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MICROSATELLITE ANALYSIS OF MEHSANA AND PANDHARPURI BUFFALOES (*BUBALUS BUBALIS*)

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1. INTRODUCTION

Microsatellite are short segment of DNA in which specific motifs of 1-6 bases is repeated upto 100 times or so (Tautz, 1993). The term microsatellite introduced by Litt and Lutty (1989) to characterize the tandemly repeat sequence motif. Microsatellite also known as simple sequence repeats (Tautz, 1989), short tandem repeats (Edwards et al, 1991) and sequence tagged microsatellite repeats (STMR).

The buffalo, aptly termed as the "Black Goldmine Of India", occupies a crucial place in the country by being a source of plethora of items, utilize and services. Domestication of the buffalo took place at an early age during Indus Valley civilization (3250 to 2750 BC). The buffalo is the first foremost a beast of labour, employed in the crop fields, pulling of carts, and working of mills and wells. Especially in India and other Southeast Asian countries it is stronger and more consistent than domestic cattle. In India ten important breeds of buffaloes have been described. The buffalo biodiversity in India constitutes 10 well-defined breeds of buffaloes apart from non-descript types. India possesses the richest source of germplasm of buffaloes and the best dairy breeds domesticated in northwestern region of the country.

It is now well-recognized and documented that today domestic buffalo (*Bubalus bubalis*) is a very important germplasm resource. However, it is in Asia that it has thrived and acted as a symbol of life, religion and endurance. Indo-gangetic plains have been its place of origin where it has played a unique role both as a much sought after hunting animal and as a much loved and patronized domestic animal. It is this native tract of Indian subcontinent where the buffalo has emerged as the main dairy animal, and from where it has spread to other parts of the world. India alone has now the privilege of having about 56.8% of the world's buffalo population (FAO, 2004). In fact, the buffalo now happens to be the mainstay of the rural economy of the small farmers in most of the developing countries in the middle East and South East Asia. According to an FAO survey (2004), about 90% the draft power for agricultural sector in the South East Asian countries is contribute by buffaloes. But it is only recently that potential of water buffalo as a valuable genetic resource has started being recognized outside Asia.

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Two breeds Mehsana buffalo from Maharashtra and Pandharpuri buffalo from Gujarat were taken for the present study for characterization and evaluation of these breeds using microsatellite markers. The objective of the study were as follows:

1. To study the heterologous microsatellite loci primers for amplification in buffaloes.
2. To study the diversity of Mehsana and Pandharpuri buffaloes.
3. To study the Hardy-Weinberg equilibrium, neutrality and linkage disequilibrium of the microsatellite loci in the buffalo populations.
4. To study the genetic distance among the two buffalo populations.
5. To assess the assignment and dispersion of the buffalo populations.
6. To analyze whether the two populations are in mutation drift equilibrium.
7. To analyze and study the various population genetic parameters of Mehsana and Pandharpuri buffaloes.

2.1 MEHSANA BUFFALOES

The home tract is centered around Mehsana and Ahmedabad district of Gujarat province of India. The rural areas of Patan, Sidhpur, Kadi Radhanpur, Kael, Banaskantha and Sabarkantha have larger herds of Mehsana buffaloes.

Mehsana animals are intermediate between Murrah and Surti. It has been postulated that Mehsana breeds arose as a result or interbreeding between Murrah and Surti breeds (Oliver, 1938). The relatively large variations in colour (Black to fawn gray) also indicate their possible lineage from Surti and Murrah. Mehsana animals are longer than local Murrah with lighter limbs but slightly longer and heavier head. Mehsana breeds are reputed for regular breeding with persistency. Population of these breed is around 0.4 million. Males are moderately tractable while female are docile. Male calves are not cared properly.

PANDHARPURI BUFFALOES

The Pandharpuri breed is native of Kolhapur, Solapur, Sagli and Satara districts in South Maharashtra. These breeds are named after the name of geographical area, i.e., Pandharpur block in Solapur district. These breeds are also found in adjoining Belgaum district of Karnataka. There are approximately 0.19 million Pandharpuri breeds. Community like Gawali and Joshi are local breeders. They maintain these breeds.

MATERIALS AND METHODS

METHODS PERFORMED

Blood Sample Collection

Two populations of buffalo Mehsana and Pandharpuri were selected and 44 blood samples from each breed were collected at random from field conditions from the breeding tract for these

populations (fig. 8 and 9). 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.

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 0.1mM

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).

3.1 POLYMERASE CHAIN REACTION FOR 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, analyze, and synthesize any specific piece of DNA or RNA. Polymerase chain reaction is an *in vitro* method for analyzing 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.

