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MICROSATELLITE ANALYSIS OF BUFFALOES OF GUJARAT STATE

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1. INTRODUCTION

The first animal to be domesticated before 6000 B.C. was sheep and goats followed by cattle (Bos) in lowlands of Mesopotamia gradually diverse animals would have come under human control in other regions also. In Indian sub-continent, there is evidence from Mohenjo Daro/Harappa indicating the domestication of sheep, cattle, buffaloes (Cleson, 1977).

India also has great diversity in domestic livestock and poultry genetic resources. There are 30 breeds of cattle, 10 breeds of river buffaloes, 43 of sheep, 20 of goats, 8 of camel, 7 of horses and 18 of indigenous poultry and a few of pigs, yaks and other livestock in the socioeconomic development of millions of rural households. India has more than 50% worlds buffalo and 20% of its cattle. The Indian dairy sector contributes a large share of the agriculture gross domestic product (GDP). Although share of agriculture to GDP as declined during last decades, the contribution of the livestock sector has increased from less than 5% in early 1980's to over 6% in the late 1990s. During 1950s and 1960s. India was one of the largest importers of dairy products importing over 40% of milk solids in the total throughput of the dairy industry.

Buffaloes have emerged as the main dairy animal, in the Indian sub-continent and now India alone has the privilege of having about 455 of the worlds buffalo population. According to the FAO survey, about 90% of draft buffaloes contribute power for agriculture sector in the South East Asian countries. Buffalo forms the backbone of agriculture and dairy industry in India. In addition to milk they provide much of the draught power for farm operations and transport meat, hides and dung throughout the India Sub-continent. Buffalo are the preponderant species among all livestock in India and account for over 75% of total output at Rs.295 billion in milk, Rs.37 billion in work draught, Rs.43 billion in dung and Rs.40 billion in meat hides and output value of Rs.470 billion and all livestock produce in 1991. In 1992 country had 83.3 million buffalo registering a growth of nearly 32% over the 1951 population in case of buffalo. Source of food and power and also buffalo form the backbone of agriculture and dairy industry in India. Probably milk production formed the basis of selection and breeding, which resulted in evolution of dairy breeds of riverine buffalo like Murrah, Kundi, Nili Ravi, Jaffarabadi, Surti, and Mehsana etc.

The present study was undertaken on the buffalo breed which is perhaps the heaviest breed of buffalo in Indian sub continent.

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Jaffarabadi: This breed of buffalo is found in Gujarat. This breed is of riverine type has different names according to its native place. As it is also called as Gir as it is found in Gir forest of Kathiawar, Jaffari, Bhawanagri in Jaffarabad and Bhavnagar respectively. This has its home tract in Junagarh and Amreli districts of Saurashtra region. They are massive in conformation and are heavy grazers. There main green fodder are maize, sorghum, groundnut fodder, sugarcane crops and dry fodders are groundnut husks and hulls, wheat straw, bhusa etc.

It is characterized with relatively long body and loose frame. A typical Jaffarabadi is black in colour but a few with white spots called Nav Chandra are also seen. Hairs are medium in length, straight and glossy. The horns are heavy and broad inclined to drop on each side of the neck and slightly ascent close to tip is an incomplete curl (A ring like structure).

On an average adult Jaffarabadi female and male weight upto 700 to 1000 respectively. Exceptionally heavy bulls may weigh upto 2000 kg. The milk yield ranges between 1800 & 2700 kg. in one lactation. Average fat is 7-8%. Males are good draught animals.

The present study was taken in Jaffarabadi with following objectives:

1. To study the heterologus microsatellite loci primers for amplification in buffalo.
2. To study the diversity of Jaffarabadi buffalo.
3. To study the Hardy –Weinberg equilibrium, neutrality and linkage disequilibrium of the microsatellite loci in the buffalo populations.
4. To study the inter-individual genetic distances.
5. To analyze whether these population is in mutation drift equilibrium.
6. To analyze and study the various population genetic parameters of Jaffarabadi buffalo.

2. MATERIALS AND METHODS

2.1 MATERIAL REQUIRED:

2.1.1 Blood Sample Collection:

Populations of buffalo Jaffarabadi was selected and 45 blood samples from each breed were collected at random from field conditions from the breeding tract for these populations (fig 6.). 10 ml of whole blood was collected aseptically from juglar vein of each animal using heparinised vacutainer tubes and transported to laboratory at 0-5^{o}C

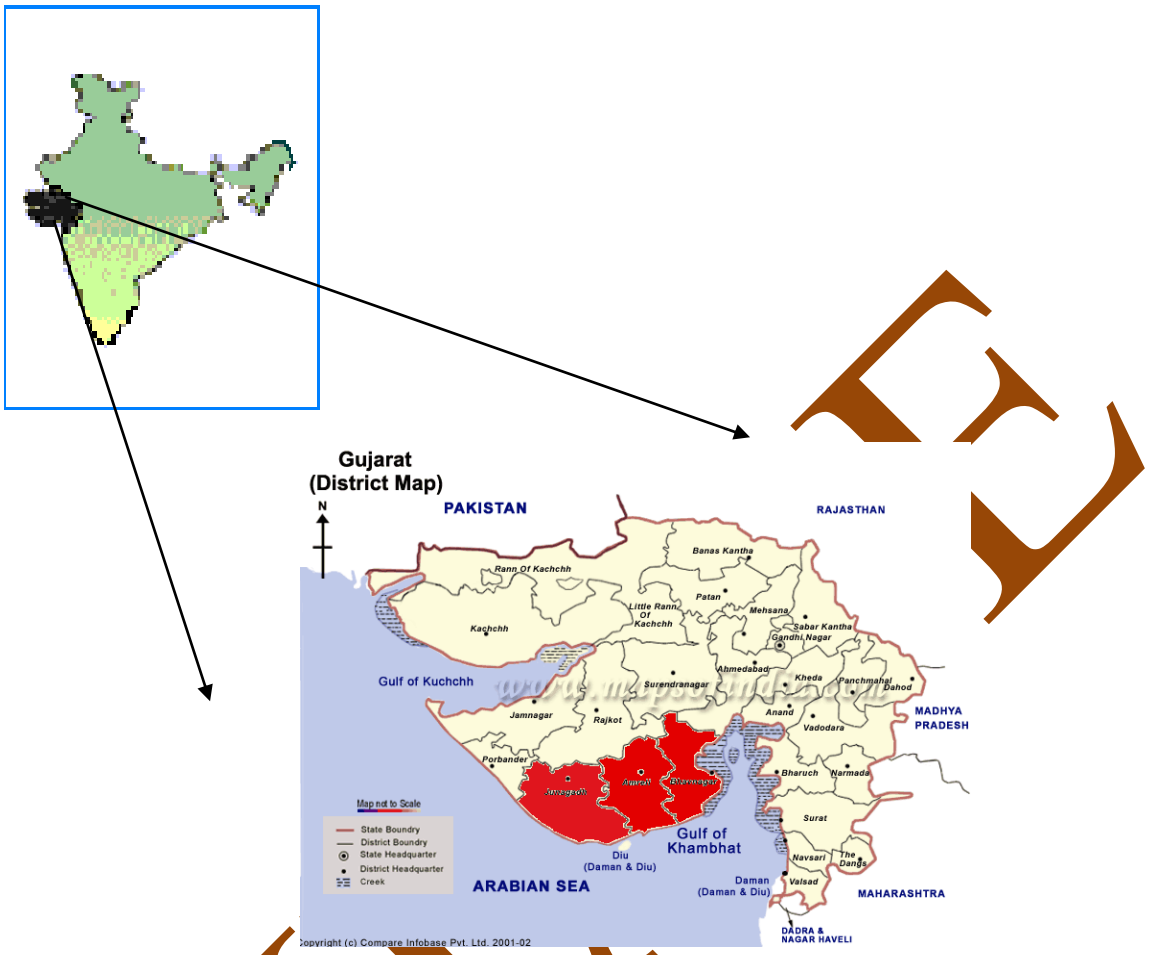

Fig.1 Geographical Distribution And Breeding Tract Of Jaffarabadi Buffaloes

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2.1.2 DNA Isolation:

For isolation of DNA from collected sample, the blood was transferred to autoclaved Oakridge centrifuged tubes. The RBCs were lysed with lysis buffer (Ammonium chloride 155 mM, Potassium bicarbonate 10 mM and EDTA 0.1 mM). In all the blood samples, double amount of lysis buffer was added. The samples were then mixed gently and kept in ice for 10 minutes. The tubes were centrifuged at 12,000 rpm for 10 minutes at 4^{o}C in the refrigerated centrifuge. The supernatant was carefully decanted and the pallet was redissolved in lysis buffer and washed three times as described above or until white pallet was obtained.

