Phenotypic and Molecular Characterization of Diara Buffalo

THESIS

SUBMITTED TO THE

BIHAR ANIMAL SCIENCES UNIVERSITY

(FACULTY OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY)

PATNA, BIHAR

In partial fulfillment of the requirements

FOR THE DEGREE OF

Master of Veterinary Science

IN

ANIMAL GENETICS & BREEDING

BY

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BIHAR VETERINARY COLLEGE
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CERTIFICATE-I

This is to certify that the thesis entitled "*Phenotypic And Molecular Characterisation of Diara Buffalo*" submitted in partial fulfillment of requirement for the degree of Master of Veterinary Science (Animal Genetics and Breeding) of faculty of Post-Graduate Studies, Bihar Animal Sciences University, Patna, Bihar is the record of bonafide research carried out by **Dr. Hitesh Purohit**, Registration No-**BVC/M/AGB/001/2017-18** under my supervision and guidance. No part of the thesis has been submitted for any other Degree or Diploma.

It is further certified that such help or information received during the course of this Investigation and preparation of the thesis have been duly acknowledged.

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We, the undersigned members of the Advisory Committee of **Dr. Hitesh Purohit**, Registration No-**BVC/M/AGB/001/2017-18,** a Candidate for the degree of Master of Veterinary Science with major in Animal Genetics & Breeding, have gone through the manuscript of the thesis and agree that the thesis entitled "*Phenotypic And Molecular Characterisation of Diara Buffalo*" may be submitted by **Dr. Hitesh Purohit**, in partial fulfilment of the requirement for the degree.

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LIST OF ABBREVIATIONS

ABBREVIATIONS

Mg	Milligram
Kg	Kilogram
µg	Microgram
Ng	Nanogram
Pg	Picogram
mL	Milliliter
µL	Microliter
Fig	Figure
No.	Number
Avr	Average
BL	Body Length
HW	Height at withers
CG	Chest Girth
PG	Paunch Girth
FL	Face Length
EL	Ear Length
HL	Horn Length
AFS	Age at First Service
AFC	Age at First Calving
CI	Calving Interval
SP	Service Period
AI	Artificial Insemination

AFLP	Amplified Fragment Length Polymorphism
Bp	Base Pairs
Cm	Centimetre
dNTP	Deoxy Nucleotide Tri phosphates
EtBr	Ethidium Bromide
DNA	Deoxyribo Nucleic Acid
EDTA	Ethylenediamine Tetra Acetic Acid
FAO	Food and Agriculture Organization
Fig.	Figure
Fis	Fixation Index
Ho	Observed Heterozygosity
He	Expected Heterozygosity
I	Shannon's information index
IAM	Infinite Alleles Model
Min.	Minutes
NaOH	Sodium Hydroxide
Na	Observed Number of Alleles
Ne	Effective Number of Alleles
OD	Optical Density
PBS	Phosphate Buffered saline
PCR	Polymerase Chain Reaction
PHA	Phyto-hemagglutinin
PIC	Polymorphic Information Content
RAPD	Random Amplified Polymorphic DNA
RBC	Red Blood Cells
RFLP	Restriction Fragment Length Polymorphism
Rpm	Rotation per Minute
RT	Room temperature
SDS	Sodium Dodecyl sulphate

SMM	Stepwise Mutation Model
SSCP	Single Strand Conformation Polymorphism
SSR	Simple Sequence repeats
TAE	Tris acetic acid EDTA
TE	Tris EDTA
TPM	Two-Phase Model
WBC	White blood cells

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ABSTRACT

In past recent years, Bihar has achieved appreciative growth rate and economic development with greater contribution of service sector in comparison to share of agriculture in State Domestic Product (SDP). The real development of Bihar lies in the economic development of rural masses those are mainly dependent on agriculture sector. The buffalo among different livestock is prominent because of more sustainable in production in rural masses in all harsh condition and low input management systems. The milk production in India and Bihar is presenting increasing trend of milk production with greater contribution of Buffalo milk. The average buffalo productivity for milk yield presented increasing trend from 3.95 kg/day (2012 – 13) to 4.3 kg/day (2016 – 17) in Bihar which is far below than national average 5.23 kg milk/day with significant high productivity 8.39 kg/day in Haryana and 8.21 kg/day in Punjab. Buffaloes are under non-descript category. Therefore, field level study was undertaken to characterize Bihar buffalo populations. Diara Buffalo is distributed under the area South and North Genetic plains of Bihar. They are well adapted to submerged condition of land in rainy season with water of the river Ganga. Thus, the present study is being taken with following objectives:

- To phenotypically characterize Diara buffalo.
- To characterise the production traits of Diara buffalo.
- To analyse genetic diversity of Diara buffalo using Short Tandem Repeats (STR) markers.

The data on aspects of breeding tract, Ecological Settings, status of buffaloes in breeding tract, Buffalo Husbandry Practices (Housing, Feeding and Breeding), Management practices, Physical characteristics, production performance and utility was collected using questionnaires, direct communication with farmers, direct observations and measurements. Characterization using 10 microsatellite markers was carried out in laboratory on 50 unrelated individual

buffaloes. Data of microsatellites was analysed using various software and estimated various parameter of diversity.

From study it was observed that Diara buffaloes are hardy, dual purpose animals reared for both milk and draught purposes. These buffaloes are able to thrive well in low input systems forming an integral part in the livelihood of farmers in the region. Sufficient genetic diversity was found to exist in the population as revealed by microsatellite data, however steps need to be taken for the genetic improvement as well as conservation of this precious germplasm of the country.

The information gathered could be utilized to plan breeding, improvement and conservation programs for this valuable Buffalo germplasm resource to exploit its unique adaptability traits. The significant level of variability in this population reflects that the local buffalo population contains a valuable and substantial amount of genetic diversity among the studied breed but the study needs to be extended to include more microsatellites in a large sample size to further validate the research.

Dr. Hitesh Purohit
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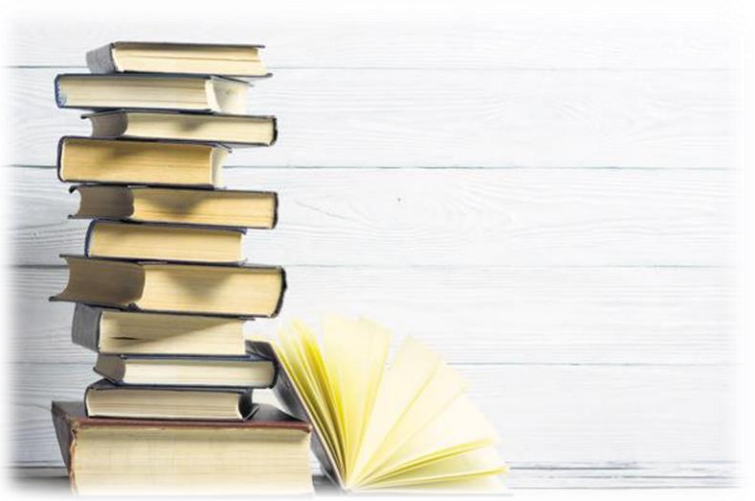

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CHAPTER No.1

INTRODUCTION

In past recent years, Bihar has achieved appreciative growth rate and economic development with greater contribution of service sector in comparison to share of agriculture in State Domestic Product (SDP). The real development of Bihar lies in the economic development of rural masses those are mainly dependent on agriculture sector. They are primarily depending on agriculture as the prime source of livelihood which is characterized by subsistence, low input-low output, technologically lagged mixed farming system and is dominated by smallholders (more than 55% land is under 0-1 hectare's category). The mixed farming system is mostly followed by the farmers with cropping of cereals in major and livestock rearing. The productiveness from cereals is largely uncertain round the year in Bihar due to heavy and least rain depending on location as a result of ever changing climate. The livestock farming ensures round the year income in rain-fed and semi-arid regions of Bihar and used as an alternative source of income. The buffalo among different livestock is found more in number because it is more sustainable in production in rural masses in all harsh condition and low input management systems.

India harbours all the recognized and high milk producing breeds of buffalo of the world. India has been the center of dispersion of good germplasm of buffaloes for improvement of the species. There are fifteen well recognized breeds of buffalo in India which constitutes about 30% of the total buffalo population in the country. However, 70% of the total buffalo population in the country is classified as non-descript because adequate efforts have not been made so far to characterize them phenotypically and genetically.

The milk production in India is currently estimated to be 165.4 million Tonnes in 2016-17 with 6.4% growth rate, of which 49.2% milk is being contributed by indigenous (35.4%) and non – descript (13.8%) buffalo population (BAHFS,2017-18). The in–milk buffalo population in India is reported to be 92.6 million Tonnes. The Bihar had presented increasing trend of milk production from 2012 to 2017 and has produced 8.7 million Tones milk (BAHFS 2017). The out of which buffalo milk 3.35 million Tonnes produced in Bihar contributed 4.1% milk to total milk 81.2 million Tonnes produced by buffaloes in India. The average milk yield per In–milk Buffalo presented increasing trend from 3.95 Kg/day (2012 -13) to 4.3 Kg/day

(2016 – 17) in Bihar. The normal average productivity of Buffaloes is 5.23 Kg/day in India with significant high productivity 8.39 Kg/day in Haryana and 8.21 Kg/day in Punjab. Bihar possess 4.93 % (2.1 million) In-milk indigenous buffaloes out of 42.56 million Tonnes In–milk Buffalo of India (BAHFS 2017). Moreover, Buffaloes of Bihar are kept under indigenous category and no Buffaloes under Descript category. While majority states in India had categorized their indigenous buffaloes into descript and non–descript Buffaloes after undertaken proper scientific study. Therefore, field level study of Buffaloes of Bihar need to be undertaken to ensure livelihood of rural people with aim to enhance their productivity.

In Bihar, buffalo milk accounts for nearly 48% of the total milk production in the state (BAHFS 2017). These are strong indications of the fact that buffaloes available in Bihar are efficient producers and thus, an important Animal Genetic Resource for augmenting milk production in the state. Although buffaloes are distributed throughout the length and breadth of the state, but the area under South and North Gangetic plains of Bihar is densely populated with clusters of buffalo, phenotypically homogenous in certain characteristics, popularly known as "Diara buffalo". They are well adapted to submerged condition of land in rainy season with water of the river Ganga. These buffalo are fully adapted to the agro-climatic and socio-economic conditions of the state under low-input management system in the Taal and Diara area of the river Ganges, Sone and Gandak. Besides these 16 well characterized buffalo breeds in India, there exist some local and non-descript germplasm which can be characterized and improved for dairying. The Diara buffaloes of Bihar has not been explored so far for phenotypic and genetic characterization using microsatellite based genetic diversity.

Genetic differentiation and diversity analysis of native breeds is an important step in genetic characterization, as it allows the evaluation of unexplored genetic variability. Assessment of genetic variability present in a breed is vital and it is a basic component for working out conservation strategies and for designing genetic improvement programs for a particular breed. Molecular markers have been comprehensively exploited, throughout the world, to access this variability as they contribute information on every region of the genome, regardless of the level of gene expression. Simple sequence repeats or Microsatellites, as coined by Litt and Luty (1989) are highly polymorphic and are presently the most favoured molecular markers, essentially owing to the option of blending their analysis with use of the polymerase chain reaction (PCR). Microsatellites have been effectively exploited to understand bovine domestication and migration pattern (Loftus *et al.* 1994; Edwards *et. al.* 2007) and to evaluate genetic diversity and

relationships among populations (Metta *et al.* 2004; Mukesh *et al*. 2004, Lei *et al.* 2005; Tantia *et al.*, 2006; Vijh *et al.,* 2008; Kathirawan *et al*., 2010; kataira *et. al.* 2010; Mishra *et al.,* 2009, Kathiravan *et al.,* 2009; Arora *et. al.* 2004, Barker *et. al.* 1997). The genetic characterization of a population and its diversity analysis is a mandatory pre-requisite for planning any breed improvement programme and for designing strategies for their improvement and conservation. 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 species over time. Hence, the present study is envisaged with the following objectives.

Objectives:-

1. To phenotypically characterize Diara buffalo.
2. To characterise the production traits of Diara buffalo.
3. To analyse genetic diversity of Diara buffalo using Short Tandem Repeats (STR) markers.

CHAPTER No.2

REVIEW OF LITERATURE

Buffalo is premier dairy animal of India and holds the greatest promise and potential for milk, meat and draught. The population of Buffalo which is characterized phenotypically and genetically, are under greater focus for their genetic improvement. The remaining population which is uncharacterized one, is under indiscriminate breeding leading further deterioration of their genetic production potential. The present study on Diara Buffalo was untaken to characterize them phenotypically and genetically. Considering this and objectives of study, available literature's were reviewed under following breeding.

1. Morphological Characterization
2. Genetic Diversity

2.1 MORPHOLOGICAL CHARACTERIZATION

2.1.1. Breeding Tract

Diara buffaloes are medium built animals distributed almost linear along both sides of banks of Ganges called Diara and its tributaries from Buxar to Bhagalpur district in Bihar and to some extent in the territories outside along this line. The Diara region of river Ganga along with its banks which is characterized by sandy alluvial and marshy soil. The animals and life of this area experienced harsh climatic conditions of all sorts viz. heavy flood rainy season, hot and humid, hot & dry summer, chill winter, scarcity of food etc. The region where Diara buffaloes survives is almost marshy in most part of the year and getting dried for cultivation only during dry-weather conditions.

2.1.2. Ecological Settings

The Bihar plain is divided into two unequal halves by the river Ganges which flows through the middle from west to east. The state of Bihar has 94,163 sq. km area. Bihar is located in the eastern part of the country and lies mid-way between the West Bengal in the east and the Uttar Pradesh in the west. Its boundary touches Nepal in north and Jharkhand state in south. Geographically, the breeding tract lies between 25°N and 26°N latitude and between 84°E and 90°E longitude in the middle Gangetic plains of India. Its average elevation above sea level is

ranged between 180 to 171 Feet. Bihar has an area of 93.6 lakh hectares, accounting for nearly 3 percent of the country's total geographical area.

Fig No. 3.1 Breeding tract of Diara Buffalo

Fig. No. 3.2. Ecological Zone of Ganga River

Primarily, the climate is sub-tropical with peak summer temperatures averaging around 40 degree Celsius during March-May and winter months during December-January recording temperatures averaging around 8 degree Celsius. Kharif, Rabi and Zaid are the three agricultural seasons in Bihar, with main crops being rice, wheat and maize, along with various horticultural crops. Northern Bihar receives water from the Himalayan rivers and is largely flood prone. The south of Bihar benefits from the rivers of central India, but it is prone to drought. The Bihar's agro-climatic zones are partitioned into three parts namely North West Alluvial Plain, North

East Alluvial Plain and South Bihar Alluvial Plain. These features determine the soil characteristics, geographical terrain, rainfall and temperature, which together influence its cropping pattern. The breeding tract of Diara buffaloes is found in major the alluvial plains of South Bihar which is generally characterized by relatively low average rainfall around 1102 mms, with 17 districts falling in this zone (Bihar Economic Survey 2018-19, Government of Bihar).

2.1.3. Status of Buffaloes in the Breeding Tract

There are sixteen well recognized breeds of buffalo in India which constitutes about 30% of the total buffalo population in the country. However, 70% of the total buffalo population in the country is classified as non-descript because adequate efforts have not been made so far to characterize them phenotypically and genetically. The milk production in India is currently estimated to be 165.4 million Tonnes in 2016 – 17 with 6.4% growth rate, of which 49.2% milk is being contributed by indigenous (35.4%) and Non–descript (13.8%) buffalo population (BAHFS,2017-18). The in–milk buffalo population in India is reported to be 92.6 million Tonnes. The Bihar had presented increasing trend of milk production from 2012 to 2017 and has produced 8.7 million Tonnes milk (BAHFS 2017). The out of which Buffalo milk 3.35 million Tonnes produced in Bihar contributed 4.1% milk to total milk 81.2 million Tonnes produced by Buffaloes in India. The average milk yield per In–milk Buffalo presented increasing trend from 3.95 Kg/day (2012 -13) to 4.3 Kg/day (2016 – 17) in Bihar. The normal average productivity of Buffaloes is 5.23 Kg/day in India with significant high productivity 8.39 Kg/day in Haryana and 8.21 Kg/day in Punjab. Bihar possess 4.93 % (2.1 million) In-milk Indigenous Buffaloes out of 42.56 million Tonnes In–milk Buffalo of India (BAHFS 2017). Moreover, Buffaloes of Bihar are kept under indigenous category and no Buffaloes under Descript category. While majority states in India had categorized their indigenous buffaloes into descript and non–descript Buffaloes after undertaken proper scientific study. Therefore, field level study of Buffaloes of Bihar need to be undertaken to ensure livelihood of rural people with aim to enhance their productivity.

Singh, R., *et. al.* (2017) reported that Water buffalo population of Bubalus bubalis species particularly is widespread in tropical and subtropical countries with hot and humid climates. Among all the domestic animal species, buffalo alone contributes highest in milk as well as meat production making India top country in the world for milk and buffalo meat production. The buffalo therefore is one of the important livestock species with significant

contribution to Indian economy. Besides the economic importance, buffalo population like Chilika of Odisha state also helps to maintain the natural ecosystem and germplasm of this population needs to be preserved. The Chilika breed, which recently has been registered, is reared under unique management conditions, having quality products, like curd.

Dhillod, S *et al.* (2017) studied to correlate the milk yield of Murrah buffaloes with certain body parts measurements on 70 lactating Murrah buffaloes maintained at Buffalo Farm, Lala Lajpat Rai University of Veterinary and Animal Science, Hisar. The value of different Traits studied are given table below.

Table-2.1: Mean of different body parts measurements along with SEs.

Body measurements	**Mean±SE**
MY (kg)	2604.77±39.47
BW (kg)	556.11±4.91
BL (cm)	152.23±0.83
MW (cm)	17.25±0.10
HW (cm)	135.78±0.46
AG (cm)	226.27±4.78
CG (cm)	214.57±1.17
TL (cm)	97.79±1.31
BDF cm)	76.23±1.27
BDR (cm)	83.58±2.14
HBD (cm)	62.24±0.48
PBD (cm)	39.03±0.46
STK (mm)	8.0±0.02

The different body measurements can be helpful as a selection tool to enhance and evaluate the production potential by setting standards of Murrah buffalo breed. BW, abdominal growth, muzzle thickness, and STK were found key factors while selecting a dairy Murrah buffalo.