3.1.1 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. They 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-1a: List of Cattle Primers Screened in Buffalo Breeds:

Primer	Forward Sequence	Reverse Sequence	Repeats	Dye	Accession Number
ILSTS87	AgCAgACATgATgACTCAgC	CTgCCTCTTTTCTTgAgAgC	$(CA)_{14}$	Ned	L37279
ILSTS59	AgTATggTAAggCCAAAggg	CgACTTgTgTTgTTCAAAgC	$(gT)_{21}$	Vic	L37266
ILSTS52	CTgTCCTTTAAgAACAAACC	TgCAACTTAggCTATTgACg	$(CA)_{11}$	Pet	L37222
CSSM29	TCTCCATTATgCACATgCCATgCT	CgTgAgAACCgAAAgCACACATTC	$(AC)_{18}$	Ned	U03807
ILSTS11	gCTTgCTACATggAAAgTgC	CTAAAATgCAgAgCCCTACC	$(CA)_{11}$	Vic	L23485
BM1818	AgCTgggAATATAACCAAAgg	AgTgCTTTCAAggTCCATgC	$(Tg)_{13}$	Pet	G18391
ILSTS72	ATgAATgTgAAAgCCAAggg	CTTCCgTAAATAATTgTggg	$(CA)_{14}$	Ned	L37272
ILSTS49	CAATTTTCTTgTCTCTCCCC	gCTgAATCTTgTCAAACAgg	$(CA)_{9}$	Pet	L37261
ILSTS05	ggAAgCAATgAAATCTATAgCC	TgTTCTgTgAgTTTgTAAgC	$(Tg)_{9}$	Vic	L23481
ILSTS58	gCCTTACTACCATTTCCAgC	CATCCTgACTTTggCTgTgg	$(gT)_{15}$	Vic	L37225
CSSM43	AAAACTCTgggAACTTgAAAAACTA	gTTACAAATTTAAgAgACAgAgTT	$(CA)_{19}$	Ned	U03824
CSSM45	TAgAggCACAAgCAAACCTAACAC	TTggAAAgATgCAgTAgAACTCAT	$(CA)_{14}$	Pet	NW_381320

Table-1b: List of Cattle Primers Screened in Buffalo Breeds:

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

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.

Cocktail Preparation:

Table-2: The cocktail for PCR one reaction (14 µl) consisted of:

Components	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.

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.

2.0

Table-3: For the specific conditions, the PCR machine is programmed at

Steps	Programme	Temperature	Time	No. of cycle
Step- 1	Initial Denaturation	95°C	5 minutes	1 cycle
Step- 2	Denaturation	94°C	45 seconds	30 cycles
Step- 3	Annealing	55°C (or standardized)	45 seconds	
Step- 4	Polymerization	72°C	45 seconds	
Step- 5	Final Extension	72°C	5 minutes	1 cycle
Step- 6	Final Temperature	4°C	Until removed from PCR machine	

3.2 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.

3.3 POST PCR MULTIPLEXING

3.3.1 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

3.3.2 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

3.4 DENATURATION

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

3. 5 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.

3.6 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 3.1. The data was exported as text file and imported into excel sheet before submitting it to further Statistical Analysis (fig 11 and 12).

3.7 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).

3.7.1 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.

3.7.2 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.

4. RESULT AND DISCUSSION

A total of 23 loci were utilized in study. All the loci selected were found to be polymorphic. The 23 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 among Mehsana and Pandharpuri buffaloes.

4.1 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. 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	3	110-118	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	157-187	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	138-178	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 R- CgTgAgAACCgAAAgCACACATTC	Ned	$(AC)_{18}$	60^0C	8	156-186	U03807	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- ∞
ILSTS11	F- GCTTgCTACATggAAAgTgC R- CTAAAATgCAgAgCCCTACC	Vic	$(CA)_{11}$	58^0C	6	258-286	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.4b: 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	12	228-278	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	4	137-149	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	8	136-198	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 R- TgTTCTgTgAgTTTgTAAgC	Vic	$(Tg)_9$	55^0C	5	175-197	L23481	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- ∞
ILSTS58	F- GCCTTACTACCATTTCCAgC R- CATCCTgACTTTggCTgTgg	Vic	$(gT)_{15}$	55^0C	12	118-146	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.4c: 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	7	221-257	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	9	98-128	NW_38132 0	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	126-166	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 R- CTTAgACAACAggggTTTgg	Vic	$(gT)_{10}$	55^0C	9	142-166	L37212	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- ∞
CSSM08	F- CTTggTgTTACTAgCCCTggg R- gATATATTTgCCAgAgATTCTgCA	Pet	$(Tg)_{15}$	55^0C	6	179-193	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.4d: 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	156-174	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	10	88-128	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	9	190-216	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 R- TgTTTTAAgCCACCCAATTATTTg	Vic	$(Tg)_{18}$	55^0C	7	125-145	AF232761	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- ∞
CSSM06	F- AgCTTCTgACCTTTAAAgAAAATg R- AgCTTATAgATTTgCACAAgTgCC	Vic	$(Tg)_{13}$	55^0C	12	199-221	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.4e: 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	7	150-164	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 R- gTCgCTggATAAACAATTTAAAgT	Pet	$(gT)_{16}$	60^0C	8	116-130	U03840	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- ∞
ILSTS38	F- GggCATTATTTTgTTTCCC R- CCACTTCTgggTAATTATCC	Pet	$(gT)_{14}$	55^0C	2	154-156	L37256	2 mM	Step 1 (1x) - 95- 5' Step 2 (30x)- 95- 45'' 55- 45'' 72- 45'' Step 3 (1x) - 72- 5' Step 4 - 10- ∞

