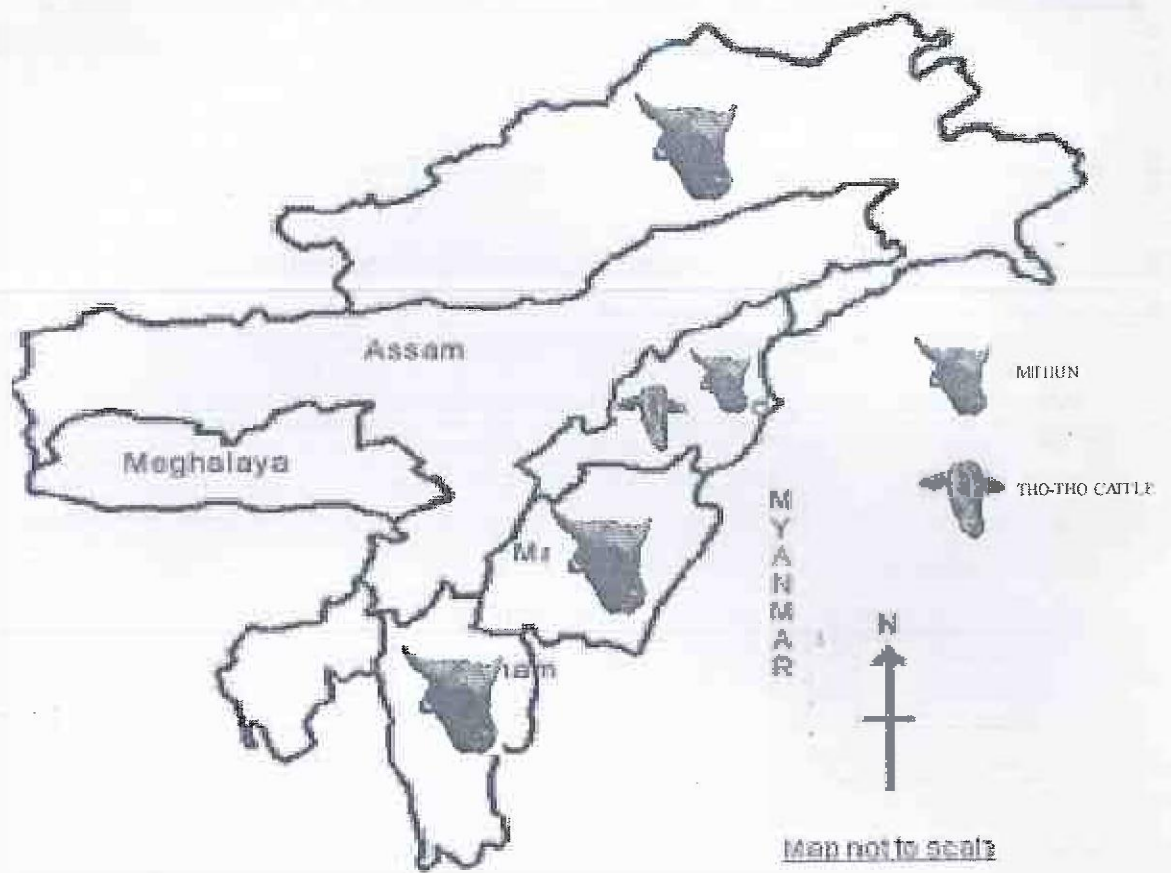


Figure 2. Habitat of Mithun and Tho-tho cattle.



Figure 3. Tho-tho male (above), Tho-tho female (below)



Figure 4. Blood sample collection (A) Field condition (B) and (C) Farm

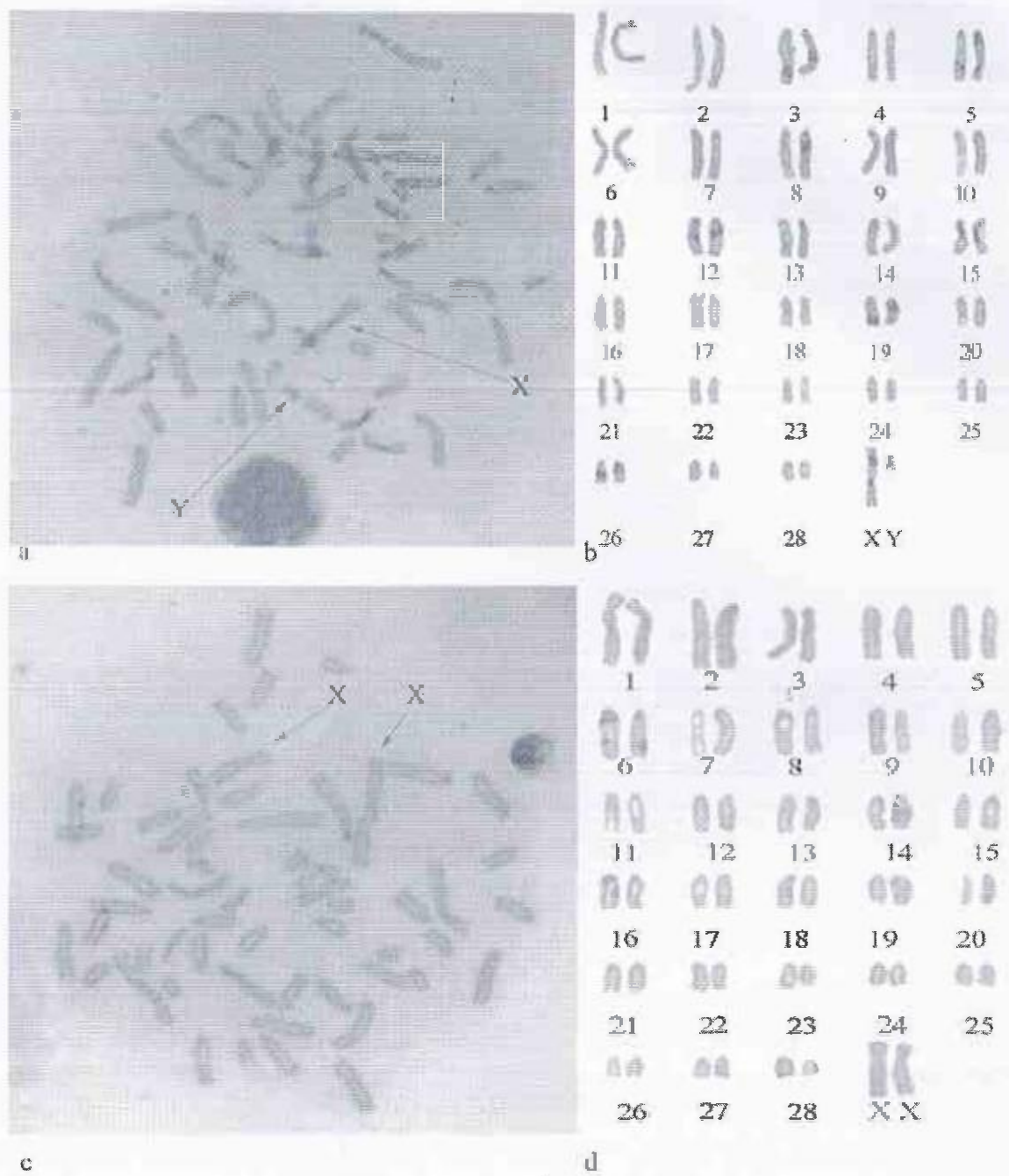
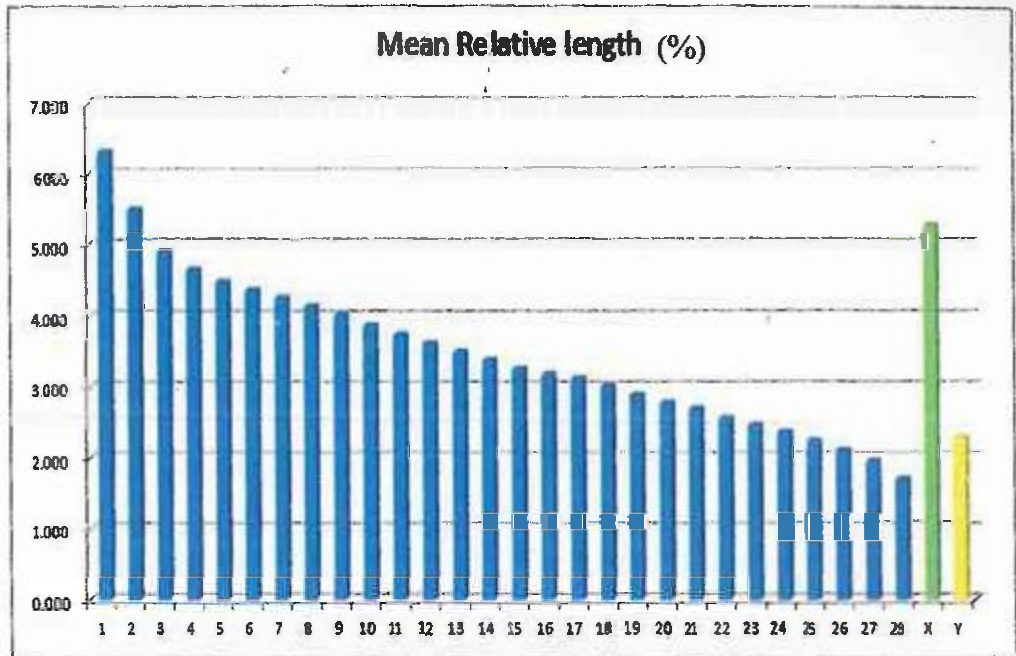


Figure 5. Metaphase spread and Karyotype of Mithun chromosome. a,b (Male), c,d (female).

Male Mithun



Female Mithun

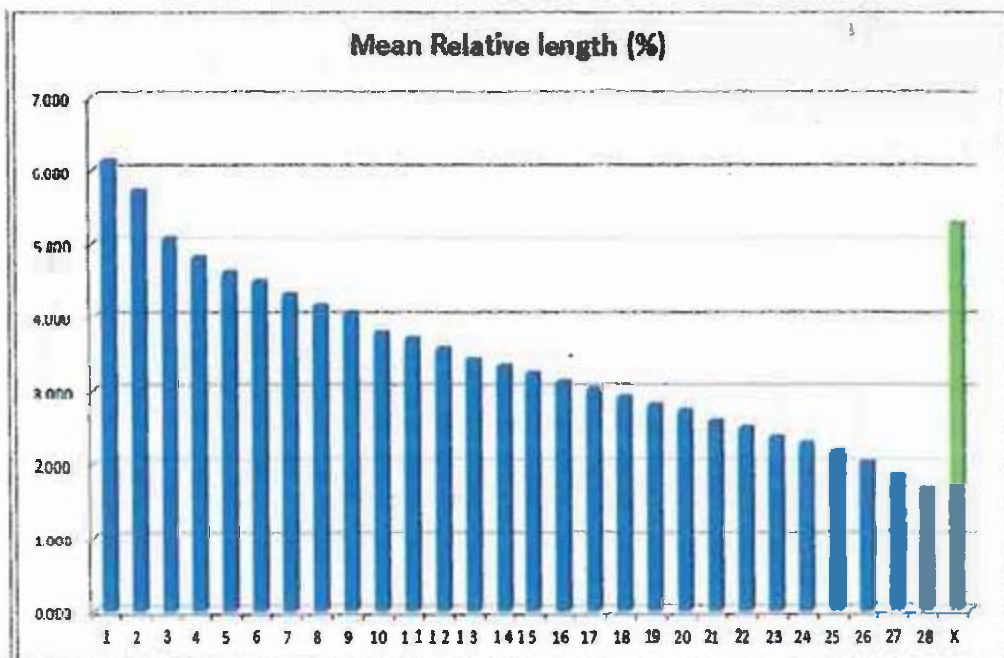
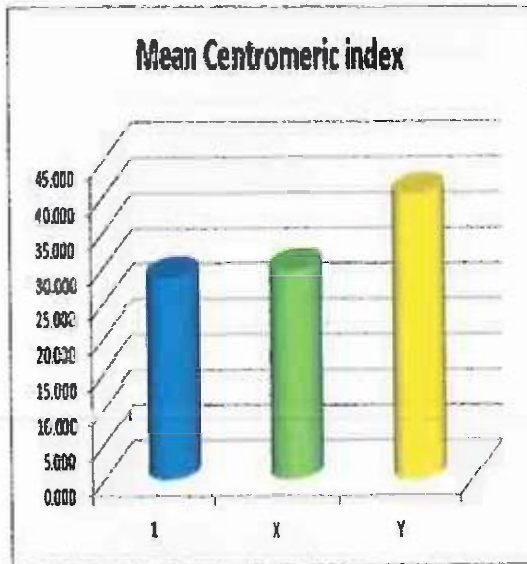
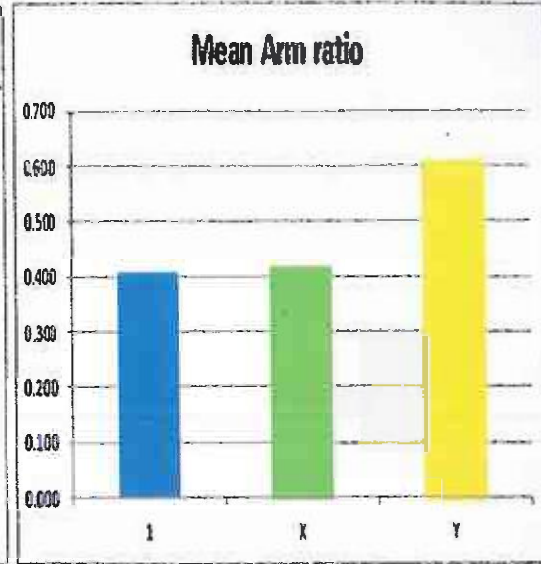


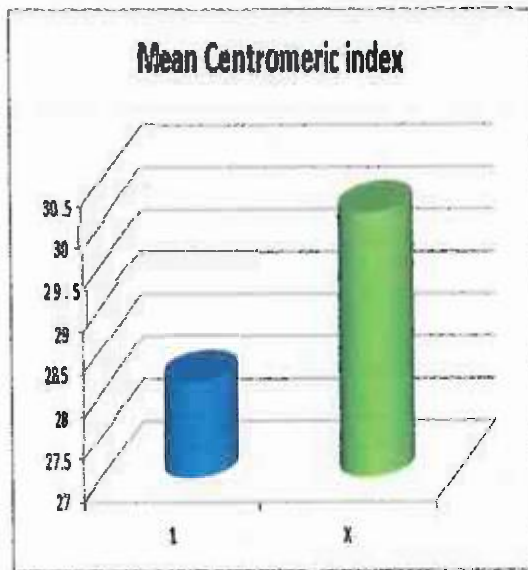
Figure 6. Relative Length of Male and Female Mithun. Autosomes indicated in blue, X chromosome indicated in green and Y chromosome indicated in yellow



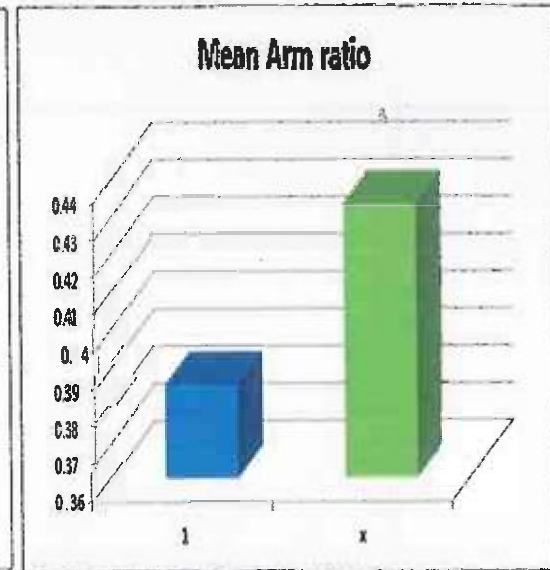
a. Centromeric index of male Mithun



b. Arm ratio of male Mithun

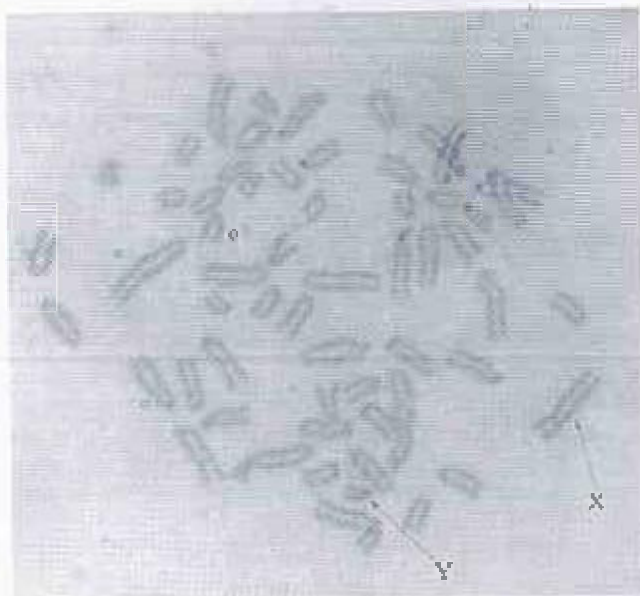


c. Centromeric index of female Mithun

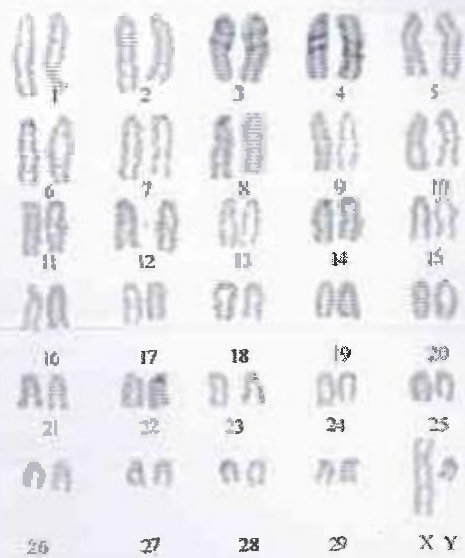


d. Arm ratio of female Mithun

Figure 7. Centromeric Index and Arm Ratio of Male and Female Mithun. Chromosome 1 indicated in blue, X chromosome indicated in green and Y chromosome indicated in yellow



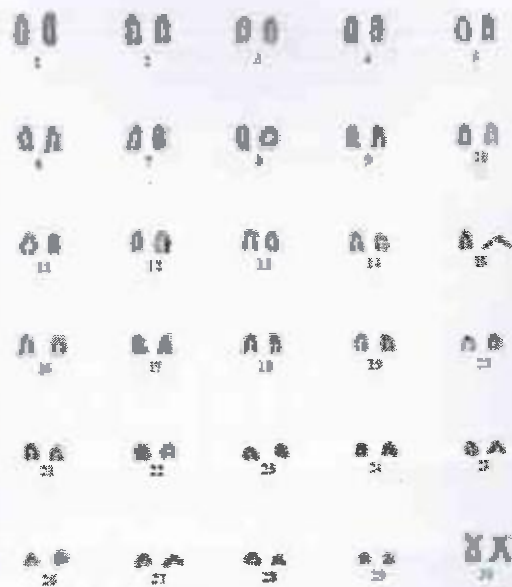
(a)



(b)



(c)



(d)

Figure 8. Metaphase spread and Karyotype of Tho-tho Cattle a,b (male) and c,d (female).