The white pellet was resuspended in 10 ml digestion buffer (Sodium chloride 75mM, Tris-Cl 1 M, pH8.0 and EDTA 0.5 mM). Vortexed gently and to it added 20% Sodium sulphate (200 µl/sample) and Proteinase K (1 mg/sample) and incubated at 57^{o}C overnight in a water bath. After incubation, digested solution was obtained to which equal amount of Tris equilibrated phenol (pH 8.0) was added, mixed gently by moving the tubes gently '8' fashions for 10 minutes and centrifuge at 12,000 rpm for 10 minutes at 25^{o}C. After centrifugation the aqueous phase and organic phase were separated. The DNA remained dissolved in aqueous phase while the protein was retained at the interphase. The aqueous phase was transferred carefully by Pasture pipette to another Oakridge tube without disturbing the interphase. To the aqueous phase, equal amount of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed by moving the tubes gently in '8' fashions for 10 minutes at 25^{o}C to separated aqueous phase and organic phase. Again the aqueous phase was carefully pipetted to another tube without disturbing the interphase. The aqueous phase was mixed with chloroform: isoamyl: alcohol (24:1). The solution was subjected to centrifugation at 12,000 rpm for 10 minutes at 25^{o}C and aqueous phase was carefully transferred to glass culture tubes. The DNA was precipitated by adding 1/10th volume of Sodium acetate (3M, pH 5.2) and 2.5 volume of aqueous phase of chilled absolute alcohol and cover the tubes with paraffin and mix slightly, the DNA get precipitated (visible as white stringy strands).

2.1.3 Washing and Storage of Isolated DNA:

The DNA was spooled out into eppendrof tubes washed twice with 70% ethanol to remove the salts. After washing the alcohol was allowed to evaporate and DNA was redissolved in 500 µl Tris EDTA buffer (Tris 10mM, EDTA 10mM, pH 8.0) and kept out eppendrof tubes at 65^{o}C for 1 hour. The stock DNA was stored at –20^{o}C.

3. AGAROSE GEL ELECTROPHORESIS FOR DILUTION OF GENOMIC DNA

The technique of electrophoresis is based on the fact that DNA is negatively charged at neutral pH due to its phosphate backbone. For this region, when an electrical potential is placed on the DNA, it will moves toward the positive pole.

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3.1 Dissolving of Agarose

- Prepared the 1X TAE buffer by diluting 50X TAE buffer.
- Weighed out 0.9 gm of agarose (0.6%) and add 150 ml 1X TAE.
- In microwave oven heat the slurry for 3 minutes in one minute incriminates, swirling the solution gently between heating cycles to release trapped air and resuspend any agarose particles caught on the side of the Erlenmeyer flask.
- Ethidium bromide was added to the warm solution and cooling the solution about 50^{o}C. [Ethidium bromide is intercalating dye and carcinogenic, handle this gel only while wearing gloves].

3.2 Casting the gel

After cooling the solution it was poured into a casting tray containing a sample comb and allowed to solidify at room temperature. The gel was 3-5 mm thick. There were no air bubbles under or between the teeth of comb. In case, if bubbles form, they can be removed by pocking them with the pointed end of pipette tips before the gel has set. After the gel has solidified, the comb was removed carefully by wriggling back and forth gently and then lifting up carefully, not rips the bottom of the well.

3.3 Preparing the sample:

While the gel was cooling, prepared the DNA sample by adding 5 µl of tracking dye to 1 µl of each DNA sample. The tracking dye contains Bromophenol Blue and Xylene cynol FF and 50% glycerol. Adding tracking dye to the sample increased its density so it falls into the well of the gel and provided a visible marker to monitor the progress of electrophoresis. Also prepared molecular size standard by mixing 5 µl of the 1 kb ladder with 1 µl of tracking dye. Bromophenol Blue migrated through agarose gel approximately 2.2 fold faster than Xylene cynol FF dye, independent to agarose gel concentration.

3.4 Loading and running the gel:

The gel on the tray, inserted horizontally into the electrophoresis chamber and flooded the top of the gel with fresh running buffer (1X TAE) to cover the gel to depth of about 1 mm. Sucked the solution (DNA sample with dye) into the pipette, placed the tip in the top of the well and gently expelled the solution into the well. The lid and power leads were placed on the apparatus, 40-80 V current is applied, and current flowing was confirmed by observing bubbles coming off from the electrodes. Run the gel until the Bromophenol blue and Xylene cynol FF was migrated the appropriate distance about three-fourth of the way across the gel.

3.5 Visualization of DNA:

Bright orange colour DNA bands were visualized by placing the tray onto High Performance UV Transilluminator.

4. POLYMERASE CHAIN REACTION AMPLIFICATION OF MICROSATELLITE LOCI

Polymerase chain reaction (PCR), is the major scientific development of the last quarter century, sometime referred to as “molecular photocopying”, that can characterize, analyse, and synthesize any specific piece of DNA or RNA. Polymerase chain reaction is an in vitro method for analysing defined sequence of DNA. Kary Mullis, while working Cetus Corporation, California invented PCR in 1985 for which he was awarded the Nobel Prize in chemistry in 1993.

There are three major steps in PCR, were carried out in the same vial but at different temperatures and are repeated for 30 cycles. This was done on an automated thermocycler, which automatically heated and cooled the tubes with the reaction mixture in a very short time.

Denaturation at 94^{o}C: During the denaturation, the first part of the process, the double strand melted, open to single stranded DNA, all enzymatic reactions stop (e.g., the extension from a previous cycle).

Annealing at 55-60^{o}C: The primers were jiggling around, caused by the Brownian motion. Ionic bonds were constantly formed and broken between the single stranded primer and the single stranded template. The polymerase enzyme attached on double stranded DNA (template and primer) and started copying the template. The vial is cooled to 55-60^{o}C. At this temperature, the primers bind or "anneal" to the end of the DNA strands.

Extension at 72^{o}C: This was the ideal working temperature for the polymerase. Primers that were on positions with no exact match get loose (because of the higher temperature) and didn't give an extension of the fragment.

4.1 Criteria For Selection of Primers

The primers were selected on the basis of recommendation given by diversity analysis group of Food and Agriculture Organization (Barker, 1995). The main criteria for selection of microsatellite loci for biodiversity analysis were:

1. The followed Mendelian heritance.
2. They were polymorphic in nature with minimum three alleles.
3. The microsatellite loci information is available in public domain.
4. They are suitable for cross amplification among related species.
5. They should be at linkage equilibrium.
6. They should be representative of whole genome.

