CHAPTER 4

RESULTS

4.1 Distribution, husbandry practices and characteristics of Siri cattle:

4.1.1 Distribution:

Siri cattle are widely distributed in Bhutan. Movement of these animals across the border from Bhutan to West Bengal and Sikkim, and vice versa is very common. The selling and purchase of animals between two countries in the above stated border areas are also frequent. So the population of the particular breed is higher in the vicinity to the border areas of Sikkim and West Bengal with Bhutan. The population gradually decreases as the distance increases from the Bhutan border. The populations of Siri cattle get diluted either by artificial insemination with exotic cattle semen or by crossing with indigenous non-descriptive local cattle population. So purity of the particular breed is not generally found in the areas which are at long distance from Bhutan border. The pure Siri cattle are now concentrated and confined in the remotest hilly areas of West Bengal and Sikkim in India.

4.1.2 Management practices:

Most animal owners take their animals for grazing in forests or in fields at the base of hills. They build temporary structure with bamboo, wood, polythene sheets and leaves in the agricultural field for temporary shelter of animals in the night and day shelter for calves. This temporary shelter is called 'Goot' by the local people (Fig-4.1B). When the grass and other common leaves decrease, they move their 'Goot' to another place. Milking and other husbandry practices are performed in the temporary shelter. Animal grazing is abandoned during rainy season.

Another husbandry practice is also noticed in these areas. In this, most of the animals of the entire village are taken to forest by one person in the morning. The animals graze on steep slopes in thick forests. Generally the cows are taken out after the morning milking leaving the calves at home. The cows in milk return home in the afternoon for feeding the calves and letting down of milk. Some male Siri graze in the deep forest and return home after 5 to 10 days interval. Generally the floor of the animal shelters is made up of mud.

Fig- 4.1 Housing and animal husbandry practices of Siri cattle.

A) Typical housing of Siri cattle

B) Temporary animal shelter (Goot)

C) Concentrate feed preparation at 'Goot'

D) Abandoned 'Goot'

Whole structure of the animal house is made up of bamboo, woods, rock stones and mud. Roof is made up of tin sheet, polythene sheet, leaves or both (Fig-4.1A). Siri cattle mainly depend on grazing at the pasture land. Some amounts of concentrates like rice, maize, millet, pulse and mustard are also offered by some owners.

4.1.3 Agriculture and husbandry practices:

Crop cultivation varies in different parts of the study regions. In the highlands of Eastern Himalayas, terrace cultivation is noticed. Here the soil is acidic in nature, the hill slopes are customized into successive steps or terraces, which are few meters broad. Different spring crops are cultivated on such terraces. Rice, maize, ginger, soya-bean, mustard and millets are the major crops produced in the study areas. In some areas, crop production is supplemented with areca nut, black piper and some spice like green cardamom cultivation. Wood cutting, hunting, home tourism and barter trades are also practiced along with agriculture and animal husbandry.

Mixed crop-livestock farming systems are practiced by majority of the farmers. Raising of livestock is incorporated into food crop production. Cattle provide milk and milk products such as cheese and ghee and act as a source of cash income. Livestock are also used for draught power for ploughing the land and also provide power for other agricultural operations such as threshing. Livestock depend mainly on fodder and grass growing on common property resources. The cow dung is generally used as main source of manure for the agricultural field.

4.1.4 Physical characters of Siri cattle:

4.1.4.1 Coat colour:

Systematic studies were done on 930 Siri cattle in four zones in West Bengal and Sikkim state to get an overall idea regarding the colour pattern of animal body parts. Siri cattle generally carry thick long hairs in whole body with a tuft of long coarse hairs in the base of horns, areas between horns and also in the upper portion of hump region. The length and compactness of hairs vary according to altitude variations. In the base region of hill, closer to plane, around 150 m above msl (mean sea level), where the temperature is not so low, the compactness and length of hairs are like other indigenous cattle except at

Fig- 4.2 Four types of coat colour patterns of Siri cattle

A) Black with white patches coat coloured Siri male

B) Brown with white patches coat coloured Siri female

C) Brown coat coloured Siri female

D) Black coat coloured Siri bull

base of horn and upper portion of hump. But at high altitude, around 2000 to 2500 m above msl, the compactness and length of the hairs are increased in all body parts. This thick long hair may be to give protection to the cattle from severe cold and heavy rain. Four types of coat colours were indentified in the Siri cattle during this study. These were i) black, ii) brown, iii) black with white patches and iv) brown with white patches in both male and female (Fig-4.2).

Majority of cattle under study were black with white patches coat colour, comprising 64.73%, followed by completely black coat colour, comprising 15.8%, brown with white patches coat colour covering 9.89% and completely brown coat colour covering 9.56% of the total population (Table- 4.1).

4.1.4.2 Muzzle colour:

The muzzle colour was observed to be black like other indigenous cattle in all categories of coat coloured Siri cattle except brown with white patches coat coloured Siri (Fig-4.3 and Table-4.1). Brown with white patches coat coloured Siri cattle carried either brownish white (8%) muzzle (Fig-4.3D) or black (92%) coloured muzzle.

4.1.4.3 Eyelid colour:

Eyes were prominent and bright in Siri cattle. All the black and black with white patches coat coloured animals carried black eyelids (Fig-4.3B). On the other hand, all the brown and brown with white patches coat coloured animals carried brown eyelid both in males and females (Fig-4.3D and Table-4.1).

4.1.4.4 Hoof colour:

The legs of the Siri cattle were very strong with very hard hoofs. Both male and female Siri cattle could climb the stiff slope of hills. The colour of hoofs of all the black and black with white patches coat coloured Siri cattle were found grey in colour (Fig-4.3A). The hoofs of all brown and brown with white patches coat coloured Siri cattle were found brownish grey in colour both in males and females (Fig-4.3D and Table-4.1).

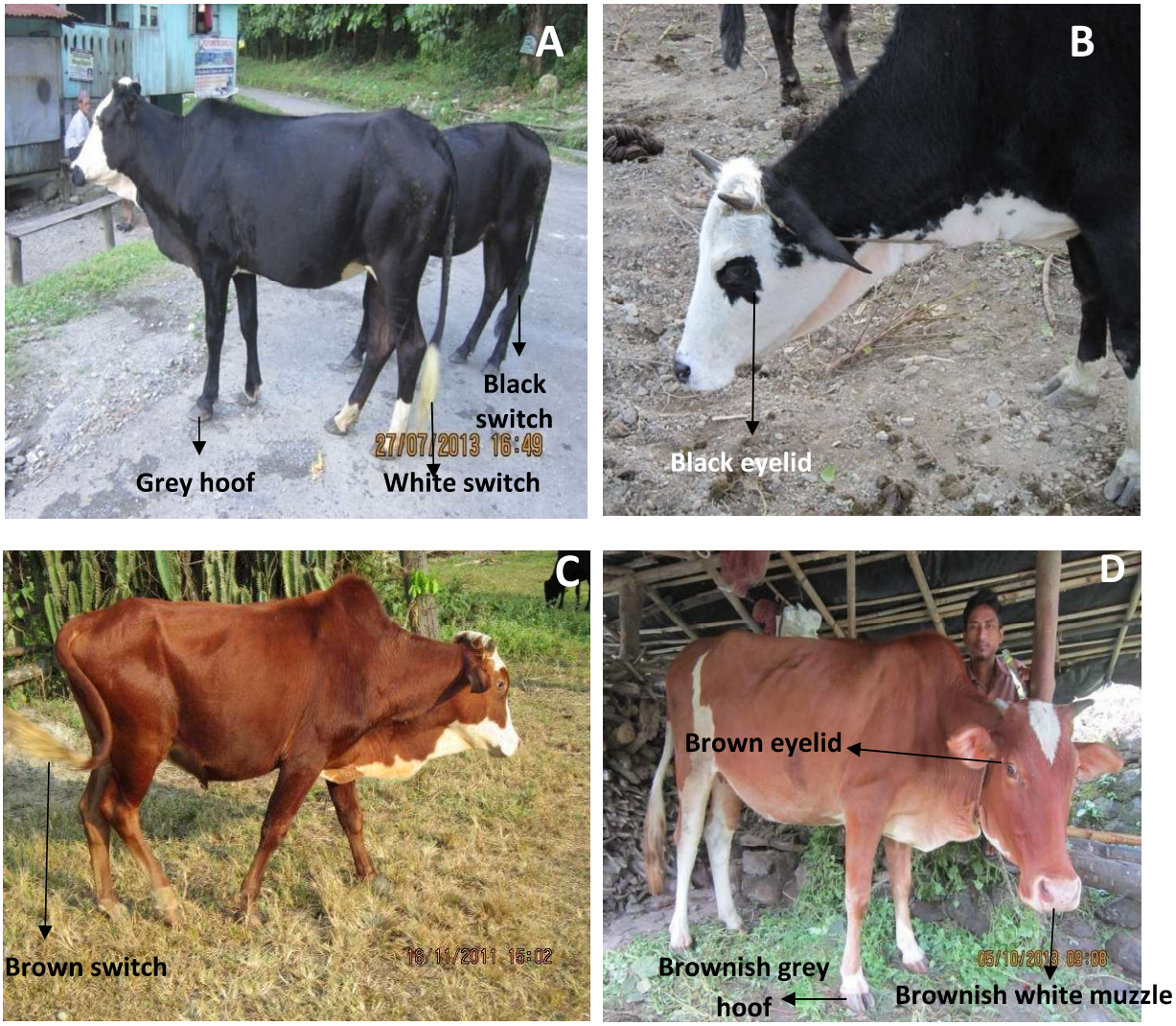

Plate- 4.3 Some phenotypic characters of Siri cattle

A) Siri cattle with grey hoof, white and black coloured tail switch

B) Siri cattle with black eyelid

C) Siri cattle with brown coloured switch

D) Siri cattle with brown eyelid, brownish grey hoof and brownish white muzzle

Table -4.1: Colour pattern of different body parts of Siri cattle

Characteristics	Types of animal in respect of coat colour (N=930)			
	Brown	Black	Brown with white patches	Black with white patches
Population percentage	9.56%	15.8%	9.89%	64.73%
Muzzle colour with percentage of animal	Black 100%)	Black (100%)	Black (92%) Brownish white (8%)	Black (100%)
Hoof Colour	Brownish grey (100%)	Grey (100%)	Brownish grey (100%)	Grey (100%)
Eyelid Colour	Brown (100%)	Black (100%)	Brown (100%)	Black (100%)
Tail switch colour	Brown (31%) Black (69%)	Black (100%)	White (54.87%) Brown (45.13%)	White (9.71%) Black (85.15%) Brown (5.145%)

4.1.4.5 Switch colour:

In the present study, the tail switch colour of all black coat coloured animals were found black (Fig-4.3A). On the other hand, tail switch of brown coat coloured animals were found either brown (31%) or black (69%) in colour. But the tail switches of the black with white patches coat coloured animal were found white (9.71%), black (85.15%) (Fig-4.3A) or brown (5.14 %) in colour both in males and females. In case of brown with white patches coat coloured animals, the tail switches were either white (54.87%) or brown (45.13%) (Fig-4.3C) colour both in male and female animals under this study (Table-4.1).