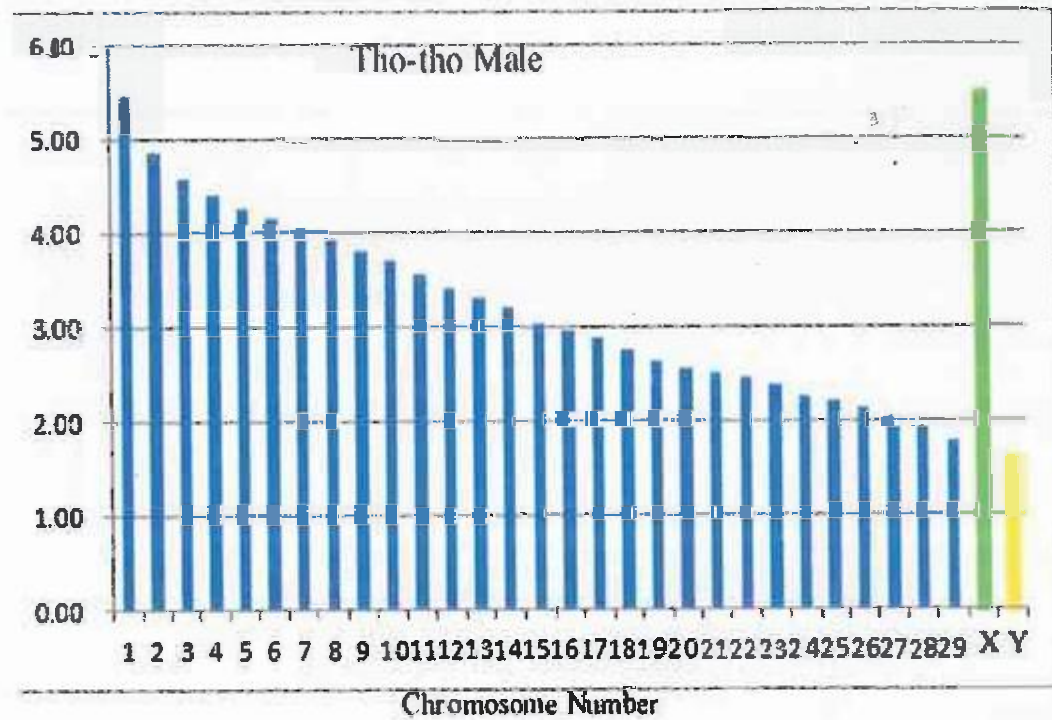
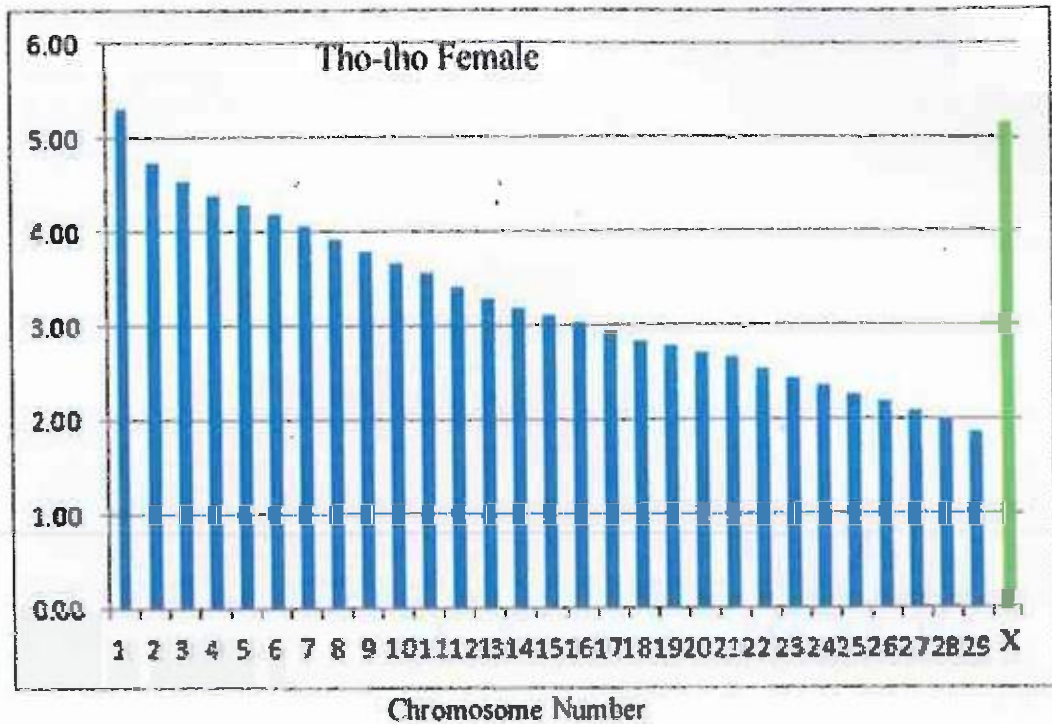
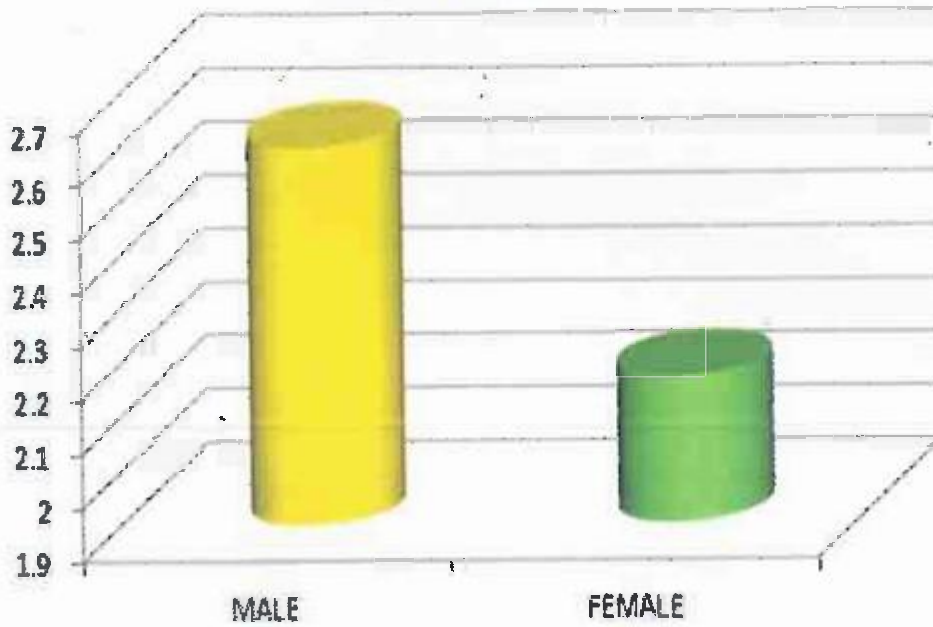


Figure 9. Relative Length of Male ($2n=60,XY$) and Female Tho-tho Cattle ($2n=60,XX$). Autosomes indicated in blue, X chromosome indicated in green and Y chromosome indicated in yellow

ARM RATIO



CENTROMERIC INDEX

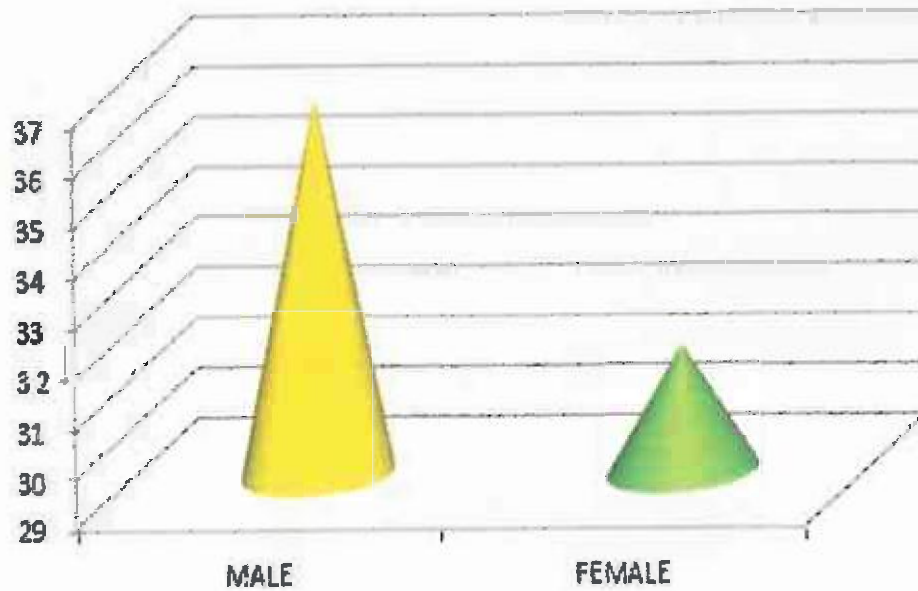


Figure 10. Arm Ratio and Centromeric Index of Male and Female Tho-tho Cattle.

Table 1. Relative Length (mean \pm SE) of Mithun (*Bos frontalis*) chromosome.

Chromosome pair number	Relative length (%)		Chromosome Type
	MALE (Mean \pm SE)	FEMALE (Mean \pm SE)	
1	6.322 \pm 0.191	6.131 \pm 0.562	Sm
2	5.508 \pm 0.092	5.741 \pm 0.406	a
3	4.918 \pm 0.055	5.069 \pm 0.300	a
4	4.667 \pm 0.042	4.814 \pm 0.242	a
5	4.492 \pm 0.031	4.625 \pm 0.242	a
6	4.376 \pm 0.028	4.483 \pm 0.250	a
7	4.271 \pm 0.024	4.318 \pm 0.200	a
8	4.144 \pm 0.020	4.167 \pm 0.125	a
9	4.023 \pm 0.027	4.054 \pm 0.131	a
10	3.878 \pm 0.022	3.783 \pm 0.409	a
11	3.741 \pm 0.040	3.702 \pm 0.123	a
12	3.623 \pm 0.044	3.569 \pm 0.128	a
13	3.499 \pm 0.040	3.426 \pm 0.182	a
14	3.388 \pm 0.032	3.332 \pm 0.181	a
15	3.262 \pm 0.032	3.228 \pm 0.189	a
16	3.193 \pm 0.026	3.115 \pm 0.165	a
17	3.127 \pm 0.025	3.011 \pm 0.105	a
18	3.017 \pm 0.031	2.919 \pm 0.112	a
19	2.888 \pm 0.030	2.791 \pm 0.119	a
20	2.784 \pm 0.026	2.713 \pm 0.089	a
21	2.701 \pm 0.029	2.588 \pm 0.099	a
22	2.567 \pm 0.028	2.490 \pm 0.126	a
23	2.473 \pm 0.031	2.383 \pm 0.119	a
24	2.368 \pm 0.025	2.281 \pm 0.099	a
25	2.247 \pm 0.032	2.163 \pm 0.093	a
26	2.112 \pm 0.036	2.035 \pm 0.130	a
27	1.955 \pm 0.045	1.833 \pm 0.170	a
28	1.705 \pm 0.040	1.666 \pm 0.146	a
X	5.284 \pm 0.153	5.276 \pm 0.282	Sm
Y	2.283 \pm 0.190		m

a= acrocentric, Sm= sub metacentric, m= metacentric

Table 2. Relative Length (mean \pm SE) of Tho-tho (*Bos indicus*) cattle chromosome

Chromosome number (pair)	Relative length (%)		Chromosome type
	Male (mean \pm SE)	Female (mean \pm SE)	
1	5.48 \pm 0.10	5.31 \pm 0.14	a
2	4.86 \pm 0.08	4.75 \pm 0.06	a
3	4.59 \pm 0.11	4.55 \pm 0.05	a
4	4.42 \pm 0.10	4.40 \pm 0.04	a
5	4.27 \pm 0.07	4.29 \pm 0.05	a
6	4.16 \pm 0.09	4.19 \pm 0.04	a
7	4.05 \pm 0.07	4.06 \pm 0.03	a
8	3.93 \pm 0.04	3.92 \pm 0.02	a
9	3.82 \pm 0.06	3.78 \pm 0.04	a
10	3.73 \pm 0.04	3.68 \pm 0.05	a
11	3.57 \pm 0.02	3.57 \pm 0.03	a
12	3.42 \pm 0.02	3.42 \pm 0.02	a
13	3.31 \pm 0.03	3.29 \pm 0.03	a
14	3.21 \pm 0.02	3.19 \pm 0.02	a
15	3.05 \pm 0.04	3.11 \pm 0.02	a
16	2.96 \pm 0.03	3.03 \pm 0.02	a
17	2.89 \pm 0.04	2.94 \pm 0.03	a
18	2.77 \pm 0.05	2.85 \pm 0.04	a
19	2.64 \pm 0.08	2.80 \pm 0.05	a
20	2.57 \pm 0.07	2.71 \pm 0.04	a
21	2.53 \pm 0.06	2.66 \pm 0.04	a
22	2.48 \pm 0.07	2.54 \pm 0.05	a
23	2.40 \pm 0.07	2.44 \pm 0.05	a
24	2.27 \pm 0.08	2.36 \pm 0.06	a
25	2.22 \pm 0.08	2.26 \pm 0.05	a
26	2.13 \pm 0.09	2.18 \pm 0.06	a
27	2.03 \pm 0.08	2.09 \pm 0.06	a
28	1.95 \pm 0.10	1.99 \pm 0.05	a
29	1.79 \pm 0.10	1.86 \pm 0.05	a
X	5.53 \pm 0.22	5.18 \pm 0.11	Sm
Y	1.63 \pm 0.05		a

*a= acrocentric, Sm= sub metacentric

DISCUSSION:

Mithun

The diploid chromosome number in Mithun was found to be 58 ($2n=58$), this result was in agreement with the earlier reports on chromosome number of the Mithun (Winter *et al.* 1984; Gupta *et al.* 1995; Qu *et al.* 2012) which include 28 pairs of autosomes and one pair of sex chromosomes. The diploid chromosome number in Mithun is similar to the Gaur that is believed to be wild ancestor of Mithun. Both the species share the same diploid number of chromosome, being the first pair of chromosome sub-metacentric chromosome and the rest autosome acrocentric chromosome. In sex chromosome the X-chromosome was sub-metacentric and Y-chromosome was the smallest metacentric chromosome. Though Y chromosome is metacentric in both the species but is perfect metacentric in gaur as compared to Mithun. The clarity of the Mithun Y chromosome was observed in chromosome spread which was harvested at early metaphase stage. Here long chromosome were observed and a clear morphology of Y-chromosome was confirmed as the centromere position was not in centre but was little more towards one end of the chromosome arm.

The finding in the present study indicated that the Y chromosome is metacentric, is in agreement with Gallagher *et al.* (1994) and Gupta *et al.* (1999). In Bovine species the metacentric nature of Y chromosome was reported in Canchim cattle herd (Antonio Junqueira Tambasco, 1990), Cambodian bandeng (Anne Ropiquet, 2008). Not in Ref.

In the present study the relative length of Mithun chromosome ranged from 6.322 ± 0.191 to 1.705 ± 0.040 in male and 6.131 ± 0.562 to 1.666 ± 0.146 in female, respectively. In sex chromosome, the X-chromosome was 5.284 ± 0.153 for male and 5.276 ± 0.282 for female. The percent relative length of Y-chromosome was 2.283 ± 0.190 .

In Mithun two pairs of sub-metacentric chromosome were observed, i.e. 1st pair autosome and X chromosome. In the present study it was important to differentiate between these two pair and placing them at the right position in the Karyotype. Measuring the relative

length made the identification of exact length for these pair of sub-metacentric chromosome and thus their position in the karyotype.

The present finding of centromeric index of chromosome number 1 in male and female Mithun was 28.378 ± 2.261 and 28.126 ± 0.940 , respectively. In sex chromosome, the centromeric index for female was $X = 30.130 \pm 1.757$ and in male $X = 29.489 \pm 1.040$ and $Y = 45.53 \pm 1.904$. Since the value of centromeric index was in the range of 25.1 to 40 (Guerra *et al.* 1986) in case of X chromosome therefore it is categorized as sub-metacentric chromosome. However, in Y chromosome the value is little above 45% which falls in the range of 40.1 to 50 (Guerra *et al.* 1986) so it is considered as metacentric chromosome.

The arm ratio was calculated in sub-metacentric chromosome i.e. chromosome 1 and sex chromosome. The value of the arm ratio for the first pair of chromosome was 2.269 ± 0.756 in male and 2.290 ± 0.930 in female which is within the range of 1.50 to 2.99 (Guerra *et al.* 1986). In female the arm ratio for the sex chromosome was 2.290 ± 0.930 and in male X chromosome was 2.390 ± 0.903 , Y chromosome was 1.180 ± 0.482 , the value of which is within 1.00 to 1.49 (Guerra *et al.* 1986).

Tho-tho cattle

The report on chromosome number of Tho-tho cattle ($2n=60$) is in agreement with the earlier report on normal chromosome number of various breeds of *Bos indicus* (Kosarcic *et al.* 2005, Balaji *et al.* 2006, Kumarasamy *et al.* 2008). The size of the chromosome variation in autosome was uniformly descending as presented in the karyotype. This can be categorized into a set of first nine pairs of chromosome as large followed by next ten pairs as medium and the last ten pairs of autosome as small size chromosomes, as observed in other cattle (Popescu *et al.* 1996; Jantarat *et al.* 2009).

In the present study it was observed that the mean relative length in autosome ranged from $5.48 \pm 0.107\%$ to $1.79 \pm 0.105\%$ in male and $5.31 \pm 0.148\%$ to $1.86 \pm 0.055\%$ in female. The largest chromosome in the genome was chromosome number 1 and all the autosomes were acrocentric chromosome. There was only one sub-metacentric chromosome i.e. X chromosome and it was the second largest chromosome. The variation of relative length observed in the present study of Tho-tho cattle was found to be similar with that of Deoni cattle breed (Balaji *et al.* 2006). The sex chromosome observed in this study was large sub-metacentric X chromosome and the Y was the smallest acrocentric. These features are similar to the report of Wurster and Benirschke (1968), Mayr and Gruber (1986) and Popescu *et al.* (1996) that revealed that Tho-tho cattle have sub-metacentric X chromosome and acrocentric Y chromosome.

Jantarat *et al.* (2009) also observed that the largest chromosome in the genome was chromosome 1 in Thai's native cattle. However, other researchers reported that in Deoni cattle breed (Balaji *et al.* 2006) X chromosome was the largest chromosome in genome with relative length measuring 5.592% and chromosome 1 measuring 5.354% and Kumarasamy *et al.* (2008) reported in Umblachery cattle breed that the X chromosome was the largest in the genome measuring $5.002 \pm 0.114\%$ as compared to the chromosome 1 which was $4.637 \pm 0.063\%$.

The average centromeric index in Tho-tho cattle was found to be $36.45 \pm 0.31\%$ in male and $31.06 \pm 0.55\%$ in female. These value falls within the range 25.1% to 40.0% (Gurra, 1986) and therefore, the bi-armed X chromosome is categorized as sub-metacentric chromosome.