Table No –1(a) List of Cattle Primers Screened in Buffalo Breeds:

Primer	Sequences	Repeats	Dye	Accession Number
ILSTS87	F- AgCAgACATgATgACTCAgC R-CTgCCTCTTTTCTTgAgAgC	$(CA)_{14}$	Ned	L37279

ILSTS59	F- AgTATggTAAggCCAAAggg R- CgACTTgTgTTgTTCAAAgC	$(gT)_{21}$	Vic	L37266
ILSTS52	F- CTgTCCTTTAAgAACAAACC R- TgCAACTTAggCTATTgACg	$(CA)_{11}$	Pet	L37222
CSSM29	F- TCTCCATTATgCACATgCCATgCT R- CgTgAgAACCgAAAgCACACATTC	$(AC)_{18}$	Ned	U03807
ILSTS11	F- GCTTgCTACATggAAAgTgC R- CTAAAATgCAgAgCCCTACC	$(CA)_{11}$	Vic	L23485
BM1818	F- AgCTgggAATATAACCAAAgg R- AgTgCTTTCAAggTCCATgC	$(Tg)_{13}$	Pet	G18391
ILSTS72	F- ATgAATgTgAAAgCCAAggg R- CTTCCgTAAATAATTgTggg	$(CA)_{14}$	Ned	L37272
ILSTS49	F- CAATTTTCTTgTCTCTCCCC R- gCTgAATCTTgTCAAACAgg	$(CA)_{9}$	Pet	L37261
ILSTS05	F- GgAAgCAATgAAATCTATAgCC R- TgTTCTgTgAgTTTgTAAgC	$(Tg)_{9}$	Vic	L23481
ILSTS58	F- GCCTTACTACCATTTCCAgC R- CATCCTgACTTTggCTgTgg	$(gT)_{15}$	Vic	L37225
CSSM43	F- AAAACTCTgggAACTTgAAAAACTA R- gTTACAAATTTAAgAgACAgAgTT	$(CA)_{19}$	Ned	U03824
CSSM45	F- TAgAggCACAAgCAAACCTAACAC R- TTggAAAgATgCAgTAgAACTCAT	$(CA)_{14}$	Ped	NW_381320

Table No-1(b) List of Cattle Primers Screened in Buffalo Breeds:

Primer	Sequences	Repeats	Dye	Accession Number
CSSM47	F- TCTCTgTCTCTATCACTATATggC R- CTgggCACCTgAAACTATCATCAT	$(gT)_{12}$	Ned	U03821
ILSTS30	F- CTgCAgTTCTgCATATgTgg R- CTTAgACAACAggggTTTgg	$(gT)_{10}$	Vic	L37212
CSSM08	F- CTTggTgTTACTAgCCCTggg R- gATATATTTgCCAgAgATTCTgCA	$(Tg)_{15}$	Pet	NW_375905
CSSM33	F- CACTgTgAATgCATgTgTgTgAgC R- CCCATgATAAgAgTgCAgATgACT	$(Tg)_{16}$	Pet	U03805
CSRM60	F- AAgATgTgATCCAAgAgAgAggCA R- AggACCAgATCgTgAAAggCATAg	$(CA)_{17}$	Vic	AF232758
ETH152	F- TACTCgTAgggCAggCTgCCTg R- gAgACCTCAgggTTggTgATCAg	$(CA)_{17}$	Ned	Z14040
CSSM19	F- TTgTCAgCAACTTCTTgTATCTTT R- TgTTTTAAgCCACCCAATTATTTg	$(Tg)_{18}$	Vic	AF232761
CSSM06	F- AgCTTCTgACCTTTAAAgAAAATg R- AgCTTATAgATTTgCACAAgTgCC	$(Tg)_{13}$	Vic	U03787
ILSTS29	F- TgTTTTgATggAACACAgCC R- TggATTTAgACCAgggTTgg	$(AC)_{19}$	Ned	L37252
CSSM57	F- TgTggTgTTTAACCCTTgTAATCT R- gTCgCTggATAAACAATTTAAAgT	$(gT)_{16}$	Pet	U03840
ILSTS38	F- GggCATTATTTTgTTTCCC R- CCACTTCTgggTAATTATCC	$(gT)_{14}$	Pet	L37256

4.1.2 Taq DNA Polymerase:

Taq DNA polymerase is obtained from the thermophilic archaea family bacteria Thermus aquaticus. It possesses a 5'→ 3' polymerase activity and a double strand specific 5'→ 3' exonuclease activity.

4.1.3 PCR Buffer

PCR Buffer (Sigma) contains Tris–Cl (100 mM, pH 8.3 at 25°C), KCl (500 mM), $MgCl_2$ (15 mM), gelatin (0.01%). It supports the activity of Taq polymerase.

4.1.4 Magnesium Chloride concentration

Magnesium concentration is a crucial factor affecting the performance of Taq DNA polymerase. Reaction components, including template DNA, chelating agents present in the sample (e.g., EDTA), dNTPs can affect the amount of free magnesium. In the absence of adequate free magnesium, Taq DNA polymerase is inactive. Conversely, excess free magnesium reduces enzyme fidelity and may increase the level of nonspecific amplification. The optimal $MgCl_2$ concentration is use for each reaction.

4.2 PRE-PCR PREPARATION:

The PCR was performed under standard condition as described by Kaul et al., 2001. The genomic DNA was diluted so as to contain about 50 ng DNA/µl.

4.2.1 Cocktail Preparation:

Table No-2: The cocktail for PCR one reaction (14 µl) consisted of:

	Volume	Concentration
10X PCR Buffer	1.5 µl	-
DNTPs	0.1 µl	200 µM
Primer (Forward)	1.0 µl	4 pmol
Primer (Reverse)	1.0 µl	4 pmol
Taq Polymerase	0.08 µl	0.4 Unit
Distilled Water	10.32 µl	-
Total	14 µl	

Beside this 2 mM $MgCl_2$ (nuclease free) was added separately for ETH152, CSSM08, ILSTS38 primers and 1.5 mM $MgCl_2$ was added for ILSTS29 primer.

4.2.2 PCR programme:

1.0 µl of the template DNA was directly added into the cocktail in each lane of the PCR plate. The PCR was carried out in Applied Bio-system and Bio-Rad thermocycler.

Table No-3 For the specific conditions, the PCR machine is programmed at:

Steps	Programme	Temperature	Time	Number of cycle
Step- 1	Initial Denaturation	95^{o}C	5 minutes	1 cycle
Step- 2	Denaturation	94^{o}C	45 seconds	30cycles
Step- 3	Annealing	55^{o}C (or standardized)	45 seconds	
Step- 4	Polymerization	72^{o}C	45 seconds	
Step- 5	Final Extension	72^{o}C	5 minutes	1 cycle
Step- 6	Final Temperature	4^{o}C	Until removed from PCR machine	

4.3 AGAROSE GEL ELECTROPHORESIS OF PCR AMPLIFIED DNA:

After completion of the PCR programme, the products were checked on 2% agarose gel for the amplification. Before loading into the well, gel-loading dye (xylene cynol FF, bromophenol blue in glycerol) was added to the sample and the samples were run under constant voltage conditions (80 V) till the two dyes were separated. Amplified product appeared as sharp orange color bands under UV Transilluminator due to the intercalation of ethidium bromide.

4.4 POST PCR MULTIPLEXING

4.4.1 Pooling Ratios

PCR products of different sizes and dyes were pooled for one capillary injection for maximizing the throughput. It was important to pool PCR products together at the correct ratios, in order to get similar florescent intensities across all loci in the pooling. The fluorescent dyes were detected with different efficiencies. The pooling ratio, or amount of each dye-labeled added with respect to the other products in the pool and was adjusted to ensure an appropriate detection of all the loci.

Dilution series was carried on each combination of pooling to determine the optimal fluorescent intensity, which gives clean sharp peaks and correct banding patterns, which was allowed for accurate allele calling. The loci in a multiplex if overlapping, were labeled with different dyes.