4.1.4.6 Different important body parts of Siri cattle:

The different body parts such as horn, ear, tail, tail switch, dewlap, hump and forehead were observed and findings were recorded. Additionally body weight, body length, height at withers and chest girth were measured. The mean results are summarized under two separate categories; coat colour wise and zone wise and listed in Table-4.2 and Table-4.3 respectively.

Horns of the four types of coat coloured animals irrespective to all zones were small to medium in length and curved outward, upward and then forward. Horn tips were pointed and sharp. Prominent long tuft of hairs at the base of horn were common both in male and female Siri cattle. Horns were mostly black in colour. In some animals, the horns were whitish grey or pale brown in colour at the base region and black in the upper portion. The horn length of adult male varied from 16 to 32 cm and in case adult female it varied from 15 to 31cm.

Ears of the male and female of all four types of coat coloured cattle were small to medium in length and horizontal in orientation. The ear length of both adult males and female varied from 18 to 20 cm.

Tail length without switch and switch length of the all four types of coat coloured animals both in males and females were measured. The tail length without switch in adult male varied from 75 to 94 cm and in case adult female, the length varied from 62 to 72 cm. The tail switch length of adult male and female varied from 40 to 47cm and from 32 to 42 cm respectively.

The forehead had distinct white patches in black with white patches and brown with white patches types of coat coloured Siri cattle. Forehead was straight in shape in all the animals under study both in males and females.

The position of hump of Siri cattle was observed to be slightly forward (cervico-thoracic position) in comparison to other zebu cattle breed. The hump of all four types of coat coloured animal in different zones were found to be prominent and covered with a tuft of long coarse hair. Hump was medium in size in male and small in size in female in all types of coats coloured Siri cattle in different study zones.

The size of the dewlap of the all four types of coat coloured animals under this study irrespective of zones was prominent and medium in size in males and small in size in females. The average width of dewlap of adult Siri male and female were 13.70±0.20cm and 10.00±0.27cm respectively.

Body conformation of Siri cattle was found to be stout and tight. The chest girth of Siri cattle varied from 168 to 182 cm and 151 to 163 cm in case of male and female respectively. The body length of the male and female Siri cattle ranged from 128 to 148 cm and from 98 to 120 cm respectively. On the other hand the height at wither of adult Siri male and female varied from 136 to 142 cm and from 120 to 124 cm respectively. Body weight of adult male and female Siri cattle ranged from 301 to 452 kg and from 207 to 294 kg respectively. Birth weight of Siri calf ranged from 18 kg to 26kg.

4.1.4.7 Morphometric parameters of reproductive features of Siri cattle:

The different reproduction related organs of both male and female such as testes, naval flap, testicular circumference, penis sheath and teat were observed and findings were recorded. The mean results are summarized under two separate categories; coat colour wise and and zone wise listed in Table-4.4 and Table-4.5 respectively.

Male:

Naval flap was very small in size (average width: 2.11± 0.05 cm) in all four coat coloured male Siri cattle irrespective of four study zones. In some animal the naval flap was almost absent. Penis sheath flap of all coat coloured male animals was very small in size irrespective of four study zones. Testicles were uniform and medium in size among all the mature four types of coat coloured non castrated Siri bulls irrespective of zones. The scrotal circumference varied from 26.5 to 30 cm (average: 28.03 ± 0.13 cm). Left testicular length varied from10.5 to 13 cm (average: 11.68 ± 0.07 cm) and the right testicular length varied from 11 to 14 cm (average: 12.36 ± 0.08 cm).

Female:

The udder was usually small in size, firmly attached and bowl shaped. Teats were observed to be cylindrical and centrally placed with rounded tips. Milk vein was not found to be prominent in the studied animals.

Variations among groups (zone wise and coat colour wise) with respect to different morphometric parameters of different body parts of Siri cattle were non-significant (P<0.05).

Table-4.2: Morphometric parameters of male and female Siri cattle observed in four different coat colour categories.

Physical Characters	Brown coat coloured animal		Black coat coloured animal		Black with white patches coat coloured animal		Brown with white patches coat coloured animal		Overall	
Sex	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Body length (cm)	140.10 ±1.26	104.75 ±1.47	138.35 ±1.29	105.05 ±1.48	139.50 ±1.31	102.90 ±1.02	135.65 ±2.18	103.10 ±1.02	138.40 ±0.79	103.95 ±0.63
Heart girth (cm)	177.50 ±0.84	154.75 ±1.11	176.25 ±0.92	155.05 ±1.01	177.10 ±0.87	154.00 ±1.02	176.45 ±1.10	153.65 ±1.04	176.82 ±0.46	154.36 ±0.51
Body weight (kg)	407.65 ±7.47	232.15 ±6.76	393.00 ±9.12	233.55 ±6.27	404.10 ±7.71	225.50 ±5.24	390.80 ±10.6	224.85 ±5.31	398.89 ±4.40	229.01 ±2.94
Wither height (cm)	139.15 ±0.46	122.20 ±0.27	138.40 ±0.46	122.20 ±0.29	139.10 ±0.49	122.25 ±0.27	139.25 ±0.41	122.50 ±0.31	138.98 ±0.22	122.29 ±0.14
Switch length (cm)	43.65± 0.48	38.70± 0.50	43.15± 0.44	38.05± 0.60	43.35±. 039	38.75± 0.51	43.15± 0.38	38.25± 0.54	43.32± 0.21	38.43± 0.26
Tail length, without switch (cm)	85.05± 1.17	67.75± 0.61	84.90± 1.48	66.20± 0.67	87.15± 1.50	67.55± 0.65	86.95± 1.57	67.25± 0.60	86.01± 0.71	67.18± 0.32
Ear length (cm)	19.45± 0.24	19.00± 0.97	19.50± 0.25	19.10± 0.21	19.75± 0.27	18.95± 0.99	19.80± 0.27	19.15± 0.23	19.62± 0.13	19.05± 0.97
Horn length (cm)	19.00± 0.90	19.30± 1.13	20.65± 1.00	18.90± 0.99	20.25± 0.75	18.55± 0.99	20.10± 3.66	19.25± 1.04	20.00± 0.43	19.00± 0.51
Width of fore head (cm)	18.55± 0.21	18.00± 0.24	18.45± 0.21	18.20± 0.23	18.60± 0.19	18.00± 0.21	18.65± 0.20	18.30± 0.20	18.56± 0.10	18.12± 0.11
Face length (cm)	46.60± 0.3	38.45± 0.29	43.70± 0.34	38.25± 0.33	43.95± 0.35	38.30± 1.41	44.10± 0.36	38.30± 1.45	43.83± 0.17	38.32± 1.40
Dewlap height (cm)	13.95± 0.56	11.05± 0.95	13.75± 0.34	9.45± 0.29	13.55± 1.57	9.95± 0.28	13.65± 0.34	9.55± 0.28	13.70± 0.20	10.00± 0.27

N = 160; Variation among groups is non-significant (P<0.05).

Table-4.3: Morphometric parameters of male and female Siri cattle recorded in the four different study zones.

Physical Characters	Zone –I		Zone –II		Zone –III		Zone –IV	
Sex	Male	Female	Male	Female	Male	Female	Male	Female
Body length (cm)	138.80 ±1.30	103.80± 1.17	139.25± 1.33	103.95± 1.36	138.15± 1.19	103.25± 1.00	136.40 ±1.72	103.00 ±1.02
Heart girth (cm)	176.60 ±0.91	155.10± 0.97	176.85± 0.88	154.80± 1.07	176.30± 0.83	154.05± 0.91	175.85 ±1.00	154.10 ±0.90
Body weight (kg)	399.85 ±7.83	230.75± 5.36	402.30± 7.84	230.40± 6.28	396.50± 7.18	226.25± 4.78	389.85 ±9.06	225.90 ±4.81
Wither height (cm)	137.55 ±0.40	122.20± 0.28	138.20± 0.35	122.20± 0.26	138.20± 0.45	122.55± 0.24	138.50 ±0.46	122.55 ±0.24
Switch length (cm)	43.45± 0.48	38.45± 0.56	43.65± 0.45	38.65± 0.55	43.70±. 0.44	38.65± 0.56	43.55± 0.43	38.90± 0.54
Tail length, without switch (cm)	84.65± 1.37	67.05± 0.67	85.35± 1.40	68.40± 0.65	86.15± 1.44	68.35± 0.66	86.55± 1.45	67.80± 0.64
Ear length (cm)	19.50± 0.25	18.90± 0.22	19.65± 0.29	18.80± 0.21	19.65± 0.26	18.80± 0.23	19.55± 0.27	18.75± 0.23
Horn length (cm)	19.80± 0.84	16.90± 0.35	19.65± 0.88	18.10± 1.01	19.50± 0.75	17.10± 0.68	19.40± 0.83	17.10± 1.68
Face length (cm)	44.00± 0.33	38.40± 0.24	43.45± 0.32	38.65± 0.26	43.85± 0.36	38.55± 0.27	43.75± 0.36	38.45± 0.27
Width of fore head (cm)	18.50± 0.23	18.10± 0.23	18.70± 0.23	18.00± 0.27	18.65± 0.23	18.05± 0.27	18.60± 0.22	18.05± 0.27
Dewlap height (cm)	13.95± 0.31	10.20± 0.24	14.10± 0.56	11.05± 0.95	13.85± 0.31	10.00± 0.27	13.85± 0.31	9.90± 0.28

N = 160; Variation among groups is non-significant (P<0.05).

Table-4.4: Different morphometric parameters of testes, naval flap and teat observed in four different coat colour categories.

Physical Characters	Brown coat coloured animal		Black coat coloured animal		Brown with white patches coat coloured animal		Black with white patches coat coloured animal		Overall	
Sex	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Testicular circumference (cm)	27.82 ±0.27	--	28.12 ±0.25	--	27.87 ±0.25	--	28.30 ±0.28	--	28.03 ±0.13	--
Right testicular length (cm)	12.30 ±0.15	--	12.45 ±0.19	--	12.32 ±0.12	--	12.37 ±0.17	--	12.36 ±0.08	--
Left testicular length (cm)	11.62 ±0.13	--	11.75 ±0.16	--	11.67 ±0.13	--	11.67 ±0.14	--	11.68 ±0.07	--
Naval flap size (cm)	2.12± 0.10	--	2.05± 0.11	--	2.12± 0.10	--	2.17± 0.11	--	2.11± 0.05	--
Teat length (cm)	--	3.60± 0.11	--	3.68± 0.13	--	3.68± 0.10	--	3.66± 0.13	--	3.66± 0.06

N = 160; Variation among groups is non-significant (P<0.05).

Table-4.5: Different morphometric parameters of testes, naval flap and teat of Siri cattle in the four different study zones.

Physical Characters	Zone –I		Zone –II		Zone –III		Zone –IV	
Sex	Male	Female	Male	Female	Male	Female	Male	Female
Testicular circumference (cm)	28.20 ±0.26	--	28.02± 0.28	--	28.07± 0.27	--	28.17 ±0.24	--
Right testicular length (cm)	12.52 ±0.20	--	12.40± 0.17	--	12.42± 0.17	--	12.37 ±0.18	--
Left testicular length (cm)	11.82 ±0.16	--	11.72± 0.14	--	11.72± 0.15	--	11.70 ±0.15	--
Naval flap size (cm)	2.17± 0.11	--	2.12± 0.11	--	2.15± 0.11	--	2.12± 0.10	--
Teat length (cm)	--	3.77± 0.15	--	3.51± 0.10	--	3.51± 0.10	--	3.51± 0.10

N = 160; Variation among groups is non-significant (P<0.05).