The chromosome 1 and sex chromosome of Tho-tho cattle revealed that the arm ratio was 2.60 ± 0.09 and 2.23 ± 0.06 for male and female, respectively. These value lies in the range within 1.50-2.99 (Gurra, 1986), which add up to the confirmation of X chromosome being sub-metacentric.

The present cytogenetic screening of Mithun in the National Research Centre on Mithun farm helped in creating a cytogenetic album which will be helpful in breeding program. The cytogenetic profile of Mithun created in the present study will be a platform for further molecular cytogenetic studies. The cytogenetic parameters of the Tho-tho breed of cattle are in agreement with the other breeds *Bos indicus*, thus confirming that karyotype profile of investigated animals do not differ from standard karyotype of bovine species. However, the present study on chromosomes of Tho-tho cattle will be a platform for further comparative studies with other breeds of same species by molecular cytogenetics and molecular techniques. The study on the chromosomal diversity and morphology in Tho-tho cattle was done since this breed is having the habitat in North-eastern region of India that is remotely located from the habitat of all other descript breed and the history of domestication is also not very far. Thus, it is required to study the chromosomal profile so that scientific and systematic breeding strategies can be followed. It is also necessary to start a cytogenetic screening of the Tho-tho and expand upon more numbers to be kept at different villages of Nagaland in order to identify animals with chromosomal abnormalities so that ~~it~~^{they} can be excluded from future breeding strategies for conservation of Tho-tho genetic resource.

CHAPTER II

**TO STUDY AND COMPARE THE CHROMOSOME
MORPHOLOGY AND STRUCTURE OF MITHUN AND
THO-THO CATTLE BY VARIOUS BANDING
TECHNIQUES**

INTRODUCTION:

Chromosome banding is the process of eliciting differences in staining appearance along the length of a chromosome there by producing a specific banding pattern. These banding patterns are reproducible, unique and are characteristics to each chromosome. They allow the identification of chromosomes, the construction of karyotypes, detailed description of structural rearrangements, and the comparison of chromosomes within a species or between closely or distantly related species. (Hayes, 2000).

Chromosome banding techniques have proved to be useful in pairing of chromosomes and thus making karyotype more precise. Any variation in chromosome number and /or structure may result in unbalanced karyotype. Thus the detection of chromosomal abnormalities in domestic animals will prevent the rapid spread of chromosomal defects in successive generations through natural and/or advanced reproductive tools.

There are three related objectives in studying mechanism of chromosome banding: obtaining and understanding of chromosome structure, understanding the behavior of chromosome in terms of their substructure and improving the reliability of banding techniques. Similarities in organism are commonly interpreted as the result of common ancestry. Since chromosomes are the carriers of heredity, similarities in chromosomes could have special significance in studying the ancestry and relationships of species. Many studies comparing chromosome banding have been conducted. Observations of heredity defects, had led cytogenetists to look for possible association between abnormalities of chromosomes and respective phenotypes, in both humans and in various livestock species for both reproductive and economically important traits.

Banding techniques

Procedure for the identification of chromosome has appeared since early seventies. The stains instead of uniformly dying the entire chromosome display characteristic bands. Due to certain treatments, stain binds preferentially to certain zones or segment of chromosomal DNA. It is owing to the chemical differences. The banding techniques

provide valuable information for precise identification of chromosomes. The banded chromosome exhibit differential staining along their length into dark prominent and light zones. Since the sequence of bands is very specific for each somatic pair, these serve as landmarks in identification and karyotyping.

R-banding

R-bands show a pattern that is reverse of G-bands i.e. light banded region of G-banded chromosomes became darkly stained and vice versa. R-bands are the results of a technique that stains regions rich in GC that are typical for euchromatin. R-bands correspond to the regions on chromosome having proteins lacking sulphur. R-banding was ~~at~~ first developed for human chromosome (Ditrillaux and Lejeune, 1971), in which the pattern is opposite to G-bands. Therefore, it is useful to investigate telomeric deletion and also sex chromosome identification due to inactivation of one of the X chromosome (Dosage compensation theory).

C-banding

This is one of first chromosome banding technique discovered as an outcome of the *in-situ* RNA/DNA hybridization (Pardue and Gall, 1970); the name is derived from centromeric or constitutive heterochromatin. In this method the chromosome undergo alkaline denaturation prior to staining leading to an almost complete depurination of the DNA after washing the probe, the remaining DNA is renatured again and stained with Giemsa solution. Heterochromatin binds a lot of the dye, while the rest of the chromosomes absorb only little of it (Hsu, 1973). C-banding of chromosome is used to stain the centromeric regions of the chromosome and it also defines the regions of constitutive heterochromatin. This type of DNA often contains highly repetitive sequences.

REVIEW OF LITERATURE:

Animal cytogenetists use giemsa's dye mixture as a conventional stain for chromosome study instead of acetocarmine or hematoxylin used by early workers. It is useful in studies ~~in~~ chromosomal anomalies, providing information on diploid number and morphological features of chromosome. But banding patterns give more information on these aspects by specifying the location of the respective anomalies (i.e. gap, break, deletion, duplication, translocation, rearrangement etc).

✓ Caspersson *et al.* (1969) were responsible for revolutionizing and heralding the era of chromosome banding with the introduction of fluorescent acridine derivative (quinacrine mustard) for staining the chromosome. They demonstrated that chromosomes were not homogenous organelles but were possessing very precise colouration qualities, which permitted identification of numerous structures and/or bands characteristic of each chromosome pair. Since then many banding techniques have been evolved to delineate the chromosomes in all species of livestock.

The enzymatic digestion of chromosome using pronase as pre-treatment which resulted in the banding in human chromosome was first demonstrated by ✓ Dutrillaux *et al.* (1971). ✓ Summer *et al.* (1971) achieved G-banding through incubation of slides in various salt solutions followed by staining with Giemsa (acetic/saline/Giemsa technique). Subsequently Drets and Shaw (1971) modified this method by treating the chromosome preparations with sodium hydroxide (alkaline/Giemsa technique). A rapid G-banding technique involving trypsin pretreatment of chromosome was proposed by Seabright (1971) which is the most common method being followed at present. The trypsin hydrolyses the protein component of nucleoproteins which was denatured during fixation and thus allowed giemsa to act on the exposed DNA (Wang and Fedroff, 1972). G-banding was also produced using protein denaturing substances like sodium dodecyl sulphate and sodium lauryl sulphate (Shiraishi and Yosida, 1972).

NOT in
Reference

A cytogenetic investigation conducted by Di Meo (2000) in five cattle, two of the Podolian breed, two Grey Alpine, and one Chianina, all of which were heterozygous carriers of three centric fusion translocations (rob-1;29, rob-4;8, rob-25;27). The use of G-, R- and C-banding patterns, combined with the FISH technique by using both type I molecular markers and some human chromosome painting probes, allowed the precise identification of chromosomes involved in the chromosomal abnormalities, compared to previous reports. While the chromosomes involved in the well known rob (1;29) were confirmed, BTA6 (not BTA4) was involved in the second translocation. Furthermore, BTA26 and BTA29 (not BTA25 and BTA27) were involved in the third translocation. C-banding patterns confirmed the mono-centric nature of rob (1;29) and revealed the dicentric nature of both rob(6;8) and rob (26;29). The importance of these marker chromosomes in bovid chromosome nomenclatures is also discussed.

NOT in
Bibliography

C-banding

C-banding was first demonstrated by Pardue and Gall (1970). It is used specifically to stain the centromeric regions and other regions containing constitutive heterochromatin i.e. the secondary constrictions and the distal segment of the Y-chromosome long arm.

A new method was developed by Arrighi and Hsu (1971) to obtain C-banding by treatment of the chromosome with sodium hydroxide. But the BSG-method (Barium-hydroxide-Saline-Giemsa) of Sumner (1972) is practiced in the laboratories worldwide for demonstrating constitutive heterochromatin in the chromosomes, though many new methods have been devised by other workers (Alfi and Menon, 1973; Hansen et al. 1974; Noda and Kasa, 1978).

Gupta et al. (1974) used C-banding to distinguish the Y-chromosome in the Sahiwal and Red Sindhi cattle. Potter and Upton (1979) confirmed this and the earlier studies of the Banteng cattle (Fischer, 1969; Ahmad, 1974). Thus C-banding is considered to be the method of choice for distinguishing the Y chromosome from the smaller autosomes in *Bos indicus*. C-banding demonstrated dark bands (C-positive) on all centromeric regions of autosomes, the representative of constitutive heterochromatin. However, there is no

dark band (light or C-negative) on the X and the Y chromosome. According to the report of

Stranzinger *et al.* (2007) which studied on the polymorphism of chromosome Y in various breeds of cattle (*Bos taurus*) in Switzerland and showed the negative X chromosome from C-banding and the positive short arm of Y chromosome.

Kumarasamy (2008) C-banding in Umblachery cattle was showed all autosomes with darkly stained centromere but the sex chromosomes were found to be C-band negative.

R-banding

R-banding was first used by Dutrillaux *et al.* (1971) in humans. It is helpful for analyzing deletions or translocations that involve the telomeres of chromosomes. R-bands are the reverse of G-bands and can be produced by thermal denaturation (Dutrillaux *et al.* 1971); formaldehyde treatment (Wyand *et al.* 1974) and antibiotics (Schweizer, 1976; Van De Sande *et al.* 1977). Gustavsson (1980) presented a review of R-band patterns obtained on the chromosomes of the domestic animals.

Berardino and Iannuzzi (1984) gave the description of RBA banding pattern of river buffalo chromosomes and its diagrammatic representation was presented as a "model" for the definition of a standard RBA-banded karyotype for the bovine species. Which change the current system of nomenclature for the Bovidae also suggested facilitating comparative studies among the members of the family.

Iannuzzi (1996) studied the G and R band of cattle (*Bos taurus*) karyotypes reporting about 500 band level and discussed in light of chromosome banding standard nomenclature discrepancies. GTG-, GBG-, RBA- and RBG-banded karyotypes were arranged according to the Reading Conference standard. The G- and R-banding patterns of small acrocentric and disputed chromosomes (25, 27, 28, and 29) were demonstrated using some bovid marker chromosomes. G- and R-banded idiogrammatic representations

using only one common banding nomenclature were also performed. His study contributed to the construction of new and clearer standard G- and R-banded karyotypes for this important species.

~~Berardino and Burguete (1998)~~ performed high resolution RBA-band in early prometaphase chromosomes of cattle (*Bos taurus*) and goat (*Capra hircus*), from thymidine synchronized lymphocyte cultures and compared at a level of 700 bands per haploid genome, with the purpose of detecting the extent of banding homologies between the two species and improving the resolution level of the ISCNDA (1989) standardized RBA-banded karyotypes. The results demonstrate that, at this level of resolution, at least 10 autosomes can be fully homologized between the two species, namely chromosomes 11, 16, 17, 18, 20, 21, 22, 23, 24 and 26, whereas the remaining autosomes show variations in some regions which require further investigations on more elongated chromosomes. Such investigations should also involve the use of GTG, GBG and RBG-banding techniques. These variations concern basically the relative distance of the bands from the centromeres or telomeres, appearance of sub bands, and clustering of some positive bands.

MATERIALS AND METHODS:

The lymphocyte culture technique was carried out as per Yadav *et al.* (1984), with slight modification. Subsequently, cultures were harvested in usual manners and slides were prepared by air-dry method. The slide was kept for aging for 3 days.

Banding Techniques

Two banding techniques were adopted for the present investigation.

1. C-banding
2. R-banding

C-banding using Barium Hydroxide [$\text{Ba}(\text{OH})_2$] treatment (Summer 1972)

1. Slides with chromosome preparations made by air drying method were treated with 0.2N HCl for one hour at room temperature followed by a rinse with GDW.
2. The slides were placed in a freshly prepared aqueous solution of $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ at 56°C for 2-8 minutes, followed by thorough rinsing with GDW in three successive times.
3. The slides were then kept in a coplin jar containing 0.2N HCl at 56°C for 10 minutes to remove traces of barium hydroxide crystals.
4. Another thorough rinsing was given in GDW.
5. The slides were incubated in 2X SSC for one hour at 60°C , followed by a rinse in GDW.
6. The slides were then stained with 2% giemsa stain for 30 minutes at room temperature.
7. After a final rinse with GDW, the slides were blotted and allowed to dry thoroughly in the incubator at 37.5°C .
8. The slide was then observed under microscope and good metaphase spread was selected and photographed.
9. Karyotype of C-banding was prepared after arranging it according to the shape and size of the chromosome.

not. Ref.

R-banding technique by Fluorescence-Photolysis-Giemsa method after BrdU incorporation (RB-FPG) following Romagnano and Richer (1984)

1. During the lymphocyte culture incubation, the cultures were incorporated with BrdU at the rate of 0.1 ml (12 μ g) per culture, 8 hours prior to harvesting.
2. Subsequently, cultures were harvested in usual manners and slides were prepared by air dry method.
3. Slides were treated in Hoechst-33258 dye solution (0.5%) for 15 minutes and rinsed with distilled water.
4. The slides were mounted with cover glass in 2X SCC and were placed under a fluorescence blue-black tube light.
5. The slides were exposed for 2 hours.
6. After light exposure, slides were rinsed with GDW and were stained with 2% giemsa staining solution for 15 minutes.
7. The slide was then observed under microscope and good metaphase spread was selected and photographed.
8. Karyotype of R-banding was prepared after arranging it according to the shape and size of the chromosome.

OBSERVATION:

Chromosome banding in Mithun

R-banding

R-banding technique produces bands on chromosome that are reverse of G-bands i.e. those regions that do not stain well by G-banding are intensely stained by R-banding procedure (Dutrillaux and Lejeune, 1971; Bobrow and Madan, 1972) not in ref.

R-banding by incorporation of a Bromodeoxyuridine (BrdU) followed by giemsa staining (RB-FPG) was used in the present study. The R-banding metaphase spread chromosome of Mithun is shown in Figure 11.

In Mithun, the R-banded chromosome show a series of strongly stained and more or less wide bands. It has been observed that the number of bands in chromosome 1 (sub-metacentric) is more as compared to the acrocentric autosome. The number of dark bands in p and q arm of chromosome number 1 pair was 22 bands. The number of bands depends upon the size of the chromosome. On an average the numbers of bands in larger acrocentric autosomes were more. Whereas the smaller acrocentric autosomes comparatively showed less number of bands. The detail number of bands per chromosome is shown in Table 3. In female, the inactivation of X- chromosome is clearly shown as the p arm of one X- chromosome shows no dark band. While the other X- chromosome shows normal banding pattern. The total number of R-band in Mithun was found to be 336.

C-banding

The C-banding technique produces selective staining of constitutive heterochromatin regions of chromosome. These bands are mostly located at the centromeric region of chromosomes, hence they are known as C-bands.

The C-banded metaphase spread of the Mithun is presented in Figure 12. All the acrocentric autosomes, i.e. from chromosome number 2 to chromosome number 28,

showed distinct staining of heterochromatin in the centromeric region. However, in chromosome number 1 and in sex chromosome, which are sub-metacentric chromosomes, staining of the centromeric region was very faint (Figure 12).

The intensity of the banding varied among the autosomes. The larger chromosome in the karyotype i.e. chromosome 2 to chromosome 15, showed a darker stained heterochromatin region as compared to the smaller chromosome in the karyotype i.e. chromosome 16 to chromosome 28.

Chromosome banding in Tho-tho cattle

R-banding

The R banded chromosomes show a series of strongly stained bands along the length of the chromosome. The numbers of R-positive bands are seen more in larger chromosomes and reduce as the size of the chromosome decreases in karyotype (Figure 13). The prominent bands are observed in the telomeric portion of all chromosomes. In female, the inactivation of X-chromosome is clearly shown as the p arm of one X-chromosome shows no dark band and the q arm shows alternating dark bands and light bands. The R-positive dark bands are the approximate bands that can be observed. The present study found that the number of R-bands for Tho-tho cattle were 496 and 499 in male and female respectively. The detail number of R-bands of each chromosome pair is given in Table 4.

C-banding

In case of Tho-tho cattle all the autosomes are acrocentric chromosomes whereas X chromosome is sub-metacentric chromosome. The C-banded metaphase chromosome showed a distinct dark stain at the centromeric region in all the acrocentric autosomes. The centromeric region of the sub-metacentric chromosome, i.e. X-chromosome, showed very faint stain. The density of the centromeric stain appears to be more in the larger chromosome and it reduces as the size of the acrocentric chromosome descends. In Tho-tho the Y chromosome which is also an acrocentric chromosome did not show banding pattern (C-negative).

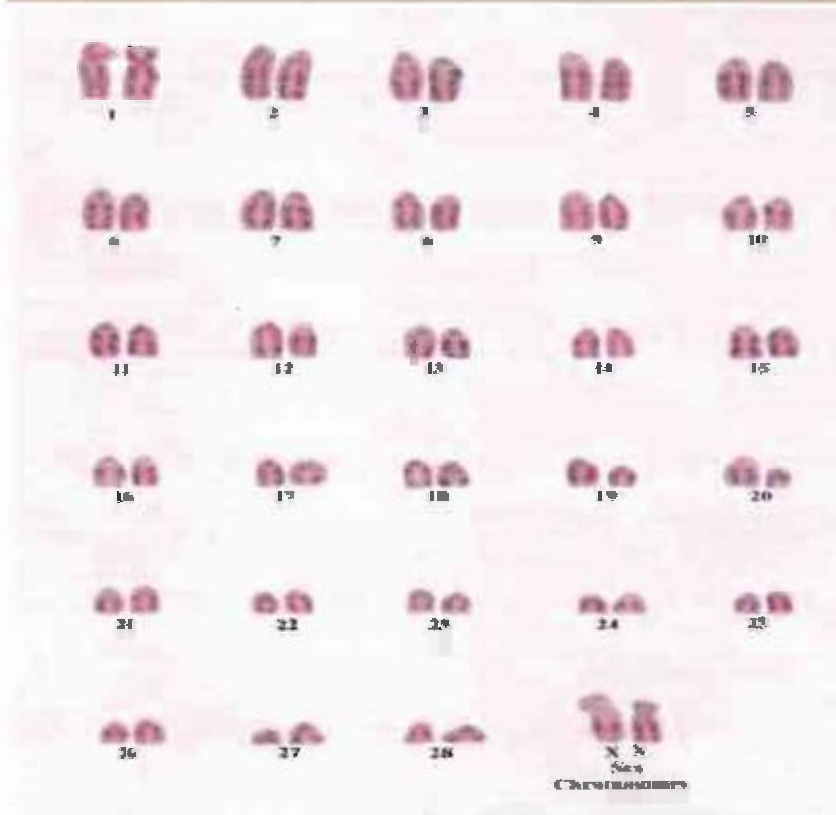
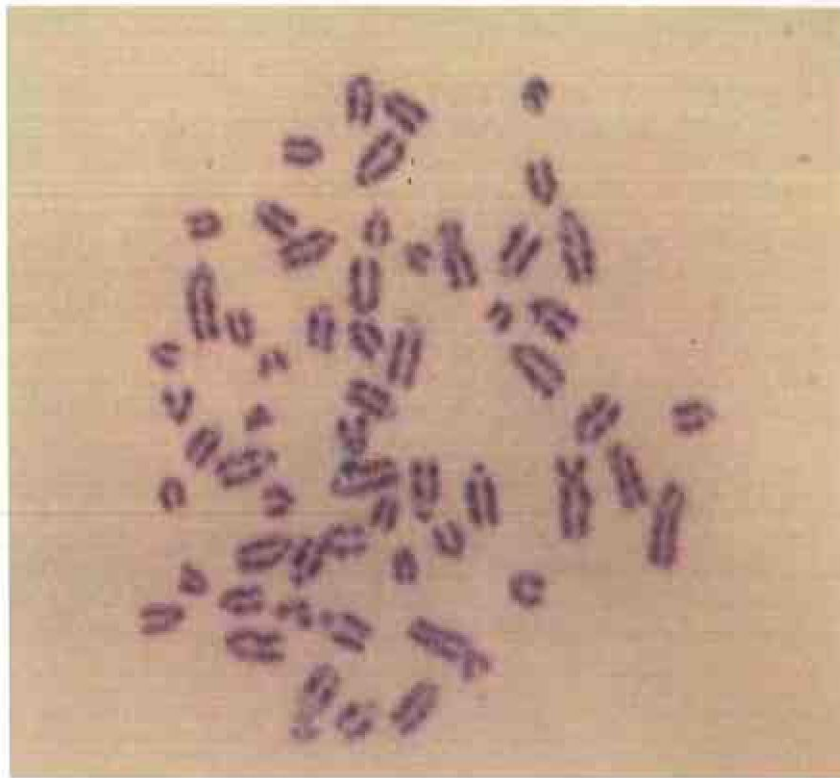


Figure 11. R-banded Metaphase spread and Karyotype of Mithun chromosome.