Vohra, V., *et. al.* (2015) characterized phenotypically taking 233 records of adult Gojri buffaloes from Punjab and Himachal Pradesh states of India for 13 body biometric traits viz. height at withers, body length, chest girth, paunch girth, ear length, tail length, length of tail up to switch, face length, face width, horn length, circumference of horn at base, distances between

pin bone and hip bone. Traits were analyzed by using varimax rotated principal component analysis (PCA) with Kaiser Normalization to explain body conformation. The value of 13 body biometric traits are given Table no 2.2. PCA revealed four components which explained about 70.9% of the total variation. First component described the general body conformation and explained 31.5% of total variation and can be used in the evaluation and comparison of body conformation in buffaloes and thus provides an opportunity to distinguish between early and late maturing to adult, based on a small group of biometric traits to explain body conformation in adult buffaloes.

Table No. 2.2: Communalities and unique factor of various biometry traits in adult female Gojri buffalo

Biometry traits	Communalities	Unique factor
Horn length	0.448	0.552
Paunch girth	0.571	0.429
Face width	0.659	0.341
Horn circumference	0.668	0.332
Height at withers	0.672	0.328
Face length	0.698	0.302
Body length	0.701	0.299
Chest girth	0.742	0.258
Ear length	0.783	0.217
Tail length	0.803	0.197
Pin bone distance	0.804	0.196
Tail length up to switch	0.827	0.173
Hip bone distance	0.835	0.165

Vohra, V., *et. al.* (2017) analyzed 18 body biometric traits in 157 adult female water buffalo from Chhattisgarh state of India using Principal component analysis (PCA) with a varimax rotation to deduce the components that control body conformation, suitable for use in buffalo breeding, and to reveal the main sources of their shared variability. First principal component explained 34.47% of total variance in body biometric traits and can be used in the evaluation and comparison of body morphology in female water buffaloes using body height, neck circumference, rump width, leg length, paunch girth, chest girth and tail length.

Table 2.3: Communalities and unique variance of biometry traits In Chhattisgarhi buffalo

Trait	Communalities	Unique variance
Horn length	0.921	0.079
Neck length	0.869	0.131
Tail up to switch	0.854	0.146
Tail length	0.823	0.177
Pin bone width	0.812	0.188
Body length	0.758	0.242
Body height	0.736	0.264
Face length	0.716	0.284
Ear length	0.700	0.300
Neck circumference	0.675	0.325
Leg length	0.665	0.335
Face width	0.635	0.365
Rump width	0.631	0.369
Chest girth	0.619	0.381
Distance between horns	0.604	0.396
Paunch girth	0.557	0.443
Horn circumference	0.538	0.462
Rump length	0.511	0.489

The measurement value of these different traits are given Table no. 2.3. The shared variability due to common variance ranged from 92% (horn length) to 51% (rump length) whereas 8 to 49% of their variation was contributed by unique variance specific for each trait in Chhattisgarhi buffaloes.

Chandran, P. C. *et. al.* (2015) studied Morphometric and body weight traits of Diara buffaloes and found that they (Diara buffaloes) are medium-sized animals with prominent

forehead and loosely curved horns. They are smaller than the heavy-sized breeds like Murrah, Jaffarabadi and Nili-Ravi. The height, length and girth of female Diara buffaloes were 94.96±0.58 cm, 92.96±0.49 cm and 115.93±0.81 cm up to 1 year of age and 133.60±0.69 cm, 138.36±0.74 cm and 200.79±0.95 cm above 7 years of age, respectively. Estimated adult body weights of Diara buffaloes pooled over ages were found to be 494.99±27.15 kg in males and 483.21±3.58 kg in females. Diara buffaloes are good milkers with an average per day milk production was found to be 7.8 litre and peak yield reached up to 10.5 litre per day. Diara population remains so far largely untouched and breed improvement programmes, probably involving selective breeding, could be undertaken to further enhance the genetic potential of these buffaloes.

Table No.-2.4 Mean of different body parts measurements along with SEs of Diara buffaloes

Traits	> 7 years male	> 7 Female
Height at withers	142.33±2.92	133.60±0.69
Body length	150.67±3.62	138.36±0.74
Chest girth	206.50±2.63	200.79±0.95
Face length	53.33±2.12	54.17±0.42
Face width	23.33±0.95	21.21±0.20
Ear length	30.67±1.67	26.11±0.18
Tail length	89.67±4.39	83.63±0.79
Horn length	31.00±0.89	28.96±0.30
Horn circumference	29.17±0.75	22.80±0.21

Figures in round parentheses are number of observations whereas figures in the square parentheses are coefficients of variation.

Mishra, B. P., *et. al.* (2009) analyzed data on 397 adult milking buffaloes of Banni buffaloes distributed in the Kachchh region of Gujarat state. Banni buffaloes are medium to large in body size with a compact body and typical coiled horns. Mean body length, heart girth and height at withers estimated during the survey were 153.7±0.4 cm, 205.5±0.6 cm and

136.7±0.2 cm, respectively. The various reproductive traits of Banni buffaloes reported the mean age at first calving to be 39.7±0.4 months, a mean service period of 66.4±1.3 days, a mean lactation length of 293.3±1.5 days and a mean peak yield of 15.7±0.1 litres.

Javed, K., *et. al.* (2013) analyzed 1180 records on linear type and body measurements traits maintained at 5 Institutional herds (Pattoki, Chack Katora, Haroonabad, Khushab, Rakh Ghulaman) in Punjab and few private breeder's farms of Pakistan. Mean score for height at sacrum (135.766±4.401cm), for bone structure (5.344±1.787), for dairy form (5.619±1.203), for horn diameter (18.646±2.059 cm), for ear length (29.5±2.118 cm), for tail length (103.515±12.551 cm), for rump length (43.516±2.582 cm) and for average score day milk yield 6.85±2.19 kg were recorded. Height at sacrum (0.26) and ear length (0.163) and rump length (0.158) were positively and significantly correlated with score day milk yield. Bone structure was found to be negatively correlated (-0.219) with score day milk yield and it was highly significant. Dairy form, horn diameter and tail length were not correlated with score day milk yield. The results of the present study indicate that most of the linear type traits and body measurements in Nili Ravi buffaloes fall under the intermediate value when compared with other buffalo breeds. All the traits had variation among the herds. Positive correlation between milk yield and other traits like height at sacrum, ear length and rump length need further investigations to reach at some conclusion to include these traits in selection strategy for improvement in milk yield.

Sapkota, S., *et. al.* (2017) conducted a field study understand the productive performance, morphometric measurements and qualitative traits of 20 adult buffaloes in Eastern Terai of Nepal. Results of present study reflected that the age at first calving was 3.75±0.14 years, whereas, total number of calves born per buffalo was observed 1.75. Similarly, number of lactations in this study was recorded 3.2 times, daily milk yield (1.6 liters), lactation length (9.5 months), and peak yield (3.8 liters). Hand milking was common practice for majority of the farm households. Majority of the buffalo population in the study areas were naturally mated. Morphometric traits such as body weight was recorded as 331.5±7.6 kgs, chest girth (170.5±4.5 cm), body length (128.5±3.0 cm), height at withers (132.5±4.1 cm), hock circumference (19.9±0.4 cm), tail length (79.6±6.0 cm), ear length (28.5±0.9 cm), and horn length (32.76±2.1 cm). Regarding to other morphological traits, majority of the buffaloes were observed plain, black and pigmented coat color, skin, muzzle, eyelid, eye, and hooves (with white spots on forehead, legs and tail switch). Similarly, almost 95% buffaloes had fixed horn attachments

with black (70%) and grey (30%) horn color. Haemorrhagic septicemia (HS), tapeworm infestation, weak legs were the major problems prevalent in the study areas. Whereas inbreeding, repeat breeding, low milk productivity and shortage of high quality local buffalo bull for breeding were other important problems for buffalo herders in the region. Thus the information observed and reported in this study would have great importance in developing effective buffalo improvement plans focusing Terai regions in the future.

Thiruvenkadan, A. K.*, et. al. (*2013) the contribution of buffalo (Bubalus bubalis) to the Indian agrarian economy is considerable by way of milk, meat and draught power production and as a source of security that requires minimum inputs. The domesticated buffaloes in Indian are mainly of river type with small number of swamp buffaloes present mainly in north-eastern part of India. India is having 56.70 percent of the world buffalo population and they supply 68.21 percent of the total milk produced around the world. The river buffaloes of the Indian sub-continent are maintained chiefly for milk production, but all of them are also dual purpose animals, exhibiting good meat characteristics. The swamp buffalo is more or less a permanent denizen of marshy lands, where it wallows in mud and feed on coarse marsh grass. India has rich repository of buffalo breeds with 13 recognized breeds and the best known breeds of buffaloes are Murrah, Nili-Ravi, Jaffarabadi, Surti and Mehsana. The germplasm of such well-defined breeds constitute a valuable genetic resource which needs to be conserved on priority basis. The rich biological diversity of this species is progressively being eroded due to unplanned breeding. Except in few organized farms which maintain small herds of pure breed, there is almost unrestricted interbreeding among different breeds and there is a marked decline in the availability of unique animals conforming to the attributes of defined breeds, particularly in their native breeding tracts. The situation is further complicated by the fact that there exists no breed societies or breed registration/ improvement societies to register animals of specific breeds, maintain herd books and ensure the purity of the breeds. Hence, proper conservation measures have to make to preserve the valuable buffalo genetic resources of India for the sustainable utilization.

Sahu, S., *et. al.* (2017) studied on records of 198 adult animals (67 male, 131 female) Sambalpuri buffalo for conformation, production and reproduction traits at farmers' level. These data were subjected to least squares analysis and Duncan's multiple range test. The overall least square means for body length, heart girth, height at withers, paunch girth, head length, horn length, tail length, body weight were found to be 132.54±0.72 cm, 177.90±0.71

cm, 125.50±0.46 cm, 189.06±0.82 cm, 50.73±0.36 cm, 55.29±0.55 cm, 81.33±0.90 cm and 411.47±5.34 kg respectively. The overall least square means for age at first calving, calving interval, gestation period, average daily milk yield, lactation length, dry period and lactation yield were 1488.42±3.24 days, 533.92±2.12 days, 314.19±0.55 days, 2.95±0.08 litres, 282.26±2.69 days, 251.15±3.46 days and 809.57±18.75 litres respectively.

Table No. 2.5: Least square means with standard error for conformation, production and reproduction traits of adult Sambalpuri buffaloes

Sl. No.	Traits	Overall
1	Body length (cm)	132.54 ± 0.72
2	Heart girth (cm)	177.90 ± 0.71
3.	Height at withers (cm)	125.50 ± 0.46
4	Paunch girth (cm)	189.06 ± 0.82
5	Head length (cm)	50.73 ± 0.36
6	Horn length (cm)	55.29 ± 0.55
7	Tail length (cm)	81.41 ± 0.79
8	Body weight (kg)	411.47 ± 5.34
9	Age at first calving (days)	1488.42 ± 3.24
10	Calving interval (days)	533.92 ± 2.12
11	Gestation period (days)	314.19 ± 0.55
12	Average daily milk yield (lt)	2.95 ± 0.08
13	Lactation length (days)	282.26 ± 2.69
14	Dry period (days)	251.15 ± 3.46
15	Lactation yield (lt)	809.57 ± 18.75

Horn length difference was found to be significant ($P<0.01$) among localities but, between sex it was non-significant. All other conformation traits were found to be non-significant among localities. However, there exists a significant difference between two sexes ($P<0.01$) in relation to all conformation traits except horn length and tail length. The effect of localities were significant ($P<0.01$) on all the production and reproduction traits except for gestation period.

Shankar and Mandal (2014) conducted experiment on 60 randomly selected dairy units consisting of 116 Graded Murrah,70 Diara type and 121 Non-descript type buffalo cows

utilizing the procedure of stratified random sampling with proportional allocation (Snedecor & Cochran,1967) in and around Patna. Genetic factors were the three different genetic groups of buffaloes viz. Graded Murrah, Diara and Non-descript types prevalent in Bihar Whereas Non-genetic factors included in the study were location of herd, faming system and sequence of lactation. The average estimates of body weight of Graded Murrah, Diara and Non-descript were found to be 508.972+3.36, 461.789+3.32 and 483.857+3.30 kg respectively. The three genetic groups of buffaloes differed significantly ($p<0.05$) among themselves with respect to their body weight. Farming system and lactation order had significant ($p<0.01$) influence on body weight. Body weight of the animals was the lowest at first parity and then increased significantly ($p<0.05$) in subsequent parities.

Singh P. K., *et. al.* (2007) documented Gangatiri breed which is an important dual-purpose cattle breed of Uttar Pradesh state of India. They reported average daily milk yield of Gangatiri cows ranged between 4-6 Liters/day with a Lactation length of 150 to 250 days. The inter-calving period varied between 14 months to 24 months. The average body length, height at withers, chest girth, face length, ear length and tail length were recorded as 110, 124, 153, 46, 25 ,85 cm, respectively, in adult cows and 121, 142, 168, 52, 27 and 9l cm, respectively, in bullocks under field conditions, Overall body measurement of the breed was lesser than that of Hariana cattle. The breed is significantly contributing to the livelihood of the people due to its good draught ability and average milk production. Therefore, sincere efforts should immediately be undertaken for its genetic improvement by using semen of the Gangatiri bulls in the breeding tract.

Singh P. K., *et. al.* (2014) studied Sanchori cattle, having good milk production potential, are maintained by the farmers of Jalore district of Rajasthan. The animals are kept in herds with size varying from 2–10 animals under semi-intensive production system and stall feeding. Age at first calving, lactation length, calving interval, dry period and service period of Sanchori cows were found in the range of 36–48 months (average 39.5 months), 8–15 months (average 10.16 months), 12–20 months (average 14.4 months), 0.5–10 months (average 4.3 months) and 2–11 months (average 5.44 months), respectively. The life span of Sanchori cattle was found as 20–25 years with 12 to 15 lifetime calving. Average daily milk yield of Sanchori cows ranged from 3.05 to 16.3 litre with an average of 9.08 litre. Keeping in view the declining

population status of Sanchori cattle, there is a need to take up suitable measures for its genetic improvement and conservation.

2.2 GENETIC DIVERSITY

Genetic diversity primarily defined as the variety of alleles and genotypes present within and between populations of organisms and this can be reflected in morphological, physiological and behavioral differences between individuals of the population and between populations. Four elements of genetic diversity are usefully distinguished; the number of different forms (alleles) ultimately found in several populations, their distribution, and the effect they have on performance and the overall distinctness between different populations. Genetic diversity also encompasses distinct populations of a particular species. Each species holds a huge amount of genetic information in the form of traits, characteristics, etc. (http://edugreen.teri.res.in/explore/life/genetic.htm).

Genetic characterization of the native animal is the first step in the breed conservation program of the animal genetic resources and it also has implications in future breeding strategies. It is the description of the genome to measure the genetic diversity and the uniqueness of the breed. There are more than 40 domestic livestock species, within this tiny slice of biological diversity. Only 14 % of those species contribute to 82 % of the world's food and agriculture production. Over the last 12000 years, these 14 species are domesticated and have evolved into separate and genetically unique breeds adapted to native environments and community needs. Domestic animal diversity is the spectrum of genetic variations among and across all breeds and species used in agriculture (World Watch List 2000).

Extinction of endangered farm animal breeds results in an irreversible loss of genetic diversity. For biological, economic and cultural reasons conservation of genetic diversity is very important. The genetic diversity found in domestic breeds permits farmers to develop new characteristics in response to changes in atmosphere, diseases or market conditions. Additionally, the loss of genetic diversity could be a loss of the history of civilization.

Analysis of genetic variability and relationship among the variety of livestock breeds is important to deal with genetic resources and their continual usage (Vijh *et al*., 2008). This is the most crucial step when the livestock species like buffalo, cattle camel, have shown a decline in the headcount during the last couple of years. The factor that is responsible for the decreasing genetic diversity of livestock are:

- Destruction of native habitats of livestock breeds.
- Genetically uniform livestock breeds development.
- Farmers or consumer preference for certain varieties and breeds.

2.2.1 Livestock Diversity

As per FAO Commission on genetic resources for food and agriculture assessments, 2019 reports 7,745 extant local breeds of livestock, 26% are at the risk of extinction. Most of the AnGR is yet to be characterized at the genetic and phenotypic level. They are altogether used in numerous ways for the production of food, to generate livelihood, to transport goods, plow fields, to fertilize fields with manure and major source of earning to low land and landless farmers, etc. Livestock diversity plays an important role in genetic improvement so this diversity must be kept for the future as well as for current use. About 17% (1458) of the world's farm animal breeds are facing the risk of extinction, while the risk status of many others breeds (58%) is still unknown due to a lack of phylogenetic data and structure of their populations (Livestock census, 2012). This enormous task has convinced FAO and other international organizations to create a domestic animal diversity information system and databases (ILRI, Domestic Animal Diversity Information System (DAGRIS)) (https://www.ilri.org/dagris). FAO and the Commission on Genetic Resources for Food and Agriculture, manage, supervise, check and support the implementation of the Global Plan of Action by: (I) Organizing intergovernmental meetings and yielding global status and current reports; (II) maintaining and establishing the breed database DAD-IS; (III) introducing an online society of practice to talk over animal genetic resources (Livestock diversity multiple functions in multiple production systems, FAO).

According to the FAO Global Databank for Farm Animal Genetic Resources (FAnGR), data on population size are available for approximately 4,183 breeds. At present, 740 breeds are reported as extinct, and 1335 (32%), are categorized as high risk of destruction that may lead to their extinction. Without appropriate action, more than 2,000 domestic animal breeds could be lost within the coming decades (Food and Agriculture Organization of the United Nations, http://www.fao.org/).