4.1.5 Female reproductive characters:

Reproductive performances like age at first estrous, age at first calving and calving interval of the four types of coat coloured animals were studied in four zones. The age at first estrous, age at first calving and calving interval varied from 30 to 50 months, 41 to 61 months and 410 to 540 days respectively, irrespective of coat colour and study zones. Average gestation length of Siri cattle was found to be 289.15 ± 0.85 days (Table- 4.6 and Table- 4.7). The animals, those were offered the concentrated feed, showed early symptom of heat. This leads to lowering of age at first estrus, age at first calving and calving interval.

4.1.6 Productive performances of female:

The daily milk yield of the Siri cattle ranged from 1.5 to 3 kg (average: 2.13 ± 0.05 kg) while the peak milk yield varied from 2.5 to 4 kg (average: 3.08 ± 0.05 kg). The lactation period of Siri cattle under study ranged in between 210 to 280 days and averaged at 239.00 ± 2.04 days (Table- 4.6 and Table- 4.7). Cows those were supplemented with concentrated feed along with normal feed produced more milk than the other cows that were not supplemented with concentrate. The solid non fat of milk of Siri cow ranged from 7.4% to 9.6 % and fat of milk varied from 3.8% to 5.2 %. The average solid non fat and fat of Siri cow milk were recorded as 8.28±0.15 % and 4.485±0.09 % respectively.

Variations among groups (zone wise and coat colour wise) in respect to different reproductive and productive characters of Siri cattle were non-significant (P<0.05).

Table 4.6: Productive and reproductive parameters of four different coat colour Siri cattle.

	Brown coat coloured animal	Black coat coloured animal	Brown with white patches coat coloured animal	Black with white patches coat coloured animal	Overall
Age at first calving (months)	50.50±1.61	51.55±1.61	53.25±1.63	50.75±1.65	51.51±0.81
Age at first estrous (months)	39.85±1.31	40.15±1.27	44.25±1.29	40.90±1.14	41.29±0.65
Gestation length (days)	287.75±1.04	288.00±1.13	287.80±1.18	288.25±1.04	287.95±0.54
Calving interval (days)	478.25±8.99	472.50±10.35	471.25±9.36	476.75±9.55	474.69±4.70
Peak milk yield (kg)	3.05±0.10	3.10±0.10	3.05±0.10	3.15±0.10	3.08±0.05
Daily milk yield (kg)	2.10±0.11	2.15±0.12	2.10±0.11	2.20±0.11	2.13±0.05
Lactation period (days)	240.0±3.40	241.75±4.73	237.00±4.49	237.50±3.83	239.00±2.04

N = 80; Variation among groups is non-significant (P<0.05).

Table 4.7: Productive and reproductive parameters of Siri cattle observed in different zones.

Economic traits	Zone-1	Zone –II	Zone –III	Zone –IV
Age at first calving (months)	51.55±1.60	50.50±1.60	50.75±1.65	53.25±1.63
Age at first estrous (months)	40.55±1.60	39.50±1.60	39.75±1.65	42.25±1.63
Gestation length (days)	289.85±0.99	289.80±1.21	290.15±1.01	289.15±0.85
Calving interval (days)	458.50±9.31	461.50±10.03	475.00±9.82	459.50±8.68
Peak milk yield (kg)	2.97±0.09	3.15±0.10	3.00±0.10	2.97±0.08
Daily milk yield (kg)	1.97±0.09	2.15±0.10	2.00±0.10	1.97±0.18
Lactation period (days)	246.25±4.67	243.0±3.56	242.50±3.61	244.25±4.18

N = 80; Variation among groups is non-significant ($P<0.05$).

4.1.7 Draught performance of male:

Most of the adult Siri males observed in this study were non-castrated and were used for natural reproductive purpose as well as for ploughing in the agricultural field. However some castrated males were noticed in the areas close to the plane land where the facilities of castration were available. The Siri were found to be very docile in nature. The non-castrated males obeyed the owner's direction at the time of ploughing. Drought tolerance of Siri was found to be excellent. The Siri male was capable of ploughing or working 10 to 12 hours per day without fatigue and was therefore very useful animal in the study areas where no facilities of ploughing of agricultural land were available. Habitually they call their owner for ploughing through shouting, thus expressing their willingness (Fig-4.4D).

Fig- 4.4 Productive performance of Siri cattle

A) New born Siri calf with Siri cow

B) Milking of Siri cow

C) Fresh Siri cow milk

D) Siri bull used for ploughing purpose

4.1.8 Heat tolerance ability:

The heat tolerance ability of Siri cattle was excellent. This cattle breed showed high degree of adaptation to wide range of temperature ranged from 0°C to 24°C in the normal distribution areas. But the bull or bullock could plough very well in the plane areas without panting or showing any symptoms of fatigue in summer when the temperature rose up to 35°C. Three Siri bulls housed in Haringhata farm showed no symptoms of panting in the summer season when temperature rose up to 42°C. Additionally there was no change in semen production.

Variations among groups (Zone wise and coat colour wise) in respect to different morphometric, productive and reproductive characters were non-significant ($P<0.05$).

4.1.9 Reproductive performances of male:

The reproductive performances of bull can be assessed by evaluating the quality of semen. For determining the quality of semen, several factors were taken into consideration and kept constant during the experiment with all the three bulls. These included health and nutritional status as well as environmental conditions (weather and surrounding environmental factors). For assessing the semen quality of the Siri bulls, neat semen volume, colour, pH, mass motility, concentration, initial motility, live-dead percentage and sperm morphology were studied.

4.1.9.1 Age at 1st semen donation:

Three Siri bulls donated semen for the 1st time at the age of 40 months, 50 months and 53 months and average was computed as 47.66 ± 3.93 months.

4.1.9.2 Volume of semen per ejaculate:

Semen volume per ejaculate directly correlated with the volume of testes. But the semen volume per ejaculate varied from bull to bull and ejaculate to ejaculate of the same bull. The semen volume per ejaculate of study animal varied between 1.2 ml to 5.4 ml and average volume was 3.51 ± 0.16 ml (Table-4.8).

4.1.9.3 Colour of the semen:

Colour of the neat semen of Siri bull was found to be creamy white in all study samples.

4.1.9.4 Mass motility:

Grade for mass motility of Siri semen ranged from 2 to 5 and average was recorded as 4.07 ±0.16 (Table-4.8).

4.1.9.5 pH of neat semen:

The pH of neat semen is one of the main indicators of quality of semen and reproductive health of bull. In this study, the pH of the neat semen was slightly acidic in nature which varied from 6.6 to 6.8 with an average of 6.72 ± 0.01(Table-4.8).

4.1.9.6 Concentration of sperm of neat semen:

Concentrations of sperm of neat semen varied widely between bulls as well as from ejaculate to ejaculate of same bull. Concentration of the sperm in neat semen ranged between 307 to 2064 million per ml and average was computed as 980.93 ± 84.33 million per ml (Table-4.8).

4.1.9.7 Initial motility of the sperm in neat semen:

Initial motility of the sperm in fresh semen varied between bull to bull and also ejaculate to ejaculate of same bull. Initial motility of the sperm in fresh semen ranged between 30% to 80 % and average was recorded as 64.65 ± 2.72% (Table-4.8).

4.1.9.8 Live and dead percentage of sperm in neat semen:

Percentage of live sperm varied among the different bulls and also among the different ejaculates of same bull. This parameter positively correlated with the individual sperm motility. Live sperm in Siri bull varied from 45% to 93% and mean value was recorded as 77.56 ± 1.91%. The dead sperm percentage ranged between 7 to 55 % and average was computed as 22.44 ± 1.91% (Table-4.8).

Table 4.8: Semen characteristics of Siri bull

Numbers of observations =43	Volume (ml)	Concentration (Million per ml)	Initial motility (%)	Mass motility (0 to 5)	pH	Live sperm (%)	Dead sperm (%)	Normal sperm (%)	Abnormal sperm	
									Primary abnormality (%)	Secondary abnormality (%)
Maximum	5.4	2064	80	5	6.8	93	55	92	5	13
Minimum	1.2	307	30	2	6.6	45	7	85	1	6
Average	3.51 ±0.16	980.93 ±84.33	64.65 ±2.72	4.07 ±0.16	6.72 ±0.01	77.56 ±1.91	22.44 ±1.91	88.35 ±0.27	2.86 ±0.15	8.79 ±0.29

4.1.9.9 Abnormality of spermatozoa:

Different types of abnormality were recorded during evaluation of Siri cattle semen and those parameters were the main indicators of bull fertility. Primary abnormalities like double, pyriform, micro and macro head were identified and varied from1 to 5% (average: 2.86 ± 0.15%). The secondary abnormalities like double tail, de-touched tail, coiled tail, proximal and distal droplet were identified. Secondary abnormalities ranged from 6% to 13% and mean value were recorded as 8.79 ±0.29 %. The percentage of normal spermatozoa in the fresh semen of Siri bull varied from 85% to 92% and average was 88.35 ±0.27% (Table-4.8.)

4.1.10 Numeric and morphometric characters of Siri cattle chromosome:

4.1.10.1 Chromosome numbers:

Cytogenetic study of Siri cattle revealed that the number of chromosomes of Siri cattle was 30 pairs i.e. 2n= 60 in all examined complete metaphase spreads. Metaphase spread, karyotype and ideogram of Siri male cattle chromosome are shown in Fig-4.5. Out of total 60 chromosomes, 58 autosomes and 2 sex chromosomes (X and Y) were identified.

4.1.10.2 Chromosome morphology:

All the autosomes and Y-chromosome were telocentric in shape. The X-chromosome however was sub-metacentric (Fig-4.5A, B).

4.1.10.3 Relative length, centromeric index and arm ratio:

The average relative longest and smallest autosome lengths were 2.71 ± 0.03 % and 0.97 ± 0.02 % respectively. The average length of Y-chromosome was 0.90 ± 0.03 % and it was found to be the smallest telocentric chromosome of the Siri cattle. The average length of X- chromosome was 2.60 ±0.05% (Table-4.9 and Fig-4.5C). Variations among the three Siri males in respect of 30 pairs of chromosome length were non-significant ($p < 0.05$). The average centromeric index of X- chromosome was 34.54±0.85%. The average arm ratio of X-chromosome was 1.79±0.08.

No chromosomal abnormality (numeric or structural) was detected under this study.

Table 4.9: Relative lengths of Siri cattle chromosomes.

Chromosome Pair numbers	Relative length (%) Mean ± SE
1	2.71±0.03
2	2.45±0.02
3	2.29±0.02
4	2.22 ±0.02
5	2.17 ± 0.01
6	2.11 ±0.02
7	2.06 ± 0.02
8	2.01 ±0.01
9	1.95 ±0.01
10	1.89 ±0.01

Contd.

Table-4.9 Contd.

Chromosome Pair numbers	Relative length (%)
11	1.84 ±0.01
12	1.76 ±0.01
13	1.69 ±0.01
14	1.63 ±0.01
15	1.57 ±0.01
16	1.53 ±0.01
17	1.51 ±0.01
18	1.48 ±0.02
19	1.43 ±0.02
20	1.39 ±0.01
21	1.36 ±0.01
22	1.32 ±0.02
23	1.27 ±0.01
24	1.24 ±0.01
25	1.19 ±0.01
26	1.13 ±0.01
27	1.07 ±0.02
28	1.02 ±0.02
29	0.97 ±0.02
X	2.60 ±0.05
Y	0.90 ±0.03

N = 60; Variation among 30 pairs of chromosome is non-significant ($P<0.05$).