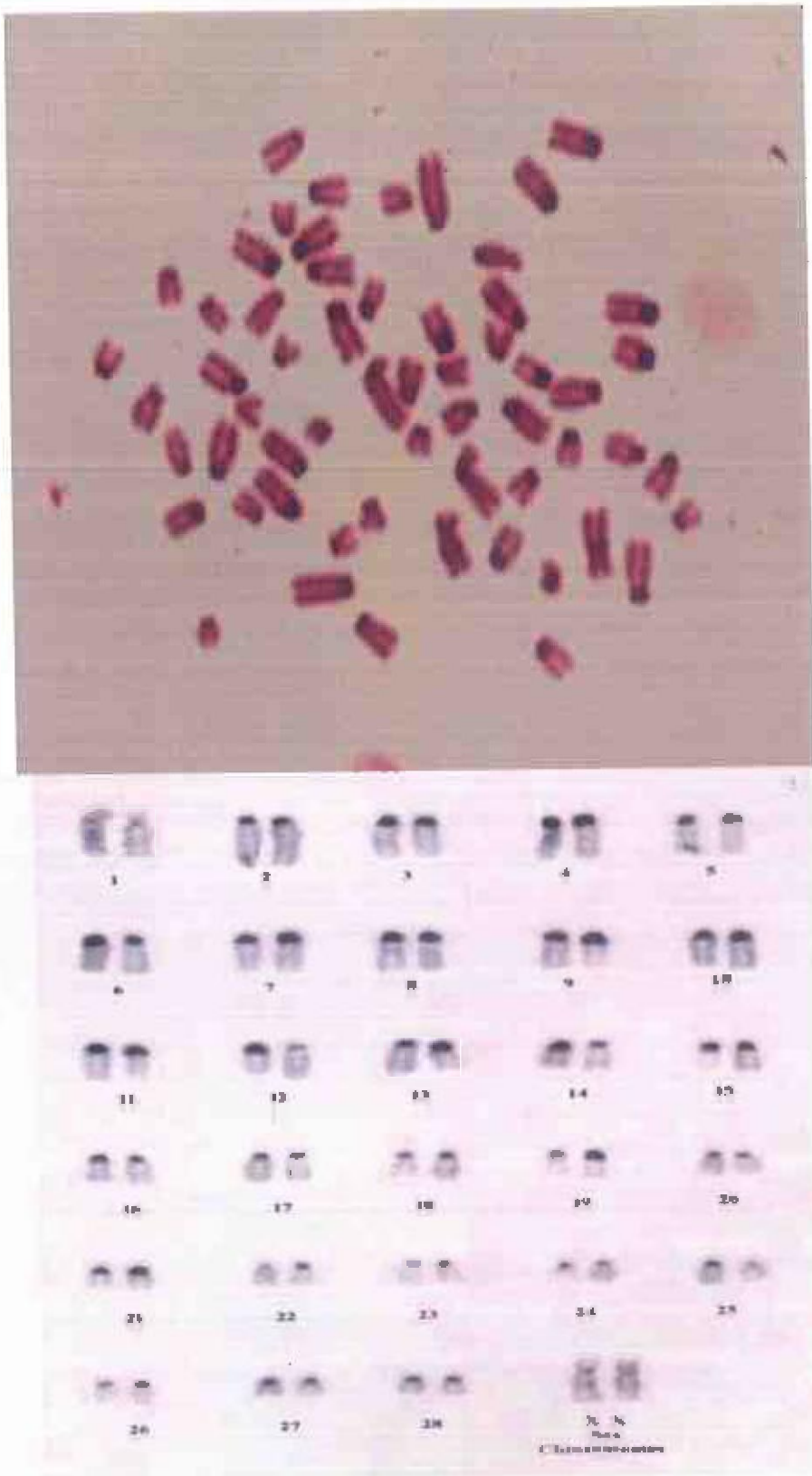
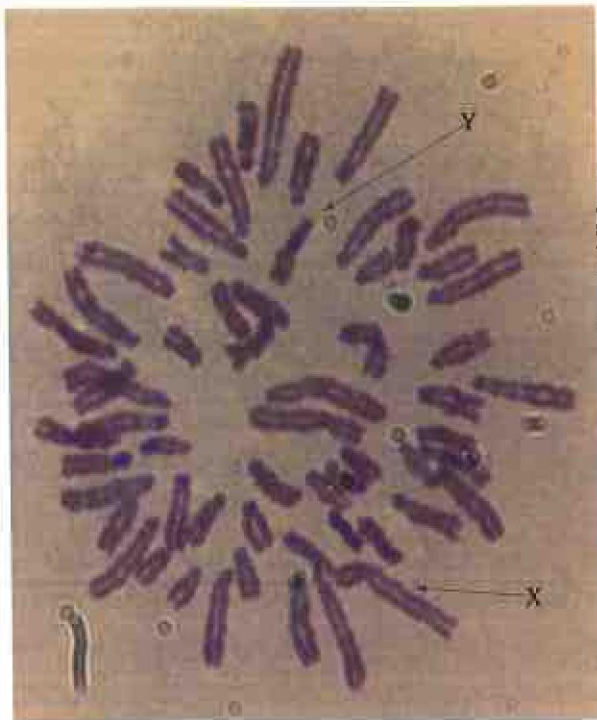
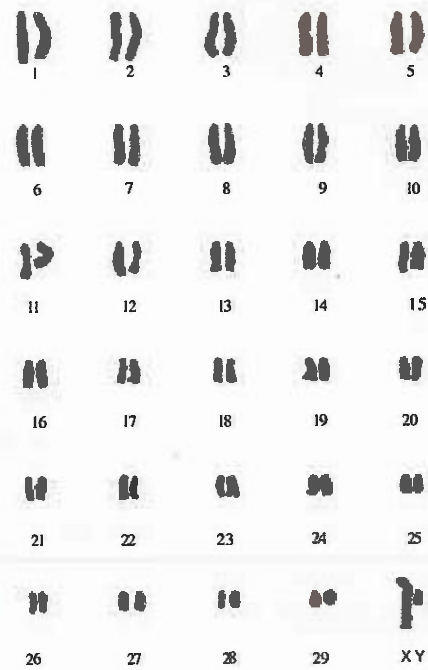


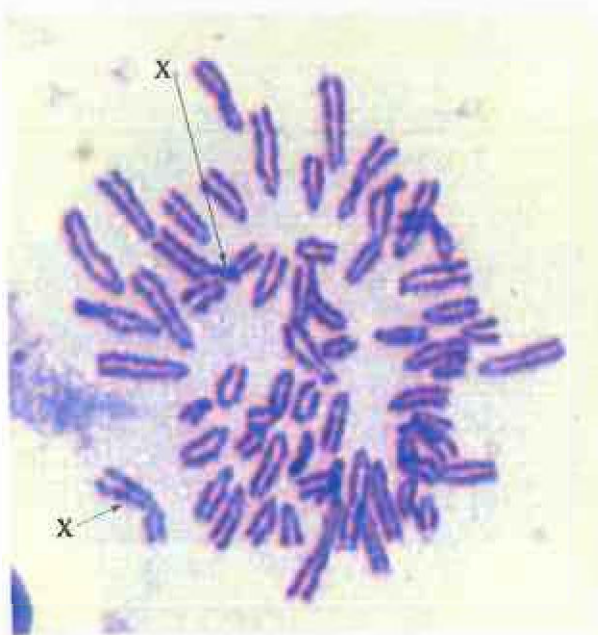
Figure 12. C-banded Metaphase spread and Karyotype of Mithun chromosome



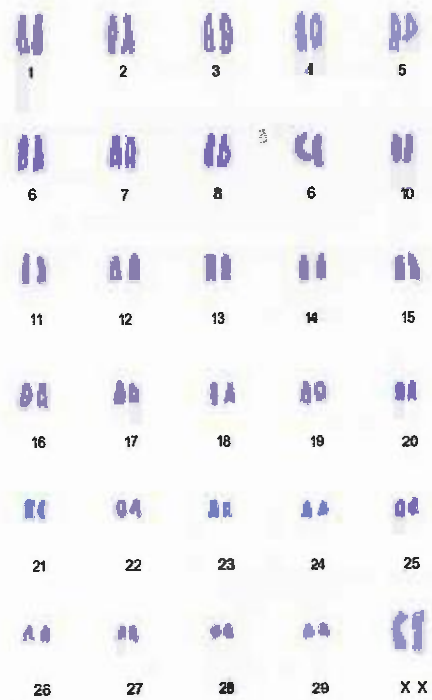
a



b



c



d

Figure 13. R-banded Metaphase spread and Karyotype of Tho-tho Cattle chromosome. a,b (male), c,d (female)

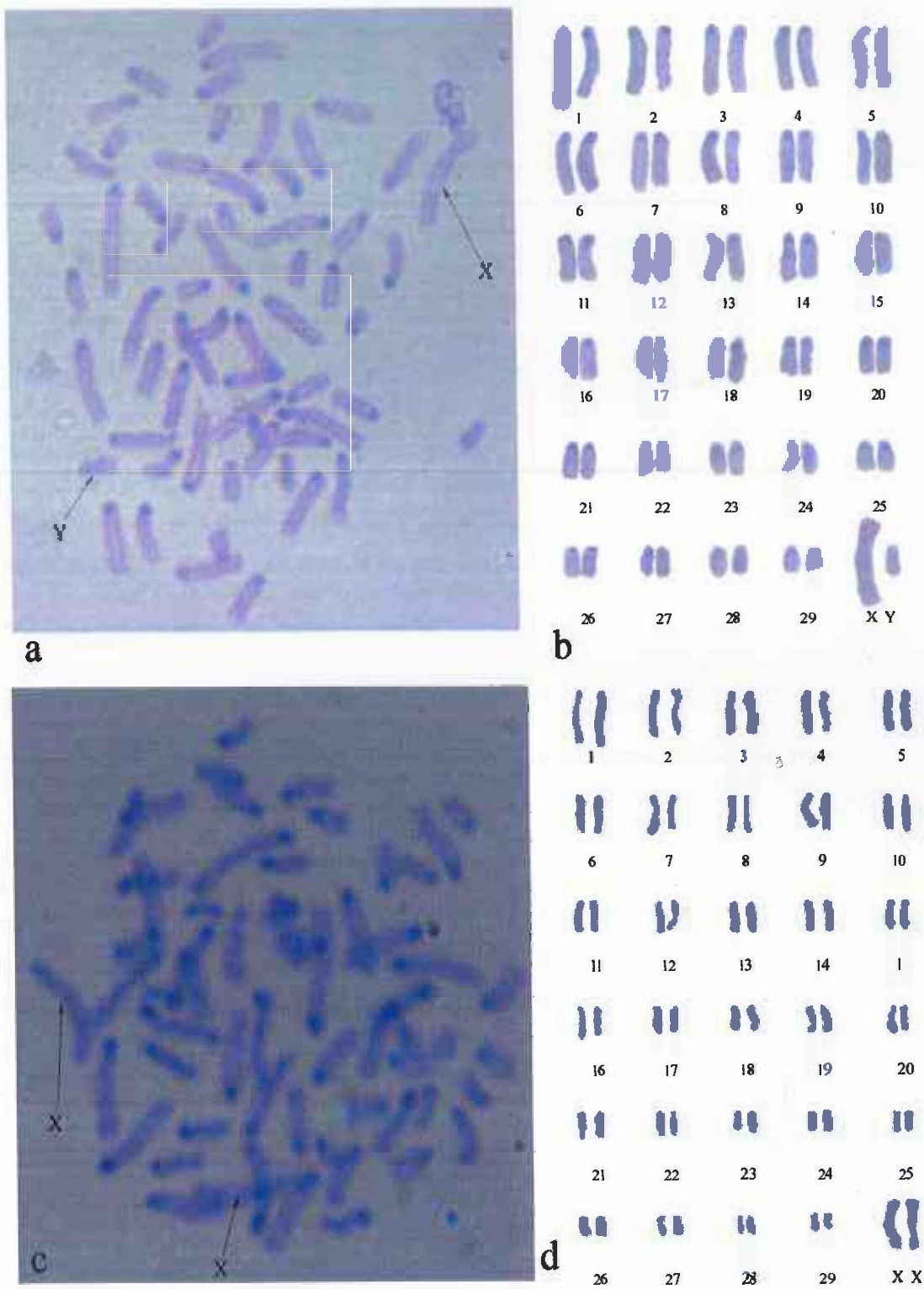


Figure 14. C-banded Metaphase spread and Karyotype of Tho-tho Cattle chromosome

Table 3. Numbers of R-bands and presence of C-band in Mithun chromosome.

Chromosome number (pair)	R-band (number per chromosome pair)	C-band (+/-)
1	22	-
2	16	+
3	15	+
4	15	+
5	15	+
6	14	+
7	14	+
8	14	+
9	14	+
10	13	+
11	14	+
12	13	+
13	13	+
14	10	+
15	8	+
16	10	+
17	9	+
18	10	+
19	11	+
20	9	+
21	9	+
22	9	+
23	10	+
24	8	+
25	8	+
26	8	+
27	6	+
28	7	+
X	12	-
Y		-

Table 4. Numbers of R-bands and presence of C-band in each chromosome of Tho-tho cattle.

Chromosome number (pair)	R-band (number per chromosome pair)		C-band (+/-)	
	Male	Female	Male	Female
1	32	28	+	+
2	29	28	+	+
3	28	27	+	+
4	24	23	+	+
5	24	22	+	+
6	24	20	+	+
7	24	24	+	+
8	21	24	+	+
9	18	20	+	+
10	19	20	+	+
11	16	20	+	+
12	16	16	+	+
13	16	20	+	+
14	16	16	+	+
15	12	15	+	+
16	16	16	+	+
17	12	16	+	+
18	12	13	+	+
19	12	13	+	+
20	12	12	+	+
21	12	12	+	+
22	12	12	+	+
23	12	8	+	+
24	12	10	+	+
25	9	12	+	+
26	4	8	+	+
27	8	8	+	+
28	8	8	+	+
29	8	8	+	+
X	20	20	-	-
Y	2		-	

DISCUSSION:

R-banding in Mithun:

The suitability of R-banding for routine cytogenetic analysis for easy identification of homologous chromosome pair (Verma and Lubs, 1975) was confirmed in our study. In the present study the band patterns observed via R-banding indicated that the smaller sub-metacentric chromosome was sex chromosome, due to the presence of light band in one of the X chromosome confirming inactivation during the embryonic development in female. The largest sub-metacentric chromosome was the autosome and occupies the first position in the karyotype.

The present study showed that the number of R-bands in Mithun varied from 22 bands in chromosome 1 to 6 bands in chromosome 27. The total number of R-bands observed in the present study was 336 bands. The total number of bands found to be less in Mithun as compared to Tho-tho cattle. This may be due to the fact that some of the sections or portions of chromosomes might have lost during the chromosomal rearrangements. This also supports the fact that the Mithun is originated from the bovine primitive karyotype. As centric fusion between two autosomes are believed to result in the formation of this new rare bovine species. The level of bands observed in the present study in Mithun were lower as compared to the other bovine studied earlier by Iannuzzi (1996) where he reported the R-band level of about 500 band level in *Bos taurus*. Higher level of bands has also been demonstrated at a level of 700 bands by Bernardino and Burguete (1998) in cattle. This suggests that the higher level of R-bands can be achieved.

C-banding in Mithun:

The C-banding in the present study indicated the heterochromatin and the centromere position in the chromosome. In all the acrocentric autosome in Mithun, very clear presence of stained centromere was observed towards one end. However, no C-band in the sub-metacentric chromosome was observed towards one third position of the chromosome. The finding of C-band negative in sub-metacentric chromosome was observed in other bovine (*Bos taurus*) by Stranzinger *et al.* (2007).

In the present study all the acrocentric chromosome showed prominent C-band. The intensity of the bands was higher in the larger acrocentric chromosomes as compared to the smaller acrocentric chromosomes. Therefore, the intensity of the C-band reduces as we move from chromosome number 2 to chromosome number 28 in karyotype.

The band pattern were studied and standardized in Mithun that can be utilized for further genetic and biotechnological intervention. The various parameters generated by cytogenetic analysis along with the band pattern defines the complete chromosomal characterization of Mithun and can be used as a basis for further exploitation in research of any kind in this species.

R-banding in Tho-tho cattle:

In the present study the R-banding pattern of the Tho-tho cattle showed about 500 band level with 490 in male and 499 in female. The present finding supports the earlier R-banding in cattle of 500 band level (Iannuzzi, 1996). The chromosome banding pattern in Tho-tho cattle confirms that there was not much variation from the other breed of the same species.

The R-bands were observed in all the chromosome of the Tho-tho cattle in the present study. The number of bands varied among the pairs of the chromosomes. The number of bands reduces as we move from chromosome number 1 to chromosome number 29 in the karyotype. The maximum number of bands observed were in chromosome number 1 pair with an average of 30 bands and the lowest number of bands observed were in chromosome number 26 pair with an average of 6 bands.

The inactivation of X chromosome was observed in one of the sex chromosome in female Tho-tho cattle. The p arm of the inactivated X chromosome showed very light stain indicating the inactivated portion.

C-banding in Tho-tho cattle:

The centromeric banding technique showed that the entire acrocentric chromosome exhibited the dark bands (C-positive) on the centromeric region. The densities of the bands were higher in the larger acrocentric chromosome and less in the smaller acrocentric chromosome. The present finding of C-banding pattern in Tho-tho was in agreement with Umblachery breed of cattle (Kumarasamy *et al.* 2008). Other researcher also showed in cattle that the chromosomes 7, 12, 14 and 15 had greater heterochromatin content than the other autosomes (Rasool *et al.* 1995).

In sex chromosome there were no dark bands on the X chromosome like all the other breeds of cattle. The negative C-banding pattern of Y-chromosome in Tho-tho cattle is in agreement with the species specific banding pattern of the *Bos Indicus* (Potter *et al.* 1979).