After determining the optimal pooling ratio and dilution ratio for a set of primers, the same dilutions were used for subsequent analyses, as PCR yields were fairly consistent. In a pool the following ratio was used:

Vic labeled PCR product - 1.5 µl

Ned labeled PCR product - 2.0 µl

Pet labeled PCR product - 2.0 µl

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4.4.2 Size Standard

Gene Scan- 500 LIZ™ Size Standard or ROX standard developed by Applied Biosystems was used for fragment sizing, LIZ size standard yield size fragments between 50- 500bp poviding16 single – standard labeled fragments of 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500 bases. Each of the DNA fragments labeled with a proprietary fluorophore, which results in a single peak when run under denaturing condition. Internal lane size standard was run with every sample for accurate sizing. The genotyping reaction components were:

Pooled PCR Product - 1 µl
Hi-Di Foramide - 8.75 µl
Liz Size standard - 0.25 µl

4.5 DENATURATION:

The above components were mixed well and denatured at 95ºC for 5 minutes. The 96 well plates were loaded in Automated DNA sequencer for genotyping.

4.6 GENOTYPING

Automated DNA Sequencer- ABI 3100 Avant, which was provided with 4 capillaries, performed Genotyping and different array sizes can be used. We used 36 cm array size for performing genotyping. The large surface area of a capillary allowed heat generated during electrophoresis to be dissipated efficiently, allowing high voltage electrophoresis. The result was rapid, high-resolution separation of DNA fragments. Polymer POP 4 (Performance Optimized Polymer) was used for sizing and separating of DNA fragments. Plate records were prepared and size standard was added to Automated DNA sequencer prior to setup of the run and then 96 well plate was linked and started the run.

4.7 DATA COLLECTION AND EXTRACTION

The data extracted from Automated DNA sequencer were sized using Gene Scan software and the sizes were extracted using Gene Mapper software version ---The data was exported as text file and imported into excel sheet before submitting it to further Statistical Analysis (fig. No-7)

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4.8 STATISTICAL ANALYSIS OF DATA

The data generated using the microsatellite loci were subjected to statistical analysis. The microsatellite data was subjected to Ewens-Watersson test of Neutrality to check for the loci being selectively neutral.

The statistical analysis was carried out using POPGENE software (Yeh et al., 1999). The heterozygosity measures were calculated using the following formulae given by Nei (1978).

4.8.1 Allele number

Alleles are a set of alternative forms of the same gene occupying the same relative position or locus on homologous chromosomes. Allele number is the total number of alleles for a given marker/locus in a population, which is counted with a non-zero frequency. The allele number for each locus can be determined manually from the silver stained gels/autoradiograms.

4.8.2 Allele Frequency/Gene Frequency

The frequency of an allele 'A' is the number of 'A' alleles in the population divided by the total number of allele/genes. It gives the indication of the most or least prevalent alleles in the population. The allele frequency is affected over time by force such as genetic drift, mutation and migration.

5. RESULTS AND DISCUSSION

A total of 22 loci were utilized in study. All the loci selected were found to be polymorphic. The 22 loci were not specific for the *Bubalus bubalis* but we are taken from the gene bank accession of microsatellite loci for cattle available in the NCBI database. These primers were amplified in the related species of buffaloes and thus were heterologous in nature, since this represented cross species amplification. The sizes of alleles are likely to be different from the size range available for cattle. The loci were amplified and scored for understanding the level of polymorphism in buffalo and to estimate gene and genetic diversity.

5.1 NUMBER OF ALLELES AND ALLELE FREQUENCY

The amplified PCR products were sized using Avant 3100 Automated DNA Sequencer with Liz 500 was taken as internal size standard. The sizing of the alleles was extrapolated from the regression curve drawn using Liz squares and genetic algorithm using Gene Mapper software (V 3.0). Since most of the loci taken in the study were dinucleotide in nature, the mutation rate is relatively of higher magnitude compared to tri and tetra nucleotide repeats. The accession number, repeat and product range of each locus is given in table no.-4(a-e). The details of the parameters for the loci are given locus wise.

Table No.4a- Standarised PCR conditions, repeats, allelic range and primer sequence is label for each microsatellite loci used in the present study.

Locus	**Sequences**	**Dye Label**	***Repeats***	**A.T.**	***Alleles***	**Allele size**	**Acc No**	**$MgCl_2$ Conc.**	*Primer Cycle*
ILSTS87	*F- AgCAgACATgATgACTCAgC R-CTgCCTCTTTTCTTgAgAgC*	Ned	$(CA)_{14}$	55^0C	5	102-114	L37279	1.5 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 55- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
ILSTS59	F- AgTATggTAAggCCAAAggg R- CgACTTgTgTTgTTCAAAgC	Vic	$(gT)_{21}$	55^0C	6	157-177	L37266	1.5 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 55- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
ILSTS52	F- CTgTCCTTTAAgAACAAACC R- TgCAACTTAggCTATTgACg	Pet	$(CA)_{11}$	55^0C	10	146-176	L37222	1.5 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 55- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
CSSM29	F- TCTCCATTATgCACATgCCATgCT	Ned	$(AC)_{18}$	60^0C	3	180-184	U03807	1.5 mM	Step 1 (1x) - 95- 5'

	R- CgTgAgAACCgAAAgCACACATTC								Step 2 (30x)- 95- 45'' 60- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
ILSTS11	F- GCTTgCTACATggAAAgTgC R- CTAAAATgCAgAgCCCTACC	Vic	$(CA)_{11}$	58^0C	5	254-268	L23485	1.5 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 58- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞

Table No.4 (b)-: Standarised PCR conditions, repeats, allelic range and primer sequence is label for each microsatellite loci used in the present study.

Locus	**Sequences**	**Dye Label**	***Repeats***	**A.T.**	***Alleles***	**Allele size**	**Acc No**	**$MgCl_2$ Conc.**	*Primer Cycle*
BM1818	F- AgCTgggAATATAACCAAAgg R- AgTgCTTTCAAggTCCATgC	Pet	$(Tg)_{13}$	58^0C	10	254-278	G18391	1.5 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 58- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
ILSTS72	F- ATgAATgTgAAAgCCAAggg R- CTTCCgTAAATAATTgTggg	Ned	$(CA)_{14}$	55^0C	9	129-169	L37272	1.5 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 55- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
ILSTS49	F- CAATTTTCTTgTCTCTCCCC R- gCTgAATCTTgTCAAACAgg	Pet	$(CA)_9$	55^0C	3	136-140	L37261	1.5 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 55- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
ILSTS05	F- GgAAgCAATgAAATCTATAgCC	Vic	$(Tg)_9$	55^0C	3	175-181	L23481	1.5 mM	Step 1 (1x) - 95- 5'

	R- TgTTCTgTgAgTTTgTAAgC								Step 2 (30x)- 95- 45'' 55- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
ILSTS58	F- GCCTTACTACCATTTCCAgC R- CATCCTgACTTTggCTgTgg	Vic	$(gT)_{15}$	55^0C	12	104-144	L37225	1.5 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 55- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞

Table No.4(c)-: Standarised PCR conditions, repeats, allelic range and primer sequence is label for each microsatellite loci used in the present study.

Locus	**Sequences**	**Dye Label**	***Repeats***	**A.T.**	***Alleles***	**Allele size**	**Acc No**	**$MgCl_2$ Conc.**	*Primer Cycle*
CSSM43	F-AAAACTCTgggAACTTgAAAAACTA R- gTTACAAATTTAAgAgACAgAgTT	Ned	$(CA)_{19}$	55^0C	8	219-255	U03824	1.5 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 55- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
CSSM45	F- TAgAggCACAAgCAAACCTAACAC R- TTggAAAgATgCAgTAgAACTCAT	Ped	$(CA)_{14}$	58^0C	5	98-116	NW_381320	1.5 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 58- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
CSSM47	F- TCTCTgTCTCTATCACTATATggC R- CTgggCACCTgAAACTATCATCAT	Ned	$(gT)_{12}$	55^0C	14	122-164	U03821	1.5 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 55- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
ILSTS30	F- CTgCAgTTCTgCATATgTgg	Vic	$(gT)_{10}$	55^0C	8	152-166	L37212	1.5 mM	Step 1 (1x) - 95- 5'

	R- CTTAgACAACAggggTTTgg								Step 2 (30x)- 95- 45'' 55- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
CSSM08	F- CTTggTgTTACTAgCCCTggg R- gATATATTTgCCAgAgATTCTgCA	Pet	$(Tg)_{15}$	55^0C	9	181-201	NW_37590 5	2 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 55- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞

Table No.-4(d): Standarised PCR conditions, repeats, allelic range and primer sequence is label for each microsatellite loci used in the present study.