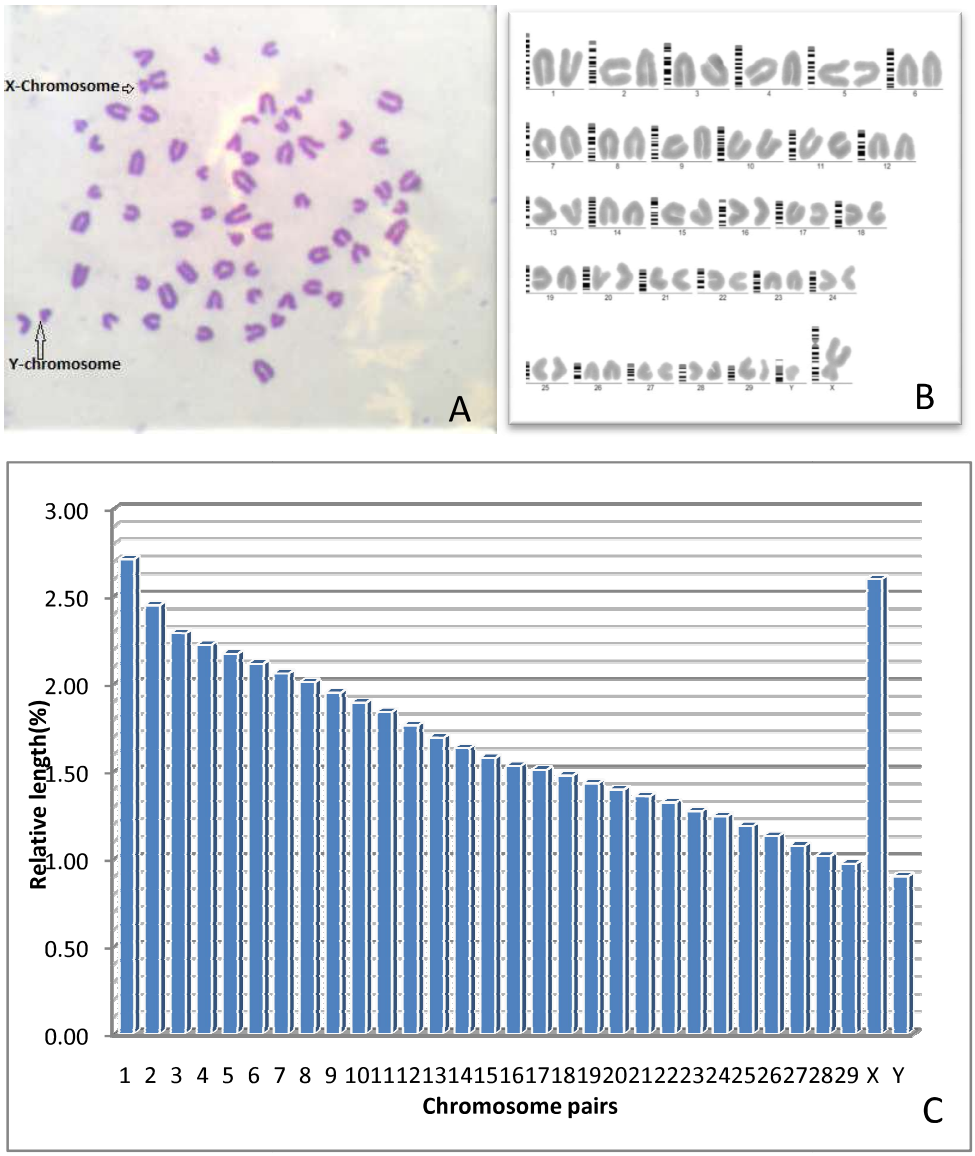

Fig- 4.5 Cytogenetic studies on male Siri cattle

A) Metaphase spread of male Siri bull

B) Karyotype of male Siri cattle

C) Ideogram representing the relative length of chromosomes of Siri cattle

4.2 Molecular identification of Siri cattle:

4.2.1 Genomic DNA from tissue of the specimens:

The genomic DNA isolated from blood of Siri cattle was run on agarose gel. This produced distinct fluorescent bands in UV trans-illuminator (Fig-4.6A). The extracted genomic DNAs were of high molecular weight (greater than 10 kb) in most of the samples. The extractions were in the range of 50 – 388 ng/µl and revealed purity in the range of 1.35 – 1.60 in terms of ratio of the absorbance at 260/280 nm. From the blood samples, the extracted genomic DNA showed smearing in few cases which may be due to hydrodynamic stress and long term handling of samples during the extraction procedure. Total 34 genomic DNA extracted from blood samples are shown in Table-4.10.

Table: 4.10 Concentration of 34 different genomic DNA samples isolated from Siri cattle.

Sl. No.	Sample	Concentration (ng/ul)
1	SKGPB-SRA	290
2	SKGPB-SRB	131
3	SKGPB-SRC	356
4	SKGPB-SRE	259
5	SKGPB-SRF	290
6	SKGPB-SRH	180
7	SKGPB-SRI	260
8	SKGPB-SRJ	323
9	SKGPB-SRK	110
10	SKGPB-SRL	162
11	SKGPB-SRM	388
12	SKGPB-SRN	190
13	SKGPB-SRO	131
14	SKGPB-SRP	166

Contd.

Table 4.10 Contd.

Sl. No.	Sample	Concentration (ng/ul)
15	SKGPB-SRQ	159
16	SKGPB-SRR	290
17	SKGPB-SRT	220
18	SKGPB-SR7	274
19	SKGPB-SR9	298
20	SKGPB-SR10	267
21	SKGPB-SR13	172
22	SKGPB-SR16	198
23	SKGPB-SR19	267
24	SKGPB-SR20	184
25	SKGPB-SR23	227
26	SKGPB-SR24	278
27	SKGPB-SR25	271
28	SKGPB-SR26	179
29	SKGPB-SRU	256
30	SKGPB-SR28	50
31	SKGPB-SRW	211
32	SKGPB-SR2	162
33	SKGPB-SR6	288
34	SKGPB-SRX	123

4.2.2 PCR amplicons of COI DNA barcode, mtDNA D-loop and gel purified products:

D-loop and COI (barcode) segment amplified successfully in PCR in all cases. A single uniform band was amplified that carried traces of primers that migrated further by-passing the PCR products. After purification, the primers were not visible, moreover none of the PCR amplicons got degraded and therefore no smearing was observed. The PCR amplification of COI gene generated an amplicon of band size about 850bp (Fig-4.6B) and for the D-loop, the amplicon size was about 1050bp (Fig-4.6C).

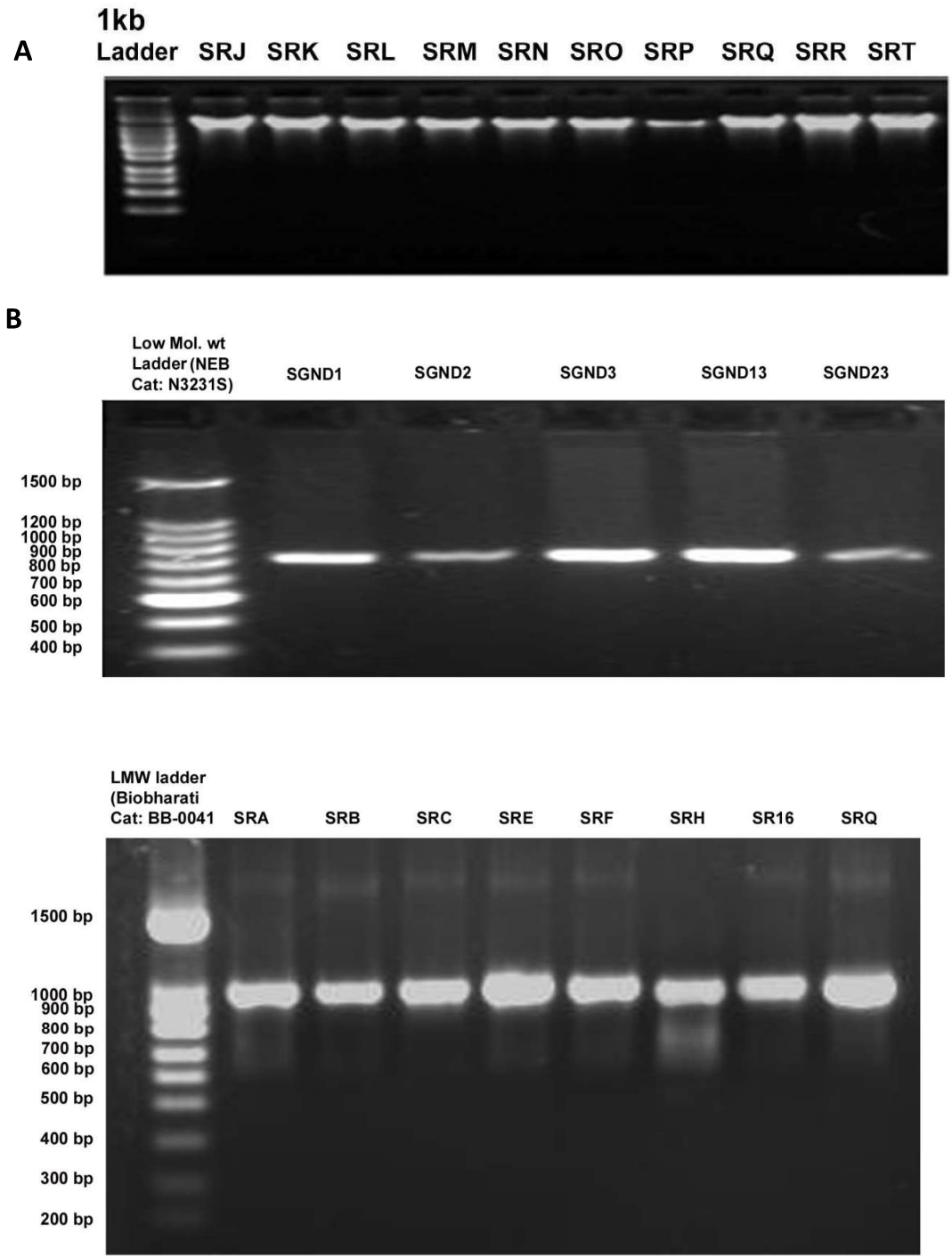

Fig-4.6. Gel electropherogram of Siri cattle DNA

A) Genomic DNA

B) PCR amplicon of COI (around 850bp).

C) PCR amplicon of D-loop (around 1050bp).

4.2.3 Raw sequences analysis:

The sequencing results were obtained in the form of two chromatograms for each sample; one of the forward strand and another for the reverse strand. The software SeqScanner (Applied Biosystems, USA) outputted the chromatograms in the form of the original sequence. The quality for basecall of each of the sequences were checked with SeqScanner and found to be on the score of 40-50 QV in all the examples, which affirmed that the sequences being 99.99% accurate. The Peak quality of the sequence chromatograms was checked position by position using BioEdit software and were found to be clean and without any background noise which were then confirmed with the reference library dataset in terms of query coverage and % identity and found to be highly accurate. Furthermore, the accuracy of the sequences was further confirmed by the amino acid sequences (only in case of *COI)* of the gene by ORF.