Cytogenetic screening of the Mithun and Tho-tho cattle population should be expanded to a larger number of animals in order to identify animals with chromosomal abnormalities and to be eliminated from the herd, so that a plan breeding strategies can be implemented for the conservation of this important bovine species. However, the accumulation of characters and genetic variation in nature is a long process and it is difficult to detect. Therefore, further analysis on these bovine species is suggested by using various molecular cytogenetic techniques, such as Fluorescent *in-situ* hybridization and molecular technique, which may reveal the variations.

CHAPTER III

UNDERSTANDING THE GENETIC DIVERGANCE OF MITHUN FROM THO-THO CATTLE BY RAPD

INTRODUCTION:

The term Genetic distance is a measure of the dissimilarity of genetic material between different species or individuals of the same species. In comparing the percentage difference between the same genes, a figure can be obtained which technically is the measure of genetic distance. Thus, depending upon the difference obtained and correcting this for known rates of evolution the genetic distance can be used as a tool to construct cladograms to depict the family tree of all living beings. (Hayes *et al.* 2009)

Population geneticists proposed several hypotheses regarding genetic diversity among the organisms (Kimura, 1989). The neutral theory of evolution proposes that diversity is the result of the accumulation of neutral substitutions. Diversifying selection is the hypothesis that two sub-populations of a species live in different environments that select for different alleles at a particular locus. Frequency dependent selection is the hypothesis that as alleles become more common, they become less fit.

The advantages of the Randomly Amplified Polymorphic DNA (RAPD) technology include (i) Suitability for work on anonymous genomes. (ii) Applicability to problems where only limited quantities of DNA are available. (iii) Efficiency and low expense. Arbitrary primers are used for this technique can be easily purchased and there is no need for initial genetic or genomic information and the unit cost per assay is low. RAPDs have the advantage that they can be obtained at a reasonable cost and will generally amplify a range of fragments of most DNA and show polymorphisms. Some primers will produce unrelated patterns between unrelated animals and identical ones for very closely related animals. Apparently, primer sites are randomly scattered along the target genome and flank both conserved and highly variable regions. Wide variation in band intensity can be shown to be reproducible between experiments, which could be the result of multiple copies of the amplified regions in the template or the efficiency with which particular regions are amplified (Kumari and Thakur, 2014).

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Genetic diversity is the basis for present day diversified living systems and future genetic improvement needs. This diversity should be properly utilized, improved and conserved.

Conservation and improvement strategies ought to be based on proper genetic characterization in association with phenotypic characterization. The objective of the study is to ~~assist~~ ^{assess} the genetic variation between Mithun and Tho-tho cattle using molecular marker.

REVIEW OF LITERATURE:

A genetic marker can be defined as any stable and inherited variation which is measurable or detectable by using a suitable method. Subsequently, it can be used to detect the presence of a genotype or phenotype other than itself, which is otherwise not measurable or difficult to detect. (Beckmann and Soller, 1987). The variations that crop up at various levels viz., morphological or anatomical, physiological, chromosomal, biochemical and DNA sequence level can serve as genetic markers. Those markers detecting polymorphism directly at DNA sequence level are termed as molecular genetic markers (DNA markers).

The development of polymerase chain reaction technique opened a new front in the field of molecular biology. The use of PCR by Saiki *et al.* (1988) to amplify a DNA sequence of interest and subsequent restriction enzyme analysis of the amplified product (PCR-RFLP) by Pinder *et al.* (1991) staged a lightning raid deep inside this field of molecular biology. Vast number of genetic polymorphisms at the DNA sequence level has bestowed upon a number of genomic markers such as Randomly Amplified Polymorphic DNA (RAPD) markers (Williams *et al.* 1990; Welsh and McClelland, 1990), Restriction Fragment Length Polymorphism (Botstein *et al.* 1980), Variable Number of Tandem Repeats (VNTR) (Jeffreys *et al.* 1985; Nakamura *et al.* 1987), microsatellites (Litt and Luty 1989; Weber and May, 1989; Beckmann and Soller, 1990; Tautz, 1990; Fries *et al.* 1990), Amplified Fragment Length Polymorphism (AFLPs) (Vos *et al.* 1995) and Single Nucleotide Polymorphism (SNPs) (Marth *et al.* 1999).

Randomly Amplified Polymorphic DNA

The development of polymerase chain reaction for DNA amplification represents one of the most important molecular techniques in the last decade. PCR technique has several advantages over traditional, *in vivo* method of cloning DNA. It enables genetic study using only a small amount of DNA. PCR technique was first established by Williams *et al.* (1990) using short dccamer primers, and called the product Randomly Amplified Polymorphic DNA, and by Welsh and McClelland (1990, 1991), who used primers of 20 to 30 nucleotides in length and named the procedure as arbitrarily primed PCR. This technique generates DNA fingerprints based on random amplification of DNA by PCR

and these fingerprint products were called as Random Amplified Polymorphic DNA markers. RAPD technique involves usage of short decamer oligonucleotide primers annealing at low temperature. The number and size of the RAPD products depend on the complementarity of sequences of the particular primer and template DNA. Different primers produce different RAPD polymorphisms. These variations may be due to differences in spacing between primer binding sites as well as point mutations, insertions, deletions and inversions which allow or abolish primer binding sites (Gwakisa *et al.* 1994). The RAPD polymorphisms presumably are based on mismatches in primer binding sites or insertion/deletion events, and therefore usually result in the presence or absence of amplified products from a single locus (Williams *et al.* 1990; Welsh and McClelland, 1991). RAPD polymorphisms may also arise, if secondary structures form around the primer annealing sites during the relatively low annealing temperature (Bowditch *et al.* 1993). RAPD-PCR is a simple technique and easy to analyze. It doesn't require radioactively labelled nucleotides. RAPD markers follow Mendelian laws of segregation. It requires very small amount (in nano grams) of genomic DNA, and is subjected to PCR using random sequence oligonucleotide primers. In RAPD-PCR, only a single random oligonucleotide primer is employed, whereas in standard two primers mediated PCR amplification, two primers are required. RAPD doesn't require any prior knowledge of nucleotide sequence since primers are chosen arbitrarily and many organisms can be mapped with the same set of primers. It is based on the principle that, when the primer is short (e.g. 8 to 10 bp), there is a high probability that the genome contains several priming sites close to one another that are in an inverted orientation. The technique essentially scans a genome ^{for} from these small inverted repeats and amplifies inverting DNA segments of variable length. Not listed

The amplification products are then resolved on agarose gel electrophoresis resulting in DNA fingerprints (bands). Since, primers of arbitrary nucleotide sequences are used in this technique to access random segments of the genomic DNA to reveal polymorphism, these markers are named as Randomly Amplified Polymorphic DNA (RAPD) (Williams *et al.* 1990). Williams *et al.* (1990) explained that nearly all RAPD markers were dominant, as DNA segments of same length were amplified from one individual but not from another. The drawback of this technique was that it was not yet fully understood how the genetic variation observed was generated and consequently, it can be difficult to

reconstruct evolutionary histories. The consistency of result was not guaranteed as minor differences in experimental conditions and different brands of *Taq* polymerases may give different amplification patterns and RAPD were dominant markers where heterozygotes were typically scored as homozygotes which decrease their information content (Meghen *et al.* 1994). Hence, RAPD may only be employed for analysis of genetic distances if reproducibility and Mendelian inheritances of the amplified DNA of interest are beyond doubt (Rothuizen and Wolferen, 1994). In spite of such considerations, it has been shown that the procedure of RAPD gives highly reproducible results under a standard protocol under optimum conditions (Welsh and McClelland, 1990; Welsh *et al.* 1991; Williams *et al.* 1990; Rothuizen and Wolferen, 1994).

RAPD in Cattle

Inheritance: Chung *et al.* (1998) studied parentage testing and conducted pedigree analysis in Holstein dairy cattle by using RAPD- PCR. The investigation revealed that the RAPD products in offspring were always present in one or both parents, indicating that the RAPD markers were inherited in Mendelian fashion (Bhattacharya *et al.* (2003)).
estimated the inbreeding in cattle (Red Sindhi, Haryana and Tharparkar) using RAPD markers. The inbreeding coefficient was highest in Haryana, although the band sharing frequency was highest in Red Sindhi. This may be due to the Haryana population being small. Animals are mostly bred within high and low-yielding groups within the population

Breed characterization: RAPD fingerprinting has been widely used for breed characterization in cattle. Gwakisa *et al.* (1994) used this technique to characterize three zebu breeds of cattle. Out of 141 random primers used, two primers could discriminate between the breed specific DNA pools. Kemp and Teale (1994) reported polymorphism in a ruminant repetitive DNA sequence which differentiated *Bos indicus* and *Bos taurus* using ILO 526 and ILO 876. Genetic variations in bovine population and their association with production traits were demonstrated by Bardin *et al.* (1995) using RAPD technique. They obtained reproducible electrophoretic patterns in Italian Friesian and Grigia Alpina bulls. Thiyagarajan (2000) used nine arbitrary primers to characterize four Indian zebu cattle breeds by RAPD technique. Out of nine primers used, only five primers gave

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reproducible DNA fingerprints. Thiagarajan and Thangaraju (2011) represented the application of RAPD-PCR technology for authentication and differentiation of Kangayam cattle by deducing breed specific fragments. Appannavar et al. (2002) used the RAPD technique for genetic characterization of Deoni breed of cattle and they reported that some primers can be used as specific primers to differentiate the three types of Deoni cattle.

Genetic Distance: Jeon et al. (1998) used the RAPD method to detect genetic variation and characteristics among the Korean Native, Holstein, Charolais, Aberdeen-Angus and Hereford cattle. The degree of band sharing between Korean native and Holstein cattle, Charolais, Angus and Hereford cattle. Ramesha et al. (2002) conducted Random Amplified Polymorphic DNA (RAPD) assay to identify polymorphic markers in Amrithmahal, Krishna Valley, Hallikar, Deoni, Khillari, Ongole and Malnad Gidda breeds of South Indian cattle. Yeo et al. (2000) In order to develop the specific genetic marker for Korean native cattle (Hanwoo), randomly amplified polymorphic DNA (RAPD) analysis was attempted and found two distinctive DNA bands specific for Hanwoo cattle. Sharma et al. (2004) carried out Random amplification of polymorphic DNA analysis using DNA samples of 30 animals of Rathi cattle and 42 animals of Tharparkar cattle. (Sid-Ahmed et al. (2007)) Random amplified polymorphic DNA was used to investigate the genetic polymorphism among and within four breeds of Sudanese Autochthonous cattle. Their study supported to the view that there are major genetic differences between these four local cattle populations. (Guneren et al. (2010)) identified the genetic relationship and variations within and between local cattle breeds of Turkey using RAPD-PCR method which needs fewer substructures when compared to other DNA fingerprinting methods.

Pasnn and Ranghoo-Sanmukhiya (2013) used RAPD analysis to differentiate the three main cattle breeds in Mauritius; the Friesian cows, the Creole cows and the Cross (Creole and Friesians) breeds. The main objective of their study was to differentiate the Creole breeds from the other two breeds thus valorizing and conserving the Creole cow which is in danger of extinction. Kim and Yoon (2014) evaluated the genetic distances and specific DNA makers by the randomly amplified polymorphic DNA method for the black-dotted nose and the normal yellow nose in Hanwoo, the Korea native cattle. The

objective of the present study was to analyze the genetic divergence between Tho-tho cattle and Mithun and within the four strains of Mithun.

MAERIALS AND METHODS:

Genetic distance analysis using molecular markers

Markers are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence which are able to differentiate between genetically distinct individuals.

Isolation of DNA

The blood sample was collected from the Mithun and the Tho-tho cattle kept at the National Research Centre at the Mithun farm and the field. Total aseptic condition was maintained during the process of sample collection. Around 6 - 8 ml of venous blood was collected from external jugular vein using vacutainer needles and vacutainer tubes containing EDTA as an anticoagulant. The samples were brought to the laboratory maintaining ice-cool temperature and stored at 4°C till further use. DNA isolation was done within 24 hours of collection to get good yield of genomic DNA. The genomic DNA was isolated using Genomic DNA Purification kit.

1. Three ml of blood sample was added to 9 ml of cell lysis solution and incubated the mixture for 10 minutes at room temperature to lyses^e the red blood cell.
2. It was then centrifuged at 2000 x g for 10 minutes and then the supernatant was discarded. This step was repeated till clear whitish pellet was observed.
3. The white blood cells were resuspended by vortexing ⁱⁿ and 3 ml of nuclei lysis solution ~~was added~~. Pipette several times to lyse the WBC's.
4. It was incubated at 37°C for 1 hour. After incubation 1 ml of protein precipitate solution was added to the nuclear lysate. Small protein clumps were visible. This protein clump was removed by centrifugation.
5. The supernatant was collected and added to equal volume of isopropanol at room temperature.
6. Gentle mixing of solution was done and white thread like strand of DNA was visible. It was centrifuged and the DNA was visible as white pellet.
7. Supernatant was removed and equal volume 70% ethanol was added to wash the DNA.
8. After removing the alcohol the tube containing DNA was kept for drying.
9. About 100 µl of DNA rehydration solution was added to tube and the DNA was rehydrated by incubating at 4° C for overnight.

DNA quality and quantity test

The concentration of DNA in samples was estimated using BiophotometerPlus (Eppendorf, Germany). Optical densities (OD) were measured at 260 nm and 280 nm of 1 µl genomic DNA. The concentration and the OD ratio were estimated automatically.

Estimation of Concentration of DNA:

Concentration of DNA in stock solution (pg/ml) = OD 260 nm x dilution factor x 50 (An OD value of one at 260 nm is equivalent to 50 µg/ml of double stranded DNA).

Purity of DNA stock solution = DNA sample was considered pure if the ratio between OD 260 nm and OD 280 nm was in the range of 1.70 to 1.90.

Testing of DNA Samples

In order to determine their quality and molecular weight, aliquots of samples containing approximately 2 to 3 µg of DNA were checked on 0.7 percent agarose gel in a horizontal gel electrophoresis unit (Figure 15).

Randomly Amplified Polymorphic DNA (RAPD) Primer selection

In the present study, the RAPD primers were taken as per the recommendation by Food and Agriculture Organization (FAO) for bovine species. A total of 40 different 10-base oligonucleotide primers with a GC content varying from 60% to 70% were obtained from Sigma Check Sigma Sheet. The stock primer was prepared by resuspending the dried oligos in TE buffer. Then the working primer was prepared by diluting the stock primer by nuclease free water. In the first few trails the concentration of primers at 10 pmol were used. However, it was standardized to 40 pmol as the later one gave improved result. Out of total 40 primers, 12 primers gave positive result with good multi bands in both the species. The primers such as OPS-17, OPR-8, OPS-3, OPR-2, OPR-1, OPM-12, OPP-14, OPP-10, OPS-11, OPM-10, OPO-18 and OPO-20 gave variation with multiple bands between Mithun and Tho-tho cattle.

Preparation of pooled DNA

Pooled DNA samples were prepared by adding equal quantities of DNA from ten Mithun for each breed and twenty Tho-tho cattle and mixed thoroughly.

RAPD-PCR Reaction

The PCR reaction was carried out in a reaction volume of 25 μ l with PCR master mix containing $MgCl_2$, dNTPs and *Taq* polymerase, primers and nuclease free water to make up the volume. In the initial trials the above mentioned concentration of promega master mix gave pale or light band amplification. Therefore, modification was done and with the addition of 1.5mM $MgCl_2$ the result was improved and good bands were observed in agarose gel. Thus, making the final concentration of $MgCl_2$ to 3mM per reaction. The PCR was carried out with a gradient PCR in Applied Biosystem (Veriti 96 well Thermal Cycler). Amplification consists of an initial denaturation at 95°C for 5 minutes followed by 35 cycle of 95°C for 30 sec, annealing temperature T_A (40°C to 50°C) for 60 sec, and 72°C for 30 sec, a final extension at 72°C for 5 mins and the sample was cooled down to 10°C.

Polyacrylamide Gel Electrophoresis (PAGE)

The PCR-RAPD product was subjected to polyacrylamide gel electrophoresis following the method of Sambrook and Russel (2001). Polyacrylamide gel was prepared at a concentration of 7% and the PCR product was run on it. The composition of polyacrylamide gel used in present investigation is given below:

Sl.No	Reagents	Quantity in 30ml
1	30% acrylamide/ bis-acrylamide mix solution (29:1) (SRL, 0124437)	7 ml
2	1XTBE	22.8 ml
3	10% Ammonium Persulphate (Promega, V313 l)	180 μ l
4	TEMED (Tetramethylethylenediamine) (Amresco, 0761)	20 μ l

Glass plates were prepared by putting the 1mm spacer in its position after proper scaling, the gel was poured into the spacer and the comb was inserted immediately. The gel was allowed to polymerize at room temperature for 2 hours. After polymerization, the gel was put in electrophoresis tank with notched plate facing towards the buffer reservoir. The reservoir of the electrophoresis tank was filled with 1X TBE, the comb was removed carefully and the wells were washed with 1X TBE. Samples were mixed with 6X loading dye and loaded into the wells. Electrophoresis was carried out at 60 Volts for 5 hours.

Silver staining was done according to Byun *et al.* (2009), in this method the gel was fixed and stained in a solution containing 10% ethanol, 0.5% acetic acid and 0.2% silver nitrate for 10 minutes. The gel was rinsed in distilled water once and was developed with a pre-warmed (55°C) solution of 3% NaOH and 0.1% HCOH, until dark staining bands appeared. It was stopped by a solution consisting of 10% ethanol and 0.5% acetic acid.

Randomly Amplified Polymorphic DNA (RAPD) Analysis

Amplified products were classified as present (denoted by 1) or absent (denoted by 0) as suggested by Yu *et al.* (2002). Polymorphic or informative fragments (bands) were also labelled according to the protocol of Yu *et al.* (2002). Analysis of the RAPD patterns involved of firstly combining the total number of bands for each animal and for all the primers. The number of bands common between any two breeds formed the basis of calculation of relatedness by the Dice algorithm (Nei and Li, 1979). The DNA profiles were analyzed using POPGENE software Version 1.31 (Yeh *et al.* 1999). Genetic distances were calculated according to the method by Nei and Li (1979).

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Please use
one pattern for
naming primers,
such as OPM2 or
OPM-12 and
so on as per farm.

OBSERVATION:

Genomic DNA was isolated from whole blood collected from 40 Mithuns and 20 Tho-tho cattle kept at the station farm of National Research Centre on Mithun and the field samples were also collected from Visvema, Kidema, Kigwema and Merima under Kohima district. The purity and concentration of DNA samples were estimated by Biophotometer Plus (Eppendorf, Germany). The optical density (OD) values at 260 nm and 280 nm were determined and subsequently used for estimating the purity and concentration of DNA. The OD ratio was in the range of 1.70 to 1.90 indicating purity of DNA.

Screening of RAPD primers

According to the breed the pool genomic DNA from 20 Tho-tho cattle and 10 Mithuns of each strains were prepared and amplified. Out of the 40 random primers used for amplification of genomic DNA, 12 primers were amplified for the Tho-tho cattle and the Mithun genomic DNA. All the primers used were found to produce consistent polymorphic banding patterns. The finger print patterns for the different primers are shown in Figs. The number of RAPD bands with the different primers in Tho-tho and Mithun were found in the range of 3 to 17 and 8 to 26, respectively. Size of the PCR products varied from 200bp to 10kb in Tho-tho cattle and 200bp to 10kb in Mithun.