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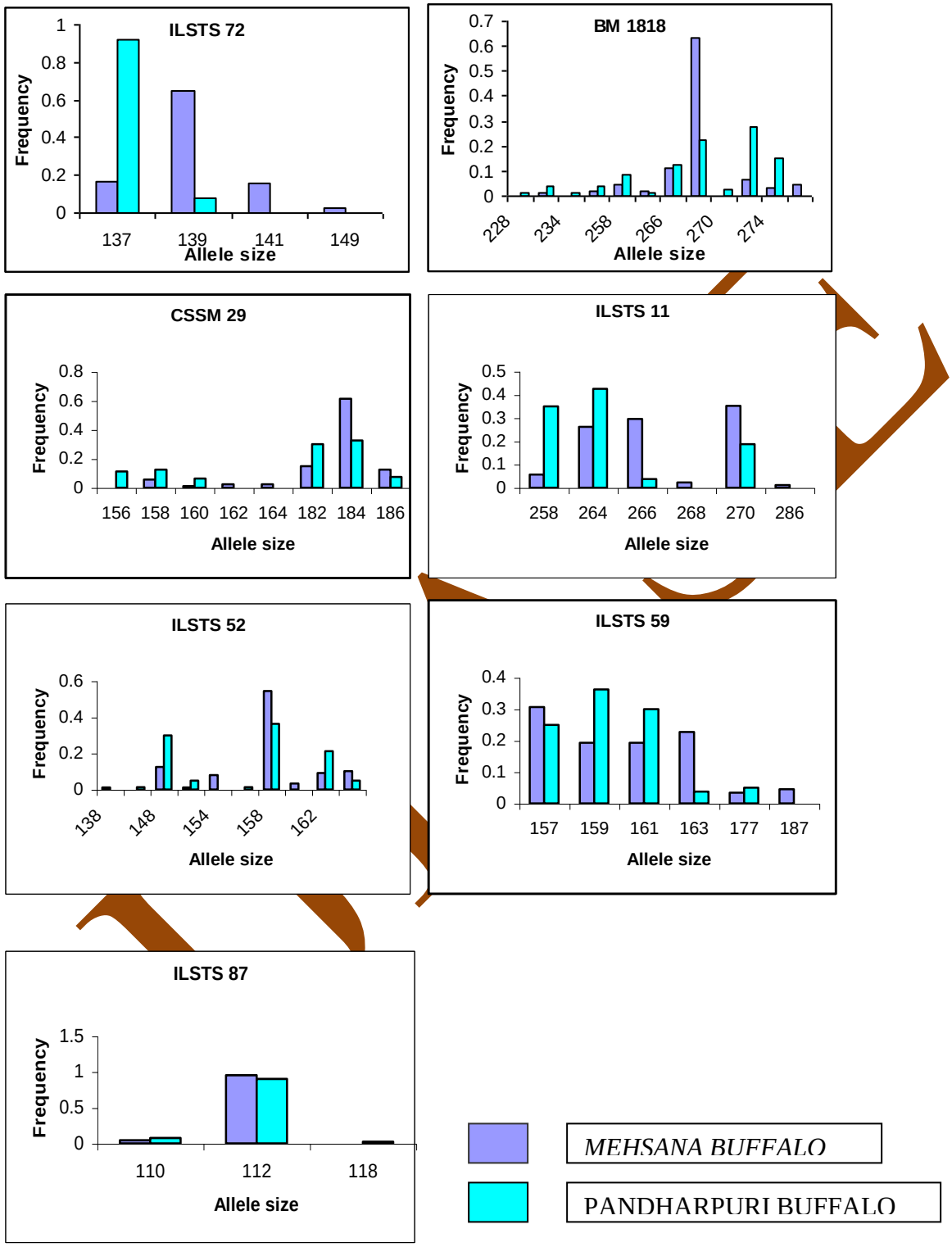


Fig 1.Representative Locus-wise allele frequency distribution in Mehsana and Pandharpuri buffaloes.