4.2.4 Final sequences after annotation of the raw sequences:

As both the forward and reverse sequence represented the same gene location of the same sample, the reverse sequence being transformed in reverse complement form (transformation of sequence of a particular strand into reverse complement form brings it in the format of sequence of the same location of the other strand with which the sequence is complementary). The match of the generated sequences with the reference sequence in all the segments confirmed the sequence being correct. On the other hand for *COI*, the aligned sequence was considered the final sequence and its translation and protein BLAST result revealed 100% homology with partial amino acid array of the COI gene. All the annotated sequences were finalized and submitted to GenBank by the name as identified and obtained valid accession numbers (Table-4.11A and B).

Table 4.11: (A) D-loop sequences of mtDNA, generated from the Siri cattle breed.

Sl No	Sample ID	Genbank Accession No.	Species	Breed	GIS location
1	SKGPB-SRA	KU682457	*Bos taurus indicus*	Siri	26.859694N & 89.394211E
2	SKGPB-SRB	KU682458	*Bos taurus indicus*	Siri	26.859694N & 89.394211E
3	SKGPB-SRC	KU682459	*Bos taurus indicus*	Siri	26.859694N & 89.394211E
4	SKGPB-SRE	KU682460	*Bos taurus indicus*	Siri	26.859694N & 89.394211E
5	SKGPB-SRF	KU682461	*Bos taurus indicus*	Siri	26.859694N & 89.394211E
6	SKGPB-SRH	KU682462	*Bos taurus indicus*	Siri	27.026836N & 88.786056E
7	SKGPB-SRI	KU682463	*Bos taurus indicus*	Siri	27.026836N & 88.786056E
8	SKGPB-SRJ	KU682464	*Bos taurus indicus*	Siri	27.026836N & 88.786056E
9	SKGPB-SRK	KU682465	*Bos taurus indicus*	Siri	27.026836N & 88.786056E
10	SKGPB-SRL	KU682466	*Bos taurus indicus*	Siri	26.738172N & 89.547694E
11	SKGPB-SRM	KU682467	*Bos taurus indicus*	Siri	26.701033N & 89.607731E
12	SKGPB-SRN	KU682468	*Bos taurus indicus*	Siri	26.701033N & 89.607731E
13	SKGPB-SRO	KU682469	*Bos taurus indicus*	Siri	26.769817N & 89.544711E
14	SKGPB-SRP	KU682470	*Bos taurus indicus*	Siri	26.769817N & 89.533297E
15	SKGPB-SRQ	KU682471	*Bos taurus indicus*	Siri	26.769817N & 89.544711E
16	SKGPB-SRR	KU682472	*Bos taurus indicus*	Siri	27.0116N & 88.78885E
17	SKGPB-SRT	KU682473	*Bos taurus indicus*	Siri	26.766217N & 89.506936E
18	SKGPB-SRU	KU682474	*Bos taurus indicus*	Siri	26.766217N & 89.506936E
19	SKGPB-SRX	KU682475	*Bos taurus indicus*	Siri	27.762286N & 89.583844E
20	SKGPB-SRW	KU682476	*Bos taurus indicus*	Siri	27.762286N & 89.583844E
21	SKGPB-SR2	KU682477	*Bos taurus indicus*	Siri	27.026086N & 88.863333E
22	SKGPB-SR6	KU682478	*Bos taurus indicus*	Siri	27.040397N & 88.873856E
23	SKGPB-SR7	KU682479	*Bos taurus indicus*	Siri	27.040397N & 88.873856E
24	SKGPB-SR9	KU682480	*Bos taurus indicus*	Siri	27.040397N & 88.873856E
25	SKGPB-SR10	KU682481	*Bos taurus indicus*	Siri	27.004697N & 88.868397E
26	SKGPB-SR13	KU682482	*Bos taurus indicus*	Siri	27.0627333N & 88.86975E
27	SKGPB-SR16	KU682483	*Bos taurus indicus*	Siri	27.004697N & 88.868397E
28	SKGPB-SR19	KU682484	*Bos taurus indicus*	Siri	27.004697N & 88.868397E
29	SKGPB-SR20	KU682485	*Bos taurus indicus*	Siri	27.004697N & 88.868397E
30	SKGPB-SR23	KU682486	*Bos taurus indicus*	Siri	27.026086N & 88.863333E
31	SKGPB-SR24	KU682487	*Bos taurus indicus*	Siri	27.05565N & 88.871033E
32	SKGPB-SR25	KU682488	*Bos taurus indicus*	Siri	27.040397N & 88.873856E
33	SKGPB-SR26	KU682489	*Bos taurus indicus*	Siri	27.026086N & 88.863333E
34	SKGPB-SR28	KU682490	*Bos taurus indicus*	Siri	27.040397N & 88.873856E

Table 4.11: (B) COI gene sequences of mtDNA, generated from the Siri cattle breed.

Sl No	Sample ID	Genbank Accession No.	Species	Breed	GIS location
1	SGND1	KX845673	*Bos taurus indicus*	Siri	27.322622N, 88.345589E
2	SGND2	KX845674	*Bos taurus indicus*	Siri	27.026086N , 88.863333E
3	SGND3	KX845675	*Bos taurus indicus*	Siri	27.079546N, 88.749721E
4	SGND13	KX845676	*Bos taurus indicus*	Siri	27.0627333N , 88.86975E
5	SGND23	KX845677	*Bos taurus indicus*	Siri	26.705990N, 88.280742E

4.2.5 Species identification through DNA barcoding:

The specimens were indentified mainly by combined approaches of similarity match and conventional method, NJ and K2P distance based approach.

4.2.5.1 Species identification based on similarity match approach:

Comprehensive species identification using five samples of the studied bovine species based on BOLD and GenBank databases is depicted in Table-4.12.

Table 4.12: Species identification by similarity match approach of the generated sequences of COI gene from collected specimens with the database sequences of Genbank and BOLD-IDS.

Sl NO	Sample (ID)	Similarity match global data base		Identified as
		Genbank (%)	BOLD-IDS	
1	SGND1	*Bos taurus (99-100)*	*Bos taurus*	*Bos taurus*
2	SGND2	*Bos taurus (99-100)*	*Bos taurus*	
3	SGND3	*Bos taurus (99-100)*	*Bos taurus*	
4	SGND13	*Bos taurus (99-100)*	*Bos taurus*	
5	SGND23	*Bos taurus (99-100)*	*Bos taurus*	

The similarity match helped us to confirm the species status of the Siri breed of cattle. The comparison of the generated species-specific COI barcode sequence with the published database sequence clearly delineated the generated sequence of Siri cattle into its respective species of *Bos taurus* significantly with the similarity percentage of 99-100%. Thus, similarity match is facilitating the straight forward identification of the species.

4.2.5.2 Species identification by Neighbour-Joining cluster and K2P genetic distance:

The species sequences generated in this study was taken along with the database sequences of the same species (Appendix - 2). The sequences from the GenBank with which our sequences showed closest match were downloaded as barcode replicates for the study. The Neighbor-Joining (NJ) tree was created as per standard barcoding protocol using 1500 bootstrap replicates. The NJ tree showed distinct clustering of the species from one another with a strong bootstrap support, the generated sequences showed an intra-specific divergence from the available database sequences. The database sequence of *Bos gaurus* showed a deep divergence within the con-specific sequences originating from a common ancestral node. On the other hand, members of *B. grunniens* and *Bison bison* clustered as a cohesive unit but as a nearest neighbor from each other as shown in Fig-4.7.

Similarly, the genetic distance based on K2P model was calculated to identify the species boundary (The Distance matrix is detailed in Appendix - 3. Within the cluster the mean K2P distance was found to be 0.0173 ± 0.003 and maximum K2P distance was 0.043.While, between the clusters, mean K2P distance was observed as 0.087 ± 0.011 with minimum K2P distance of 0.024 as summarized in Table 4.13.

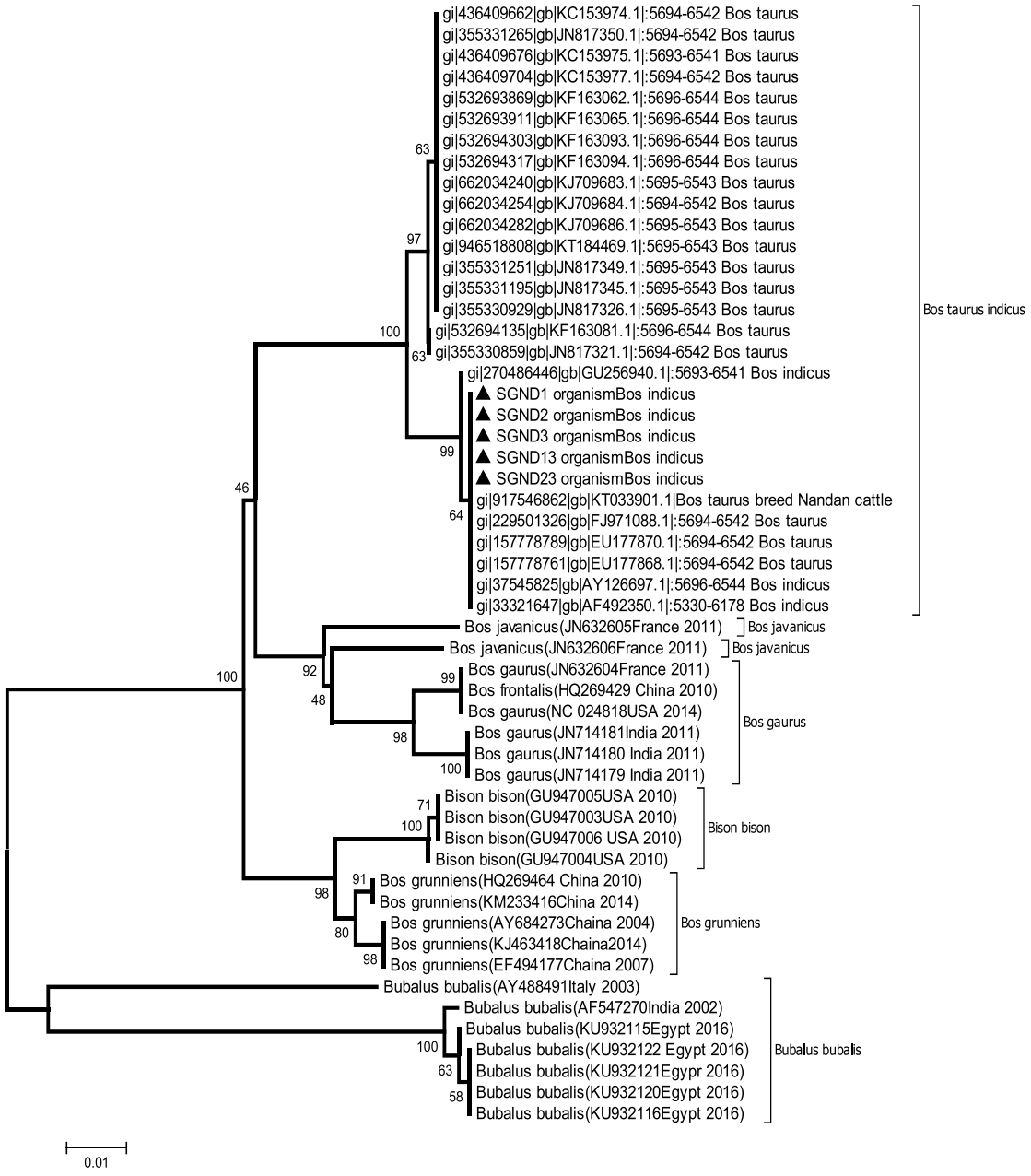

Figure 4.7 Neighbour-Joining tree from the *COI* barcode sequences of the Siri breed of cattle (*Bos taurus indicus*), marked as triangle along with the different conspecific sequences and other related sequences from different species from database.