The primer OPS3 produced the highest (26) number of RAPD bands while the primers OPP10 produced the least (3) number of bands. Total numbers of RAPD markers obtained from the 12 primers were 131 in Tho-tho cattle and 183 in Mithun.

Primer OPM12

The primer OPM-12 showed a high number of amplification and the variation in the number of amplified bands between Tho-tho cattle and Mithun was high. The number of bands was 9 for Tho-tho cattle while in the case of Mithun, the numbers of bands were 20, 16, 19 and 20 for Nagaland, Arunachal, Mizoram and Manipur strains, respectively. In the Tho-tho cattle there were two prominent bands i.e. OPM12-5 size between 750-1000bp and OPM12-13 size between 5kb-6kb. In the case of Mithun strains the

prominent bands were observed in the mid area of the gel, with sizes ranging from 500bp to 1500bp. The bands in the upper and lower area showed faint bands. However, all the visible bands were taken into consideration for the analysis. All the 9 bands which were found in the Tho-tho cattle were also observed in Mithun. However, there were 11 bands which were seen only in Mithun. The band number OPM-12.18 with a size of 500bp was found in the Arunachal strain and this band was the unique band within the Mithun strains.

Primer OPS3

The RAPD primer OPS-3 was observed to be moderate in variation of band between Tho-tho cattle and Mithun with high number of bands amplified. The size of the band ranged from 500bp to 3000bp in Tho-tho and 300bp to 3000bp in Mithun. The numbers of the bands were 17 in Tho-tho cattle and 26, 21, 24 and 21 in Nagaland, Arunachal, Mizoram and Manipur strains of Mithun respectively. The prominent bands were in the range of 500bp to 1000bp in all the strains of Mithun and Tho-tho cattle. There were 11 bands which ^{were} observed in Mithun but not in Tho-tho cattle. In case of Tho-tho there were two unique band OPS3.13 and OPS3.18 which were not seen in Mithun. The unique bands were also observed within the Mithun strains. The polymorphic bands were OPS3.23, OPS3.24 and OPS3.25.

Primer OPO18

The primer OPO18 showed a band amplification of 15 fragments in Tho-tho cattle and 18 fragments in all the strains of Mithun. The band size ranged from 300bp to 1300bp in Tho-tho cattle and 250bp to 1300bp in Mithun. However, the prominent bands were in the size ranging from 250bp to 500bp. Six bands were detected which were common in Mithun strains but absent in Tho-tho cattle. However, no polymorphic bands were seen within the strains of Mithun. There were 3 unique bands, OPO18.2, OPO18.4 and OPO18.17, which were observed in Tho-tho cattle but ~~was~~ not seen in Mithun.

Primer OPO20

The primer OPO20 showed a high variation in the band amplification with 10 bands in Tho-tho cattle, 17 bands in Manipur Mithun and 16 bands in the rest of the Mithun strains. The amplified fragments ranged from 350bp to 2500bp in both bovine species. There were 8 bands which ~~was~~^{were} found in Mithun but not in Tho-tho cattle. A unique band was observed in Tho-tho cattle, OPO20.6. The primer OPO20 showed a polymorphic band within the strains of Mithun, OPO20.12 (750bp), which was seen in Manipur strain.

Primer OPP10

The primer OPP10 was observed to be highly polymorphic within the strains of Mithun and showed a high rate of variation in the number of band amplification ~~with~~ⁱⁿ Tho-tho cattle. The band fragment size range ~~from~~^{to} 800bp to 1500bp in Tho-tho cattle, 250bp to 5000bp in Arunachal strain of Mithun and 350bp to 6kb in the rest of the Mithun strains. The bands amplified in the Tho-tho cattle were OPP10.6 (2000bp), OPP10.7 (1500bp), OPP10.10 (750bp). A total of 17 bands were observed to be specific for Mithun which ~~was~~^{were} not seen in the Tho-tho cattle. The rate of polymorphism was high within the strains of Mithun. Each strain showed variation in the banding pattern. Among the four strains of Mithun the most polymorphic was Arunachal strain and was observed to have 7 unique bands. They are OPO10.2, OPO10.3, OPO10.5 and their size ~~range~~^{were} ranging from 3kb to 6kb, OPO10.11, OPO10.12, OPO10.16 and OPO10.20, sizes ranging from 250bp to 750bp.

Primer OPM10

Primer OPM10 showed a moderate variation of band fragments between Tho-tho cattle and Mithun. The band size ranged from 350bp to 5000bp in Tho-tho cattle, 300bp to 6kb in Arunachal strain of Mithun and 250bp to 6kb in the rest of the Mithun strains. The number of bands amplified were 9 in Tho-tho cattle, 11 in Nagaland, Arunachal, and Manipur strains of Mithun and 12 in Mizoram Mithun. The bands which were observed in Tho-tho cattle were also seen in Mithun. However, there were 7 bands which were observed in Mithun but not in Tho-tho cattle. Among the strains of Mithun, Arunachal strain showed 3 unique bands which ~~was a~~^{were} polymorphic band within the Mithun strains i.e. OPM10.3 (3kb), OPM10.12 (600bp) and OPM10.13 (500bp).

Primer OPP-14

This primer showed a low number of band fragment amplification with 5 in Tho-tho cattle, 8 each in Nagaland, Arunachal and Manipur Mithun and 10 in Mizoram Mithun. The band size ranged from 800bp to 8000bp in Tho-tho cattle and all strains of Mithun. There was only one prominent band observed in Tho-tho cattle, OPP14.10 (750bp). The bands observed in Tho-tho cattle was also seen in Mithun. Mizoram Mithun showed polymorphic band within the strains of Mithun, with OPP.14.8 (2000bp) and OPP14.9 (1000bp). The Mithun of Nagaland, Arunachal and Manipur strains exhibited a similar pattern of band.

Primer OPS-17

The primer OPS17 showed an amplification of 15^{bands} in Tho-tho cattle, 17 each in Arunachal, Mizoram and Manipur Mithun and 19 in Nagaland Mithun. The size of the fragment ranged from 350bp to 3000bp in both the species. The bands such as OPS17.3 (3kb), OPS17.4 (2kb), OPS17.14(700bp) and OPS17.15 (550bp) were observed in Mithun which were unique to their species and not seen in Tho-tho cattle. Within the strains of Mithun, Nagaland strain showed two unique bands, OPS17.13 (about 750bp) and OPS17.16 (500bp), which was specific for ^{this} ~~the~~ strain. However, the rest of the Mithun strains showed almost similar banding pattern.

Primer OPR-1

The primer OPR1 showed moderate amplification fragments with 12 in Tho-tho cattle, 13 each in Mizoram and Manipur Mithun, 16 in Nagaland Mithun and 17 in Arunachal Mithun. The size of the band ranged from 600bp to 8kb in Tho-tho and 200bp to 8kb in all Mithun strains. A total of 6 bands were observed to be unique for Mithun which was absent in Tho-tho cattle. However, Tho-tho cattle showed 1 unique Band OPR1.8 (1300bp) which was not observed in Mithun strains. In case of Mithun strains Nagaland and Arunachal was found to be similar in banding pattern and that of Mizoram and Manipur was similar. The band number OPR1.10 (800bp) was found to be very prominent in Tho-tho cattle whereas Mithun strains showed very light band.

Primer OPR-2

The primer OPR2 showed ~~an~~ amplification of 9 fragments in Tho-tho cattle and 8 fragments in Mithun. The band size ranged from about 370bp to 900bp in Tho-tho cattle and about 350bp to 900bp in all the Mithun strains. The Tho-tho cattle showed two unique bands OPR2.4 (600bp) and OPR2.10 (370bp) which ~~was~~ ^{WENC} not observed in Mithun whereas in Mithun the banding pattern ^{was} almost identical in all the strains.

Primer OPR-8

The band amplification in this primer showed 13 fragments in Tho-tho cattle with band size ranging from 200bp to 2300bp. In Mithun the fragments were 13 in Arunachal Mithun, 14 in Mizoram Mithun and 15 each in Nagaland and Manipur Mithun. The band size ranged from 200bp to 2300bp in all the strains of Mithun. This primer showed a unique band in Tho-tho cattle, OPR8.15 (400bp), which was not seen in Mithun. The Mithun of Nagaland and Manipur strain showed identical banding pattern.

Primer OPS-11

The primer OPS11 showed a low rate of variation in the number of band amplification within Tho-tho cattle and Mithun. The band fragment size range from 200bp to 1500bp in Tho-tho cattle and 150bp to 1500bp in all the Mithun strains. Two bands were observed to be specific for Mithun which ~~was~~ ^{is} not seen in Tho-tho cattle i.e. OPS11.16 and OPS11.9. Tho-tho cattle also showed two unique bands i.e. OPS11.4 and OPS11.5 which ^{are} not seen in Mithun. All the strains of Mithun showed similar banding pattern. The band OPS11.13 was very prominent in Tho-tho cattle, Arunachal strain, Mizoram strain and Manipur strain of Mithun. However, this band was faint in Nagaland Mithun.

The genetic similarity and the genetic distance between four strains of Mithun and Tho-tho cattle was calculated by using Popgene software version 1.31. Genetic Distance was found to be the highest between Tho-tho Cattle and Arunachal strains of Mithun and also with Mizoram strain Mithun ($D= 0.5170$) while it was lowest between Nagaland Mithun and Mizoram Mithun ($D= 0.0862$). The genetic identity was found highest ($I= 0.9174$) between Nagaland and Mizoram strains of Mithun. The number of polymorphic loci ^{was} ~~it~~ was 117 (53.67%).

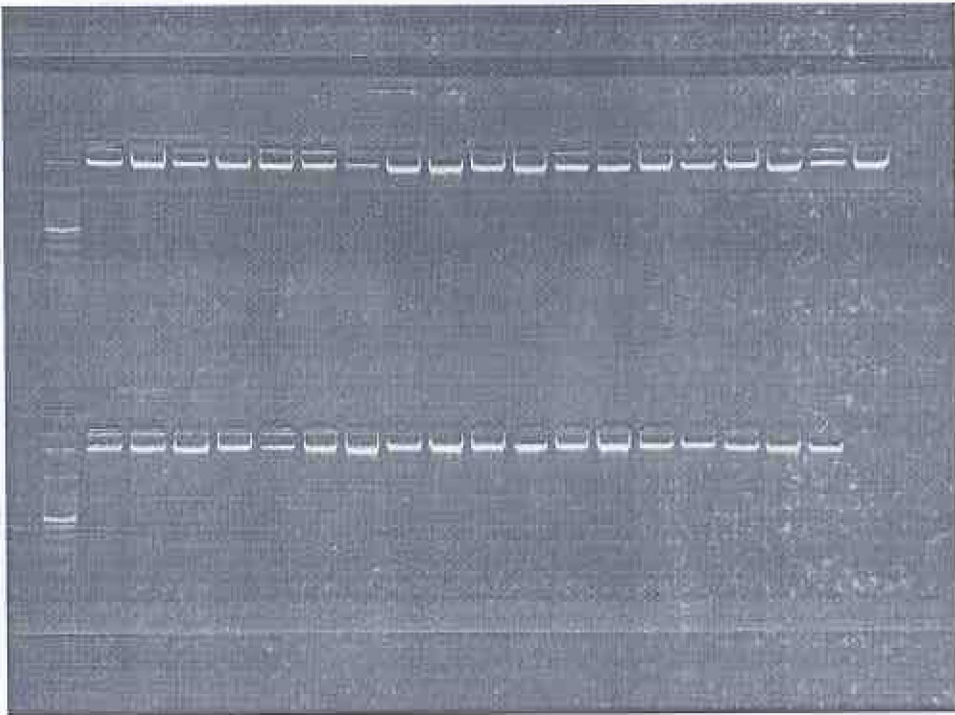


Figure 15. Checking the quality of genomic DNA. 2 μ g of DNA was loaded on 0.7% agarose gel.

Table 5. Pair-wise genetic distance between Tho-tho cattle and Mithun stains

Population	Tho-tho cattle	Nagaland Mithun	Arunachal Mithun	Mizoram Mithun	Manipur Mithun
Tho-tho cattle	—	0.6055	0.5963	0.5963	0.6881
Nagaland Mithun	0.5017	—	0.7706	0.9174	0.8532
Arunachal Mithun	0.5171	0.2605	—	0.7982	0.7982
Mizoram Mithun	0.5171	0.0862	0.2254	—	0.8807
Manipur Mithun	0.3739	0.1587	0.2254	0.1270	—

Nci's genetic identity (above diagonal) and genetic distance (below **diagonal**)

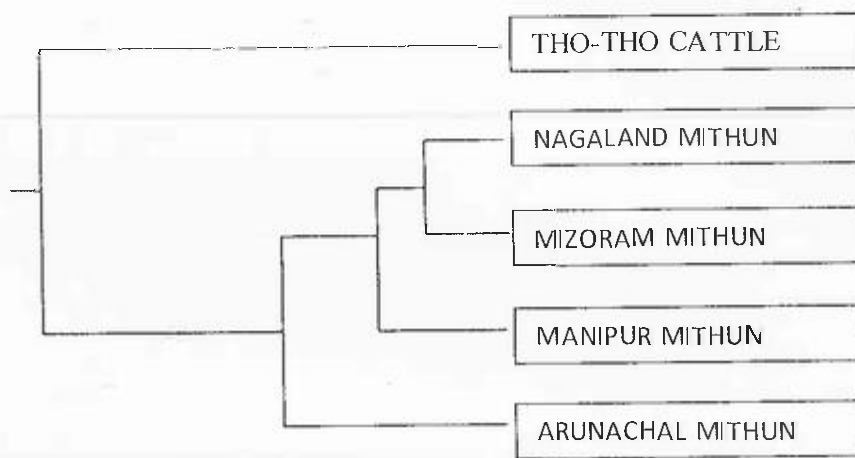


Figure 16. Dendrogram based on Nei (1978) genetic distance method, summarizing the data on genetic differentiation between Tho-tho cattle and Mithun

Table 6. RAPD primer and sequence along with average band size in Tho-tho cattle and Mithun

SL. N	PRIMER	SEQUENCES 5' TO 3'	THO-THO CATTLE		SAGALAND MITHUN		ARUNACHAL MITHUN		MISORAM MITHUN		MANIPUR MITHUN	
			NUMB OF BAND S	SIZE RANGES (bp)	NUMBE R OF BANDS AMPLI FIED	SIZE RANGES (bp)	NUMBE R OF BANDS AMPLI FIED	SIZE RANGES (bp)	NUMBE R OF BANDS AMPLI FIED	SIZE RANGES (bp)	NUMBE R OF BANDS AMPLI FIED	SIZE RANGES (bp)
1	OPM12	GGGCTTGG C	20	250- 1000	16	300-1000	19	250- 1000	20	250- 1000		
2	OPB2	CAGTGGTCC C	26	300-3000	21	300-3000	24	300-3000	21	300-3000		
3	OPM15	AGGAGGGG C	13	400-1200	18	250-1200	15	250-1200	18	250-1200		
4	OPM20	TGGGCGTTC C	12	350-2500	12	350-2500	16	350-2500	17	350-2500		
5	OPM10	TCCCGCTTA C	22	350-6000	20	250-5000	23	350-6000	20	350-6000		
6	OPM10	TCTGGCGCA C	22	200-6500	22	200-6000	22	250-6000	21	250-6000		
7	OPM14	TGAGCGGAA C	8	1500-9000	16	300-8000	10	300-8000	8	300-8000		
8	OPM17	TGGGGTGG C	19	350-3000	17	350-3000	17	350-3000	17	350-3000		
9	OPM1	TGGGATGCT C	15	200-8000	17	200-8000	18	200-8000	18	200-8000		
10	OPM3	CACAGCTGGC C	8	250-900	8	250-900	8	250-900	8	250-900		
11	OPM6	CCCTGCTGCT C	15	200-2500	13	200-2500	14	200-2500	15	200-2500		
12	OPM11	ACTGCGTGG C	24	150-1500	23	150-1500	22	150-1500	22	150-1500		

Nucleotide sequence of RAPD primers along with average number of bands amplified and their size ranges in Tho-tho Cattle and Four strains of Mithun.

number of bands and size

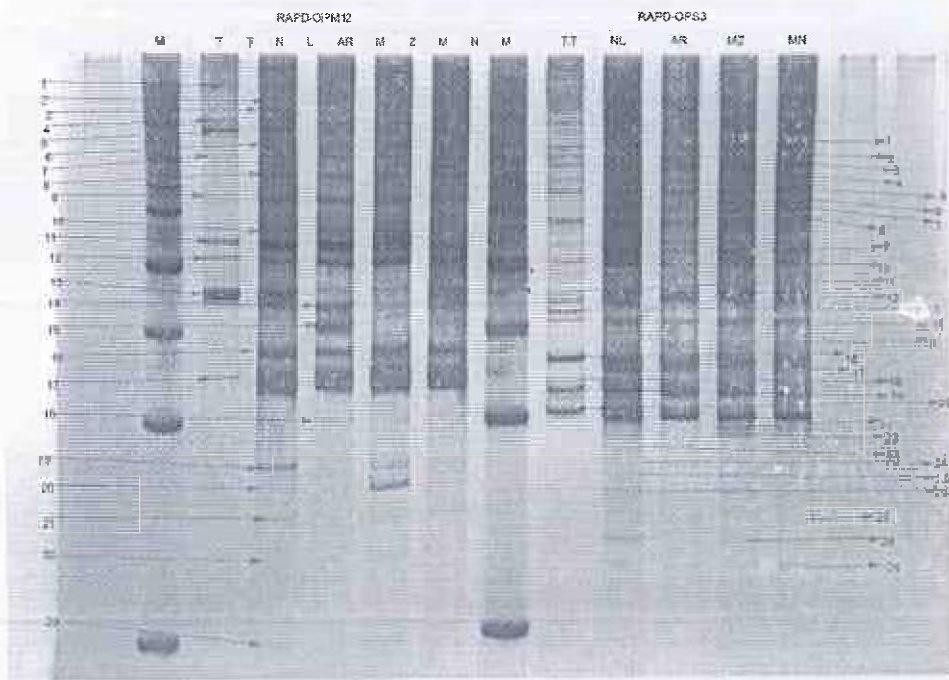


Figure 17. RAPD-PAGE profile of OPM12 and OPS3 prime. M-1Kb DNA ladder, TT.Tho.tho cattle, NL-Nagaland Mithun, AR-Arunachal Mithun, MZ-Mizoram Mithun, MN. Manipur Mithun

Table 6. RAPD score sheet of OPM12 and OPS3 primer

PRIMER NO.	RAPDPRIMER	ANIMAL STRAIN	SCORE
1	RAPD-OPM12	Tho-tho cattle	1001101010110001000000
		Nagaland Mithun	1111111111111001101111
		Arunachal Mithun	11111111111011110000
		Mizoram Mithun	111111111111001101111
		Manipur Mithun	11111111111111011101
2	RAPD-OPS3	Tho-tho cattle	1111001011111010111000000110
		Nagaland Mithun	111111111110111101111111011
		Arunachal Mithun	111111111110111101110000101
		Mizoram Mithun	111111111111011110111000111
		Manipur Mithun	111111111110111101110000101

*Presence of band is scored as 1, absent as 0.