4.2 GENIC VARIATION

The genic variation of the two populations Mehsana and Pandharpuri are depicted in table no. 5. Number of alleles observed in each of the two populations has also been given in table no. 6 along with the effective number of allele of two populations. It is evident from the table that the effective number of alleles are almost half of the total number of alleles observed. This is due to the fact that a large number of alleles are present at very low frequency (fig. 14). The mean number of alleles (na) over 23 loci were found to be 7.13 while the mean effective number of alleles (ne) were 3.36 in Mehsana buffaloes and 5.74 and 3.39 for Pandharpuri buffaloes respectively. The effective numbers of alleles depict 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 pooled data set

Locus	Sample size	na*	ne*	I*
CSSM06	166	12	3.3679	1.7223
CSSM19	168	7	3.9585	1.5938
CSSM57	168	8	3.6222	1.4548
ILSTS38	166	2	1.7412	0.6169
ILSTS29	168	7	1.5479	0.8220
ILSTS30	168	9	4.8412	1.7780
ETH152	168	9	5.1579	1.8275
CSSM47	168	14	6.5913	2.0916
CSSM33	168	7	3.3488	1.5313
CSSM08	168	6	2.6187	1.1857
CSRM60	168	10	4.2328	1.7395
CSSM43	168	7	3.3127	1.3397
CSSM45	168	9	3.6991	1.4881
ILSTS05	168	5	2.7873	1.1787
ILSTS49	168	8	1.9350	0.8318
ILSTS58	168	13	8.1619	2.2371
ILSTS72	168	4	2.3345	0.9642
BM1818	168	12	3.9876	1.7755
CSSM29	168	8	3.3568	1.5153
ILSTS11	168	6	3.8663	1.4276

ILSTS52	168	10	3.5131	1.5663
ILSTS59	168	6	4.2764	1.5489
ILSTS87	168	3	1.1548	0.2895
Mean	168	7.913	3.6267	1.4142
St. Dev		3.059	1.5788	0.4662

* na = Observed number of alleles
* ne = Effective number of alleles [Kimura and Crow (1964)]
* I = Shannon's Information index [Lewontin (1972)]

Table No. 6: Geneic Variation of all loci in two buffalo populations

	MEHSANA BUFFALO				**PANDHARPURI BUFFALO**			
Locus	**Sample Size**	**na***	**ne***	**I***	**Sample Size**	**na***	**ne***	**I***
CSSM06	86	10	2.9466	1.5874	80	10	3.6655	1.6562
CSSM19	88	7	3.5328	1.5334	80	5	3.8462	1.4415
CSSM57	88	6	3.5885	1.4360	80	6	3.5359	1.3796
ILSTS38	88	2	1.4824	0.5066	78	2	1.9538	0.6813
ILSTS29	88	7	1.7194	0.9289	80	4	1.3658	0.5605
ILSTS30	88	9	5.5552	1.8903	80	6	3.8278	1.4640
ETH152	88	8	4.5986	1.7425	80	8	5.4237	1.8268
CSSM47	88	13	5.9296	2.0494	80	9	6.0721	1.9231
CSSM33	88	7	3.7124	1.5878	80	6	2.9331	1.3902
CSSM08	88	6	2.7578	1.217	80	5	2.1993	1.0448
CSRM60	88	9	3.6806	1.6394	80	8	4.7904	1.7674
CSSM43	88	7	3.2981	1.3885	80	3	2.6756	1.0322
CSSM45	88	7	3.3322	1.3890	80	5	2.5869	1.1169
ILSTS05	88	5	2.5094	1.1019	80	4	2.7875	1.1517
ILSTS49	88	8	1.9959	0.9441	80	2	1.8594	0.6548
ILSTS58	88	11	7.9835	2.2040	80	11	6.0377	2.0391
ILSTS72	88	4	2.1078	0.9613	80	2	1.1611	0.2664
BM1818	88	9	2.3311	1.3369	80	11	5.6838	1.9511
CSSM29	88	7	2.3901	1.2279	80	6	4.2838	1.5997
ILSTS11	88	6	3.5264	1.3783	80	4	2.9439	1.1681
ILSTS52	88	8	2.9445	1.4600	80	7	3.6782	1.4673
ILSTS59	88	6	4.4711	1.5901	80	5	3.4745	1.3485

ILSTS87	88	2	1.0950	0.1849	80	3	1.2251	0.3813
Mean	88	7.1304	3.3691	1.3603	80	5.7391	3.3918	1.2745
St. Dev		2.5460	1.5683	0.4587		2.7339	1.4840	0.5052

* na = Observed number of alleles
* ne = Effective number of alleles [Kimura and Crow (1964)]
* I = Shannon's Information index [Lewontin (1972)]