Locus	**Sequences**	**Dye Label**	***Repeats***	**A.T.**	***Alleles***	**Allele size**	**Acc No**	**$MgCl_2$ Conc.**	*Primer Cycle*
CSSM33	F- CACTgTgAATgCATgTgTgTgAgC R- CCCATgATAAgAgTgCAgATgACT	Pet	$(Tg)_{16}$	58^0C	7	156-76	U03805	1.5 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 58- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
CSRM60	F- AAgATgTgATCCAAgAgAgAggCA R- AggACCAgATCgTgAAAggCATAg	Vic	$(CA)_{17}$	60^0C	14	88-138	AF232758	1.5 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 60- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
ETH152	F- TACTCgTAgggCAggCTgCCTg R- gAgACCTCAgggTTggTgATCAg	Ned	$(CA)_{17}$	60^0C	14	190-232	Z14040	2 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 60- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
CSSM19	F- TTgTCAgCAACTTCTTgTATCTTT	Vic	$(Tg)_{18}$	55^0C	10	125-147	AF232761	1.5 mM	Step 1 (1x) - 95- 5'

	R- TgTTTTAAgCCACCCAATTATTTg								Step 2 (30x)- 95- 45'' 55- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
CSSM06	F- AgCTTCTgACCTTTAAAgAAAATg R- AgCTTATAgATTTgCACAAgTgCC	Vic	$(Tg)_{13}$	55^0C	9	203-221	U03787	1.5 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 55- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞

Table No.-4(e): Standarised PCR conditions, repeats, allelic range and primer sequence is label for each microsatellite loci used in the present study.

Locus	**Sequences**	**Dye Label**	***Repeats***	**A.T.**	***Alleles***	**Allele size**	**Acc No**	**$MgCl_2$ Conc.**	*Primer Cycle*
ILSTS29	F- TgTTTTgATggAACACAgCC R- TggATTTAgACCAgggTTgg	Ned	$(AC)_{19}$	60^0C	6	154-64	L37252	1.5 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 60- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
CSSM57	F- TgTggTgTTTAACCCTTgTAATCT	Pet	$(gT)_{16}$	60^0C	6	118-134	U0384	1.5	Step 1 (1x) - 95- 5'

	R- gTCgCTggATAAACAATTTAAAgT						0	mM	Step 2 (30x)- 95- 45'' 60- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞
ILSTS38	F- GggCATTATTTTgTTTCCC R- CCACTTCTgggTAATTATCC	Pet	$(gT)_{14}$	55^0C	3	154-158	L3725 6	2 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 55- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞

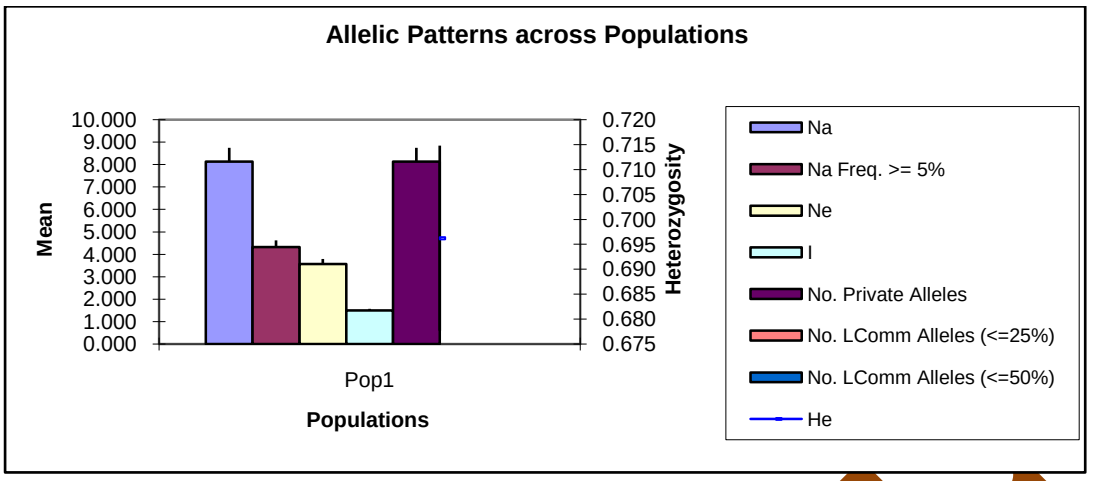

Fig.-2 Allelic patterns of Jaffarabadi buffalo populations

The allelic distributions of various alleles over the various loci taken in this study have been depicted in the form of a graph (Fig.9). The graph represents the overall view over the 22 loci.

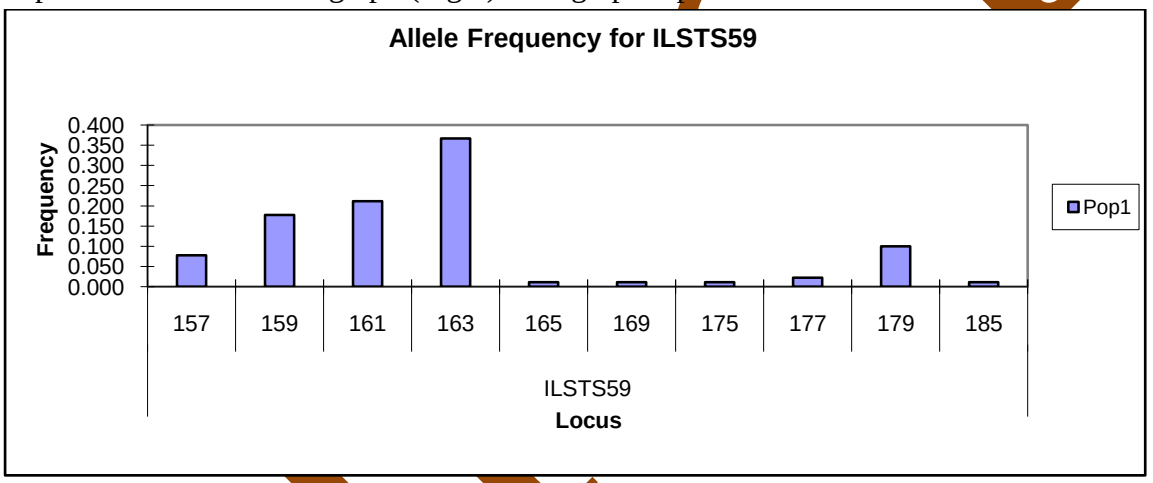

Fig.-3 Representative diagram of the allele frequecy at a locus of microsatellite.

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5.2 GENIC VARIATION

The genic variation of the population Jaffarabadi is depicted in table number-5of alleles observed in population has also been given in Table no.5 along with the effective number of allele of the population. It is evident from the table No.-5 that the effective number of alleles is almost half of the total number of alleles observed (fig. No.-11). This is due to the fact that a large number of alleles are present at very low frequency. The mean number of alleles (na*) over 22 loci was found to be 8.14 while the mean effective number of alleles (ne*) was 3.40 in Jaffarabadi buffalo. The effective numbers of alleles depicted the number that shall be available and shall not be lost from the population by chance. The Shannon's Information Index, which represents the relative abundance of information of a specific locus to the total information available over all loci. The Shannon's Information Index is thus a function of number of alleles observed for a specific locus.