It is important to characterize them based on genetic and phenotypic polymorphism for the process of selecting and designing breeding and conservation strategies of livestock species. The markers used to evaluate polymorphism previously were morphological markers,

biochemical markers or chromosomal markers. Based on physical appearance such as pigmentation, animals were selected in morphological markers, and the disadvantage is a lack of polymorphism. Subsequently, the focus was on selecting animals using biochemical markers such as Hb (hemoglobin), amylase, blood groups, etc. because they were sex-limited, age-dependent, environment-influenced and, moreover, covering less than 10% of the genome. Chromosomal markers, which select the animals based on structures such as deletions, insertions, etc., and numerical variations of chromosomes like trisomy, monosomy, nullisomy, also had the disadvantage of low polymorphism. In the 1960s, considering the organization of eukaryotic genomes (Britten and Kohne 1968), the main procedure for estimating differences at the actual genomic DNA level was produced.

Then DNA-DNA hybridization came into the picture depending on the thermodynamic re-annealing properties of single-stranded heterologous DNA arrangements. Direct approaches to the DNA sequence overcame the hypothetical and practical difficulties of the hybridization of DNA-DNA (Avise *et al*., 1994). Subsequently, molecular markers such as RFLP (restriction fragment length polymorphisms) revolutionized the field of genetic characterization following the discovery of endonucleases (REs) of bacterial restriction that split into specific restriction sites (Meselson and Yuan 1968).

RFLP markers were first shot in the genome revolution and used for the analysis of diversity and phylogenetic molecular analysis (Botstein *et al*., 1980). These are codominant markers and low polymorphism is the only disadvantage. The previously used Southern blotting technique was based on separating bands first and then hybridizing on paper with nitrocellulose. Later PCR (polymerase chain reaction) invented by Mullis, K., in 1986 replaced Southern blotting in RFLP analysis and several PCR-based markers such as RAPD, AFLP, VNTR, STR, etc.

Among PCR based procedures, DNA polymorphisms were tested using a technique developed by Williams et al., in1990 called RAPD (Random Amplification of Polymorphic DNA), using arbitrary single primers (about 10 nucleotides). RAPD procedures use random amplification of genetic segment, where the primer binds hence known as RAPD (Random amplified polymorphic DNA) markers. Low polymorphism is a major disadvantage, and the dominant RAPD markers are based on genetic variations at primary binding sites. The RAPD technique is popular in the plant population geneticist community, but due to lack of specificity

and repeatability, it has failed to gain ground in a wider context. Mother PCR-based technique developed during the 1990s was AFLPs (Amplified Fragment Length Polymorphism) which is dominant than RFLP and RAPD markers. Due to time-consuming, the use of radioactive labels or special staining techniques such as silver staining, these are not widely used for characterization. Also not very popular is the bi-allelic and co-dominant gel based resolution of DNA fragments called SSCP (Single Stranded Conformation Polymorphism). These methods were not widely applied in evolutionary studies for one or the other reasons. The focus was later on a new class of hypervariable DNA regions, first revealed in the genome by Southern blot analysis of repetitive elements known as minisatellites (Jeffrey *et al*., 1985). Due to differences in the number of repeats, these are also known as VNTR (variable number of tandem repeats). This change in repeat copy numbers due to the high rate of unequal crossover during meiosis results in hyper-variable patterns. The repetitive sequence detection samples have been developed and the methodology surrounding this technique has been called DNA fingerprinting (especially for humans). The complexity of the gel profiles, which contain 20 or more scorable bands, the high rate of mutation to new variants of length, and the difficulty of achieving consistent results, have generally precluded their wide applications in evolutionary genetics. Microsatellite markers have become prominent among the genetic marker types used to study population genetic relationships between closely related individuals or populations in recent years. The main reason for their popularity is because of the presence, even within a population, of a large number of alleles at a single locus. These are highly polymorphic and are widely distributed throughout the genome, enabling closely related animals to be distinguished at the DNA level.

2.2.2 Microsatellite Markers

Microsatellite markers have been developed for human genetic mapping (Litt and Lutty1989, Weber and May 1989). These are also referred to as simple tandem repeats motifs of 1-6 length bases, short tandem repeats of two, three or four nucleotides, and are found throughout the genome. To date, the type most thoroughly studied is dC-dA type repeats. Also known as simple sequence repeats (SSRs), short tandem repeats (SSTRs), simple sequence tandem repeats (SSTRs), variable tandem repeats (VNTRs), simple sequence length polymorphisms (SSLPs), sequence tagged microsatellites (STMSs) (Teneva *et al.*, 2009). Because the number of tandem repeats can vary greatly at a locus, SSR markers tend to be among the most polymorphic types of genetic markers. For example, one allele could have 10

copies of the CA tandem repeat (CA) 10, where another would have 11 copies (CA) 11, 12 copies (CA) 12 and so on. The occurrence frequency of SSR in the mammalian genome is one SSR per 6-10 Kb of genomic DNA (50,000-100,000). Due to different numbers of repeats, polymorphism is due to the high rate of mutation. By differentiating even a single nucleotide, their short length makes them suitable for PCR amplification and allele resolution. The polymerase chain reaction (Weber, *et al*., 1990) can amplify alleles at microsatellite loci, even from small amounts of genomic DNA, and the alleles are separated and precisely sized on a polyacrylamide gel as one or two bands or fragment analysis by means of an automated DNA sequencer and used to quantify genetic variations within and between species populations. Specific microsatellite markers are amplified using the specific primers designed from the preserved adjacent region (Saiki *et al*., 1988). For population genetic studies of different animal species, these markers have been widely used (Azhar *et al*., 2018).

2.2.3 Microsatellite markers based genetic diversity in buffalo.

In the Indian context, Buffalo has particular importance for both dairy and meat production. With contributions from 16 registered breeds, which make up approximately 57 % of the country's total 108 million buffalo population and several unregistered and graded populations, India ranks top in both sectors. It is most important to analyze the genetic structure before designing any conservation and breeding policies for any livestock breed or population. Microsatellite was widely used among the various markers discussed above to characterize different breeds/populations of buffalo.

Barker *et al*., (1997) analyzed genetic diversity in 11 Asian water buffalo populations using 21 microsatellites compared to 25 polymorphic protein-coding loci, 8 showing significant departures from the equilibrium of Hardy-Weinberg. Based on their findings, they concluded that at least 10,000-15,000 years ago riverine and swamp bifurcated. Swamp and river types of water buffaloes were significantly differentiated. Microsatellite markers based on genetic diversity in Buffalo.

Van Hooft *et al*., (1999) used 168 bovine microsatellite markers to study seven African buffalo populations and observed amplification in 139 markers, and 91 markers were found to be polymorphic and concluded there suitability for use in characterizing African buffalo genetic diversity. Subsequently, Van Hooft *et al*. (2000) investigated the effects of Rinderpest

epidemics and habitat fragmentation on nine African buffalo (Syncerus caffer) populations using 14 microsatellites. Based on the data analysis, the workers have been able to conclude population dynamics and migration as well as the differentiation of subspecies between African buffaloes. The effect of habitat fragmentation was investigated by O'Ryan *et al*., 1998 in African buffaloes from four populations in South Africa. The genetic variation measured was 105. Seven polymorphic microsatellite markers have been used to quantify population levels of heterozygosity, allelic diversity, and genetic differentiation. There was a significant correlation between the amount of genetic variation and population size and differentiation was detected among all populations measured by F_{ST} and R_{ST} Based on their results, the workers were able to draw inferences about the conservation management of fragmented buffalo populations, especially where natural gene flow was no longer possible. (Moioli *et al*., 2001) evaluated genetic diversity in three Mediterranean, Italian, Greek, and Egyptian buffalo populations by using 13 microsatellites and detected 2 to 19 alleles per locus. The main difference is present in 5 different loci in 3 populations of buffalo and the degree of differentiation observed was greater in Egyptian than in Italian and Greek buffalo. They concluded that microsatellites are also useful in finding geographic population distribution. In a panel of 25 buffaloes, (Navani *et al*., 2002) used 108 microsatellite primers from cattle and found 75% discrete amplification and in that 50% showed polymorphic bands and concluded that cattle-specific primers could be used successfully in the buffalo genome analysis.

Arora *et al*., (2004) used a set of heterologous bovine specific microsatellite markers to evaluate genetic diversity in two Bhadawari and Tarai Northern India buffalo populations. The average number of alleles in both populations across all loci was found to be 4.7 and the PIC value for these markers was 0.54. For both populations, the average observed and expected heterozygosity was 0.59 and 0.64 respectively. They found genetic variability in both populations by seeing common alleles with varying allele frequencies, but low population differentiation was observed. Their data show that bovine microsatellite markers are suitable for the analysis of the genetic diversity of buffalo.

Tantia *et al*., (2006) produced data from 3 buffalo populations on 24 microsatellite loci. Bhadawari, Tarai, and Kerala populations were analyzed for genetic distance estimation. Phylogenetic analysis revealed that the populations of Tarai and Bhadawari were close to each other and this was possible due to the continuity of their breeding tract, which resulted in increased gene flow.

Kumar *et al*., (2006) classified the Toda, Jaffarabadi and Pandharpuri breeds as one lineage: Bhadawari, Nagpuri, Surti, Mehsana and Murrah breeds as admixture based on 27 microsatellite marker diversity analysis. The use of AMOVA rendered morphological and physiological classification invalid and concluded that genetic analysis provides a more suitable classification system than the physiological and morphological classification. The genetic distance (Ds) between the breed of Mehsana and Bhadawari was the maximum (0.29) and the lowest Ds of 0.05 between the breeds of Jaffarabadi and Nagpuri. The time of divergence between Mehsana and Bhadawari breeds was 1318 years, while among the Jaffarabadi and Nagpuri breeds it was found to be the lowest (272 years).

Zhang *et al*., (2007) analyzed the genetic diversity and evolutionary relationships among 18 indigenous Chinese swamp buffalo populations using 30 microsatellite markers and found that patterns of genetic differentiation and genetic relations between Chinese buffalo populations were consistent with their geographic distribution. By using 22 microsatellite markers, Vijh *et al*., (2008) investigated diversity among 12 buffalo breeds and found 8 different clusters from 12 populations by genetic distance analysis and concluded that different breeds were domesticated for various purposes at different locations. They found buffalo from Kalasthi (South India) genotypically similar to the endangered breed of Toda buffalo.

Aminafshar *et al*., (2008) used 14 microsatellite markers to analyze diversity in Guilan buffalo in Iran's South and Southwest Caspian Sea area and reported high genetic diversity in Guilan buffalo.

Sraphet *et al*., (2008) used cattle microsatellite markers to analyze diversity on 105 Thai swamp buffaloes and found 16 markers out of 34 markers to successfully amplify DNA buffalo with a range of 2 to 9 per locus. They concluded that cattle markers may not be ideal for studying the Thai swamp buffalo's genetic diversity.

Mishra *et al*., (2009a) used cytogenetic, microsatellite and mitochondrial D-loop sequencing to characterize Chilika buffalo and concluded that Chilika buffalo is a type of riverine rather than a type of swamp. By using 25 microsatellite markers on 45 random Chilika buffalo samples, the workers found a total of 117 separate alleles. It was found that the highest number of alleles was 8(ILSTS052 and ILSTS029) and that the lowest was 2 (ILSTS073). The number of alleles observed was higher than the effective number of alleles. Compared to Murrah (5.16) and Nagpuri (5.28), the workers found Chilika showing lower mean allelic

diversity, but slightly higher than Toda (4.64) buffalo values. Chilika resembled genetically closer to Nagpuri based on microsatellite data, followed by interbreeding analysis of Murrah and Toda buffaloes.

Kataria *et al.*, (2009a) found heterologous bovine microsatellite markers useful for assessing genetic variability in buffalo breeds using a set of 25 microsatellite markers on silver stained PAGE gel. The PIC values ranged from 0.10 (ILSTS019 locus) to 0.81 (ILSTS058 locus) with an average of 0.53 for the microsatellite loci. They noted that no bottleneck existed in Nagpuri, located in central India. Diversity indices suggested sufficient genetic variability for future breeding and conservation strategies with useful in Nagpuri buffalo.

South India's Toda buffalo was assessed for its genetic variability using a set of Kataria *et al.*, (2009b) of 25 bovine specific heterologous microsatellite markers. A total of 25 STR markers are used on 48 samples and 105 alleles have been detected in Toda buffalo with an effective mean number of alleles being 2,661. In the recent past, the 3 models IAM (Infinite Allele Model), TPM (Two-Phase Model) and SMM (Step-Wise Mutation Model) were used to analyze data.

Rupinder *et al.*, (2009) reported heterozygosity of 0.49 and 0.53 respectively with an average of 0.51 in Bhadawari and Murrah breeds. In Bhadawari, the average PIC value over all loci was found to be 0.51 and in Murrah breeds 0.52. The standard genetic distance (Ds) and genetic identity were 0.13 and 0.88 respectively between Bhadawari and Murrah breeds. Private alleles were observed in Bhadawari and Murrah i.e. four races in Bhadawari and three races in Murrah.

Kathiravan *et al.*, (2009a) used 24 microsatellite markers in the Nili-Ravi and Murrah buffaloes, located in northern India, for diversity analysis and found that the inbreeding coefficient is high in both breeds. The neighbor-joining tree showed distinct clustering and confirmed by PCA analysis and the workers concluded that these markers had a significant impact on conservation and biodiversity issues based on the study.

Mishra *et al.*, (2009b) analyzed genetic diversity in the breed of Jaffarabadi buffalo using 25 microsatellite markers and found sufficient diversity of alloys with a mean number of 0.536. Different test types, i.e., three quantitative tests, sign test, standardized differential test and Wilcoxon sign rank test, and a qualitative test performed to confirm that the population had

undergone genetic bottleneck in the recent past and concluded that there was no past genetic bottleneck in the population.

Mishra *et al*., (2009c) analyzed genetic diversity using 15 Banni buffalo heterologous bovine markers distributed in Gujarat state's Kachchh region. They found a high degree of polymorphism showing a total of 81 alleles with an average of 5.4 and also found a population deficiency of heterozygotes. Seven out of 15 microsatellite loci have deviated from the equilibrium of Hardy-Weinberg.

In Banni and Murrah buffaloes, Mishra *et al*., (2009d) used 24 microsatellite markers to analyze diversity. They found a total of 138 alleles in two populations, and both populations had a heterozygous deficiency. The lack of genetic bottleneck by qualitative graphical testing was observed in Banni buffaloes. There was a differentiation between the two populations and diversified about 7286 years ago, concluding that the population of Banni buffalo is on decline and needs programs for conservation.

Kathiravan *et al*., (2009b) analyzed genetic diversity in Marathwadi buffalo, a registered breed in central India, using 24 microsatellite markers. They found the presence of allelic diversity with a total of 109 alleles across different loci and also reported a lack of genetic bottleneck in the recent past as confirmed by made qualitative shift testing.

Using 571 microsatellite markers on 24 unrelated buffaloes, Nagarajan et al., (2009) found 498 polymorphic with average heterozygosity of 0.51. Due to the high conservation of cattle microsatellite loci in water buffalo, the workers claimed the usefulness of cattle microsatellite markers on buffalo and also concluded the use of microsatellite markers for radiation hybrid mapping.

Kataria *et al*., (2010) used twenty-four heterologous microsatellite markers to analyze mutation-drift balance and recent bottleneck in Indian riverine buffalo breeds. Infinite allele model of microsatellite evolution showed significant heterozygosity in all seven breeds compared, but this was reversed in a step-by-step mutation model and found that Mehsana was not in two-stage model mutation-drift equilibrium, confirmed by all three statistical methods. Standardized differences testing found excess heterozygosity and Wilcoxon signed-rank revealing possible cryptic demographic bottleneck in Western India's Mehsana buffaloes.

Workers evaluated diversity within Kanarese buffalo by using 23 STR markers and observed an average of 180 alleles of 7.83 per locus. The estimate of inbreeding within the population was significantly positive due to lower than expected observed heterozygosity. They also found that the mutation-drift equilibrium analysis lacked any recent genetic bottleneck and concluded that the conservation of this important germplasm is needed (Kathiravan *et al*., 2010).

Mishra *et al*., (2010b) used 23 microsatellite markers in Assamese buffaloes to observe 133 alleles across different loci. Assamese buffaloes ' genetic diversity analysis showed moderate to high levels of variability within the population. They also found a low deficiency in heterozygosity and no recent genetic bottleneck in the Assamese buffalo population was reported by bottleneck analysis. Berthouly *et al*., (2010) used 17 microsatellite markers to characterize swamp buffalo, which is used primarily in farming systems in Vietnam's Ha Giang provinces. Results showed the need for conservation and improvement for future householders to be high in Ha Giang inbreeding value and genetic diversity. Other workers reproached the presence of large numbers of polymorphic loci and sufficient variability Murrah breed by the presence of sufficient heterozygosity, both observed and expected above 0.5 heterozygosity (Bhuyan *et al*., 2010). Another study showed significant genetic variability within breeds in the breed of Mehsana buffalo based on 11 microsatellite markers (Jakhesara *et al*., 2010). Mishra et al., (2010c) found that buffaloes from Banni and Jaffarabadi were distinct from other riverine buffaloes.

When Phylogenetic tree was built using chord distance estimates, pair-wise MDS display F_{ST} values revealed the proximity of Mehsana, Surti and Murrah buffaloes while Banni and Jaffarabadi buffaloes were placed separately. This genetic structure was further supported by Bayesian clustering analysis, which revealed three inferred clusters, each forming separate clusters with Banni and Jaffarabadi, while the remaining three breeds viz. A single cluster branded Mehsana, Surti, and Murrah together. The results thus revealed Banni buffalo's genetic uniqueness among other regional buffalo breeds.