Table 4.13: Genetic Distance of the collected species of bovine sample on *COI* barcode sequences.

Group	Mean K2P	Minimum	Maximum
Within Species	0.0173 ± 0.003	0.001	0.043
Between Species	0.087 ± 0.011	0.024	0.15

4.2.6 Nucleotide composition of *COI* barcode sequences:

Nucleotide compositions at all codon positions of the bovidae family were analyzed (Table 4.14). The base frequencies for each sequence and for total barcode length were calculated by MEGA6. The analysis revealed that nucleotide composition at third codon position showed significant variations among the species while for first and second codon positions nucleotide composition were almost uniform across different species (Figure 4.8). At 3rd codon position, all the species showed variation in all four nucleotides. A tendency toward low G content was observed and at 3rd codon position, it was significantly lower in all the collected species. At the 2nd and 3rd codon positions, there was a bias towards AT over GC (Figure 4.9).

Comparing the correlation of average GC content with GC content at each codon position, in different species of bovine animals, it was observed that GC content at the 3rd codon position was strongly positive correlated (r = 0.79) to the overall GC content of the barcode region (Figure 4.10). At second codon position, the GC content was positively correlated (R = 0.73) to overall GC content and at first codon position, it was negatively correlated (R = -5.57E-15) to the overall GC content. To obtain a closer perception of GC content distribution, the correlation between GC content at each codon position and average GC content in species of different orders of bovine species was plotted (Figure 4.10). Each point represents correlation of average GC content in an order at a given codon position with average GC content. Each codon position is marked with a different marker as given in the figure legend.

Table 4.14: Nucleotide compositions of COI gene of some species of Bovidae family.

Species	T	C	A	G	T-1	C-1	A-1	G-1	T-2	C-2	A-2	G-2	T-3	C-3	A-3	G-3
Bos taurus indicus	28.8	26.3	28.1	16.7	22.1	22.2	26.3	29.4	42.0	25.5	17.0	15.5	22.4	31.2	41.2	5.3
Bos grunniens	27.6	27.7	28.0	16.7	21.6	22.3	26.4	29.7	42.2	25.7	16.7	15.4	19.0	35.0	41.0	5.0
Bos gaurus	27.0	28.6	27.3	17.1	21.3	22.5	26.2	30.0	42.3	27.0	16.0	14.7	17.5	36.2	39.7	6.7
Bos frontalis	27.0	28.6	27.3	17.1	21.3	22.5	26.2	30.0	42.3	27.0	16.0	14.7	17.5	36.2	39.7	6.7
Bos javanicus	28.2	27.1	27.6	17.2	22.1	21.8	26.4	29.6	42.2	25.7	16.8	15.4	20.2	33.7	39.5	6.6
Bison bison	27.8	27.4	28.2	16.6	22.1	21.8	26.7	29.5	42.1	25.3	17.2	15.4	19.2	35.2	40.6	5.0
Bubalus bubalis	28.9	26.5	26.8	17.8	20.4	23.7	25.6	30.2	41.8	26.8	16.5	15.0	24.5	28.8	38.4	8.2

In addition to AT-GC content, mitochondrial genomes also vary in their patterns of strand asymmetry (usually measured as GC skew and AT skew). Figure 4.11 shows the plot of AT and GC skew for different bovine species at each codon position in the barcode and for the total barcode region. Strand asymmetry in the total barcode region showed a different pattern than in each codon position. Complete barcode region of all the studied species (Figure 4.11) showed overall negative GC skew, in most of the cases AT showed mostly negative skew whereas positive AT skew only for few samples. At first codon position, (Figure 4.12) both AT and GC showed positive skew while at second codon position, (Figure 4.13) AT and GC skew showed negative values. The third codon position showed a positive AT skew whereas GC skew showed negative values (Figure 4.14).

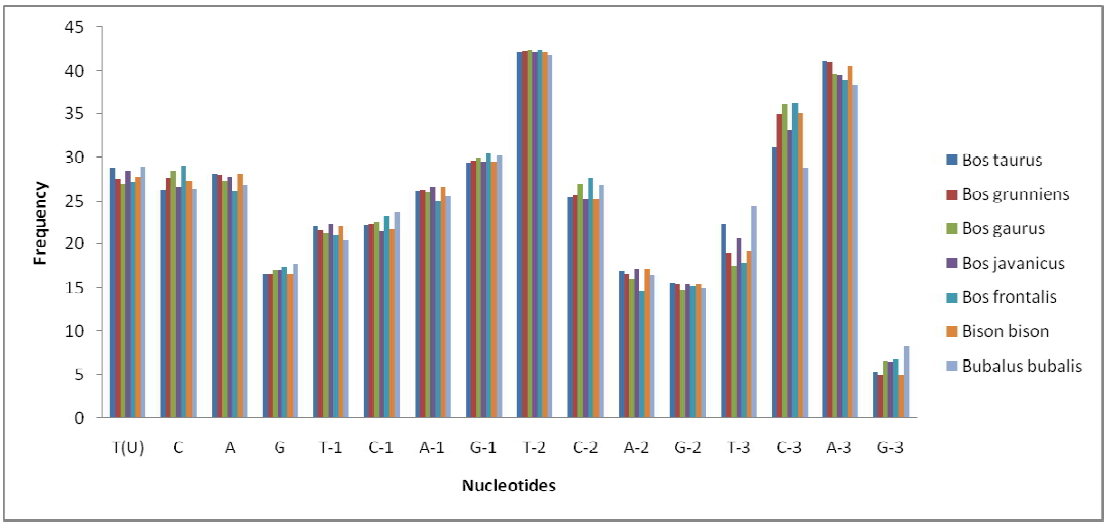

Figure 4.8: Nucleotide composition of different bovine samples (different colour bars represent different bovine species).

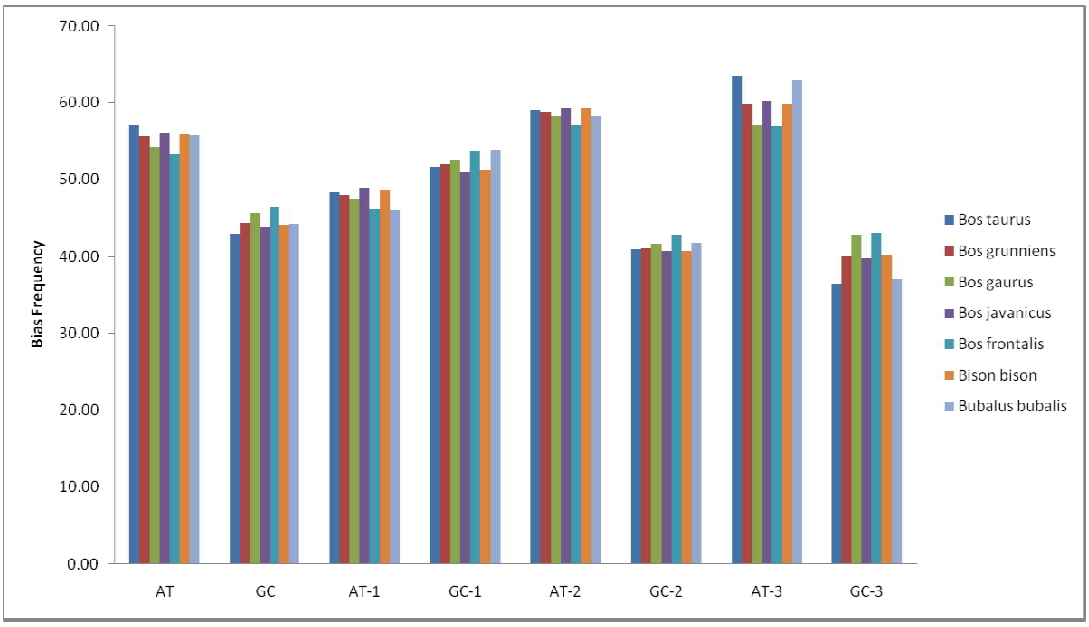

Figure 4.9: AT-GC bias in different bovine sample.

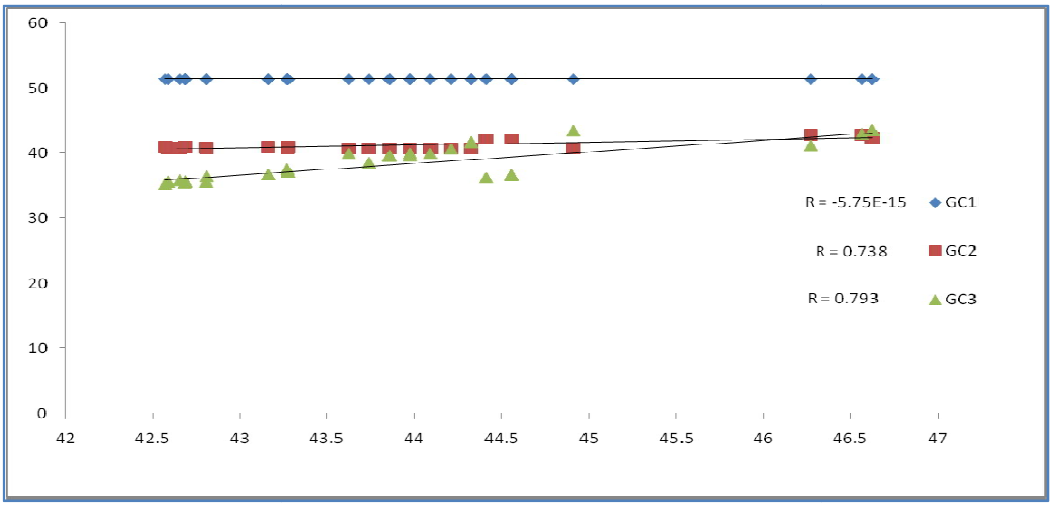

Figure 4.10: Correlation of average GC content with GC content at each codon position of different bovine species.

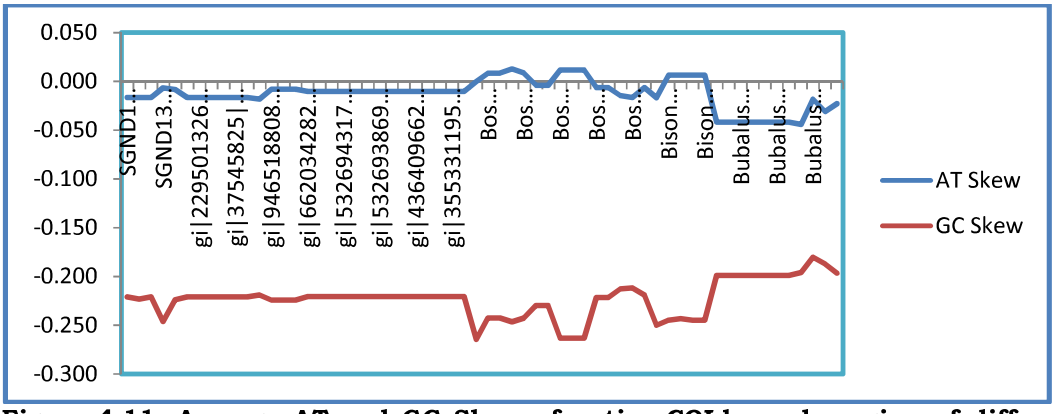

Figure 4.11: Average AT and GC Skew of entire *COI* barcode region of different bovine species under study.