Table No. 5: Genic Variation Statistics for all loci in Jaffarabadi data set.

Locus	**Sample Size**	**na***	**ne***	**I***
BM1818	90	7	4.1242	1.59
CSSM29	90	4	2.4324	1.07
ILSTS52	90	8	3.5526	1.62
ILSTS59	90	10	4.3926	1.72
CSRM60	90	11	4.2722	1.85
CSSM43	90	9	2.3262	1.31
ILSTS05	90	6	3.8868	1.45
ILSTS49	90	4	2.024	0.78
ILSTS72	90	3	2.3303	0.96
CSSM08	90	6	3.4675	1.4
CSSM47	90	13	6.2693	2.12
CSSM57	90	6	2.4545	1.09
ILSTS11	90	7	2.7439	1.23
ILSTS30	90	6	3.2297	1.31
CSSM19	88	8	3.2786	1.57
CSSM45	86	12	3.2581	1.63
ILSTS29	82	8	3.1807	1.53
ILSTS38	80	11	3.1968	1.59
CSSM06	90	13	3.9784	1.78
CSSM33	88	8	4.3702	1.68
ETH152	90	10	5.5708	1.93
ILSTS58	90	9	4.0339	1.67
Mean	89	8.14	3.5624	1.5
St. Dev		2.83	1.0507	0.33

na* = Observed number of alleles; ne* = Effective number of alleles (Kimura & Crow,1964)

I* = Shannon's Information index (Lewontin, 1972)

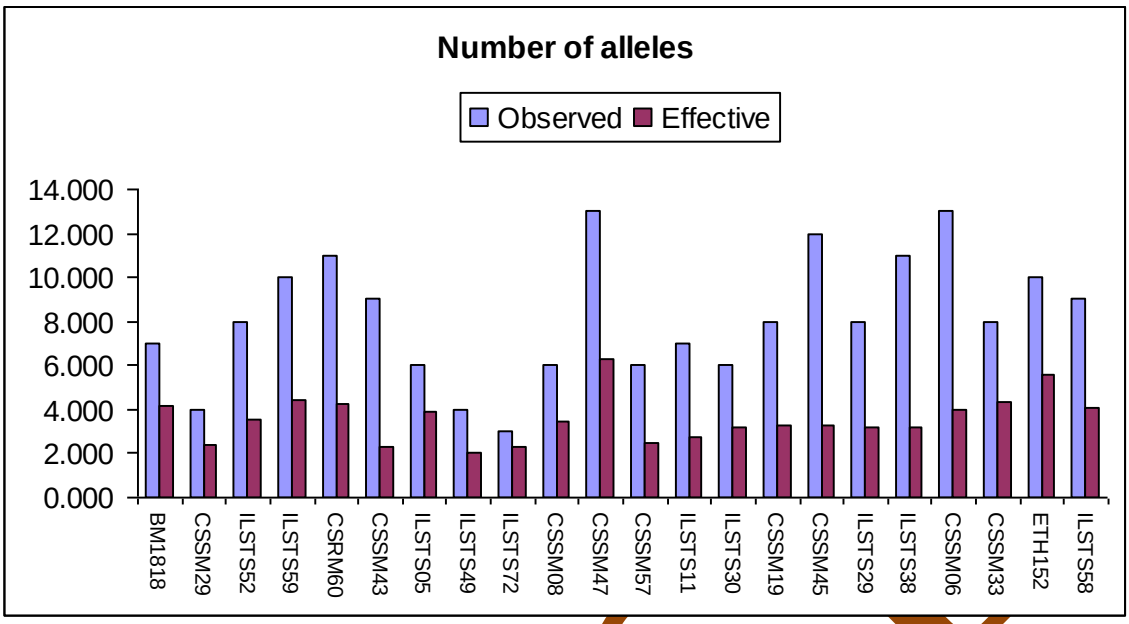

Fig No. 4-Graphical representation of comparision of number of alleles (na*) & effective number of alleles(ne*)

5.3 HETEROZYGOSITY AND POLYMORPHISM INFORMATION CONTENT

The observed homozygosity and heterozygosity, expected homozygosity and heterozygosity, average heterozygosity table no. 6 for pooled data set of buffalo population. The observed and expected homozygosity and heterozygosity are given in table no. 6 for Jaffarabadi buffalo. The average number of samples was 89 for Jaffarabadi population (applied genome). The observed heterozygosity ranged from 0.450 (ILSTS38) and 0.822 (CSSM47). The mean observed heterozygosity was found to be 0.583 with a standard error of 0.025. The expected heterozygosity, which is a function of alleles and its frequency, was highest for locus CSSM47 (0.85) and minimum for locus ILSTS49 (0.51). The PIC, which is estimated from deduction of expected homozygosity and cross homozygosity from 1.0. The PIC gives the information that the locus is likely to provide in terms of gene diversity. The values are slightly less then the expected heterozygosity values (fig. 12). The locus with least PIC was ILSTS87 (0.25) and highest PIC was ILSTS58 (0.86). The Nei's average heterozygosity also given in table for Jaffarabadi buffalo. The PIC of these loci was 0.65, which is quit high, and thus reaffirm the utility of these 22 loci for genetic diversity analysis.

Table No. 6: Heterozygosity estimates for loci a measure of variability of microsatellite loci in Jaffarabadi Buffaloes.

Locus	Sample Size	Obs_Hom	Obs_Het	Exp_Hom*	Exp_Het*	Nei**	Ave_Het
BM1818	90	0.4667	0.5333	0.234	0.766	0.7575	0.7575
CSSM29	90	0.4222	0.5778	0.4045	0.5955	0.5889	0.5889
ILSTS52	90	0.3111	0.6889	0.2734	0.7266	0.7185	0.7185
ILSTS59	90	0.0889	0.9111	0.219	0.781	0.7723	0.7723
CSRM60	90	0.4444	0.5556	0.2255	0.7745	0.7659	0.7659
CSSM43	90	0.8444	0.1556	0.4235	0.5765	0.5701	0.5701
ILSTS05	90	0.2889	0.7111	0.2489	0.7511	0.7427	0.7427
ILSTS49	90	0.7556	0.2444	0.4884	0.5116	0.5059	0.5059
ILSTS72	90	0.7333	0.2667	0.4227	0.5773	0.5709	0.5709
CSSM08	90	0.5111	0.4889	0.2804	0.7196	0.7116	0.7116
CSSM47	90	0.1778	0.8222	0.1501	0.8499	0.8405	0.8405
CSSM57	90	0.4444	0.5556	0.4007	0.5993	0.5926	0.5926
ILSTS11	90	0.4889	0.5111	0.3573	0.6427	0.6356	0.6356
ILSTS30	90	0.2667	0.7333	0.3019	0.6981	0.6904	0.6904
CSSM19	88	0.2727	0.7273	0.297	0.703	0.695	0.695
CSSM45	86	0.3256	0.6744	0.2988	0.7012	0.6931	0.6931
ILSTS29	82	0.2927	0.7073	0.3059	0.6941	0.6856	0.6856
ILSTS38	80	0.55	0.45	0.3041	0.6959	0.6872	0.6872
CSSM06	90	0.3333	0.6667	0.2429	0.7571	0.7486	0.7486
CSSM33	88	0.4773	0.5227	0.22	0.78	0.7712	0.7712
ETH152	90	0.4667	0.5333	0.1703	0.8297	0.8205	0.8205
ILSTS58	90	0.2	0.8	0.2395	0.7605	0.7521	0.7521
Mean	89	0.4165	0.5835	0.2958	0.7042	0.6962	0.6962
St. Dev		0.1894	0.1894	0.088	0.088	0.087	0.087

*Expected homozygosity &heterozygosity were computed using levene (1949)