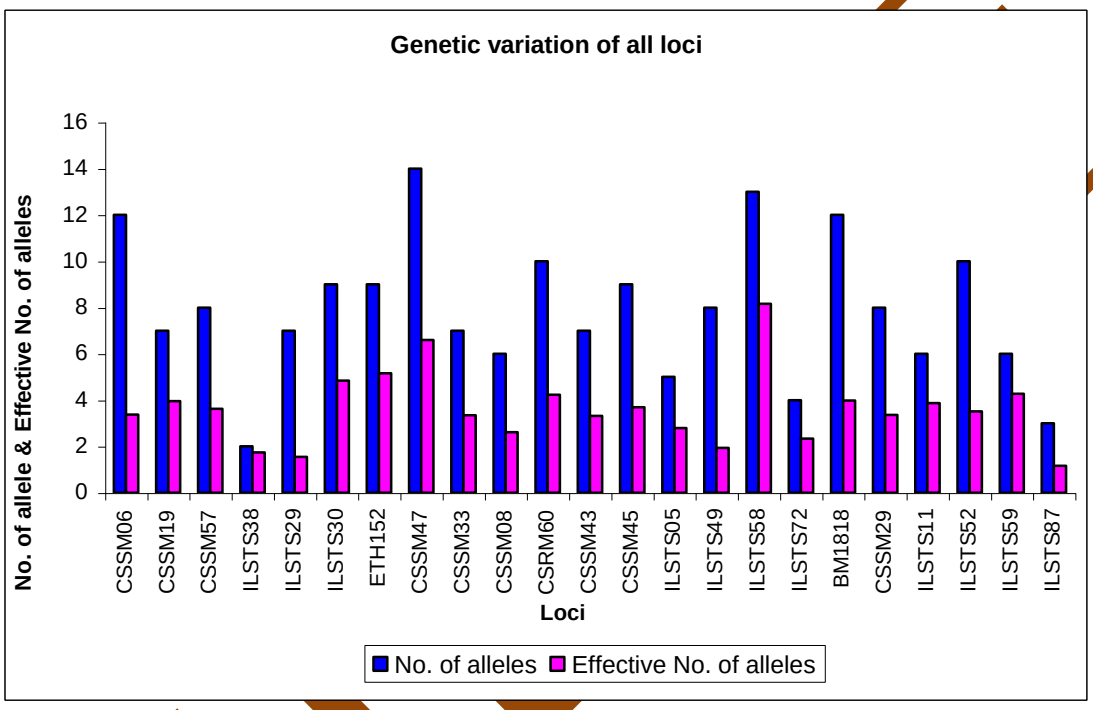


Fig 2. Graphical representation of number of alleles and effective number of alleles of pooled data set of Mehsana buffaloes and Pandharpuri buffaloes

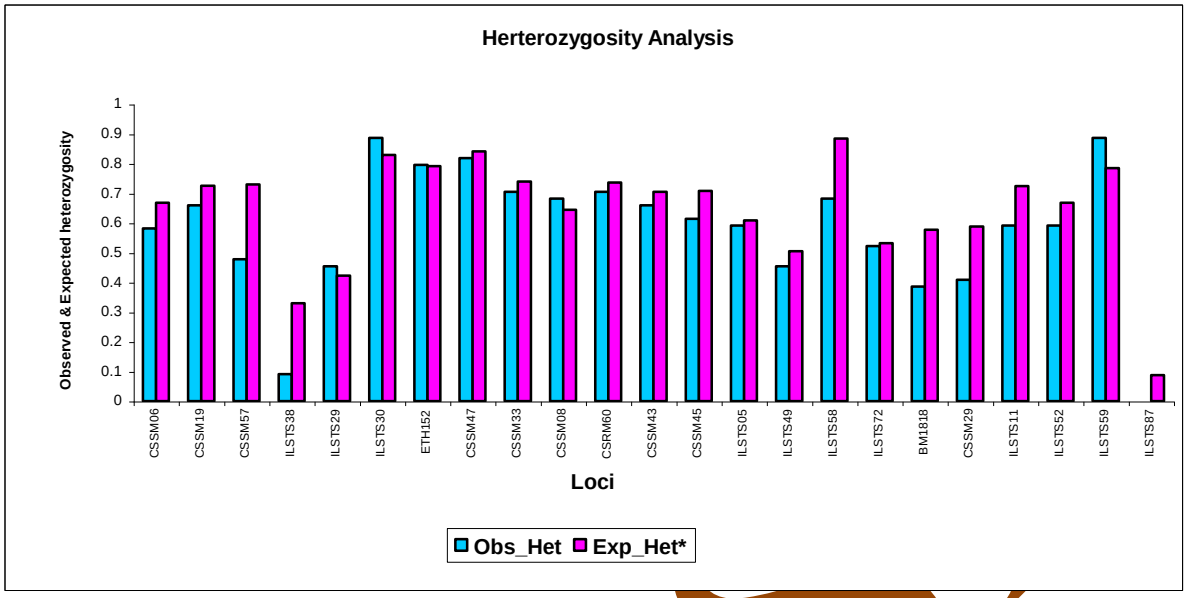


Fig 3. Graphical representation of observed and expected heterozygosity of Mehsana buffalo