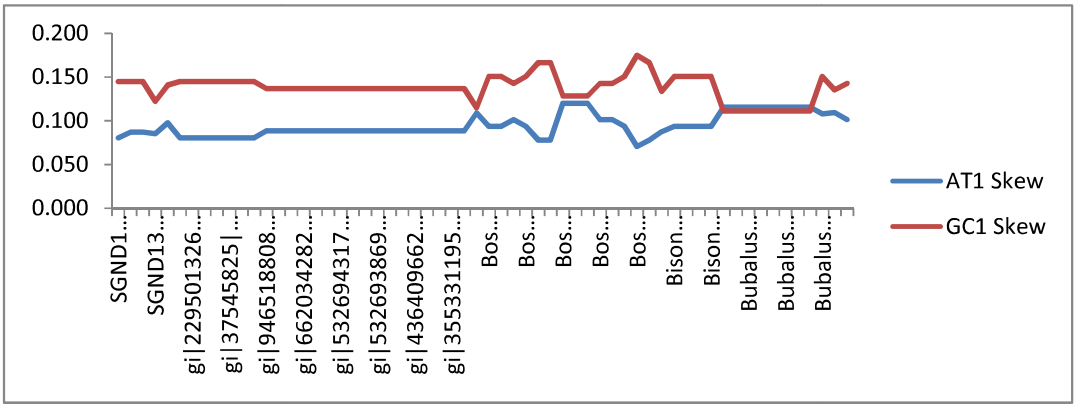

Figure 4.12: AT and GC Skew of *COI* barcode region of different bovine species at 1st codon position.

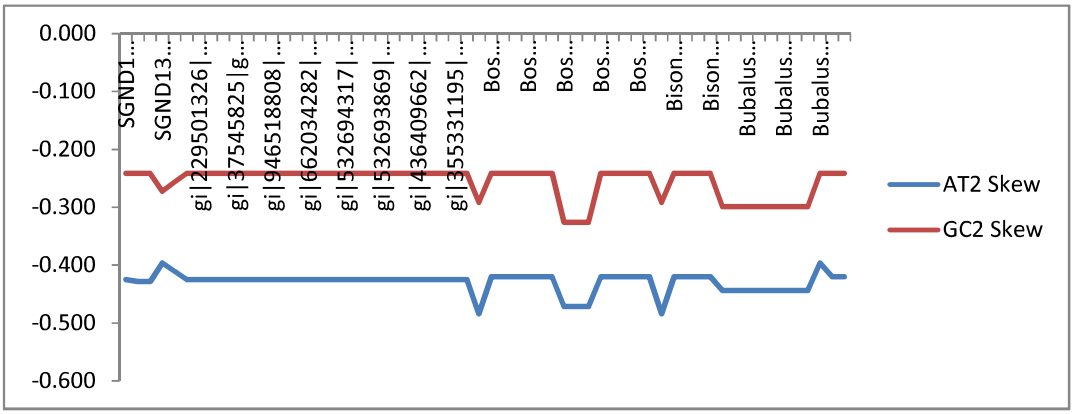

Figure 4.13: AT and GC Skew of *COI* barcode region of different bovine species at 2nd codon position.

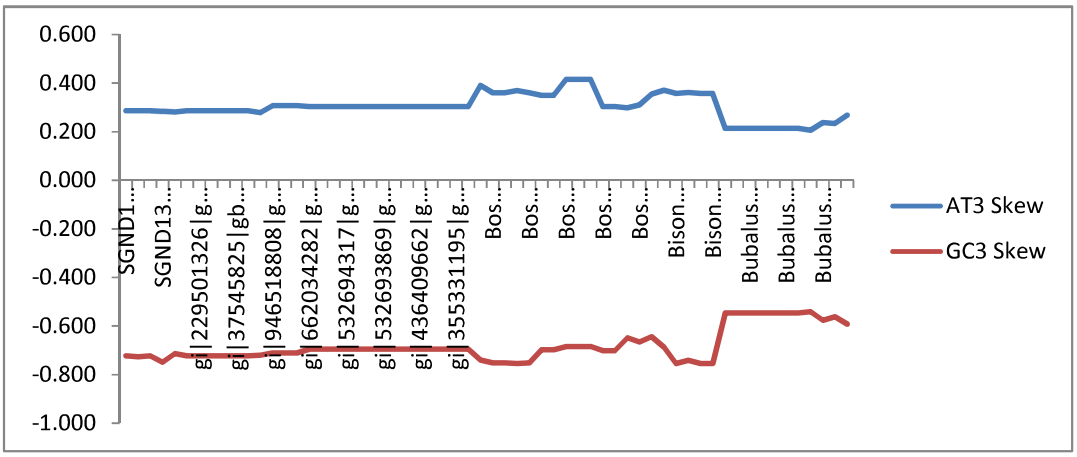

Figure 4.14: AT and GC Skew of *COI* barcode region of different bovine species at 3rd codon position.

4.2.7 Amino acid composition:

The amino acid composition of the *COI* (barcode) region was calculated using MEGA6 for all the bovine sample species collected along with the global dataset as barcode replicates. The study showed highest frequency of Leucine followed by Glycine and Alanine. On the other hand lowest frequency was observed for Cystein and Lysine among the studied samples as given in Figure 4.15.

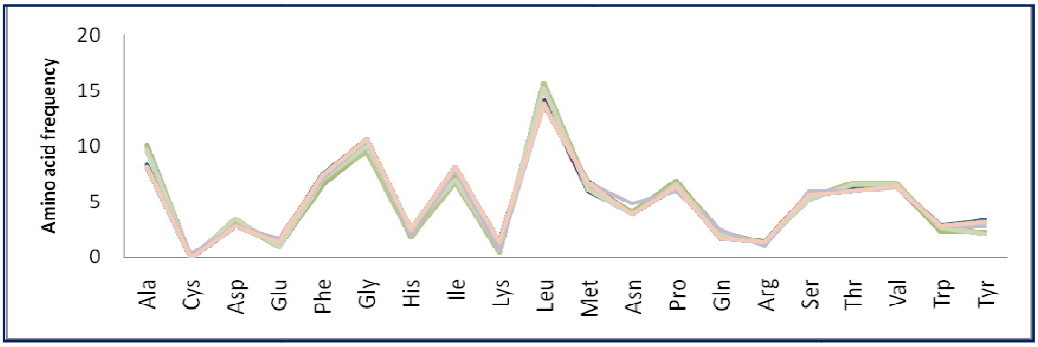

Figure-4.15: Amino acid composition of the different bovine species based on *COI* barcode sequences.

4.2.8 Codon usage:

There are 64 possible codons that code for 20 amino acids (and stop signals) so one amino acid may be encoded by several codons. It is therefore interesting to know the codon usage for each amino acid. The numbers of the 64 codons used in a gene is computed for all examined sequences. In addition to the codon frequencies, the relative synonymous codon usage (RSCU) statistic is also computed. Many amino acids are coded by more than one codon; thus multiple codons for a given amino acid are synonymous. However, many genes display a non-random usage of synonymous codons for specific amino acids. A measure of the extent of this non-randomness is given by the Relative Synonymous Codon Usage (RSCU). The RSCU for a particular codon (i) is given by RSCUi = Xi / ∑(XI / n), Where Xi is the counting numbers of i^{th} codon for a given amino acid; ∑XI is the sum of the occurrence numbers for all the synonymous codons in a certain amino acid; and n is the number of synonymous codons for a specific amino acid (Sharp *et al.* 1986).

Table 4.15: Codon Usage of *COI* barcode sequences of Bovidae family

Amino acid	Codon	Count	RSCU
Phenylalanine	UUU	6.6	0.66
	UUC	13.3	1.34
Leucine	UUA	10	1.58
	UUG	0.4	0.06
	CUU	3.4	0.54
	CUC	6.8	1.07
	CUA	15.1	2.38
	CUG	2.4	0.38
Isoleucine	AUU	11	1.07
	AUC	9.5	0.93
Methionine	AUA	14.1	1.57
	AUG	3.9	0.43
Valine	GUU	4.4	1.02
	GUC	1.3	0.3
	GUA	9.7	2.24
	GUG	1.9	0.43
Serine	UCU	2.8	1.1
	UCC	3.2	1.26
	UCA	6.6	2.59
	UCG	0.7	0.26
Proline	CCU	6.1	1.39
	CCC	8.6	1.97
	CCA	2.1	0.48
	CCG	0.7	0.16
Threonine	ACU	1.1	0.27
	ACC	8.5	2.04
	ACA	7	1.69
	ACG	0	0
Alanine	GCU	6	1.05
	GCC	5.8	1.02
	GCA	10.9	1.91
	GCG	0.1	0.02
Tyrosine	UAU	2.8	0.67
	UAC	5.5	1.33

Contd.

Table 4.15 Contd.

Amino acid	Codon	Count	RSCU
** (stop codon)	UAA	0	0
	UAG	0	0
Histidine	CAU	2.5	0.75
	CAC	4.1	1.25
Gutamine	CAA	4.8	1.9
	CAG	0.2	0.1
Asparagine	AAU	2.8	0.52
	AAC	7.9	1.48
Lysine	AAA	2.6	1.49
	AAG	0.9	0.51
Aspartic acid	GAU	3.2	0.82
	GAC	4.7	1.18
Glutamic acid	GAA	3.6	1.99
	GAG	0	0.01
Cysteine	UGU	0	0
	UGC	0	2
Tryptophan	UGA	6.2	1.64
	UGG	1.4	0.36
Arginine	CGU	0.1	0.06
	CGC	1.8	1.91
	CGA	1.2	1.21
	CGG	0.8	0.82
Serine	AGU	0	0
	AGC	2	0.79
** (stop codon)	AGA	0	0
	AGG	0	0
Glycine	GGU	5.3	0.74
	GGC	4.2	0.6
	GGA	16.5	2.33
	GGG	2.4	0.33

The codon usage is calculated (Table 4.15) using vertebrate mitochondrial codon table as template for the bovine specimen. The sequences under analysis did not contain any stop codon. Differences in the frequency of occurrence of synonymous codons in coding sequences were observed as summarized in Table 4.15.

4.3 Phylogenetic study of the Siri breed of cattle using mitochondrial D-loop sequences:

4.3.1 Genetic diversity of Siri cattle with respect to other indigenous cattle breeds:

The 34 generated sequences; from individual Siri cattle of different geographical location within the Eastern India was aligned and compared with 10 other described breeds from India and Bangladesh. Two out groups were taken (*Bos primigenius* which is now extinct and the other is *Bison bison).* The details of the other Indian breeds taken in the study are summarized in the Table 4.16.