Cacho *et al*., (2013) found only 10 markers polymorphic out of 45 amplified markers using 110 cattle microsatellite primers on water buffaloes in the Philippines. The mean number of alleles per locus was 5.45 and the mean heterozygosity values observed and expected were 0.56 and 0.50, with an average PIC of 0.5174, respectively. They found Philippine Carabao grouping into a separate cluster, reflecting the breeding history, from American Murrah buffalo

and Bulgarian Murrah buffalo. Acosta *et al*., (2014) also used 16 bovine microsatellite markers to characterize the Cuban water buffalo population and observed a total of 87 alleles with an average of 5.44 alleles per locus. The heterozygosity values observed and expected were 0.46 and 0.54 respectively. For these markers, the overall PIC value was 0.495 and concluded that these markers can be used in the study of genetic diversity and differentiation of our population with others. Twenty microsatellite loci were utilized to define genetic diversity among 56 water buffalo samples in Northern Turkey, North Turkey, and Eastern by Ozkan *et al*., (2014). Three to ten different alleles were identified per microsatellite locus in the total of 103 alleles documented. The PIC values for the microsatellite loci analyzed range from 0.14 (CSSM32) to 0.82 (CSSM47) with a mean of 0.4945.

Bakr *et al*., (2016) analyzed molecular genetic variability from six different locations within and between Egyptian buffalo groups. Genetic diversity was analyzed using 9 markers on 312 samples of 6 breeds and a total of 139 alleles were found with an overall mean of 0.890 ± 0.048 and 0.923 ± 0.017, respectively. The mean fixation index was found to be 0.024 ± 0.017 indicating a wide genetic variation at the molecular level in the Egyptian buffalo. Singh *et al*., (2017) characterization of a local buffalo population performed in Jammu and Kashmir's northern temperate region of India using 15 microsatellite markers on 50 samples and found 103 separate alleles. The workers reported a maximum number of alleles in CSSM13 and CSSM61 markers with an overall value of 0.7913±0.008 to be nine. They concluded that there were sufficient diversity and use of these markers in other indigenous buffaloes for analyzing diversity. Uffo *et al*., (2017) used 30 cattle microsatellite markers for buffalo characterization and reported amplification of 28 markers with monomorphic ETH10 marker in Cuban buffalo populations. A total of 143 alleles with an average number of alleles per Locus to 5.04 were observed. These markers showed an average PIC value of 0.482 and positive inbreeding was found at 14 loci. They concluded that cattle microsatellite markers are suitable for analyzing diversity in buffaloes and using data for future breeding and conservation strategies.

Hussain *et al*., (2017) reported the first genetic diversity based on microsatellite markers in Pakistan's 5 native water buffalo breeds, using 8 microsatellite markers and finding a total of 42 alleles across 6 polymorphic loci were 2 loci monomorphic. The average heterozygosity values observed and expected in all studied buffalo breeds across all polymorphic loci were 0.43 and 0.53 with a total PIC value of 0.53. They concluded data on

genetic diversity supporting the phenotypic differentiation between the breeds of the studied buffalo.

Soysal *et al.*, (2007) used 11 cattle microsatellite loci to characterize Anatolian water buffalo breed and found 4 markers that successfully amplified genomic DNA buffalo with several alleles ranging from 3 to 9. The heterozygosity observed and expected ranged from 0.550 to 0.775 and 0.494 to 0.815 respectively, with Fes ranging from -0.101 to 0.205. They concluded that this buffalo population seemed to be in the expectation of Hardy-Weinberg based on their results.

Marques, *et al.*, (2011) used a panel of 25 microsatellite markers to analyze genetic diversity in Brazilian buffaloes (Carabao, Jafarabadi, Mediterranean, Murrah, and Baio). Baio and Carabao Populations are stable in the equilibrium of Hardy-Weinberg and confirmed that Carabao is a member of a different subspecies through genetic distances, factor analysis, and individual assignment values, forming a cluster separate from Baio, Mediterranean and Murrah buffalo.

Swathi *et al.*, (2018) explored genetic variability in Murrah buffaloes using a set of FAO recommended 30 microsatellite markers. All the loci successfully amplified in Murrah buffalo and the number of alleles observed ranged from 4 to 12 with 239 alleles in total. The overall mean observed heterozygosity and expected heterozygosity values respectively 0.82.6 and 0.841, and all loci were found to be polymorphic with PIC ranging from 0.656 to 0.879 and highly informative. The mean inbreeding coefficient (FIS) recorded was 0.483 and out of 30 markers, 29 differed significantly from the equilibrium of Hardy Weinberg due to animal selection over generations.

2.2.4 Microsatellite Markers based genetic diversity in other species

Using bovine specific microsatellite markers to evaluate genetic diversity among Indian three cattle breeds, Mukesh *et al*., (2004) found an estimated mean allelic diversity of 5.2, 6.5 and 5.9 respectively in Sahiwal, Mariana and Deoni breeds, and a total of 167 alleles. The observed heterozygosity value was lower than expected in Sahiwal compared to two other breeds, which were speculated to be due to the relatively small population size. F_{ST} estimates showed 88.7 % of total genetic variation in breeds Hariana and Deoni showed close genetic

similarity to Sahiwal phylogenetically. Nguyen *et al.*, (2005), tested the suitability of 131 bovine microsatellite markers for Swiss yak genetic characterization found amplification in124 markers yielding 476 alleles, 117 of which were polymorphic. The number of alleles per locus ranged from two to nine among the polymorphic markers. Seven loci did not amplify yak genomic DNA, however. The workers found polymorphic content of information ranging from 0.355 to 0.752 when tested on the other 51 animals and observed heterozygosity from 0.348 to 0.823. A set of 13 markers, the probability of exclusion of 0.995 was assessed for parentage testing.

Analysis of genetic diversity in Tharparkar, an indigenous race belonging to Rajasthan, revealed a high degree of diversity and no bottleneck in the recent past by using 25 microsatellite markers. There was no loss of rare alleles and the population, which was rich in genetic diversity, concluded suitability for using these microsatellite markers for analysis of diversity in other Indian cattle breeds (Sodhi *et al.*, 2006).

Zhang *et al.*, (2007) used a set of 30 microsatellite primers to analyze diversity in 30 yellow cattle breeds, 27 native cattle breeds of China and three outside introduced cattle breeds. The mean number of alleles per locus was 9.093 for native breeds and 6.885 for the three introduced breeds were observed in these 480 microsatellite alleles in 30 breeds. Three clades are observed by phylogenetic analysis, one formed by 3 introduced breeds and another two formed by indigenous cattle, one cluster belonging to humped breeds and one less humping breed.

Assessment of eight western-central Indian cattle breeds, by using 22 microsatellite markers, Shah *et al*., (2013) revealed the presence of genetic variation within and between breeds and also the presence of inbreeding due to close matching. Based on their results, the workers concluded that microsatellite makes it possible to reveal the unknown aspects of animal population genetic structure, thus helping to design future strategies to improve, conserve and breed management.

Analysis of genetic diversity using 12 microsatellite markers among 3 dwarf breeds; of Indian cattle, viz. Malnad Gidda, Punganur, and Vechur revealed the highest genetic diversity in the population of Malnad Gidda compared to the breeds of Punganur and Vechur (Ramesha *et al*., 2016). Workers also studied native zebu cattle Vechur and taurine crossbred cattle

populations in the state of Kerala using 14 microsatellite markers and found high genetic diversity in both populations and low levels of Vechur inbreeding (Radhika *et al*., 2018).

By using 50 microsatellite loci SanCristobal *et al*., (2006), the selection of approximately 50 individuals per breed analyzed diversity in 58 European pig breeds. Significant variability within breeds was observed, with the expected average heterozygosity and the number of alleles per locus respectively being 0.56 and 0.45.

They found homozygous excess in 12 out of 15 populations whose genetic frequencies departed from the expectations of Hardy-Weinberg ($P<0.01$). Genetically very distinct were the European breeds, with a Wright F_{ST} index value of 0.21. Drawn from the Reynolds distances between the breeds, the Neighbour-Joining tree shows that the national varieties of major breeds and the commercial lines cluster mostly around their reference breeds. By contrast, except for the Iberian breeds, local breeds exhibited a star-like topology. The results helped to determine European pig breeds recent evolution.

Kakoi *et al*., (2007) used 27 microsatellite markers for diversity analysis and found an average number of 9.6 alleles per locus in 318 native horses and found that Japanese native populations showed lower diversity within populations and higher differentiation between populations compared to foreign populations. By using 15 microsatellite markers to evaluate genetic variation in seven West Balkans Pramenka Sheep typed Cinkulov *et al*., (2008) found that heterozygosity and allelic were expected to mean richness over the microsatellite loci and sheep types were 0.78 and 7.9, respectively. They found that the largest panmictic population was formed by Serbian, Kosovan, Bosnian, Montenegrin and Albanian types. Agha *et al*., (2008) used 7 microsatellite markers for analyzing genetic diversity in three (Egyptian Baladi, Barki and Zaraibi) and two Italian (Maltese and Montefalcone) goat breeds. They found more than 0.5 PIC (Polymorphic information content) value at most of the loci, useful in accessing within and between-breed variability. The expected heterozygosity of the breeds varied from 0.670 to 0.792. They found Egyptian breeds were separated from Italian breeds by genetic distances and population structure and the Mediterranean breeds, sampled from Africa and Europe differentiated from each other and there was only little genetic exchange between genetically isolated species.

CHAPTER No.3

MATERIALS & METHODS

The data for morphometric characterizations on aspects of breeding tract, ecological settings, status of buffaloes in breeding tract, buffalo husbandry practices (Housing, Feeding and Breeding), management practices, physical characteristics, production performance and utility were collected using questionnaires, direct communication with farmers, direct observations and measurements. The molecular characterization using FAO recommended microsatellite markers were carried out in the laboratory on 50 unrelated individual buffaloes. The Diara buffaloes belonging to the breeding tract lies between 25°N and 26°N latitude and between 84°E and 90°E longitude in the middle Gangetic plains of India were taken into present study.

3.1 MORPHOMETRIC CHARACTERIZATIONS

Surveys were conducted in 3 districts of the Bihar state i.e. Patna, Ara and Buxar. A total of 120 farmers from 16 villages were interviewed to record information on various management practices opted by the livestock owners in the state. Diara Buffaloes were randomly selected in a range from first to fifth parity on the basis of availability at farmers. Farmers were interviewed to know the habitat, status, management, utility and performance of the cattle available. Farmers were also enquired about choice of breed, sale and purchase of animals, animal housing, feeding, breeding and prevalent diseases in the area. Performance traits like age at first calving, age at first service, daily milk yield, calving interval, lactation yield, peak yield, lactation length, dry period, service period and calving interval were collected by conversing with the farmers from the surveyed villages using structured questionnaire.

Different body measurements and physical characteristics were recorded on 132 animals of different age and sex. All measurements were recorded twice by the same recorder to minimize the error and to avoid between recorder effects. The circumference measurements were taken by a measuring tape while the other measures were taken by a mapping stick. The body measurements (body Height or height at withers, leg length, neck length, neck circumference, body length, heart girth, paunch girth, face length, face with, ear length, horn length, horn circumference, distance between horns, hip bone height, distance between hip bone, pin bone height, distance between pin bones, distance between pin and hip bones, tail

length without switch and tail length up to switch) were recorded. The physical characteristics were recorded for hair colour and length, coat colour, skin colour, muzzle colour, hoofs colour, tail switch colour, horn shape, horn size, horn orientation, head shape, head polls, naval flap, penis sheath flap, temperament, udder shape, udder size, teat shape, teat tip and milk vein.

3.1.1 Body Parts Measurements

Buffaloes were randomly selected in a range from first to fifth parity on the basis of availability at farm. The various body parts measurements were recorded along with MY. Body length (BL) was measured as the distance from the point of shoulder to the point of pin bone. Chest girth (CG) was measured as the circumference of body over the chest of animal just behind the elbow. Abdominal girth (AG) recorded as the circumference of the body over the flank just in front of the udder. The wither height was measured when the animal was in standing position evenly on the ground with normal posture. The muzzle width (MW) was measured with help of big horn caliper and full MW was recorded. The distance between two hip bones and pin bones was measured with help of measuring tape. The HG tape measured the circumference of the chest just behind the forelegs. HW (Height at weather) was measured as the distance from the base of the hoof to the highest point of the withers (Ugur, 2005).

All these body dimensions taken at different age groups and sex were measured by using a specially designed graduated wood stick fitted on support on base and a sliding stick on the top in centimetres. To ensure a consistent methodology, all measurements (Figure 3.1) were carried out by the same person. The body measurements data were analysed for Descriptive statistics using MS-Excel (2010). The survey included 8 male and 90 female animals. The Survey included 10 calves it below 2 weeks age, 12 calves of below one year, 12 calves of age between 1 to 3 years, 8 male and 90 females of age more than 3 years.

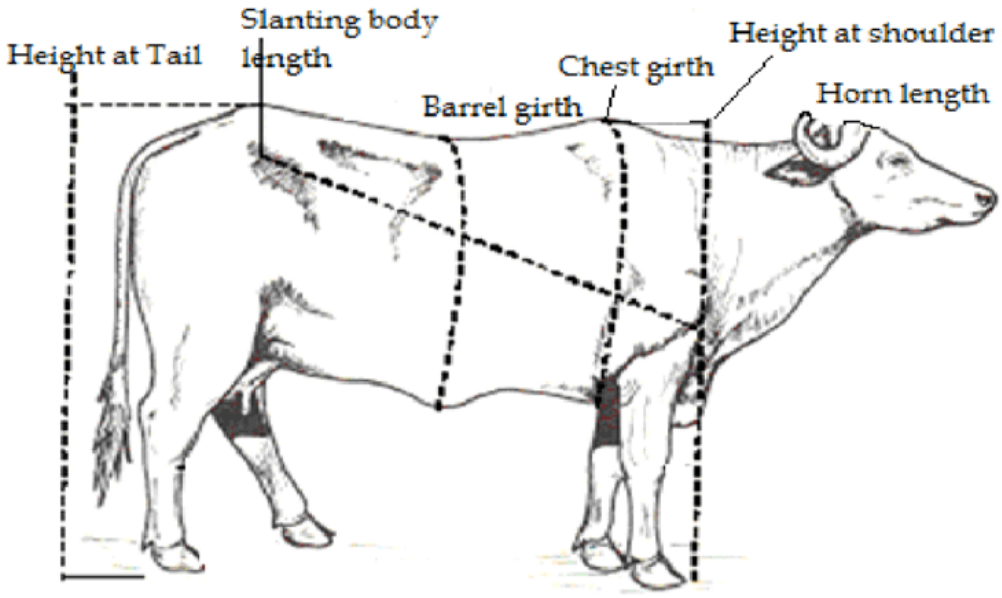

Fig No.3.3 A View of Body Part Measurements of Buffalo

3.2 MOLECULAR CHARACTERIZATION

The molecular characterization using FAO recommended microsatellite markers were carried out in laboratory on 50 unrelated individual buffaloes. For which blood samples of 50 randomly selected Diara Buffaloes were collected from breeding tract Patna, Ara and Buxar districts of the Bihar state.

3.3 MATERIALS

3.3.1 Equipment

The equipment was used as per the requirement during the research work. Major equipment used and their make were – Measuring Tape, Vernier Clipper, Measuring Stand, autoclave, incubator and incubator shaker (Innova 4230), electronic balance (for weighing the chemicals) (Mettler), magnetic stirrer, vortex mixer, pH meter (Orion), horizontal electrophoresis unit (Sci-Plas/BioRad), microwave oven, water purification system (Elga), centrifuge (Sigma-SVI, Hermle, Fisher Scientific, Remi), micropipettes (Eppendorf), gel documentation system (UVP), water bath (Poly science), thermal cycler (BioRad, C-1000/Eppendorf), nanodrop (use for DNA concentration) (Thermo Scientific ND1000), -20°C deep freezer (Vestfrost), -80°C deep freezer (Haier MLT freezer), spinner (for mixing properly).

3.3.2 Chemicals and Reagents

All the chemicals and biological used in this study were molecular biology grade procured from Amresco, Sigma-Aldrich, Biogene, Promega, IDT, Invitrogen, Bangalore-Genei, MBI Fermentas, Invitrogen, Ambion, New England BioLabs, Merck India, SD- Fine Chemicals, etc.

3.3.3 Plastic and Glassware

Plastic wares used for the present study were procured from Imperial Bio-Medics, Axygen, and Fisher Scientific. All the plastic wares used for research work were certified nuclease-free and sterile before use. Glassware were from Schott Duran Riviera.

3.3.4 Ethical Approval

During collection of blood samples from local buffalo population, attention had been paid to minimize pain to the animals and all the samples collection was carried out in accordance with the guidelines laid down by the Institutional Animal Ethics Committee (IAEC) and prevailing local laws and regulations. The approval for carrying out this study was taken from the Institutional Animal Ethics Committee.

3.3.5 Blood Samples Collection

The blood samples of Diara buffalo were collected from their native breeding tract in Patna, Ara and Buxar districts of Bihar. Blood samples were collected from 50 buffaloes consisting of 9 males and 41 female animals from different parts of their breeding tracts in Patna, Ara and Buxar districts of Bihar of both sexes from various villages in the breeding tract for microsatellites profiling. The samples are collected into vacutainer with heparin or EDTA coated, from jugular vein by 20-gauge needle and Samples were transported to the lab within 24 to 48 hours under cold conditions before extraction of DNA from blood. The samples were stored at 4°C until starting for DNA isolation. The stored samples were transported to ICAR-National Bureau of Animal Genetic Resources (NBAGR), Karnal, Haryana for further processing and analysis.

3.4 ISOLATION OF GENOMIC DNA

DNA was isolated from blood samples of Diara buffaloes by using the standard protocol of SDS-Proteinase-K described by (Sambrook and Russell, 2001). DNA isolation involved mainly four parts:

- Lysis of cells using a detergent such as Sodium Dodecyl Sulphate (SDS).
- Protein digestion with Proteinase-K, released from cell lysis.
- Extraction of DNA with Phenol: Chloroform: Isoamyl alcohol.
- DNA precipitation with ethanol and sodium acetate.