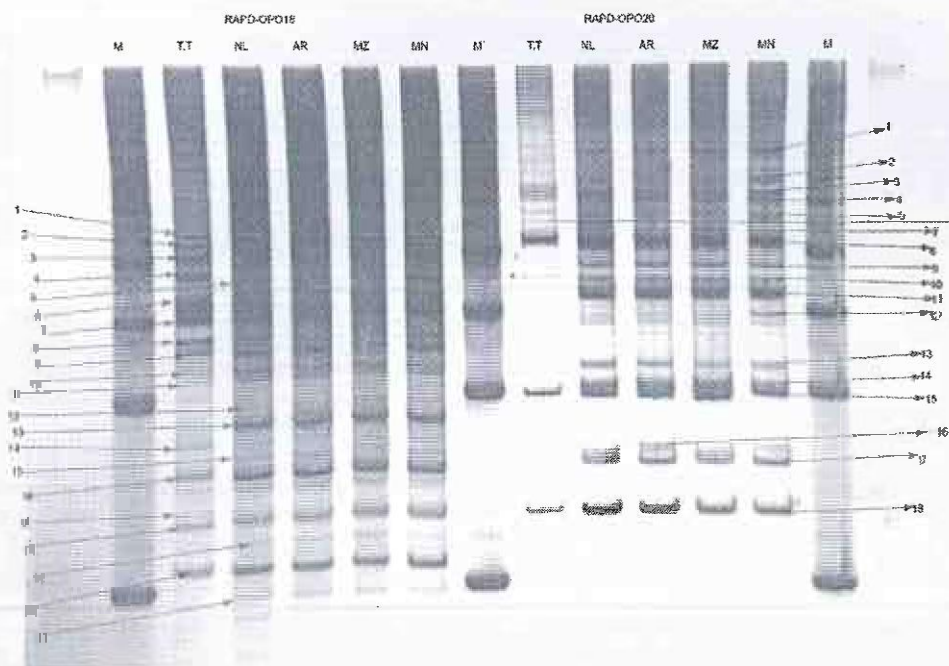


Figure 18. RAPD-PAGE profile of OPO18 and OPO20 primer. M-1Kb DNA ladder, TT-Tho-tho cattle, NL-Nagaland Mithun, AR-Arunachal Mithun, MZ-Mizoram Mithun, MN- Manipur Mithun

Table 7. RAPD score sheet of OPO18 and OPO20 primer

PRIMER NO.	RAPDPRIMER	ANIMAL STRAIN	SCORE
3	RAPD-OPO18	Tho-tho cattle	111101111110010111010
		Nagaland Mithun	101011111111111101111
		Arunachal Mithun	101011111111111101111
		Mizoram Mithun	101011111111111101111
		Manipur Mithun	101011111111111101111
4	RAPD-OPO20	Tho-tho cattle	11111010100001001
		Nagaland Mithun	1111101111101111111
		Arunachal Mithun	1111101111101111111
		Mizoram Mithun	1111101111101111111
		Manipur Mithun	1111101111111111111

*Presence of band is scored as 1, absent as 0.

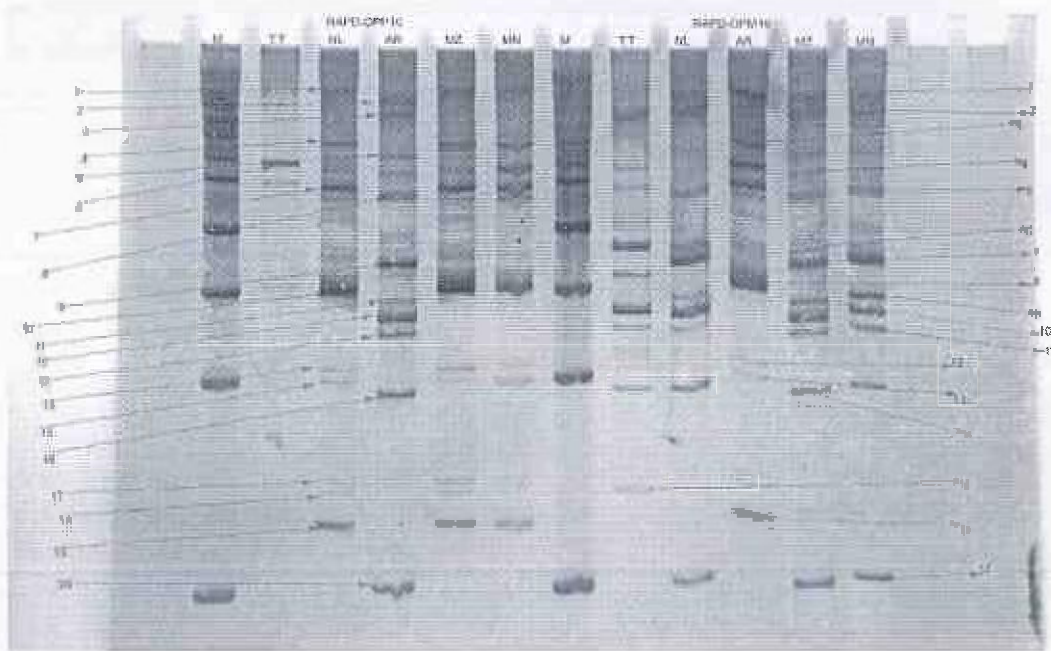


Figure 19. RAPD-PAGE profile of OPP10 and OPM10 primer. M-1Kb DNA ladder, TT-Tho-tho cattle, NL-Nagaland Mithun, AR-Arunachal Mithun, MZ-Mizoram Mithun, MN- Manipur Mithun

Table 8. RAPD score sheet of OPP 10 and OPM10 primer

PRIMER NO.	RAPDPRIMER	ANIMAL STRAIN	SCORE
5	RAPD-OPP10	Tho-tho cattle	00000110010000000000
		Nagaland Mithun	10010111110001101110
		Arunachal Mithun	01101111101110010001
		Mizoram Mithun	10010111110001101110
		Manipur Mithun	10010111010000100010
6	RAPD-OPM10	Tho-tho cattle	01011101011001100
		Nagaland Mithun	11011110111001001
		Arunachal Mithun	11111101000110110
		Mizoram Mithun	11011110111001011
		Manipur Mithun	11011110111001001

*Presence of band is scored as 1 and absence as 0

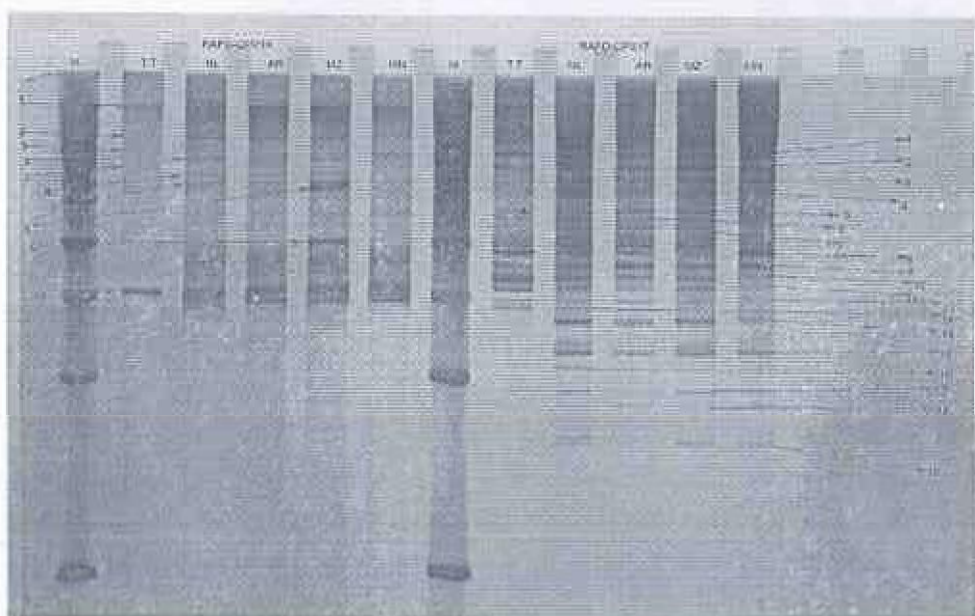


Figure 20. RAPD-PAGE profile of OPP14 and OPS17 primer. M-1Kb DNA ladder, TT-Tho-tho cattle, NL-Nagaland Mithun, AR-Arunachal Mithun, MZ-Mizoram Mithun, MN- Manipur Mithun

Table 9. RAPD score sheet of OPP14 and OPS17 primer

PRIMER NO.	RAPDPRIMER	ANIMAL STRAIN	SCORE
7	RAPD-OPP14	Tho-tho cattle	1110100001
		Nagaland Mithun	1111111001
		Arunachal Mithun	1111111001
		Mizoram Mithun	1111111111
		Manipur Mithun	1111111001
8	RAPD-OPS17	Tho-tho cattle	1100111111111001111
		Nagaland Mithun	1111111111111111111
		Arunachal Mithun	111111111110110111
		Mizoram Mithun	111111111110110111
		Manipur Mithun	111111111110110111

*Presence of band is scored as 1, absent as 0.

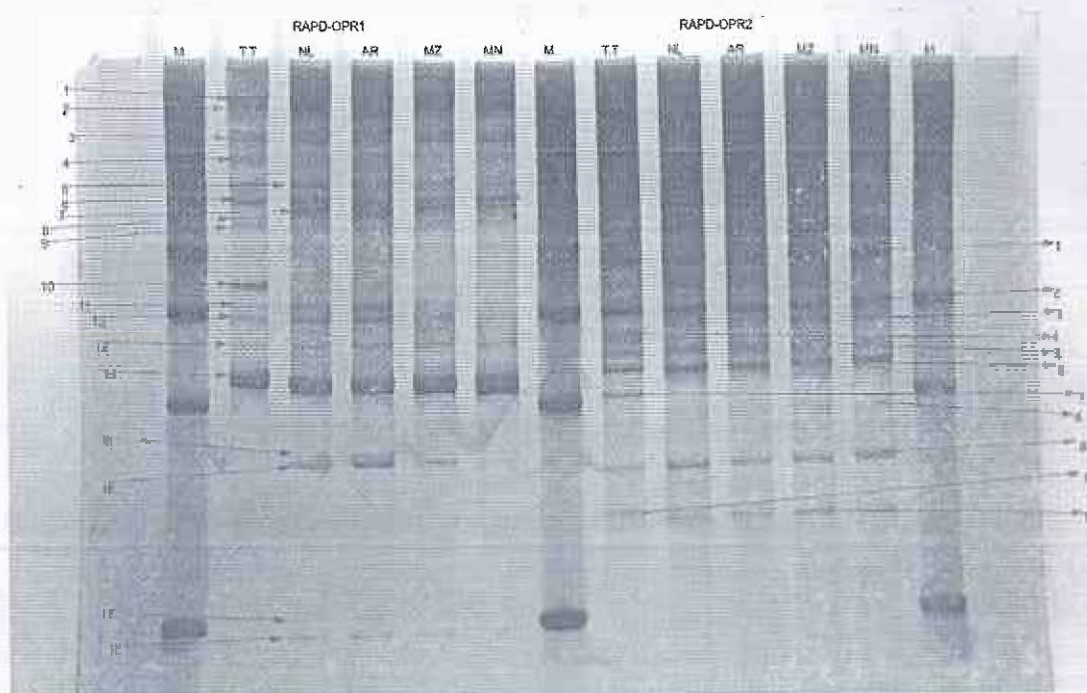


Figure 21. RAPD-PAGE profile of OPR1 and OPR2 primer. M-1Kb DNA ladder, TT-Tho.tho cattle, NL-Nagaland Mithun, AR-Arunachal Mithun, MZ-Mizoram Mithun, MN- Manipur Mithun

Table 10. RAPD score sheet of OPR1 and OPR2 primer

PRIMER NO.	RAPDPRIMER	ANIMAL STRAIN	SCORE
9	RAPD-OPR1	Tho-tho cattle	11110101111110000
		Nagaland Mithun	11111101011111111
		Arunachal Mithun	11111101111111111
		Mizoram Mithun	111011101011110101
		Manipur Mithun	111011101101110101
10	RAPD-OPR2	Tho-tho cattle	11011111110
		Nagaland Mithun	11101101101
		Arunachal Mithun	11101101101
		Mizoram Mithun	11101101101
		Manipur Mithun	11101101101

*Presence of band is scored as 1, absent as 0.

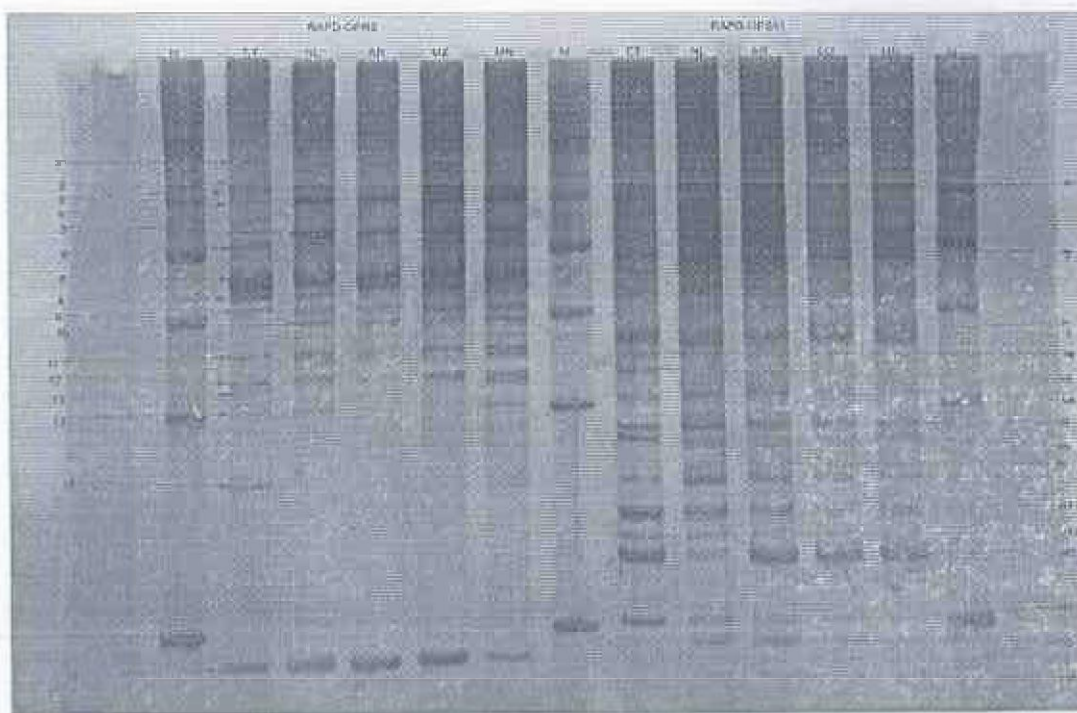


Figure 22. RAPD-PAGE profile of OPR8 and OPS1 primer. M-1Kb DNA ladder, TT-Tho-tho cattle, NL-Nagaland Mithun, AR-Arunachal Mithun, MZ-Mizoram Mithun, MN- Manipur Mithun

Table 11. RAPD score sheet of OPR8 and OPS11 primer

PRIMER NO.	RAPDPRIMER	ANIMAL STRAIN	SCORE
11	RAPD-OPR8	Tho-tho cattle	1101011110111111
		Nagaland Mithun	1111111111111101
		Arunachal Mithun	1111111011110101
		Mizoram Mithun	1111111011111101
		Manipur Mithun	1111111111111101
12	RAPD-OPS1	Tho-tho cattle	1111111101111110
		Nagaland Mithun	1110011111111111
		Arunachal Mithun	1110011111101111
		Mizoram Mithun	1110011101101111
		Manipur Mithun	1110011101101111

*Presence of band is scored as 1, absent as 0.

DISCUSSION:

Random amplification of polymorphic DNA (RAPD) is a PCR based technique. The PCR technique using 40 randomly selected decamer short nucleotide primers was employed to generate polymorphic DNA fingerprints. In the present study the pooled genomic DNA from 20 Tho-tho cattle and 10 Mithun of each strains were prepared and amplified. Here the pooling of DNA was done so that the variation in the genome of Mithun and Tho-tho cattle can be determined.

Out of the 40 random primers used for amplification of genomic DNA, 12 primer ~~were~~ amplified ^{random sequences} for Tho-tho cattle and Mithun genomic DNA. All the primers used were found to produce consistent polymorphic banding patterns. The number of RAPD bands in Tho-tho and Mithun were found in the range of 3 to 17 and 8 to 26, respectively. Size of the PCR products varied from 200bp to 10000bp in Tho-tho cattle and similarly also in Mithun. The primer OPS3 produced highest (26) number of RAPD bands while the primers OPP10 produced the least (3) number of bands. The total number of RAPD markers obtained from the 12 primers was 218 loci, the number of polymorphic loci was found to be 117 (53.67%). Similar to the present study, Joshi *et al.* (2007) also showed high number of loci (196) in his study between two Indian cattle breeds Kankrej and Gir. Nei and Roychoudhury (1974) confirmed theoretical conclusion that a relatively reliable estimate of average heterozygosity can be obtained from a small number of individuals if a large number of loci are examined. NOT
Sited

Devrim and Kaya (2006) demonstrated within and between the four cattle breeds in the province of Kars and found out that ^{6 out of 89} of 89 amplified loci, 78 were polymorphic and ~~89~~ ~~loci were amplified out of which 78 were polymorphic~~. They found that the percentage of the polymorphism was 87.6% and it was sufficient to genetically differentiate between and within these cattle breeds. Though the percentage of polymorphism is comparatively less as compared to the above study but the number of polymorphic loci was high enough to give a good genetic variation analysis between Tho-tho cattle and Mithun and within the four strains of Mithun.

In the present study random bovine primers ^{are} used to differentiate between the Tho-tho cattle and the Mithun wherein many unique species specific bands were found. The unique bands observed between Tho-tho cattle and Mithun in the present study can be used for species identification. There were few unique bands that were observed in the Tho-tho cattle ~~which were found to be~~ species specific. The bands OPS3.13, OPS3.18, OPO13.2, OPO18.4, OPO18.17, OPR2.4, OPR2.10, OPR8.15, OPS11.4 and OPS11.5 were unique for Tho-tho cattle. Similarly, Kemp and Teale (1994) reported polymorphism using RAPD by revealing the specific unique band for *Bos indicus* of African origin and not in *Bos taurus*.

Similarly unique bands were observed among the four strains of Mithun. These strain specific bands can be used as a marker for strain identification and selection in the breeding program of Mithun. The polymorphic bands were OPS3.23, OPS3.24 and OPS3.25. Within the strains of Mithun, Nagaland strain showed two unique bands, OPS17.13 and OPS17.16, which ^{are} specific for these strains. In the case of Manipur strains of Mithun, the primer OPO20 showed a polymorphic band, OPO20.12, which was specific for this strain. Mizoram Mithun strain also showed polymorphic band as OPP14.8 and OPP14.9. The present study also found out that the maximum numbers of polymorphic bands within the strains of Mithun were observed in Arunachal strains of Mithun. The band ~~numbers are~~ ^{were} OPM-12.18, OPO10.2, OPO10.3, OPO10.5, OPO10.11, OPO10.12, OPO10.16 and OPO10.20, OPM10.3, OPM10.12 and OPM10.13. This finding was ~~in~~ similar to the report of Thiyagarajan (2000) ^{who} characterized four Indian zebu cattle breeds by RAPD technique and recorded unique band for each breed. Ramesha *et al.* (2002) reported a high number of breed specific loci among the south Indian breeds of Zebu cattle using RAPD markers.