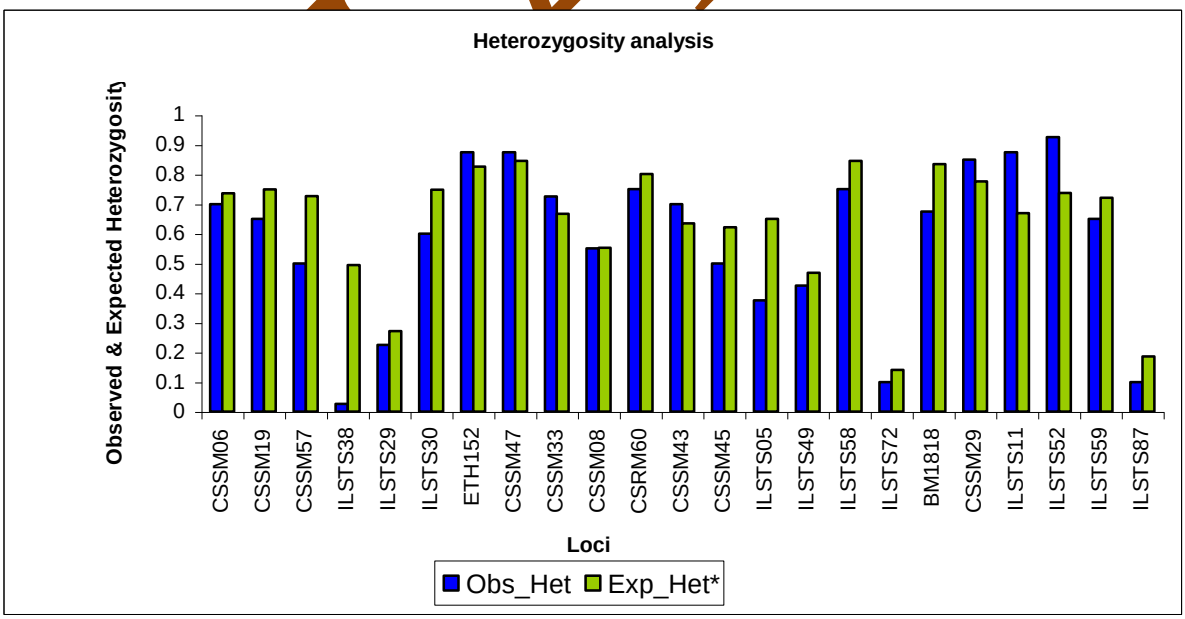


Fig.4 Graphical representation of observed and expected heterozygosity of Pandharpuri buffaloes

4.3 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. Two methods (statistics) were applied to test for the population of Mehsana and Pandharpuri. They were χ^2 test and G^2 or likelihood ratio test. The values obtained for each locus and populations have been depicted in table no.10 and table no.11 in Mehsana and Pandharpuri buffaloes respectively. Eight loci (CSSM19, CSSM57, ILSTS38, ILSTS49, ILSTS58, BM1818, CSSM29 and ILSTS87) in Mehsana deviated from Hardy Weinberg equilibrium using χ^2 test while five loci (CSSM57, ILSTS38, BM1818, CSSM29 and ILSTS87) using likelihood ratio test deviated from Hardy Weinberg equilibrium.

Similarly 12 loci (CSSM57, ILSTS38, ILSTS29, ILSTS30, CSSM08, CSRM60, ILSTS05, ILSTS58, BM1818, ILSTS11 and ILSTS87) in Pandharpuri deviated in χ^2 test and 7 loci deviated in G^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.

Table No. 7: Testing Mehsana population for Hardy Weinberg Equilibrium using Chi square test and Likelihood test

Locus	Degree of Freedom	Chi square Test		Likelihood Ratio Test	
		Chi square	Probability	G^2	Probability
CSSM06	45	59.268478	0.075233	37.343760	0.784234
CSSM19	21	36.082046	0.021406*	29.664794	0.098934
CSSM57	15	39.849188	0.000478*	40.540872	0.000376*
ILSTS38	1	24.198563	0.000001*	20.461442	0.000006*
ILSTS29	21	17.709345	0.667316	12.175812	0.934749
ILSTS30	36	35.554984	0.489594	38.648604	0.350860
ETH152	28	26.958021	0.520534	24.120860	0.675140
CSSM47	78	72.837571	0.643975	49.728974	0.994763
CSSM33	21	24.259515	0.280655	21.834940	0.409075
CSSM08	15	9.767762	0.834088	10.924276	0.757944
CSRM60	36	40.004841	0.296845	36.603432	0.440665
CSSM43	21	14.176756	0.861888	13.860312	0.875524
CSSM45	21	12.917699	0.911479	14.205765	0.860600
ILSTS05	10	15.299188	0.121529	13.076003	0.219453
ILSTS49	28	177.080301	0.000000*	25.096387	0.622584