**Nei's (1973) expected heterozygosity

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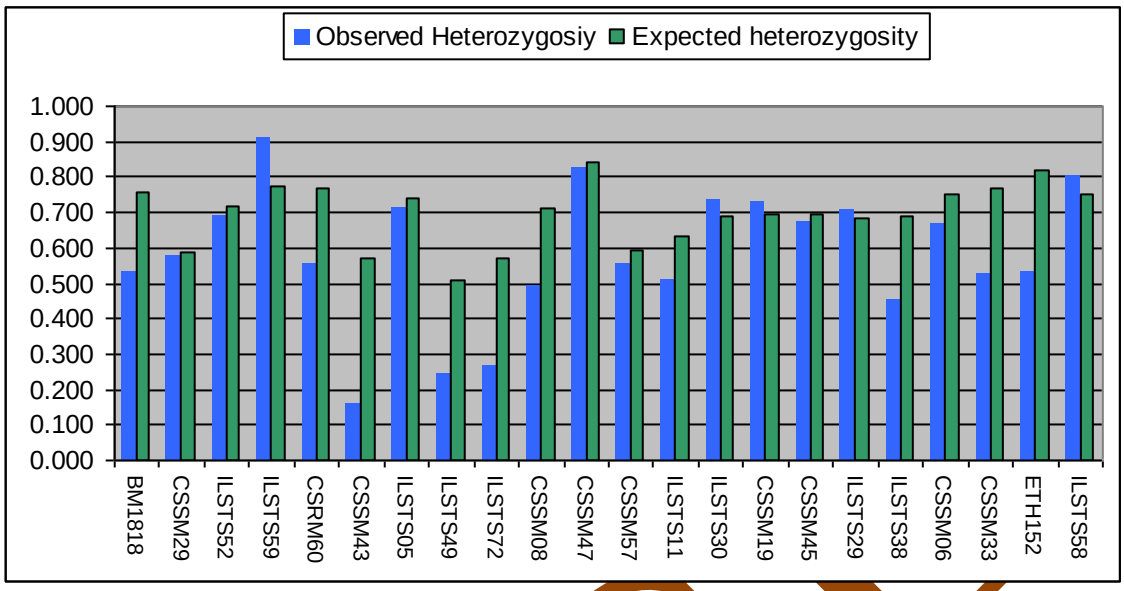

Fig. No.5-Graph predicting the observed & expected heterozygosity in Jaffarabadi buffaloes.

5.4 HARDY WEINBERG EQUILIBRIUM

The Hardy Weinberg equilibrium states that in a large random mating population, the gene and genotypic frequency does not change from generation to generation in the absence of mutation, migration and selection. The method (statistics) applied to test for the population of **Jaffarabadi** was χ^2 test .The values obtained for each locus and populations have been depicted in table no.7 in Jaffarabadi buffaloes. **Fourteen loci (CSSM06, CSSM57, ILSTS30, ILSTS38, ETH152, CSRM60, ILSTS05, ILSTS58, BM1818, CSSM45, ILSTS11, ILSTS59 and ILSTS87) deviated from Hardy Weinberg equilibrium using χ^2 test The deviation from Hardy Weinberg equilibrium point may be either towards small population size, mutation, migration or selection. All these factors have to be analyzed in details for** pinpointing the deviations.

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Table No. 7: Testing Jaffarabadi Buffalo population for Hardy-Weinberg Equilibrium using Chi square test.

Locus	Degree of Freedom	ChiSquare values	Probability	Significance
BM1818	21	42.872	0.003	**
CSSM29	6	2.168	0.904	ns
ILSTS52	28	25.462	0.603	ns
ILSTS59	45	71.213	0.008	**
CSRM60	55	107.131	0.000	***
CSSM43	36	207.010	0.000	***
ILSTS05	15	9.031	0.876	ns
ILSTS49	6	15.954	0.014	*
ILSTS72	3	25.230	0.000	***
CSSM08	15	61.367	0.000	***
CSSM47	78	64.636	0.861	ns
CSSM57	15	92.702	0.000	***
ILSTS11	21	73.535	0.000	***
ILSTS30	15	14.130	0.516	ns
CSSM19	28	23.961	0.684	ns
CSSM45	66	78.935	0.132	ns
ILSTS29	28	81.231	0.000	***
ILSTS38	55	172.630	0.000	***
CSSM06	78	122.297	0.001	**
CSSM33	28	91.750	0.000	***
ETH152	45	91.489	0.000	***
ILSTS58	36	42.593	0.209	ns

F- STATISTICS

It provides the analysis pertaining to nonrandom union of gametes i.e., inbreeding as well as population differentiation. The F_{IS} values provide the nonrandom union of gametes in the population i.e., the mating among the individuals in the populations is more/less related than the average relationship among the individuals. The more the value of F_{IS} the more the inbreeding coefficient and thus the individuals are more closely related to one another. The negative values of F_{IS} point towards out breeding i.e., the mating of individuals who are less related than the average relationship of the population.

In case of Jaffarabadi the F_{IS} values are significantly different from zero and ten loci point towards the outbreeding while thirteen loci point (table no-9) towards mating among the individuals of the population more closely related than the average relationship**.** In case of Jaffarabadi buffaloes 5 microsatellite loci show negative values meaning thereby that there is outbreeding at these 5 loci . the loci exhibiting the negative values are (ILSTS59, ILSTS30,

CSSM19, ILSTS58, ILSTS29).the rest of the loci show quite high values which points towards the non random mating or union of gametes. It means existence of a population structure.

Table No.8: Wright's Fixation Index in Jaffarabadi buffaloes

Locus	Sample No	F_{IS}
BM1818	45	0.296
CSSM29	45	0.019
ILSTS52	45	0.041
ILSTS59	45	-0.180
CSRM60	45	0.275
CSSM43	45	0.727
ILSTS05	45	0.043
ILSTS49	45	0.517
ILSTS72	45	0.533
CSSM08	45	0.313
CSSM47	45	0.022
CSSM57	45	0.062
ILSTS11	45	0.196
ILSTS30	45	-0.062
CSSM19	44	-0.046
CSSM45	43	0.027
ILSTS29	41	-0.032
ILSTS38	40	0.345
CSSM06	45	0.109
CSSM33	44	0.322
ETH152	45	0.350
ILSTS58	45	-0.064

5.5 GENETIC DISTANCES

The interindividual genetic distance estimated and the constitution of phylogenetic tree was carried using Nei's Genetic distances & Cavalli S'Forza genetic distances. The two algorithms viz. UPGMA & Neighbour Joining was used for the construction of tree. Diagrams one each using UPGMA &NJ have been depicted in fig. (14a-e).