The protocol followed involved the following steps-

1. The blood sample (10 ml) of each animal was transferred to a 50 ml Oakridge tube separately.
2. Added twice the volume of chilled lysis buffer (NH4Cl-155mM, KHCO3- 10mM, EDTA- 0.1mM) to the blood sample and mixed, then incubated in ice for 10 min.
3. The above mixture (blood mixed with lysis buffer) was centrifuged at 12000xrpm at 4°C for 10 min.
4. The supernatant was discarded carefully and the steps 2 and 3 were repeated until WBC pellet left was clear (2 to 3 washes were sufficient). 5 ml of extraction buffer (NaCl-75mM, Tris-Cl-10mM, EDTA-0.1mM) was added to clear pellet and mixed gently.
5. SDS (20%) at a final conc. 0.5% and Proteinase-K (stock-20 mg/ml) final conc 100µg/ml were added and mixed gently.
6. The samples were incubated at 56°C for 6 hours or overnight in a water bath.
7. Tris-equilibrated phenol (pH 8.0) was added in an equal volume of sample and mixed well for 5-10 min. by inverting the tubes gently until it formed a uniform suspension and the samples were centrifuged at 10000 rpm for 10 min. at 25ºC.
8. The phase containing DNA i.e., upper was collected in separate Oakridge tube carefully and added an equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) then mixed well by inverting the tubes for 5-10 min. and were centrifuged at 10000 rpm for 10 min at 25°C.
9. The separated upper aqueous phase from the above-centrifuged tube into fresh Oakridge tube carefully. An equal volume of Chloroform: Isoamyl alcohol (24:1) was added

(approx. 5 ml) and mixed well for 5-10 min by inverting the tubes gently to avoid disintegration of genomic DNA and centrifuged at 10000 rpm for 10 min at 25°C.

10. Transferred upper aqueous phase into a glass tube and added 1/9 volume of 3M sodium acetate pH 5.2 (final concentration 0.3M) followed by addition of two volumes of 100% chilled ethanol. The sample was mixed gently and DNA was precipitated (visible as white stringy strands), which was spooled out and transferred to a microfuge tube.
11. The DNA pellet was washed with 1 ml of 70% ethanol leaving for 1-2 min and centrifuged at 5000 rpm for 5 min at 25°C, ethanol decanted off and tube was left open until the DNA pellet dried off.
12. To the DNA pellet, 500µl of TE buffer (10 mM Tris-HCl and 1mM EDTA) was added to dissolve it and the isolated DNA samples were stored at -80˚C or -20˚C until further use.

3.5 ASSESSMENT OF QUANTITY AND QUALITY OF DNA

3.5.1 Agarose Gel Electrophoresis

The quality of DNA samples was checked by mixing each sample of 1 µl of DNA sample with 0.3 µl of tracking dye and was loaded slowly into the wells of 0.6% agarose gel using a micropipette. Electrophoresis was carried out at 5 V/cm for an hour in 0.6% agarose gel using 1X TAE buffer. After completion of electrophoresis, the gel was examined under UV trans-illuminator and photographed using a gel-doc system (UVP).

3.5.2 UV Spectroscopy

The actual concentration and purity of DNA were also checked by measuring OD (Optical density) of samples at 260 and 280 nm, using a NanoDrop (ND-1000 Thermo Scientific). The ratio of OD260/OD280 was used to check DNA purity and the ratio of around 1.8 was considered acceptable. The quantity of DNA was measured by taking 1.0 OD 260 equivalent to 50 ug/ml of DNA. Based on gel electrophoresis and OD based concentration, the working DNA samples were prepared at approximately 50 ng/µl concentration from the stock DNA samples by adding TE (10mM Tris+1mM EDTA) buffer.

3.6 MICROSATELLITE GENOTYPING

3.6.1 Standardization of PCR

Using DNA from 50 samples each of Diara buffaloes, 10 microsatellite markers were PCR amplified in each animals using the primer sets already described by Kataria et al., 2009a, with a given annealing temperature using gradient PCR. Primer sequences with respective annealing temperatures and allele size range are given in Table 3.1. The forward primer of each set was labeled with any of the four fluorescent dyes, FAM, NED, VIC or PET for detection in automated DNA Sequencer.

Table no. 3.1 Details of microsatellite primers used to study genetic diversity in Diara buffalo.

Locus	Primer sequences (5'-3')	Fluorescent Dye	Temperature
ILSTS89	GAGCAAGGTGTTTTTCCAATC CATTCTCCAACTGCTTCCTTG	VIC	56.0
CSSM47	ATTTGCACAAGCTAAATCTAACC AAACCACAGAAATGCTTGGAAG	PET	56.0
ILSTS033	CAACAGCTATTTAACAAGGA AGGCTACAGTCCATGGGATT	PET	54.0
ILSTS060	CAAGACAGGTGTTTCAATCT ATCGACTCTGGGGATGATGT	PET	64.0
ILSTS019	ACACAAATCCTTTCTGCCAGCTGA AATTTAATGCACTGAGGAGCTTGG	FAM	55.0
ILSTS025	GTTCAGGACTGGCCCTGCTAACA CCTCCAGCCCACTTTCTCTTCTC	NED	58.4
ILSTS056	CACTGTGAATGCATGTGTGTGAGC CCCATGATAAGAGTGCAGATGACT	NED	61.0
HEL13	GATCACCTTGCCACTATTTCCT ACATGACAGCCAGCTGCTACT	NED	58.0
ILSTS028	TGTCTGTATTTCTGCTGTGG ACACGGAAGCGATCTAAACG	FAM	65.0
ILSTS058	GCTTGCTACATGGAAAGTGC CTAAAATGCAGAGCCCTACC	NED	54.0

The concentration of various reaction components and cycling conditions were optimized. The following reaction mixture was found to be having all components optimum for amplification of different alleles in a 20 µl reaction (Tables 2 and 3). PCR amplification was carried out in a 96 well plate.

Table No. 3.2 Reaction mix for PCR amplification of different microsatellite primers.

Items / Ingredients	**Volume/ quantity**
Genomic DNA	2.0 µl (~50-150 ng/µl)
*Forward Primer (10 pmol/µl)	0.5 µl
Reverse Primer (10 pmol/µl)	0.5 µl
dNTPs (10 mM)	0.5 µl
Taq DNA Polymerase (Bangalore GeNei 3units/µl)	0.3 µl (1 unit)
10x PCR buffer	2.0 µl
Nucleus free water	14.2 µl
Total	20.0 µl

*Fluorescent dye labeled

The reaction mixture prepared based on above amount was subjected to PCR amplification with the following PCR cycling conditions.

The amplified PCR products, using above conditions were checked by gel electrophoresis, loading 3µl of PCR product adding 0.5µl of 6x loading dye on a 1.5% agarose gel using 1X TAE buffer, along with standard molecular size marker. The ethidium bromide was added in gel preparation which was helpful in visualization and photographed using UVP gel-doc system.

Table 3.3 PCR cycles followed for amplification of microsatellite markers.

Name of process	Temperature	Time	No. of cycle
Initial Denaturation	95.0ºC	3 minutes	
Denaturation	94.0ºC	30 sec.	32 cycles
Annealing	variable*	30 sec.	
Extension	72.0ºC	30 sec.	
Final Extension	72.0ºC	5 minutes	

*Annealing of respective primers as given in Table No. 3.1.

3.6.2 Microsatellite Fragment Analysis

Based on expected allele size and dye labels, multiplexing of PCR products representing different microsatellite markers carried out. Each set had 4 markers and during multiplexing, these 4 different markers' PCR products were mixed in a single well of 96 well plates i.e. PCR products of 4 markers of the same animal. Depending upon dye label, PCR products' volumes were mixed as given below-

FAM - 1.0 µl

NED - 2.0 µl

VIC - 1.0 µl

PET - 2.0 µl

The forward primer for each marker was fluorescently labeled with FAM, NED, VIC or PET dyes or differed in size if the same dye was used for two markers in a multiplex for ease of scoring. During the genotyping, in an automated DNA sequencer GS500LIZ (Applied Biosystems, USA) as an internal lane size standard was added to each multiplex along with PCR products.

From the above mixture took 0.5 µl and mixed with a GS500LIZ (0.1 µl) and HiDi (9.5 µl), making to 10 µl of final volume. Samples were run on ABI 3100XL Genetic Analyzer, 16 capillary automated DNA sequencer after spinning for 1 min to mix all the components and

denaturing at 95ºC for 5 minutes before genotyping in a 96 well plate. After completion of run the data was retrieved and analyzed further.

3.6.3 Microsatellite Data Analysis

After post fragment analysis on automated DNA sequencer, GENEMAPPER software (Imle, 2005) was used to extract allele size data from raw data and further GenAlEx 6.5 software (Peakall and Smouse, 2012) used to calculate allele frequency, observed number of alleles (No), effective number of alleles (Ne), observed heterozygosity (Hobs) and expected heterozygosity (Hexp) across two buffalo populations. Polymorphism information contents (PIC) values for each marker were calculated using the observed number of alleles and allelic frequency of each marker by a formula given by Botstein et al. (1980). Sign, Standardized differences and Wilcoxon sign rank tests under three models (IAM—infinite allele model, SMM—stepwise-mutation model, TPM—two-phased model) and mode shift test were performed with BOTTLENECK v.1.2.02 (Piry *et al*., 1999) in order to detect mode shift to ascertain whether given breed has undergone any bottleneck in recent past by estimating the presence of minor alleles frequencies in the populations. In order to infer the information on admixture analysis and draw the population structure, STRUCTURE software 2.3.4 was used for differentiation of different breeds based on allelic data at different K values (Pritchard *et al*., 2000). Length of Burnin Period set for population structure analysis was 10000 and number of MCMC Replicates after Burnin was set at 50000 with 20 reiterations. Maximum support K value for structure analysis was derived through the analysis of data using Pophelper 2.2.6 software (http://royfrancis.github.io/pophelper/).

CHAPTER No.4

RESULTS AND DISCUSSIONS

4.1 BUFFALO HUSBANDRY PRACTICES

4.1.1 Housing

Diara buffaloes were surveyed in their breeding tract at farmers houses where it was observed that Animals are housed close to the human dwellings. In most cases, closed housing is provided (85%). In most instances (75%), the animals and humans are housed in different parts of the same building. In the remaining cases, a separate structure was provided to animals away from farmer's house. Most of the constructions are permanent (85%) with thatched roofs covered with paddy straw or tiled roofs. Asbestos roof was also observed in 5% animal houses. Floors are generally uneven without proper drainage facilities. In peri-urban areas, the animals are overcrowded with less than the minimum required floor space of 3.5 square meters being provided. In rural areas, the practice of allowing the animals to wallow in the nearby water sources is prevalent (90.5 %). Mostly the animals wallow around noon after grazing in the fields under a hot sun. Animals are kept in night in closed house. In day time, animal was feed in open area of house where their Mengers are located. After morning feeding and milking, animals are either kept in open area away of house or let loose to graze in field and wallow. Similarly, in evening, animals were given feeds in their mangers and after milking they are brought in closed house. The region where Diara buffaloes survives is almost marshy in most part of the year and getting dried for cultivation only during dry-weather conditions.

4.1.2 Herd structure:

Rearing of cattle and buffaloes in India is mostly in hand of small and marginal farmers and part of small holder production system. In rural area of India, major livestock population is found where disorganized farming system is prevalent. The livestock reared in urban and peri-urban area constitute scanty population out of total population where to some extent organized farming systems is found. The management of Diara buffaloes by the farmers in the breeding tract is no exception to it. The herd size of Diara buffaloes is very small with an average being 3.1 which is in agreement with chandran *et al.* 2015. 2. The herd structure included almost nil adult males, 2.0 adult females, 0.2 male calves and 0.8 female calves. The disparity between

male and female calves might be due to immediate disposal of male calves by the farmers once dam's milk yield ceases or even during milking period. Farmers take less interest in better caring of male because they find uneconomical to rear them. Due to mechanization of agriculture systems, drought power of male Diara buffalo has also become unimportant. Adult male buffaloes are even almost unavailable at farmer's house evening for breeding.

Fig. 4.1 Herd management of Diara Buffalo

4.1.3. Feeding

Paddy straw, dry mixed grasses and green grasses are the main sources of roughage. Wheat bran, linseed oil cake, mustard oil cake and rice bran are given as concentrates. About two third of the farmers provide concentrates to the milking animals; 0.5 to 2 kg of concentrate is usually given to the lactating animals at the time of milking. Some farmers even feed the animals with kitchen wastes and hotel wastes; this practice is more prevalent in the urban areas.

Fig. No. 4.2 Feeding management of Diara Buffalo

4.1.4. Breeding

Breeding of buffaloes is highly disorganized in the breeding tract. Natural service is primarily practiced by the farmers in the urban, peri-urban and rural areas using breeding bulls. A.I. is now occasionally used due to unavailability of breeding bulls and practical difficulties of farmers. Therefore, Natural service is now presenting decreasing trend in urban and peri-urban areas. Breeding bulls those used for breeding in rural area are free ranged animals. Their ears are generally cut for certain rituals of local peoples after which they are let loose to field for open grazing. Although A.I. services are available in urban and peri-urban areas, semen of Diara buffaloes is not available and the farmers have to opt for either Murrah semen or other breeds. As a result, the proportion of graded Diara buffaloes and non-descript animals are more common in the urban areas.

4.1.5. Physical characteristics

Diara buffaloes are well-built medium-sized animals. The coat colour varies from Black to grey. Their skin is black. The black coloured Diara buffaloes were found more efficient producer and popular among farmers in comparison to grey coloured one. The head is fairly long with a broad forehead. Ears are moderately long and erect. The neck is moderately long with almost negligible dewlap.

Horns are medium sized, flat, corrugated and curved, projecting backward, sideward and upward at half of the neck. Shoulders are long and slope smoothly with the body. The barrel is well built and medium in size with a straight and wide back. Legs are strong with hard hooves. The udder is moderately developed with teats of medium size and squarely placed between the hind legs. The tail is fairly long, thin and flexible ending in a black, black and white switch. Figures 4.5 and 4.6 show typical male and female Diara buffaloes, respectively.

Fig. No. 4.3 Male Diara Buffalo

Fig No. 4.4 Female Diara Buffalo

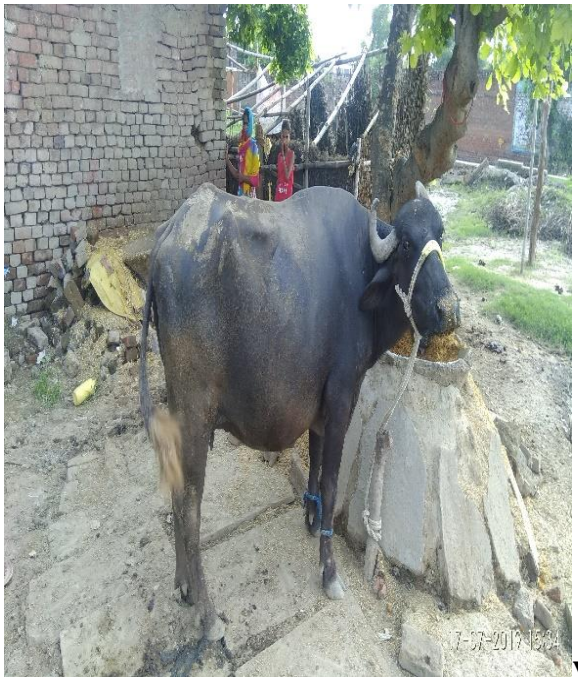


Fig. No. 4.5 **Fig. No. 4.6**

Typical Forehead Feature of Diara Buffalo


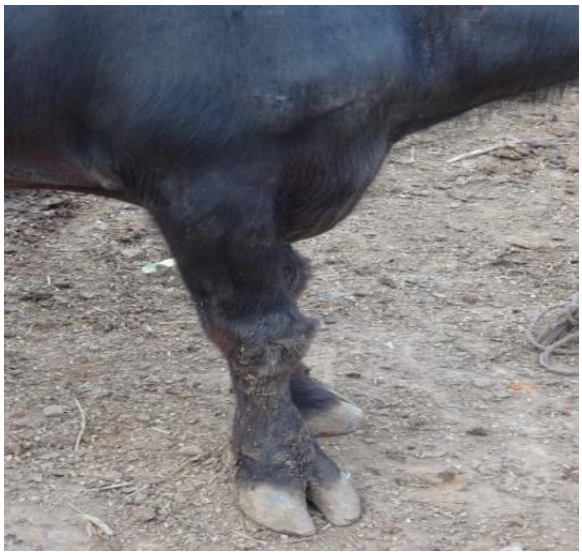

Fig. No. 4.7 **Fig. No 4.8**

Different Typical feature of Body Color & Shoulder of Diara Buffalo

4.1.6. Body measurements

The mean and standard error of eight different body measurements in different age groups are presented in Table 4.1.

The Height at wither (cm), Body length Horizontal (cm), Body length Oblique (cm), Heart girth (cm), Paunch Girth (cm), Leg (cm), Neck (cm), Neck Circumstance (cm), Face Length (cm), Face Width (cm), Ear Length (cm), Hip Bone (cm), Hip Height (cm), Pin Bone (cm), Distance between Hip and Pin Bone (cm), Tail length and Tail Length up to Switch for the calves below the age of 7 days were found to be 80±2, 52.5±3.5, 57.5±4.5, 80.5±2.5, 81.5±2.5, 53.5±2.5, 15±1, 45±1, 21±4, 9.5±0.5, 15.5±2.5, 16.5±1.5, 75.19±3.83, 8.5±0.5, 22±2, 37.5±4.5 and 43.5±4.5, respectively. The Height at wither (cm), Body length Horizontal (cm), Body length Oblique (cm), Heart girth (cm), Paunch Girth (cm), Leg (cm), Neck (cm), Neck Circumstance (cm), Face Length (cm), Face Width (cm), Ear Length (cm), Horn Length (cm), Horn Circumstance (cm), Distance between horns (cm), Hip Bone (cm), Hip Height (cm), Pin Bone (cm), Distance between Hip and Pin Bone (cm), Tail length and Tail Length up to Switch for young stock of age between one week to one year were found to be 94.25±5.009, 76.37±6.89, 87±5.69, 117±10.12, 137±10.78, 60±1.81, 30.87±1.75, 62.37±7.02, 31.87±1.65, 13.87±0.58, 22±0.96, 8.14±2.56, 11.28±1.10, 18.71±1.59, 22.37±1.06, 106±16.5, 12.25±1.57, 26.37±1.96, 60±4.73, 70.25±5.75, respectively. The Height at wither (cm), Body length Horizontal (cm), Body length Oblique (cm), Heart girth (cm), Paunch Girth (cm), Leg (cm), Neck (cm), Neck Circumstance (cm), Face Length (cm), Face Width (cm), Ear Length (cm), Horn Length (cm), Horn Circumstance (cm), Distance between horns (cm), Hip Bone (cm), Hip Height (cm), Pin Bone (cm), Distance between Hip and Pin Bone (cm), Tail length and Tail Length up to Switch for young stock of age between one year to three year were found to be 111.8±7.48, 89.2±7.51, 101.20±9.24, 150.8±15.24, 167.2±14.48, 69±4.33, 37.8±2.41, 72.6±9.9, 36.8±2.2, 17.4±0.97, 25±0.89, 21.2±3.24, 18±1.37, 23.6±2.65, 32.4±3.52, 112±16, 21.2±4.95, 31.2±2.47, 68±5.54 and 77.8±5.01, respectively.