ILSTS58	55	98.646496	0.000277*	66.271959	0.141947
ILSTS72	6	7.160931	0.306221	9.341828	0.155247
BM1818	36	100.015287	0.000000*	61.487369	0.005114*
CSSM29	21	48.890175	0.000519*	37.102814	0.016382*
ILSTS11	15	10.952129	0.755980	11.191950	0.738872
ILSTS52	28	27.435794	0.494624	25.411410	0.605363
ILSTS59	15	9.717124	0.837146	12.522852	0.639100
ILSTS87	1	58.024096	0.000000*	17.422854	0.000030*

* Deviation from Hardy Weinberg Equilibrium (<0.05)

Table 8: Testing Pandharpuri buffalo population for Hardy Weinberg Equilibrium using Chi square test and Likelihood test

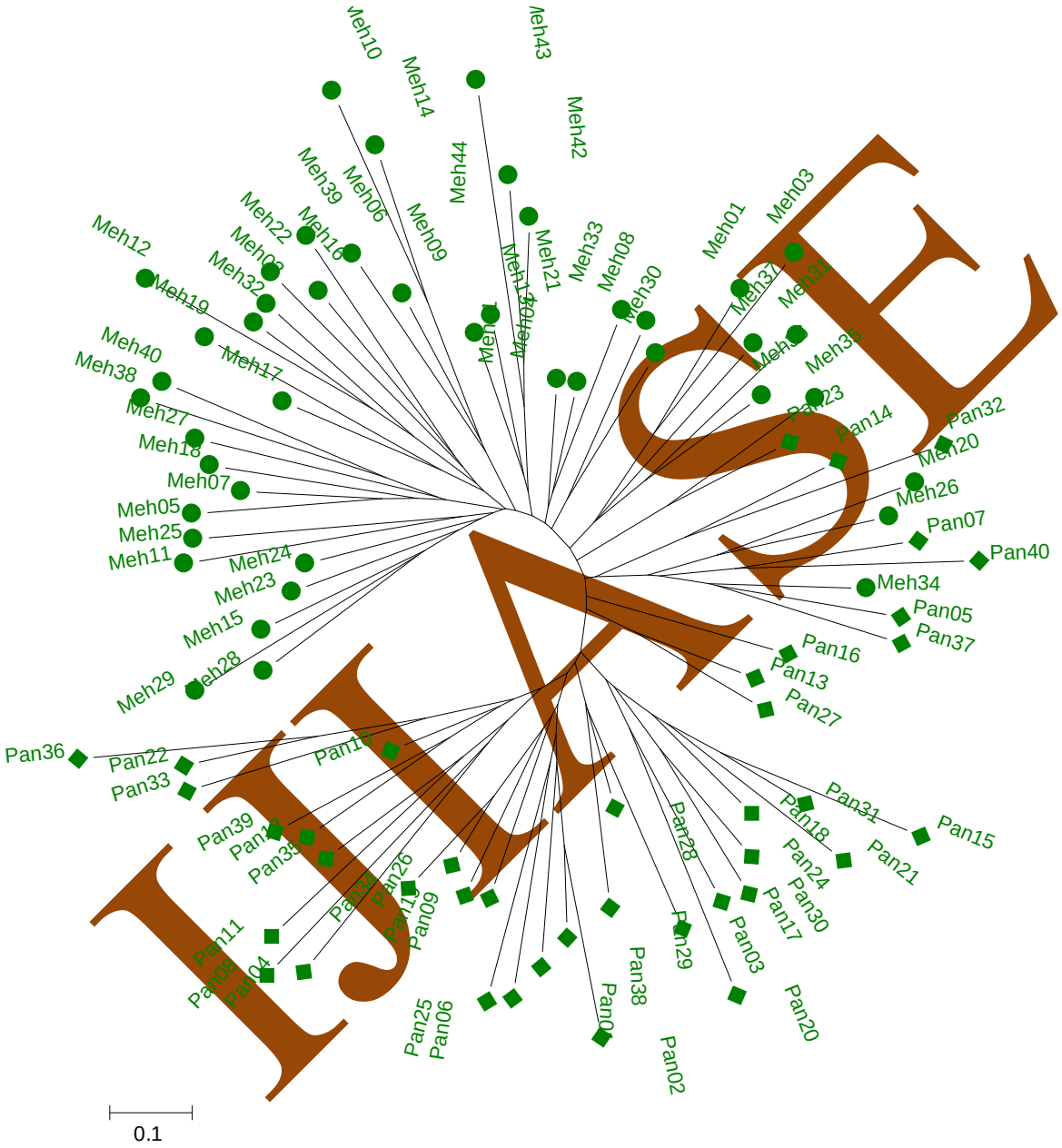
Locus	**Degree of Freedom**	**Chi square Test**		**Likelihood Ratio Test**	
		Chi square	**Probability**	**G^2**	**Probability**
CSSM06	45	39.163329	0.716650	32.242522	0.923064
CSSM19	10	17.805573	0.058333	22.716354	0.011843*
CSSM57	15	30.573380	0.010014*	37.040945	0.001248*
ILSTS38	1	36.029630	0.000000*	44.829091	0.000000*
ILSTS29	6	17.196393	0.008588*	9.093857	0.168368
ILSTS30	15	93.273364	0.000000*	30.034690	0.011797*
ETH152	28	28.615545	0.432216	33.324908	0.223987
CSSM47	36	41.541130	0.241977	38.428859	0.360064
CSSM33	15	15.362594	0.425628	18.316827	0.246376
CSSM08	10	27.417105	0.002236*	9.524360	0.483165
CSRM60	28	101.688570	0.000000*	35.206664	0.163931
CSSM43	3	1.824886	0.609535	2.948861	0.399577
CSSM45	10	9.824795	0.455997	9.140817	0.518794
ILSTS05	6	33.534068	0.000008*	24.990936	0.000343*
ILSTS49	1	0.348374	0.555035	0.345232	0.556825
ILSTS58	55	141.093492	0.000000*	50.167759	0.659416
ILSTS72	1	3.942836	0.047071*	2.241436	0.134356
BM1818	55	111.686683	0.000010*	55.705458	0.448074
CSSM29	15	16.104214	0.375160	19.854958	0.177568
ILSTS11	6	15.687758	0.015532*	20.315528	0.002433*
ILSTS52	15	27.105348	0.167393	28.720886	0.120865
ILSTS59	10	4.887113	0.898590	5.755545	0.835366
ILSTS87	3	19.721857	0.000194*	9.13943	0.027493*