The Genetic distance was highest between Tho-tho Cattle ^{and} ~~to that of~~ Arunachal strains of Mithun and Mizoram strain Mithun ($D= 0.5170$) and lowest between Nagaland Mithun and Mizoram Mithun ($D= 0.0862$). High value of genetic distance was also observed by Serrano *et al.* (2004) between the Brazilian native bovine breeds. They found that the genetic distance between Nellore (*Bos indicus*) and Holstein Friesian (*Bos primigenius*)

Not listed
in Ref.

was $D=0.4260$. Tahrous et al. (2010) also reported a high genetic distance between the horse breeds with a value of $D=0.5442$ between Arabian breed and Thoroughbred breed.

Mufti et al. (2009) studied the genetic variation among the Red Chittagong Cattle breed of Bangladesh and found that the pair-wise mean genetic distance value ranged from 0.0092-0.0279. Kumar et al. (2008) employed RAPD to find the genetic variability among sheep breeds and found that the genetic distance was highest between Malpura and Garole ($D=0.1428$) and lowest ($D=0.0612$) between Avikalin and Chokla breed.

Radwan et al. (2014) carried out a genetic variability ^{study} between the freshwater Crayfish in Egypt. They found that the genetic distances among the population ranged from 0.0076 to 0.1735 and concluded that there is a high level of genetic variation in the population. Therefore, it can be suggested that the genetic variation in the present study between Tho-tho cattle and the four breeds of Mithun is high as compared to the degree of genetic variation observed from other studies.

The study has explored the extent of genetic variation among four strains of Mithun. The trend is as following: Highest genetic variation ^{was} found to be between Mithun of Arunachal strain and Nagaland strain ~~has the highest variation~~ ($D=0.2605$). On the other hand the lowest genetic distance was between Mizoram Mithun strain and Manipur Mithun strain ($D=0.1270$).

The genetic identity was found highest ($I=0.9174$) between Nagaland and Mizoram strains of Mithun which was followed by Manipur strain and Mizoram strain ($I=0.8807$). High genetic identity values were also demonstrated between the breeds of cattle in Kars province by Devrim and Kaya (2006). They found that the value of genetic identity was $I=0.9333$ between East Anatolian Red cattle breed (EAR) and Simmental cattle breed (SIM). The high value of genetic identity between the strains of Mithun suggests that they are very closely related between the strains. These observations are evident from the Dendrogram following the method of Nei (1978).

The dendrogram shows the different branch between Tho-tho cattle and the strains of Mithun. It can be assumed from the present study that there is divergence in the evolutionary path between these two species of bovine. In case of the Mithun strains, Nagaland Mithun strain and Mizoram Mithun strain were in a different branch followed by Manipur Mithun strain and Arunachal Mithun strain. This shows that the Mithun of Nagaland strain and Mizoram strain are more related among these four strains.

RAPD analysis of the present study shows that Mithun and Tho-tho cattle are genetically divergent which is justifiable from their divergent evolutionary patterns. Among the four strains of Mithun, Nagaland strain and Mizoram strain are genetically closer and ^{Manipur} Nagaland strain and Arunachal strain are genetically distant. Present study will help to plan the fruitful breeding programme among the four different strains of Mithun belonging to Arunachal, Manipur, Nagaland and Mizoram.

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

The present study consisting of cytogenetic and Randomly Amplified Polymorphic DNA in Mithun and Tho-tho cattle was carried by taking samples from 115 Mithun and 28 Tho-tho cattle. Mithun (*Bos frontalis*) is a heavily built semi domesticated bovine species found in north eastern hill region in four states of India. Mithun are classified into four strains based on their geographical habitat and phenotypic features as Arunachal, Manipur, Mizoram and Nagaland. Tho-tho cattle is short to medium stature black coloured animal available in different hilly pockets of Nagaland, especially in Kohima and Phek districts. These bovine species are less explored cytogenetically and it is very essential to identify and understand the nature of chromosome before starting any biotechnological interventions including genome analysis in this species, as karyotype features allow individual species to be distinguished.

Cytogenetic analysis consists of karyotype construction, estimate of relative length, arm ratio, centromeric index, number of bands and idiogram of the chromosome. These parameters were used not only for the construction of karyotype and idiogram, but also required for morphology description of the chromosome.

The diploid chromosome number in Mithun was found to be 58 ($2n=58$), which include 28 pairs of autosomes and one pair of sex chromosomes. The normal karyotype of Mithun shows, the first pair of autosome is the largest and sub-metacentric chromosome and the rest autosome were acrocentric. Among the sex chromosome the X-chromosome was sub-metacentric and Y-chromosome was the smallest metacentric chromosome. The relative length (%) of the first pair was 6.322 ± 0.191 and 6.131 ± 0.562 in male and female, respectively. The relative length of the smallest chromosome was 1.705 ± 0.040 in male and 1.666 ± 0.146 in female. In sex chromosome, the X-chromosome was 5.284 ± 0.153 for male and 5.276 ± 0.282 for female. The percent relative length of Y-chromosome was 2.283 ± 0.190 . The average centromeric index of chromosome number 1 in male and in female was 28.378 ± 2.261 and 28.126 ± 0.940 , respectively. In sex chromosome the centromeric index for female was $X=30.130 \pm 1.757$ and in male $X=29.489 \pm 1.040$ and $Y=45.53 \pm 1.904$. The value of the arm ratio for the first pair of

chromosome was 2.269 ± 0.756 and 2.290 ± 0.930 in male and female, respectively. In female the arm ratio for the sex chromosome was 2.290 ± 0.930 and in male X chromosome was 2.390 ± 0.903 , Y chromosome was 1.180 ± 0.482 .

Cytogenetic analysis was done for both male and female Tho-tho cattle and found that the mean relative length in autosome ranged from $5.48 \pm 0.107\%$ to $1.79 \pm 0.105\%$ in male and $5.31 \pm 0.148\%$ to $1.86 \pm 0.055\%$ in female. In Tho-tho cattle the largest chromosome was chromosome number 1. The average centromeric index was found to be $36.45 \pm 0.31\%$ in male and $31.06 \pm 0.55\%$ in female. The arm ratio was 2.60 ± 0.09 and 2.23 ± 0.06 for male and female Tho-tho cattle.

Chromosome banding is the process of eliciting differences in staining manifestation along the length of a chromosome thereby producing a specific banding pattern. In the present study banding methods employed were R-banding and C-banding. In Mithun the R-banded chromosome show a series of strongly stained and more or less wide bands. It has been observed that the number of bands in chromosome 1 (sub-metacentric) is more as compared to the acrocentric autosome. The number of dark bands in p and q arm of chromosome number 1 pair was 22 bands. The number of bands depends upon the size of the chromosome. On an average the numbers of bands in larger acrocentric autosomes were more. Whereas, the smaller acrocentric autosomes comparatively showed less number of bands. In female, the inactivation of X- chromosome is clearly shown as the p arm of one X- chromosome shows no dark band. The total number of R-band in Mithun was found to be 336.

In Tho-tho cattle the R banded chromosome show a series of strongly stained bands along the length of the chromosome. The prominent bands are observed in the telomeric portion of all chromosomes. In female, the inactivation of X- chromosome is clearly shown as the p arm of one X- chromosome shows no dark band and the q arm shows alternating dark bands and light bands. The present study found that the number of R-bands for Tho-tho cattle were 496 and 499 in male and female, respectively. The C-banded metaphase chromosome showed a distinct dark stain at the centromeric region in all the acrocentric

autosome. The centromeric region of the sub-metacentric chromosome, i.e. X-chromosome, showed very faint stain. The density of the centromeric stain appears to be more in the larger chromosome and it reduces as the size of the acrocentric chromosome descent. In Tho-tho the Y chromosome which is also an acrocentric chromosome did not show banding pattern (C-negative).

Genetic diversity is the basis for present day diversified living systems and future genetic improvement needs. The present study was carried out with an aim to find the genetic variation between Mithun and Tho-tho cattle using molecular marker. Genetic diversity study was done based on PCR technique. Random amplification of polymorphic DNA-PCR technique using 40 randomly selected decamer short nucleotide primers was employed to generate polymorphic DNA fingerprints. Genomic DNA was isolated from whole blood collected from 40 Mithun and 20 Tho-tho cattle kept at the station farm of National Research Centre on Mithun and the field samples were also collected from Visvema, Kidema, Kigwema and Merima under Kohima district.

According to the breed, the pool genomic DNA from 20 Tho-tho cattle and 10 Mithun of each strains were prepared and amplified. Out of the 40 random primers used for amplification of genomic DNA, 12 primer were amplified for Tho-tho cattle and Mithun genomic DNA. All the primers used were found to produce consistent polymorphic banding patterns. The number of RAPD bands with the different primers in Tho-tho and Mithun were found in the range of 3 to 17 and 8 to 26, respectively. Size of the PCR products varied from 200bp to 10000 bp in Tho-tho cattle and 200bp to 10000 bp in Mithun. The primer OPS3 produced highest (26) number of RAPD bands while the primers OPP10 produced least (3) number of bands. Total numbers of RAPD markers obtained from the 12 primers were 131 in Tho-tho cattle and 183 in Mithun.

The primer OPM-12 showed a high number of amplification ^{ed products} and the variation in number of amplified bands between Tho-tho cattle and Mithun was high. The number of bands was 9 for Tho-tho cattle and the size ranged from 600bp to 10,000bp. In case of Mithun,

the numbers of bands were 20, 16, 19 and 20 for Nagaland, Arunachal, Mizoram and Manipur strains, respectively. The RAPD primer OPS-3 was observed to be moderate in variation of bands between Tho-tho cattle and Mithun with high number of bands amplified. The numbers of the bands were 17 in Tho-tho cattle and 26, 21, 24 and 21 in Nagaland, Arunachal, Mizoram and Manipur strains of Mithun, respectively. The primer OPO18 showed a band amplification of 15 fragments in Tho-tho cattle and 18 fragments in all the strains of Mithun. The band size ranged from 300bp to 1300bp in Tho-tho cattle and 250bp to 1300bp in Mithun. The primer OPO20 showed a high variation in the band amplification with 10 bands in Tho-tho cattle, 17 bands in Manipur Mithun and 16 bands in the rest of the Mithun strains. The amplified fragments ranged from 350bp to 2500bp in both bovine species. The primer OPP10 was observed to be highly polymorphic within the strains of Mithun and showed a high rate of variation in the number of band amplification with Tho-tho cattle. The band fragment size range from 800bp to 1500bp in Tho-tho cattle, 250bp to 5000bp in Arunachal strain of Mithun and 350bp to 6000bp in the rest of the Mithun strains. Primer OPM10 showed a moderate variation of band fragments between Tho-tho cattle and Mithun. The band size ranged from 350bp to 5000bp in Tho-tho cattle, 300bp to 6000bp in Arunachal strain of Mithun and 250bp to 6000bp in the rest of the Mithun strains. The number of bands amplified were 9 in Tho-tho cattle, 11 in Nagaland, Arunachal, and Manipur strains of Mithun and 12 in Mizoram Mithun. These primer showed a low number of band fragment amplification with 5 in Tho-tho cattle, 8 each in Nagaland, Arunachal and Manipur Mithun and 10 in Mizoram Mithun. The band size ranged from 800bp to 8000bp in Tho-tho cattle and all strains of Mithun. The primer OPS17 showed an amplification of 15 in Tho-tho cattle, 17 each in Arunachal, Mizoram and Manipur Mithun and 19 in Nagaland Mithun. The size of the fragment ranged from 350bp to 3000bp in both the species. The primer OPR1 showed moderate amplification fragments with 12 in Tho-tho cattle, 13 each in Mizoram and Manipur Mithun, 16 in Nagaland Mithun and 17 in Arunachal Mithun. The size of the band ranged from 600bp to 8000bp in Tho-tho and 200bp to 8000bp in all Mithun strains. The primer OPR2 showed an amplification of 9 fragments in Tho-tho cattle and 8 fragments in Mithun. The band size ranged from 370bp to 900bp in Tho-tho cattle and 350bp to 900bp in all the Mithun strains. The band amplification in the primer OPR8 showed 13 fragments in Tho-tho cattle with band size ranging from 200bp to 2300bp. In Mithun the fragments were 13 in Arunachal Mithun, 14 in Mizoram Mithun and 15 each in Nagaland and Manipur Mithun. The band size ranged from 200bp to 2300bp in all the

strains of Mithun. The primer OPS 11 showed a low rate of variation in the number of band amplification within Tho-tho cattle and Mithun. The band fragment size range from 200bp to 1500bp in Tho-tho cattle and 150bp to 1500bp in all the Mithun strains.

The genetic similarity and the genetic distance between four strains of Mithun and Tho-tho cattle was calculated by using Popgene software version 1.31. Genetic Distance was found to be highest between Tho-tho Cattle and Arunachal strains of Mithun and also with Mizoram strain Mithun ($D= 0.5170$). Genetic Distance was lowest between Nagaland Mithun and Mizoram Mithun ($D= 0.0862$). The genetic identity was found highest ($I= 0.9174$) between Nagaland and Mizoram strains of Mithun. The number of polymorphic loci was 117 (53.67%).

The present cytogenetic screening of Mithun in the NRCM farm helped in creating a cytogenetic album which will be helpful in breeding program. The cytogenetic profile of Mithun created in the present study will be a platform for further molecular cytogenetic studies. The cytogenetic parameters of the Tho-tho breed of cattle are in agreement with the other breeds *Bos indicus*, thus confirming that karyotype profile of investigated animals do not differ from standard karyotype of bovine species. However, the present study on chromosomes of Tho-tho cattle will be a platform for further comparative studies with other breeds of same species by molecular cytogenetics and molecular techniques. The study on the chromosomal diversity and morphology in Tho-tho cattle was done since this breed is having the habitat in North-eastern region of India that is remotely located from the habitat of all other descript breed and the history of domestication is also not very far. Thus, it is required to study the chromosomal profile so that scientific and systematic breeding strategies can be followed. It is also necessary to start a cytogenetic screening of the Tho-tho and expand upon more numbers to be kept at different villages of Nagaland in order to identify animals with chromosomal abnormalities so that it can be excluded from future breeding strategies for conservation of Tho-tho genetic resource.

The R-banding showed a series of dark (R-band positive) and light bands along the length of the chromosome, with the number of band decreasing as the size of the chromosome

decreases. The heterochromatin content was observed by C-banding in all the acrocentric autosome but not in sub-metacentric chromosomes. Cytogenetic screening of the Mithun population should be expanded to a larger number of animals in order to identify animals with chromosomal abnormalities and to be eliminated from the herd, so that a plan breeding strategies can be implemented for the conservation of this important majestic bovine called Mithun. The chromosome banding revealed the presence of heterochromatin with dark banding pattern at the centromeric region of all autosome. However, Y-chromosome and X-chromosome did not show any band. The R-banding pattern showed the banding pattern at 500 band level, which was similar to the standard banding pattern described earlier in other breed of *Bos indicus*. The accumulation of characters and genetic variation in nature is a long process and it is difficult to detect. Therefore, further analysis on these cattle is suggested by using various molecular cytogenetic techniques, such as FISH Fluorescent *in-situ* hybridization and molecular technique, which may reveal the variations of Tho-tho cattle from other *Bos indicus* breeds.

The RAPD markers in the present study were found to be useful in distinguishing between Mithun and Tho-tho Cattle. Information on polymorphic loci can be employed to detect population specific alleles, to measure the amount of genetic diversity in each species and to evaluate the change in variation in breeds over time periods. Therefore, the unique band found in the present study can be used as a tool in developing a specific genetic marker for strain identification in Mithun. Genetic diversity is the basis for present day diversified living systems and future genetic improvement needs. This diversity should be properly utilized, improved and conserved.

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APPENDIX

APPENDIX

Ba(OH)₂ solution (5%):

Ba(OH) ₂ · 8H ₂ O	- 5 gm
GDW	- 100 ml

BrdU solution:

BrdU	- 10 mg
Fluorodeoxyuridine (FUdR)	- 0.05 mg
GDW	- 10 ml

Colcemid solution (0.5 µg/ml):

Colcemid solution (10 µg/ml)	- 1.0 ml
GDW	- 20 ml

10% ethanol:

Ehtanol	- 10 ml
GDW	- 90 ml

Fixative:

Methanol – absolute (3 parts)	- 75 ml
Acetic acid – glacial (1 part)	- 25 ml

Giemsa solution (2%):

GDW	-47 ml
Sorensen's buffer (pH 7.0)	- 2.0 ml
Giemsa stain	- 1.0 ml

Hypotonic solution (0.075M KCl):

KCl	- 5.6 gm
GDW	- 1.0 L

0.056 M KCl

KCl	-4.2gm
GDW	- 1.0 L

HCl solution (0.2N):

2NHCl	- 10 ml
GDW	- 100 ml

Hoechst 33258 dye solution (0.5%):

Hoechst 33258	- 5.0 mg
GDW	- 100 ml

Penicillin:

Penicillin (lyophilized)	-
Millipore water	-

Pokeweed Mitogen (2.5 µg/ml):

Pokeweed Mitogen (lyophilized) - 0.5 gm

Millipore water - 3.5 ml

Use: 1 ml/50 ml of media (i.e. 2.5 µg/ml)

Sodium Bicarbonate (4.4%):

NaHCO₃ - 4.4 gm

GDW - 100ml

2 X Sodium Saline Citrate solution (2 X SSC):

Sodium chloride (NaCl) - 17.5 gm

Sodium citrate 2-hydrate (Na₃C₆H₅O₇·2H₂O) - 8.8 gm

GDW - 1 L

Sorensen's Buffer (pH 7.0):

Potassium phosphate monobasic (KH₂PO₄) - 5.26 gm

Sodium phosphate dibasic (Na₂HPO₄) - 8.65 gm

GDW - 1 L

Streptomycin:

Streptomycin - 200 mg

Millipore water - 10 ml

Ammonium Persulphate 10%:

Ammonium persulphate - 0.05 gm

GDW - 0.45 ml

Glacial acetic acid 10%

Acetic acid - 30 ml

GDW - 300 ml

Conference/seminar/symposia/workshop attended

1. National seminar on “Emerging challenges and prospective strategies for Hill Agriculture in 2050” organized by Indian Association of Hill Farming, held at ICAR research complex Jharnapani, Nagaland from January 23-25, 2014.
2. Training on “Principles of PCR and its Application in Research” organized by National Research centre on Mithun, ICAR from November 15-17, 2013.
3. National Training on “8TH TECHNOLOGY LED ENTREPRENEURSHIP PROGRAMME” organized by HRDG (Council of Scientific and Industrial Research) at ICT, Hyderabad from July 4-18, 2011.
4. Presented Ph.D Research Poster entitled “COMPARATIVE GENETIC STUDY ON MITHUN (*Bos frontalis*) AND THO-THO (*Bos indicus*) CATTLE IN NAGALAND” at N.U Research Poster Competition held at Lumami Campus on September 6, 2009.