The Height at wither (cm), Body length Horizontal (cm), Body length Oblique (cm), Heart girth (cm), Paunch Girth (cm), Leg (cm), Neck (cm), Neck Circumstance (cm), Face Length (cm), Face Width (cm), Ear Length (cm), Horn Length (cm), Horn Circumstance (cm), Distance between horns (cm), Hip Bone (cm), Hip Height (cm), Pin Bone (cm), Distance between Hip and Pin Bone (cm), Tail length and Tail Length up to Switch for adult male Diara buffalo of age above the three year were found to be 138.12±4.5, 130.26±5.1, 141.25 ±5.2, 208±2.5, 228±2.89, 81.21±1.9, 53.26±3.9, 95.16±5.9, 44.25±1.34, 23.89±3.84, 27.20±1.29, 34.3±1.98, 21.16±1.01, 29.4±1.2, 55.30 ±3.5, 135.46±2.75, 26.9±1.95, 40.23±2.3, 88.45±3.9 and 97.79±3.5, respectively. The Height at wither (cm), Body length Horizontal (cm), Body

length Oblique (cm), Heart girth (cm), Paunch Girth (cm), Leg (cm), Neck (cm), Neck Circumstance (cm), Face Length (cm), Face Width (cm), Ear Length (cm), Horn Length (cm), Horn Circumstance (cm), Distance between horns (cm), Hip Bone (cm), Hip Height (cm), Pin Bone (cm), Distance between Hip and Pin Bone (cm), Tail length and Tail Length up to Switch for adult male Diara buffalo of age above the three year were found to be 129.77±2.24, 117.77±3.54, 129±3.50, 192.31±5.67, 19.09±6.12, 76.09±1.61, 44.63±1.43, 87±2.11, 40.22±1.96, 19.6±0.69, 26.09±0.95, 32.1±2.08, 18.47±1.12, 26.80±0.92, 47.14±2.17, 126.41±1.95, 25.4±1.61, 37.3±1.54, 85.05±2.59, 95.65±3.33, respectively.

Table No. 4.1 body measurements of Diara Buffalo

Parameters	Young Calves (< 2 weeks) (10)	Calves (6-12 months) (12)	Young Stock (> 1 to < 3 Years) (12)	Adults	
				Male (8)	Female (90)
Height at wither (cm)	80±2	94.25±5.009	111.8±7.48	138.12±4.5	129.77±2.24
Body length	52.5±3.5	76.37±6.89	89.2±7.51	130.26±5.1	117.77±3.54
Body length Oblique	57.5±4.5	87±5.69	101.20±9.24	141.25 ±5.2	129±3.50
Heart girth (cm)	80.5±2.5	117±10.12	150.8±15.24	208±2.5	192.31±5.67
Paunch Girth (cm)	81.5±2.5	137±10.78	167.2±14.48	228±2.89	219.09±6.12
Leg (cm)	53.5±2.5	60±1.81	69±4.33	81.21±1.9	76.09±1.61
Neck (cm)	15±1	30.87±1.75	37.8±2.41	53.26±3.9	44.63±1.43
Neck Circumstance	45±1	62.37±7.02	72.6±9.9	95.16±5.9	87±2.11
Face Length (cm)	21±4	31.87±1.65	36.8±2.2	44.25±1.34	40.22±1.96
Face Width (cm)	9.5±0.5	13.87±0.58	17.4±0.97	23.89±3.84	19.6±0.69
Ear Length (cm)	15.5±2.5	22±0.96	25±0.89	27.20±1.29	26.09±0.95
Horn Length (cm)	-	8.14±2.56	21.2±3.24	34.3±1.98	32.1±2.08
Horn Circumstance	-	11.28±1.10	18±1.37	21.16±1.01	18.47±1.12
Distance between	-	18.71±1.59	23.6±2.65	29.4±1.2	26.80±0.92
Hip Bone (cm)	16.5±1.5	22.37±1.06	32.4±3.52	55.30 ±3.5	47.14±2.17
Hip Height (cm)	75.19±3.83	106±16.5	112±16	135.46±2.75	126.41±1.95
Pin Bone (cm)	8.5±0.5	12.25±1.57	21.2±4.95	26.9±1.95	25.4±1.61
Distance between Hip	22±2	26.37±1.96	31.2±2.47	40.23±2.3	37.3±1.54
Tail length (cm)	37.5±4.5	60±4.73	68±5.54	88.45±3.9	85.05±2.59
Tail Length up switch	43.5±4.5	70.25±5.75	77.8±5.01	97.79±3.5	95.65±3.33

There was no significant difference found between male and female Diara buffalo for morphometric traits. This is in agreement with Chandran *et. al*. (2015).

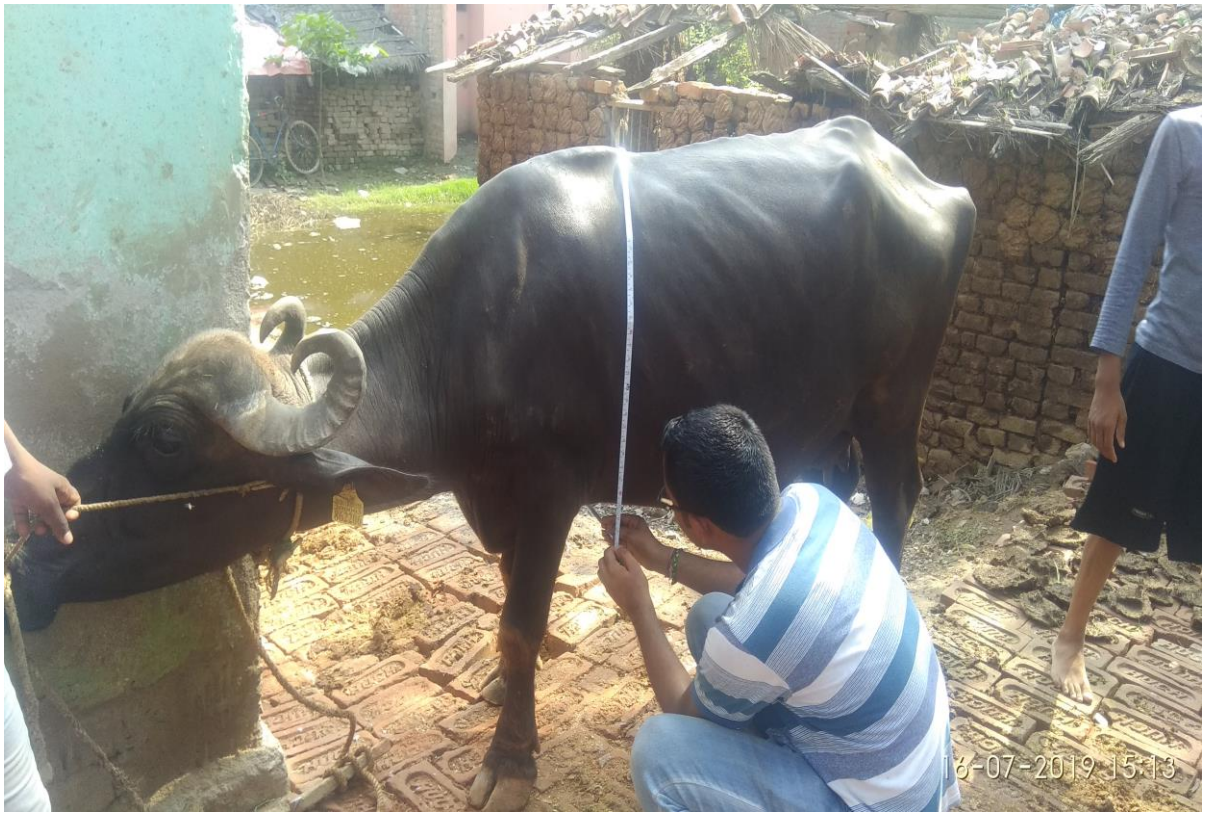

Fig. No 4.9 Body measurements of Diara Buffalo

4.1.7. Production performance

Diara buffaloes are moderate milk producers and normally give four to nine litres of milk daily. Some animals in villages reach a peak yield of more than 9.65 litres per day, however. The average daily milk yield was 4.9±0.4 litres as reported by the farmers. The length of lactation varied from 210 to more than 340 days with an average of 301.6±10.3 days. The lactation milk yield varied from 1008.4±95.7 to 1635.6±112 litres with a mean of 1450.87±28.7 litres. Diara buffaloes have relatively long productive life spans as demonstrated by animals with more than five calvings commonly found in the villages. Age at first calving and calving interval was estimated to be 46.27 ± 0.63 months and 14.4 ± 0.13 months, respectively. The Dry period, average age at first service and Service period was estimated to be 89.87±4.25 days, 34.86±0.78 months and 131.31±3.06 days. The different traits estimated above are in agreement with Chandran *et. al*. (2015) in Diara Buffalo.

Table No. 4.2 Production & Reproduction Performance of Diara buffalo

Milk Production traits of Diara Buffalo	
Lactation milk yield (Liters)	1450.87 ± 28.12
Daily Milk Yield (Liters)	4.9±0.4
Lactation length (days)	301.67 ± 12.87
Peak yield (Liters)	9.65 ± 0.33
Reproduction traits of Diara Buffalo	
Dry period (days)	89.87±4.25
Calving interval (months)	14.4±0.13
Age at first service (months)	34.86±0.78
Age at first calving (months)	46.27±0.63
Service period (days)	131.31±3.06

4.1.8 Utility

Diara buffaloes are dual purpose animals used for milk production as well as agricultural operations in wet fields. They are better suited than are local cattle to ploughing and puddling the wet fields meant for paddy cultivation. They are active, fast moving, hardy and can work continuously for four to six hours in the wet fields. Generally, males are used for the purpose, males are preferred (Figures 4.5).

4.2 MICROSATELLITE GENOTYPING

4.2.1. PCR Amplification of DNA

The DNA obtained from the sample were subjected to PCR amplification using a set of labeled primers and these primers are based on polymorphism to amplify 10 microsatellite markers already identified for buffalo genetic resources characterization (Mishra *et al*., 2009). The markers used in this study were dinucleotide repeats which are more polymorphic than tri-

nucleotides (Metta et al., 2004). The amplified products are clearly seen in the Agarose gel, and no non-specific amplification or PCR failure was observed.

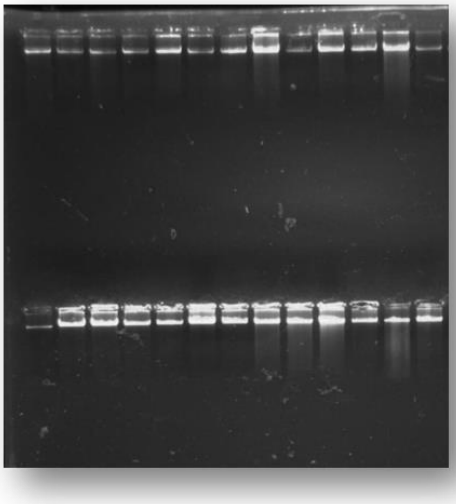

Fig. No. 4.10

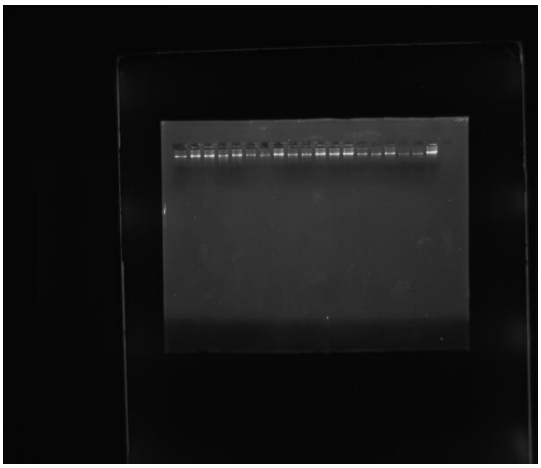

Fig. No. 4.11

Figure : Genomic And Working Gel images of Diara buffalo

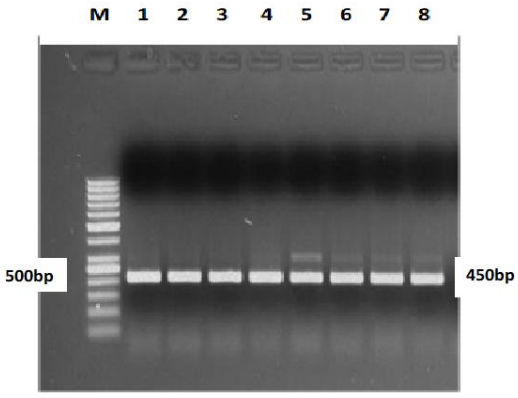

Fig. 4.12 PCR product of Diara buffalo

MW – 100bp DNA Ladder

L1 to L8 – DNA sample Showing (+)ve result

Fig No. 4.12

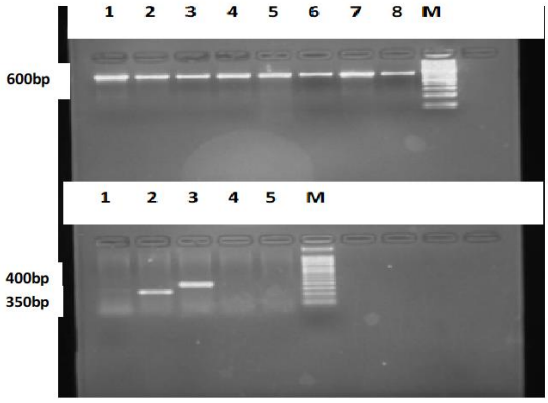

Fig. 4.13 PCR product of Diara buffalo

MW – 100bp DNA Ladder

A. L1 to L8 – DNA sample Showing (+)ve result

B. L2, L3 - DNA sample Showing (+)ve result
L1,4,5 - DNA sample Showing (-)ve result

Fig No. 4.13

Figure: Gel picture of PCR amplified products of Diara Buffalo.

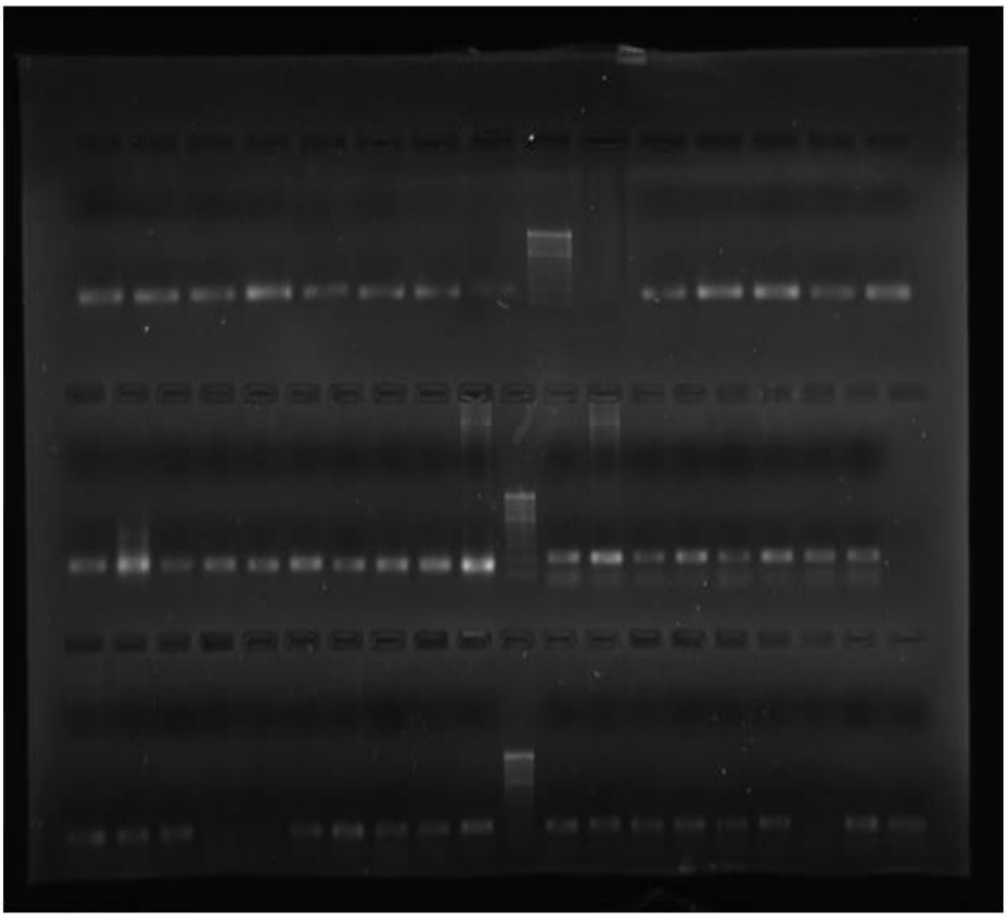

Fig No. 4.14 Multiplex PCR Product of Diara Buffalo Sample

After multiplexing of different dye-labeled amplified markers, the pooled samples were run on ABI automated DNA sequencer along with internal control LIZ standard. The data was extracted using GENEMAPPER software documenting the allele sizes for each marker in each animal.