* Deviation from Hardy Weinberg Equilibrium (<0.05)

4.4 GENETIC DISTANCES

The interindividual genetic distance estimated and the constitution of phylogenetic tree can also used as an assignment method (fig. 17a-d). In case of Mehsana and Pandharpuri, the two individuals of Mehsana clubbed with Pandharpuri while three individuals of Pandharpuri clubbed with Mehsana. In all there were five wrong assignments based on the phylogenetic tree constructed. This contributes approximately 5.7% wrong assignment meaning there by the two populations are quite distinctive in terms of their genotypes.

Neighbour joining tree using Inter individual genetic distance following Nei's Standard Genetic distance.



Radiation tree of the two populations using Nei's Standard genetic distance and Neighbour joining algorithm of Phylogenetic tree construction.

SUMMARY

1. The 23 heterologus microsatellite loci selected for this study in Mehsana and Pandharpuri breeds of buffaloes were found to be highly polymorphic with allele numbers ranging from 2 to 13 in Mehsana breed and 2 to11 in Pandharpuri breed respectively. This shows the utility of heterologus microsatellite loci of cattle in buffaloes, which can be utilized for diversity analysis.

2. The two populations Mehsana and Pandharpuri buffaloes taken in this study showed variations in terms of allelic frequencies and the population were found to be quite distinct from one another.

3. The microsatellite studied in the present study were 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.

4. The microsatellite loci selected were highly polymorphic with a large number of alleles at lower allelic frequencies. This is depicted by quite large variation between the number of alleles and effective number of alleles at these loci.

5. Most of the loci had very high heterozygosity values with mean 0.576 in Mehsana and 0.583 in Pandharpuri buffalo breed. The heterozygosity values in Mehsana population ranged for 0.3864 to 0.8864 in and 0.1 to 0.925 in Pandharpuri population, except ILSTS87, which was homozygous for the alleles, observed.

6. The Hardy Weinberg equilibrium of the two buffalo populations was tested using chi square and G^2 statistics and 8 loci out of 23 loci were not in Hardy Weinberg equilibrium in Mehsana buffalo population and 12 loci in Pandharpuri 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).

7. The F_{IS} values were significantly different from zero in both the buffalo population meaning there by that the population structure exists in the two populations.

8. The F_{ST} value in two buffalo population were 0.0462 meaning there by that the two buffalo population were poorly differentiated at DNA level even though the two buffalo population Mehsana and Pandharpuri were morphologically distinct.

9. The Analysis of Molecular Variance (AMOVA) revealed that the between population variation was 4.62% while within the population variation was quite high 95.38% which means that the inter individual distances are more pronounced than the interpopulation differences.

10. The value of number of migrants between the populations was quite high, it being 5.16 migrants per generations depicting large amount of gene flow among the buffalo populations.

11. The interindividual genetic distance based on Nei's genetic measure were utilized for the assignment of the individuals to their respective populations using neighbour joining and UPGMA algorithms. 5.7% wrong assignment was found in Mehsana and Pandharpuri buffaloes, which is attributed to similarity in allele frequencies.

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