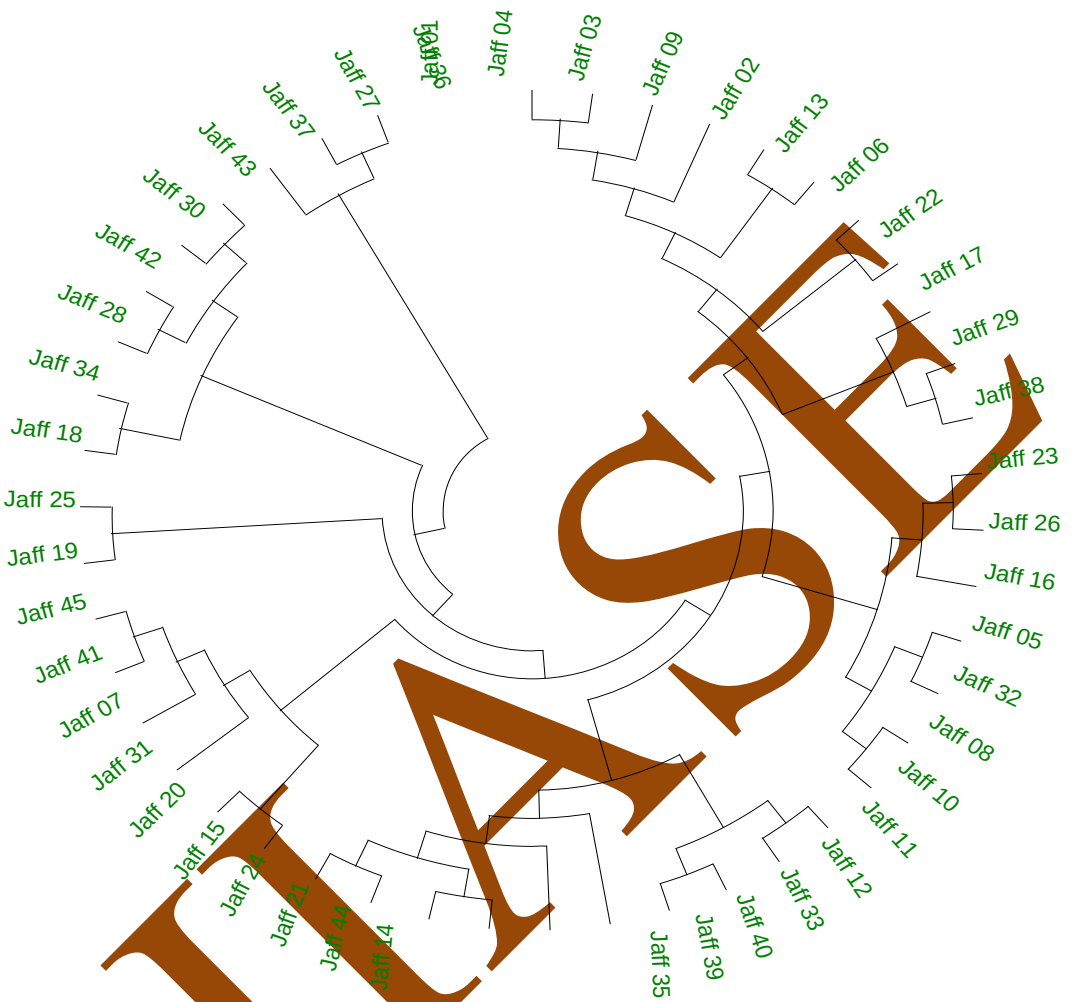

Fig. No 6a Circle Tree of Cavalli S Forza's Interindividual genetic distance using Neighbour joining Algorithm

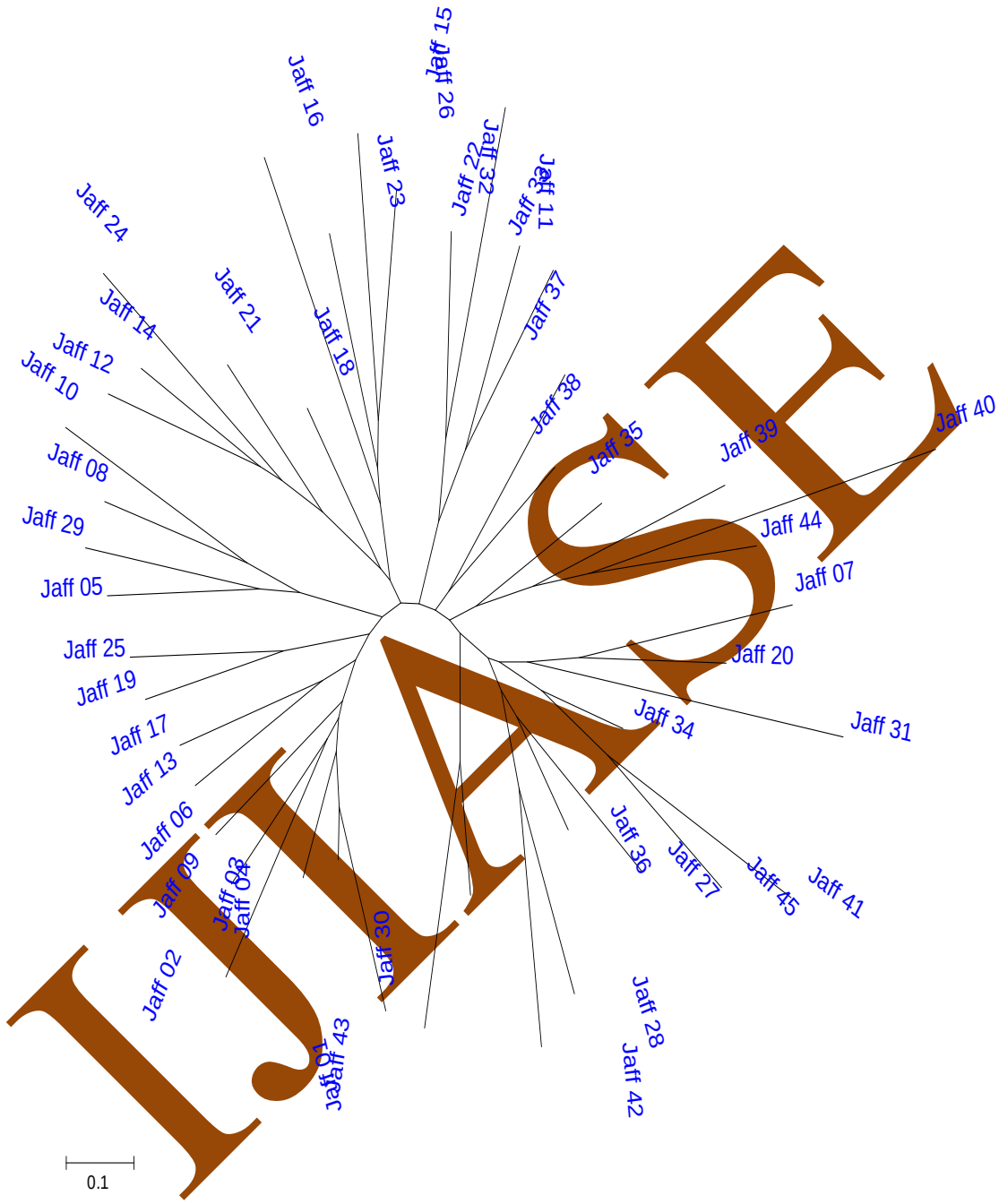

Fig. No 6b NEI'S Neighbor Joining Radiation tree of Jaffarabadi Buffaloes

SUMMARY OF RESULTS

1. The 22 heterologus microsatellite loci selected for this study in Jaffarabadi breed of buffalo was found to be highly polymorphic with allele numbers ranging from 3-13. This shows the utility of heterologus microsatellite loci of cattle in buffaloes for diversity analysis.

2. The microsatellite studied in the present study was neutral in nature as evidenced by Ewens Watterson test of neutrality. This implies that the loci have not been subjected to selection and thus are fit for population genetic analysis.
3. The microsatellite loci selected were highly polymorphic with a large number of alleles at lower allele frequencies. This is depicted by quite large difference between the number of alleles and effective number of alleles.
4. Most of the loci had very high heterozygosity values with mean 0.583 Jaffarabadi breed. The heterozygosity values were however very less in ILSTS38 although the number of alleles observed in the population were 11.The heterozygosity was however upto 0.85 for the loci CSSM47.
5. The Hardy Weinberg equilibrium of the two buffalo populations was tested using chi square 14 loci out of 22 loci were not in Hardy Weinberg equilibrium in Jaffarabadi buffalo population. It means that the gene and genotypic have changed with respect to the loci not in Hardy Weinberg equilibrium by factors like mutation, migration, selection and drift (small population size).
6. The F_{IS} values were significantly different from zero in Jaffarabadi buffalo population meaning there by that the population structure exists in this population.
7. The inter-individual genetic distance based on Nei's genetic measure were utilized for the construction of trees based on UPGMA as well as Neighbor Joining algorithm.
8. The mutation drift equilibrium and bottleneck studies revealed that Jaffarabadi population were found not to have undergone recent genetic bottleneck by graphical method as well as SMM of microsatellite evolution

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