All 10 microsatellite loci amplified successfully in the 50 samples from Diara buffaloes and produced definite banding patterns from which individual genotypes could be ascertained. Different measures of genetic variation estimated in Diara buffaloes are presented in **Table No. 4.3**.

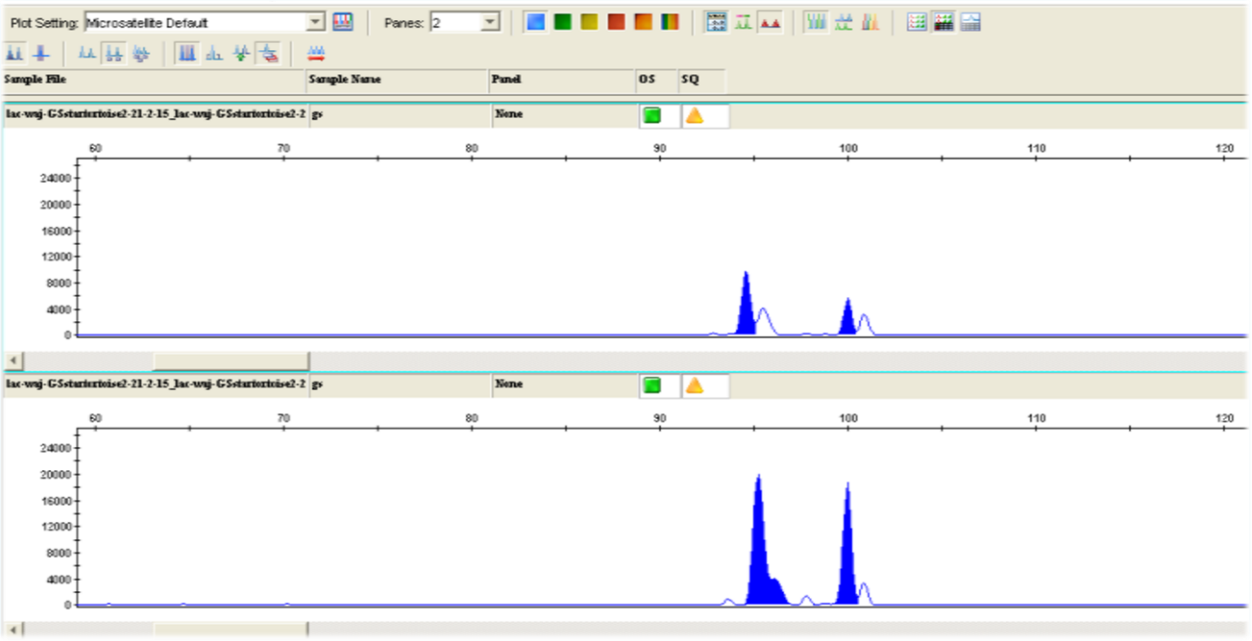

Figure No. 4.15: Graphs showing the different allele size of different microsatellite markers.

4.2.2. Allelic Diversity analysis

The data extracted were analyzed using GenAlex software to document allelic diversity and other parameters derived from allelic diversity. Further, microsatellite loci which were amplified in all the samples of Diara buffalo and had a minimum of four alleles were only considered for analyses of genetic diversity. Across the 10 microsatellites studied, a total of 74 alleles were identified. The mean observed number of alleles per locus (7.4 ± 0.2) for Diara buffaloes was marginally lower than that of other Indian buffaloes as reported by Kumar *et al.* (2006). These microsatellites exhibited a high level of polymorphism as revealed by a wide range of alleles varying from 6 (ILSTS33, 25, 56) to 9 (HELO13 and ILSTS89 & 60) in Diara buffalo population of Bihar which significantly corroborated with the findings of Navani *et al*. (2002), Kumar *et al*. (2006) and Vijh *et al*. (2008) for Indian riverine buffaloes.

The allele size ranged between 96 bp (ILSTS56) to 238 bp (ILSTS19). The mean effective number of alleles (Ne) was 5.77446±0.2. The mean effective number of alleles was less than the observed values across all loci and ranged from 4.1771 (ILSTS60) to 6.7845 (ILSTS89). This is in agreement with local buffalo of Jammu region as reported by Singh et al. (2017). The overall allelic diversity considered to be a reasonable indicator of genetic variation

within the population displayed high genetic variation in local buffalo population. A large number of alleles at low frequency were responsible for the significantly less effective numbers of alleles than the number of actually observed alleles. There should be overall 5 different alleles per locus for estimation of genetic differences between breeds as suggested by FAO. The present study showed mean number of alleles per locus to be more than the FAO recommended (http://www.fao.org), thus suitable for the estimation of genetic differences between and within breeds (Metta, et al., 2004). All 10 microsatellite markers studied, exhibited ample polymorphism for evaluating intra-population genetic variability in Local buffalo population. The results obtained in Diara buffalo almost corroborated with Pandharpuri with the observed number of alleles having mean value of 5.86±0.23 and Bhadawari and Surti Buffaloes having mean value of 7.1±0.19 (Vijh *et al*., 2008; Arora *et al*., 2004).

The mean Shannon's index value was high (1.81741±0.03) in Diara buffalo population and ranged from 1.5413 (ILSTS60) to 2.0230 (ILSTS89) thus showing higher gene diversity in the both the existing population. This is in agreement with local buffalo of Jammu region as reported by Singh et al. (2017).

Table.No. 4.3 Markers wise allelic diversity in Diara buffalo population.

Locus	Na	Ne	I	Obs het	Exp het	Ave het	Allele size (bp)	Nei	PIC
ILSTS8	9.0000	6.7845	2.0230	0.6600	0.8513	0.8407	162-174	0.8328	0.8537
CSSM4	7.0000	5.9162	1.8379	0.6800	0.8395	0.8227	208-224	0.8311	0.8096
ILSTS3	6.0000	6.0224	1.9519	0.7400	0.8517	0.7658	100-126	0.8334	0.8134
ILSTS6	9.0000	4.1771	1.5413	0.7200	0.7682	0.8118	163-188	0.7664	0.7226
ILSTS1	8.0000	5.5828	1.6765	0.7400	0.8464	0.7858	216-238	0.8221	0.7961
ILSTS2	6.0000	6.2415	1.8657	0.6200	0.7886	0.8208	182-198	0.8312	0.8256
ILSTS5	6.0000	6.5544	1.9854	0.6800	0.8177	0.7985	96-192	0.8372	0.7833
HEL013	9.0000	5.2845	1.7308	0.8200	0.8237	0.7905	124-138	0.8108	0.8156
ILSTS2	7.0000	6.1752	1.8647	0.5800	0.7746	0.7973	168-178	0.8002	0.7876
ILSTS0	7.0000	5.0060	1.6969	0.6000	0.8537	0.8296	169-188	0.7678	0.7704
Mean	7.4	5.7744	1.8174	0.684	0.8215	0.80635		0.8133	0.7977
Std	0.2	0.2	0.003	0.001	0.007	0.004		0.0007	0.008

Na= Observed number of alleles, Ne= Effective number of alleles, Ho= Observed heterozygosity, He= Expected heterozygosity

4.2.3. Heterozygosity

Heterozygosity is an appropriate measure of genetic variability within and between populations when populations are expanding. Therefore, heterozygosity values were used as an estimate for variability of studied Diara buffalo population of Bihar.

The observed and expected heterozygosity values on the basis of allele frequency are presented in Table No. 4.3 HEL013 marker showed the highest observed heterozygosity which was 0.8200, whereas the lowest was at ILSTS058 loci with the mean value of 0.684. The expected heterozygosity ranged from 0.7682 (ILSTS60) to 0.8537 (ILSTS058). The average heterozygosity represents the genetic diversity and randomness of a particular breed/population. The average observed heterozygosity was 0.684 whereas the average of expected heterozygosity was found to be 0.82154 which significantly corroborated with the result findings in Murrah, Mehsana, Toda, Surati, Pandharpuri and Nagpuri buffaloes as revealed by (Kumar *et al.,* 2006) and in Nilli Ravi, Murrah, Tarai, Jaffrabadi as revealed by (Vijh *et al*., 2008) and (Arora *et al*., 2004) in Bhadawari and Tarai where the observed heterozygosity was less than that of expected heterozygosity. The mean observed heterozygosity of Diara buffalo population is slightly lower or comparable to that of other Indian buffalo breeds. The average observed heterozygosity estimation in this study thus shows that buffaloes are harbouring a good amount of genetic variation. The low Ho reveals presence of more homozygote individual in the samples analyzed. Though few loci exhibited lower heterozygosity values, most of the loci showed relatively higher expected heterozygosity except at one loci i.e., ILSTS28, that might be due to low selection pressure, large population size and immigration of new genetic materials.

The test for Hardy-Weinberg equilibrium (HWE) showed that all the 10 loci deviated significantly (Table No. 4.3). Departure from HWE is mostly due to heterozygote deficiency which may result from one or more of the following reasons: 1. presence of null alleles; 2. small sample size; and 3. Wahlund effect i.e. presence of fewer heterozygotes in a population than predicted on account of population subdivision. In farm animal species, the prevalence of sire lines selected for economic traits leads to increased consanguinity. Such a breeding system produces reduced heterozygosity within a sub-population in a breed. The availability of very few breeding bulls in the tract might have contributed to increased consanguinity. This is further supported by the estimated mean value of FIS (Fixation Index) in the population which was

positive and equal to 0.09. Thus, the shortage of breeding bulls in the population and confinement of these buffaloes to a small geographical area could be the possible reasons for the deficiency of heterozygotes.

The genetic variability of a population is usually measured as average heterozygosity per locus while the gene differences between two populations may be measured by Nei's standard genetic distance. The average heterozygosity ranged between 0.8407 (ILSTS89) and 0.7658 (ILSTS33) with mean of 0.8296. The values for Nei's measures varied from 0.7664 (ILSTS60) to 0.8372 (ILSTS56) with mean genetic distance of 0.8133±0.007 in Diara buffalo population. These values for heterozygosity and Nei's measures in Diara buffalo were in agreement with the values of buffalo of Jammu region (Singh et. al. 2017).

4.2.4. Polymorphic Information Content (PIC)

Polymorphic information content (PIC) data showed the usefulness of microsatellite locus analyzed in population as it is an ideal index to measure polymorphism of allele fragment in any population. PIC gives us the information of a particular marker for its suitability to include in genetic diversity analysis. The PIC values, which denotes the statistical assessment of informativeness of a marker were high and ranged from 0.7226 (ILSTS60) to 0.8537 (ILSTS89) with mean PIC of 0.7977±0.008 (Table No. 4.3) in Diara buffalo of Bihar. All the 10 loci had Polymorphism Information Content (PIC) values of more than 0.5, suggesting that they are informative for population genetic analysis (Botstein *et al.*1980). This may be due to the fact that there was increased level of heterozygosity and allele Ne Value in the population which are the good indicators of genetic polymorphism in present study on Diara buffalo population of Bihar. These values are indicative of the fact that the markers used were highly informative for analysis of genetic diversity in Diara buffalo population of Bihar. The genetic marker showing PIC values higher than 0.5 are normally considered as informative in population genetic analysis. Mean PIC value of 0.53 and 0.669 lower than the values obtained in the present study, was earlier reported in the Nagpuri Buffalo (Kataria *et al.,* 2010) and Karnese Buffalo of South kanara region (Kathivaran *et al.,* 2009) respectively and higher than mean PIC value of 0.933 as reported in Egyptian Buffalo (Abou-Bakr *et al*., 2014). The present PIC values were comparable with Egyptian Buffalo Breeds (0.736 -0.862.) as reported by *El-Kholy et al.* (2007) using bovine microsatellite markers and mean value of >0.5 in Iraqi water buffaloes (Jaayid and Maytham 2014). In contrast, Acosta *et al*. (2014) obtained lower PIC

values for Cuban water buffalo (0.495), Gullian Buffaloes of Iran (0.61) by Aminaafshar *et al*. (2008).

4.2.5. Bottleneck Analysis

Microsatellite data are the best marker that subjected to statistical analysis to test whether the populations have undergone a recent genetic Bottleneck because of their generally high level of variability. Methods for detecting bottleneck do not require information on historical population size or levels of genetic variations. Cornuet and Luikart (1996) described the quantitative methods suitable for the analysis of microsatellite data for the detection of recent bottlenecks in (100-200) generations. The allele frequency spectrum visualized by the qualitative graphical method is shown in the figure. The distribution followed the normal L-shaped form suggesting that the breed had not encountered a genetic bottleneck in the recent past. Thus the test for Bottleneck did not show any significant reduction of effective population size in recent past.

Bottleneck analysis (BOTTLENECK SOFTWARE) in Diara buffalo was employed by using different approaches in this study to detect the change in effective population size, which affects the allele deficiency and heterozygosity excess. The bottleneck analysis was under the assumption of mutation-drift equilibrium, where the allele frequency shows that the given population not being in equilibrium, due to a recent reduction of the effective population size. Any population that experienced a recent bottleneck will show higher than expected (equilibrium) heterozygosity for a large number of loci.

The qualitative test was done for mode shift analysis by using 10 microsatellite markers which showed the low allelic frequency by showing normal L-shaped curve of distribution of allelic proportions in different allele frequency classes indicating absence of recent bottleneck in Diara buffaloes. The test for BOTTLENECK did not show any significant reduction of effective population size in the recent past.

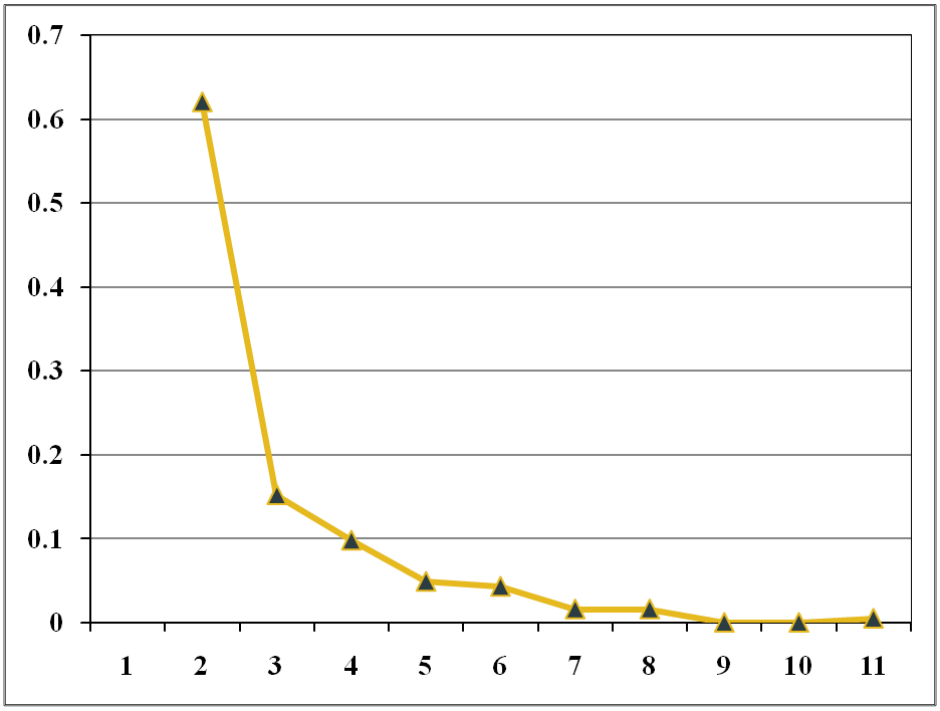

Figure No. 4.16 Mode shift analysis represents normal L- shaped structure in Diara buffalo.

4.2.6. Structure Analysis

Genetic structure of populations was analyzed by using a method developed by Pritchard et al. (2000), utilizing the software STRUCTURE (https:\\web.stanford.edu\group\Pritchard lab\ structure.html). The program implements a model-based clustering method to infer population structure, assigning individuals to populations and identifying migrants and admixed individuals using multi-locus genotype data, like microsatellite data, independent of prior population information. The approach implemented in STRUCTURE assumes a model in which there are K populations (where K may be unknown), each of which is characterized by a set of allele frequencies at each locus. Individuals in the sample are assigned probabilistically to a single population or jointly to two or more populations if their genotypes indicate them to be admixed.

In farm animal species, the prevalence of sire lines selected for economic traits leads to increased consanguinity. Such a breeding system produces reduced heterozygosity within a sub-population in a breed. However in our case, although selection and use of extensive A.I. are absent, the availability of very few breeding bulls in the Breeding tract might have contributed to increased consanguinity. This is further supported by the estimated mean value

of FIS in the population which was positive Thus, the shortage of breeding bulls in the population and confinement of these buffaloes to a small geographical area could be the possible reasons for the deficiency of heterozygotes.

CHAPTER No.5

SUMMARY AND CONCLUSIONS

5.1 SUMMARY

Diara buffaloes are medium built animals distributed almost linear along both sides of banks of Ganges called Diar and its tributaries from Buxar to Bhagalpur district in Bihar and to some extent in the territories outside along this line. The region where Diara buffaloes survives is almost marshy in most part of the year and getting dried for cultivation only during dry-weather conditions.

Geographically, the breeding tract lies between 25°N and 26°N latitude and between 84°E and 90°E longitude in the middle Gangetic plains of India. Its average elevation above sea level is ranged between 180 to 171 Feet. Bihar has an area of 93.6 lakh hectares, accounting for nearly 3 percent of the country's total geographical area. Primarily, the climate is sub-tropical with peak summer temperatures averaging around 40 degree Celsius during March-May and winter months during December-January recording temperatures averaging around 8 degree Celsius. The breeding tract of Diara buffaloes is found in major the alluvial plains of South Bihar which is generally characterized by relatively low average rainfall around 1102 mms.

5.1.1 Status of Buffaloes in The Breeding Tract

There are fifteen well recognized breeds of buffalo in India which constitutes about 30% of the total buffalo population in the country. However, 70% of the total buffalo population in the country is classified as non-descript because adequate efforts have not been made so far to characterize them phenotypically and genetically.

The milk production in India is currently estimated to be 165.4 million Tonnes in 2016 – 17 with 6.4% growth rate, of which 49.2% milk is being contributed by indigenous (35.4%) and Non – descript (13.8%) buffalo population (BAHFS,2017-18). The in–milk buffalo population in India is reported to be 92.6 million Tonnes. The Bihar had presented increasing trend of milk production from 2012 to 2017 and has produced 8.7 million Tonnes milk (BAHFS 2017). The out of which Buffalo milk 3.35 million Tonnes produced in Bihar contributed 4.1%

milk to total milk 81.2 million Tonnes produced by Buffaloes in India. The average milk yield per In–milk Buffalo presented increasing trend from 3.95 Kg/day (2012 -13) to 4.3 Kg/day (2016 – 17) in Bihar. The normal average productivity of Buffaloes is 5.23 Kg/day in India with significant high productivity 8.39 Kg/day in Haryana and 8.21 Kg/day in Punjab. The Diara buffalo daily milk yield was estimated 4.9 liters per day which is below the national average productivity of buffalo in India.

Diara buffaloes are housed close to the human dwellings. In most cases, closed housing is provided (85%). In most instances (75%), the animals and humans are housed in different parts of the same building. In the remaining cases, a separate structure was provided to animals away from farmer's house. Most of the constructions are permanent (85%) with thatched roofs covered with paddy straw or tiled roofs. Asbestos roof was also observed in 5% animal houses. Floors are generally uneven without proper drainage facilities. In peri-urban areas, the animals are overcrowded with less than the minimum required floor space of 3.5 square meters (ICAR, 2002) being provided. In rural areas, the practice of allowing the animals to wallow in the nearby water sources is prevalent (90.5 %). Mostly the animals wallow around noon after grazing in the fields under a hot sun. Animals are kept in night in closed house. In day time, animal was feed in open area of house where their Mengers are located. After morning feeding and milking, animals are either kept in open area away of house or let loose to graze in field and wallow. Similarly, in evening, animals were given feeds in their mangers and after milking they are brought in closed house. The region where Diara buffaloes survives is almost marshy in most part of the year and getting dried for cultivation only during dry-weather conditions.

Rearing of cattle and buffaloes in India is mostly in hand of small and marginal farmers and part of small holder production system. In rural area of India, major livestock population is found where disorganized farming system is prevalent. The livestock reared in urban and peri-urban area constitute scanty population out of total population where to some extent organized farming systems is found. The herd structure included almost nil adult males, 2.0 adult females, 0.2 male calves and 0.8 female calves. The disparity between male and female calves might be due to immediate disposal of male calves by the farmers once dam's milk yield ceases or even during milking period. Farmers take less interest in better caring of male because they find uneconomical rear them. Due to mechanization of agriculture systems, drought power of male

Diara buffalo has also become unimportant. Adult male buffaloes are even almost unavailable at farmer's house for breeding.

Paddy straw, dry mixed grasses and green grasses are the main sources of roughage. Wheat bran, linseed oil cake, mustard oil cake and rice bran are given as concentrates. About two third of the farmers provide concentrates to the milking animals; 0.5 to 2 kg of concentrate is usually given to the lactating animals at the time of milking. Some farmers even feed the animals with kitchen wastes and hotel wastes; this practice is more prevalent in the urban areas.

Breeding of buffaloes is highly disorganized in the breeding tract. Natural service is occasionally in practiced of the farmers in the urban and peri-urban areas using breeding bulls. Here, artificial insemination is primarily used for breeding. In the rural areas A.I. is now getting priority due to unavailability of breeding bulls and practical difficulties of farmers. Therefore, Natural service is not very common in rural farmers also. Breeding bulls those used for breeding in rural area are free ranged animals. Their ears are generally cut for certain rituals of locals' people after which they are let loose to field for open grazing. Although A.I. services are available in urban and some rural areas, semen of Diara buffaloes is not available and the farmers have to opt for either Murrah semen. As a result, the proportion of graded Diara buffaloes and non-descript animals are more common in the urban areas.

Diara buffaloes are well-built medium-sized animals. The coat colour varies from Black to grey. Their skin is black. The black coloured Diara buffaloes were found more efficient producer and popular among farmers in comparison to grey coloured one. The head is fairly long with a broad forehead. Ears are moderately long and erect. The neck is moderately long with almost negligible dewlap. Horns are medium sized, flat, corrugated and curved, projecting backward, sideward and upward at half of the neck. Shoulders are long and slope smoothly with the body. The barrel is well built and medium in size with a straight and wide back. Legs are strong with hard hooves. The udder is moderately developed with teats of medium size and squarely placed between the hind legs. The tail is fairly long, thin and flexible ending in a black and white switch. The Height at wither (cm), Body length Horizontal (cm), Body length Oblique (cm), Heart girth (cm), Paunch Girth (cm), Leg (cm), Neck (cm), Neck Circumstance (cm), Face Length (cm), Face Width (cm), Ear Length (cm), Hip Bone (cm), Hip Height (cm), Pin Bone (cm), Distance between Hip and Pin Bone (cm), Tail length and Tail Length up to

Switch for the calves below the age of 7 days were found to be 80±2, 52.5±3.5, 57.5±4.5, 80.5±2.5, 81.5±2.5, 53.5±2.5, 15±1, 45±1, 21±4, 9.5±0.5, 15.5±2.5, 16.5±1.5, 75.19±3.83, 8.5±0.5, 22±2, 37.5±4.5 and 43.5±4.5, respectively. The Height at wither (cm), Body length Horizontal (cm), Body length Oblique (cm), Heart girth (cm), Paunch Girth (cm), Leg (cm), Neck (cm), Neck Circumstance (cm), Face Length (cm), Face Width (cm), Ear Length (cm), Horn Length (cm), Horn Circumstance (cm), Distance between horns (cm), Hip Bone (cm), Hip Height (cm), Pin Bone (cm), Distance between Hip and Pin Bone (cm), Tail length and Tail Length up to Switch for young stock of age between one week to one year were found to be 94.25±5.009, 76.37±6.89, 87±5.69, 117±10.12, 137±10.78, 60±1.81, 30.87±1.75, 62.37±7.02, 31.87±1.65, 13.87±0.58, 22±0.96, 8.14±2.56, 11.28±1.10, 18.71±1.59, 22.37±1.06, 106±16.5, 12.25±1.57, 26.37±1.96, 60±4.73, 70.25±5.75, respectively. The Height at wither (cm), Body length Horizontal (cm), Body length Oblique (cm), Heart girth (cm), Paunch Girth (cm), Leg (cm), Neck (cm), Neck Circumstance (cm), Face Length (cm), Face Width (cm), Ear Length (cm), Horn Length (cm), Horn Circumstance (cm), Distance between horns (cm), Hip Bone (cm), Hip Height (cm), Pin Bone (cm), Distance between Hip and Pin Bone (cm), Tail length and Tail Length up to Switch for young stock of age between one year to three year were found to be 111.8±7.48, 89.2±7.51, 101.20±9.24, 150.8±15.24, 167.2±14.48, 69±4.33, 37.8±2.41, 72.6±9.9, 36.8±2.2, 17.4±0.97, 25±0.89, 21.2±3.24, 18±1.37, 23.6±2.65, 32.4±3.52, 112±16, 21.2±4.95, 31.2±2.47, 68±5.54 and 77.8±5.01, respectively.

The Height at wither (cm), Body length Horizontal (cm), Body length Oblique (cm), Heart girth (cm), Paunch Girth (cm), Leg (cm), Neck (cm), Neck Circumstance (cm), Face Length (cm), Face Width (cm), Ear Length (cm), Horn Length (cm), Horn Circumstance (cm), Distance between horns (cm), Hip Bone (cm), Hip Height (cm), Pin Bone (cm), Distance between Hip and Pin Bone (cm), Tail length and Tail Length up to Switch for adult male Diara buffalo of age above the three year were found to be 138.12±4.5, 130.26±5.1, 141.25 ±5.2, 208±2.5, 228±2.89, 81.21±1.9, 53.26±3.9, 95.16±5.9, 44.25±1.34, 23.89±3.84, 27.20±1.29, 34.3±1.98, 21.16±1.01, 29.4±1.2, 55.30 ±3.5, 135.46±2.75, 26.9±1.95, 40.23±2.3, 88.45±3.9 and 97.79±3.5, respectively. The Height at wither (cm), Body length Horizontal (cm), Body length Oblique (cm), Heart girth (cm), Paunch Girth (cm), Leg (cm), Neck (cm), Neck Circumstance (cm), Face Length (cm), Face Width (cm), Ear Length (cm), Horn Length (cm), Horn Circumstance (cm), Distance between horns (cm), Hip Bone (cm), Hip Height (cm), Pin Bone (cm), Distance between Hip and Pin Bone (cm), Tail length and Tail Length up to Switch

for adult male Diara buffalo of age above the three year were found to be 129.77±2.24, 117.77±3.54, 129±3.50, 192.31±5.67, 19.09±6.12, 76.09±1.61, 44.63±1.43, 87±2.11, 40.22±1.96, 19.6±0.69, 26.09±0.95, 32.1±2.08, 18.47±1.12, 26.80±0.92, 47.14±2.17, 126.41±1.95, 25.4±1.61, 37.3±1.54, 85.05±2.59, 95.65±3.33, respectively. There was no significant difference found between male and female Diara buffalo for morphometric traits.

Diara buffaloes are moderate milk producers and normally give four to nine liters of milk daily. Some animals in villages reach a peak yield of more than 9.65 litres per day, however. The average daily milk yield was 4.9±0.4 litres as reported by the farmers. The length of lactation varied from 210 to more than 340 days with an average of 301.6±10.3 days. The lactation milk yield varied from 1008.4±95.7 to 1635.6±112 litres with a mean of 1450.87±28.7 litres. Diara buffaloes have relatively long productive life spans as demonstrated by animals with more than five calving's commonly found in the villages. Age at first calving and calving interval was estimated to be 46.27 ± 0.63 months and 14.4 ± 0.13 months, respectively. The Dry period, average age at first service and Service period was estimated to be 89.87±4.25 days, 34.86±0.78 months and 131.31±3.06 days. Diara buffaloes are dual purpose animals used for milk production as well as agricultural operations in wet fields. They are better suited than are local cattle to ploughing and puddling the wet fields meant for paddy cultivation. They are active, fast moving, hardy and can work continuously for four to six hours in the wet fields. Generally, males are used for the purpose, males are preferred.

Sufficient genetic diversity was found to exist in the population as revealed by microsatellite data, however steps need to be taken for the genetic improvement as well as conservation of this precious germplasm of the country.

In the present investigation, an attempt has been made to genetically characterize Buffalo germplasm of Diara region (Bihar) using suitable FAO specific Microsatellite markers in its breeding tract and its adjoining areas using 10 FAO recommended buffalo specific microsatellite markers. Microsatellite analysis revealed high level of polymorphism and informativeness of studied microsatellite markers in genetic diversity analysis in Local Buffalo Population. The significant level of variability in this population reflects that the local buffalo population contains a valuable and substantial amount of genetic diversity among the studied breed but the study needs to be extended to include more microsatellites in a large sample size to further validate the research.

Diara buffaloes are reared for milk purposes. These buffaloes are able to thrive well in low input systems forming an integral part in the livelihood of farmers in the region. Sufficient genetic diversity was found to exist in the population as revealed by microsatellite data, however steps need to be taken for the genetic improvement as well as conservation of this precious germplasm of the country.

In the present investigation, an attempt has been made to genetically characterize Buffalo germplasm of Diara region (Bihar) using suitable FAO specific Microsatellite markers in its breeding tract and its adjoining areas using 10 FAO recommended buffalo specific microsatellite markers. Microsatellite analysis revealed high level of polymorphism and informativeness of studied microsatellite markers in genetic diversity analysis in Local Buffalo Population. The high PIC values as observed in the study are indicative of high informativeness of studied markers for genetic diversity analysis in Population. Most studied microsatellite markers had desired neutrality, thus proving to be good candidates for genetic characterization and diversity analysis. The information gathered could be utilized to plan breeding, improvement and conservation programs for this valuable Buffalo germplasm resource to exploit its unique adaptability traits. The significant level of variability in this population reflects that the local buffalo population contains a valuable and substantial amount of genetic diversity among the studied breed but the study needs to be extended to include more microsatellites in a large sample size to further validate the research.

5.2 CONCLUSIONS

- Diara buffaloes are hardy, dual purpose animals reared for both milk and draught purposes.
- There is need to organise the breeding systems of Diara buffaloes by making availability of male germ plasm.
- Diara buffaloes are reared for milk and draught purposes. These buffaloes are able to thrive well in low input systems forming an integral part in the livelihood of farmers in the region.
- Sufficient genetic diversity was found to exist in the population as revealed by microsatellite data, however steps need to be taken for the genetic improvement as well as conservation of this precious germplasm of the country.

- Microsatellite analysis using 10 FAO recommended revealed high level of polymorphism and informativeness of studied microsatellite markers in genetic diversity analysis in Local Buffalo Population.
- The significant level of variability in this population reflects that the local buffalo population contains a valuable and substantial amount of genetic diversity among the studied breed but the study needs to be extended to include more microsatellites in a large sample size to further validate the research.

CHAPTER NO. 6

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CHAPTER-7

APPENDIX (ces)

7.1 APPENDIX-1

Preparation of commonly used stock solutions:

Solution	Method of preparation	Comments
Deoxy ribonucleotide triphosphate (dNTPs)	Dissolve each dNTP in H_2O at an approximate concentration of 100mM. Using 0.05 M Tris base and a micropipette, adjust the pH of each of the solutions to 7.0 (use pH paper to check the pH). Dilute an aliquot of the neutralized dNTP appropriately. Calculate the actual concentration of each dNTP. Dilute the solutions with H_2O to a final concentration of 50 mm dNTP. Store each separately at -70˚C in small aliquots. 100 mm stock solutions of each dNTP are commercially available (Pharmacia) if you do not want to prepare our own.	
0.5 M EDTA (pH 8.0)	Add 186.1g of disodium ethylenediaminetetra-acetate. 2 H_2O to 800 ml of H_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets) Dispense into aliquots and sterilize by autoclaving.	The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approximately 8.0 by the addition of NaOH.

Ethidium bromide (10mg/ml)	Add 1g of ethidium bromide to 100 ml of H_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminium foil or transfer the solution to a dark bottle and store at room temperature.	Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain the dye and a mask should be worn when weighing it out. After use, these solutions should be decontaminated
Phenol-chloroform	Mix equal amounts of phenol and chloroform. Equilibrate the mixture by extracting several times with 0.1 M Tris - CI (pH 7.6). Store the equilibrate mixture under an equal volume of 0.01 M Tris-CI (pH 7.6) in 4˚C in dark glass bottles.	Caution: Phenol is highly corrosive and can cause severe burns. Wear gloves protective clothing, and safety glasses when handling phenol. All manipulations should be carried out in a chemical hood. Any area of skin that comes into contact with phenol should be rinsed with a large volume of water.
1M Potassium acetate (pH 7.5)	Dissolve 9.82 g of potassium acetate in 90 ml of pure H_2O (Milli-Q or equivalent). Adjust the pH to 7.5 with 2 M acetic acid. Add pure H_2O to 100ml. Divide the solution into aliquots and store them at -20°C.	
3M Sodium acetate (pH 5.2 and pH 7.0)	Dissolve 408.1g of sodium acetate $3H_2O$ in 800ml of H_2O. Adjust the pH to 5.2 with glacial acetic or adjust the pH to 7.0 with dilute acetic acid. Adjust the volume to 1 liter with H_2O.Dispense into aliquots and sterilize by autoclaving.	

5M NaCl	Dissolve 292.2 g of NaCI in 800 ml of H_2O. Dispense into aliquots and sterilize by autoclaving.	
10% sodium dodecylsulfate (SDS) (also called sodium laurylsulfate)	Dissolve 100 g of electrophoresis-grade SDS in 900 ml of H_2O. Heat to 68°C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCI. Adjust the volume to 1 liter with H_2O. Dispense into aliquots.	Wear a mask when weighing SDS and wipe down the weighing area and balance after use because the fine crystals of SDS disperse easily. There isno need to sterilize 10% SDS.
1M Tris	Dissolve 121.1 g of Tris base in 800 ml of H_2O. Adjust the pH to the desired value by adding concentrated HCI pH HCI 7.4 70 ml 7.5 60 ml 8.0 42 ml Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving.	If the 1 M solution has a yellow color, discard it and obtain better quality Tris. Although many types of electrodes do not accurately measure the pH of Tris solutions, suitable electrodes can be obtained from most manufacturers. The pH of Tris solution is temperature dependent and decreases approximately 0.03 pH units for each 1°C increase in temperature.
6X gel loading Dye	Bromo phenol blue 0.25%(w/v) Xylene cyanol 0.25% (w/v) Glycerol in water 30% (v/v)	
TAE buffer (50X)	Tris base : 242 g Glacial acetic acid : 57.1 ml EDTA (0.5M. pH 8.0) : 100 ml Added double distilled water to a volume of 1000ml, filtered and autoclaved.	

7.2 APPENDIX – II

Sr. No.	Technique used
1	Polymerase Chain Reaction
2	Genotyping
3	Gel Electrophoresis

Sr. No.	Bio-Informatics technique used
1	DNASTAR Software (Lasergene)
2	Primer3
3	NCBI (National Center for Biotechnology information)
4	Bottleneck
5	GenAleX 6.5
6	Structure
7	PopGene
8	Networking

BRIEF BIO-DATA OF THE STUDENT

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ACADEMIC QUALIFICATION

Qualification / Discipline	Passing Year	School / College	Board / University	Percentage / OGPA with Division
10th	2008	Gayatri Vidhya Mandir Sr. Secondary School, Sanchore, Dist.- Jalore (Raj)	BSER, Ajmer	54.00% (Second Division)
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B. V. Sc. & A. H.	2017	M. B. Veterinary College, Dungarpur (Raj)	RAJUVAS, Bikaner	7.12 OGPA (First division)
M. V. Sc. (Animal Genetics & Breeding)	Appearing	Bihar Veterinary College, Patna	Bihar Animal Sciences University, Patna	8.639