It was found that the maximum variation resided in HVR-1 (Hyper variable region–I) of mtDNA D-loop. The analysis was carried out by taking the HVR-1 region of the Siri cattle along with the HVR-1 region of the other Indian breeds. The genetic diversity in terms of the distance was calculated using K2P model of distance estimation. Within the breed, the mean K2P distance was found to be 0.0166 ±0.008 and maximum K2P distance was 0.041. While, between the breeds, mean K2P distance was observed as 0.065 ± 0.002 with maximum K2P distance of 0.018 and the minimum distance between the breeds was 0.006.

In NJ clustering, generated sequences and other indigenous breed sequences mined from database showed typical clustering pattern. Many of the Indian breed sequences clustered cohesively with Siri, while some of the database sequences and many other Siri breed of cattle showed distinct clustering from rest of the database sequence. This cohesive clustering among the different breeds might be due to the cross breeding between different breeds or presence of mislabeled sequences. Nevertheless, the NJ clustering clearly showed that Siri breed of the cattle are completely distinct with respect to other

Indian breed although some of the generated sequences showed close clustering with the database sequences, as shown in Figure 4.16.

Table 4.16: Lists of Indian breeds of cattle with one cattle breed, Red Chittagong along with the accession number (mitochondrial D-loop) taken in the study along with the two outgroups *Bos primigenius* and *Bison bison.*

Sl No	Accession	Breeds	No. of sequences of each breed
1	L27732.1, L27733.1	Sahiwal	2
2	L27722.1, L27723.1	Hariana	2
3	AY378133.1, Y378134.1, AY378135.1, AY378136.1	Ongole	4
4	DQ985396.1, DQ985430.1, DQ985408.1, DQ985434.1, DQ985407.1, DQ985400.1	Red Chittagong	6
5	KP223258.1, KP223259.1, KP223260.1	Bachr	3
6	KP223261.1, KP223262.1, KP223263.1	Gangatiri	3
7	KP223266.1, KP223268.1	Kenkatha	2
8	KP223269.1, KP223270.1, KP223271.1	Kheriharh	3
9	HQ234741.1, HQ234740.1, HQ234733.1	Tharpakar	3
10	HQ234723.1, HQ234724.1, HQ234736.1	Red Sindhi	3
11	JN632601	*Bison bison*	1
12	JQ269333.1, JQ269331.1, JQ269332.1	*Bos primigenius*	3

4.3.2 Genetic diversity of Siri cattle with respected to other global cattle breeds:

The generated D-loop sequences of the Siri cattle (4.11A) were compared with the database sequences with similarity match approach for diversity assessment. Altogether 71 database sequences with which the generated sequences showed similarity as Nearest Neighbours (NN) were retrieved from GenBank in FASTA format. The Accession numbers and country of origin are summarized in Table 4.17.

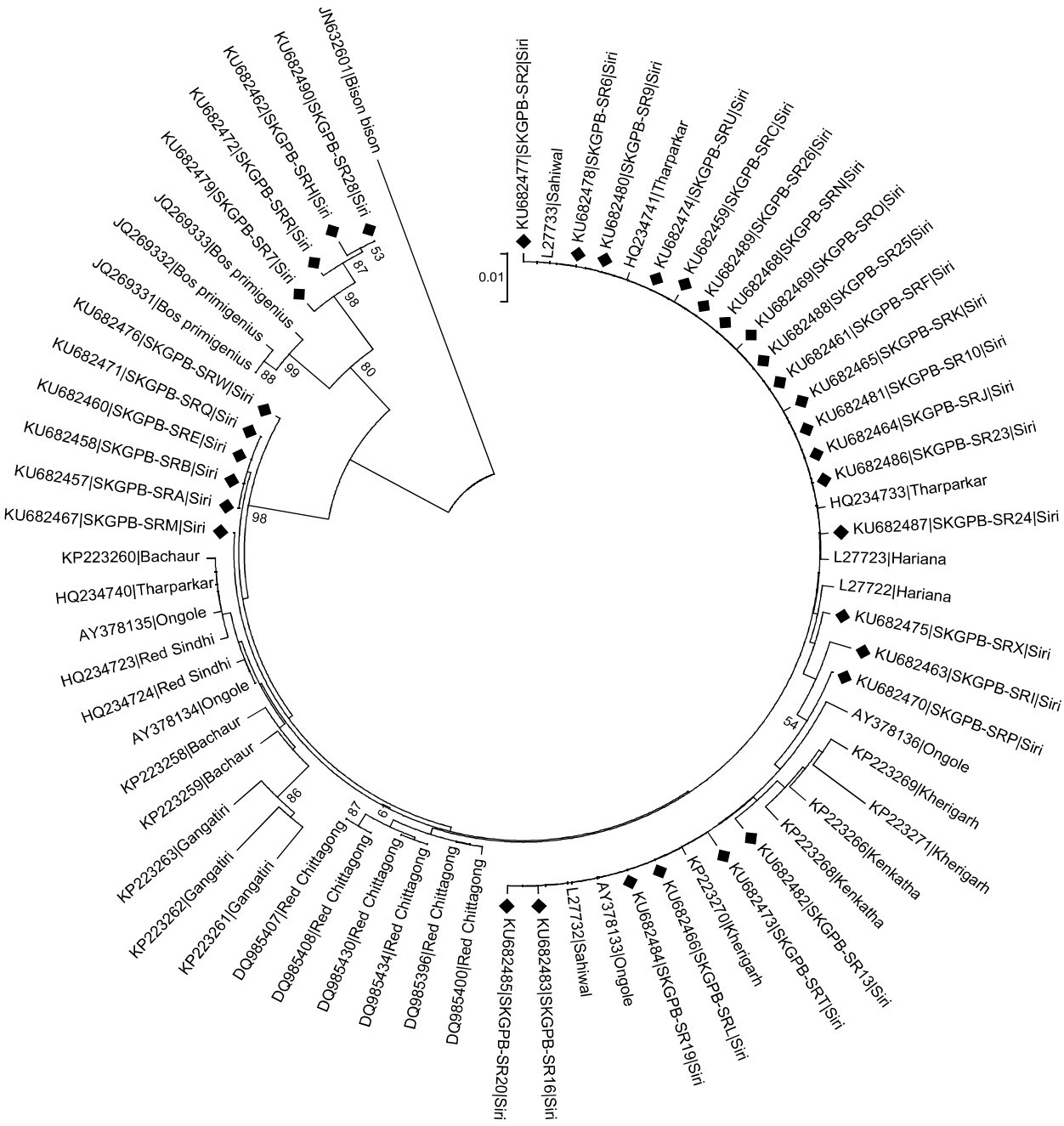

Figure 4.16 Neighbour-Joining unrooted tree with bootstrap support (1000 replicates) showing molecular clustering of indigenous Siri cattle diversity based on D-loop sequences. The black dots represent the generated sequences while rests are mined from NCBI.

Table 4.17: Sequences retrieved from GenBank (NCBI) as replicates*.

Sl No	Accn No	Country	Breed
1	HQ234736	India	Tharpakar
2	AY029265.1	New Zealand	Enderby Island Shorthorn
3	HQ234735	India	Tharpakar
4	EU281535	China	Zhaotong
5	EU281418	China	Enshi
6	EU281410	China	Enshi
7	AB268580	Bhutan	Bhutanese Native
8	AY029263	New Zealand	Enderby Island Shorthorn
9	AF389181	New Zealand	New zealand Jersey
10	EF524135	China	-
11	AY515654	China	Sinan
12	AY378134	India	Ongole
13	AY521128	China	Yunnan
14	HQ234724	India	Red Sindhi
15	EU281543	China	Zhaotong
16	EU281486	China	Weizhou
17	EU281464	China	Nandan
18	EU281431	China	Guangfeng
19	EU281412	China	Enshi
20	EU281372	China	Aletai
21	EU281370	China	Aletai
22	AB268579	Bhutan	Bhutanese Native
23	AB268567	Bhutan	Bhutanese Native
24	AB268566	Bhutan	Bhutanese Native
25	AY521085	China	Bashan

Contd.

Table 4.17 Contd.

26	EU281531	China	Zhaotong
27	EU281497	China	Wenling
28	EU281482	China	Weizhou
29	EU281461	China	Nandan
30	EU281442	China	Longlin
31	EU281437	China	Longlin
32	EU281416	China	Enshi
33	EU281406	China	Enshi
34	EU281389	China	Dabieshan
35	EU281388	China	Dabieshan
36	AF361451	Austia	Holstein-Friesian
37	L27733	USA	Sahiwal
38	AY515615	China	Liping
39	AY378137	India	Tharpakar
40	AY378119	China	Tibetan Yellow
41	HQ234741	India	Tharpakar
42	HQ234740	India	Tharpakar
43	HQ234733	India	Tharpakar
44	HQ234723	India	Red Sindhi
45	EU281484	China	Weizhou
46	EU281481	China	Weizhou
47	EU281480	China	Weizhou
48	EU281474	China	Rikaze
49	EU281404	China	Diqing

Contd.

Table 4.17 Contd.

50	EU281357	China	Apeijiaza
51	AB268565	Bhutan	Bhutanese Native
52	AB268564	Bhutan	Bhutanese Native
53	AB268563	Bhutan	Bhutanese Native
54	DQ166074	China	-**
55	AY029267	New Zealand	Enderby Island Shorthorn
56	AF389180	New Zealand	New zealand Jersey
57	EU281504	China	Wenling
58	EU281499	China	Wenling
59	EU281498	China	Wenling
60	EU281485	China	Weizhou
61	EU281445	China	Longlin
62	EU281462	China	Nandan
63	EU281459	China	Nandan
64	EU281429	China	Guangfeng
65	EU281427	China	Guangfeng
66	EU281458	China	Nandan
67	EU281438	China	Longlin
68	EU281421	China	Guangfeng
69	EU281415	China	Enshi
70	EU281390	China	Dabieshan
71	EF524125	China	-

* The database sequences with which the generated sequences showed similarity as Nearest Neighbours (NN) was retrieved.

** '-' = breed not known.

It was found that there were considerable nucleotide variations within as well as among the total numbers of sequences under the study (92 variable sites) out of which maximum variation resided in HVR-1. Further analysis was carried out considering the HVR-1 region. The genetic diversity in terms of the distance was calculated using K2P model of distance estimation. Within the breed, the mean K2P distance was found to be 0.005 ±0.002 and maximum K2P distance was 0.029. Between the breeds, mean K2P distance was observed as 0.007 ±0.002 with minimum K2P distance of 0.002.

In NJ clustering, global breed sequences deposited from worldwide and generated sequences of Siri showed similar type of clustering pattern as was observed in indigenous breeds. Many of the global sequences clustered cohesively while some of the database sequences and most Siri breed of cattle showed distinct clustering from rest of the database sequences. From the NJ clustering, it is clearly evident that Siri breed of the cattle are completely distinct from other global cattle breeds (Figure 4.17).

4.3.3 Haplotype diversity:

In addition to 34 generated sequences, another 106 sequences (Table 4.17 and Table 4.16) of D-loop from different geographic regions were mined from GenBank (http://www.ncbi.nlm.nih.gov/ genbank/). The final data set (140 D-loop sequences) were aligned using MUSCLE and subsequent phylogenetic analysis on HVR-I of D-loop containing first 293 nucleotide positions resulted 71 (71/293) variable sites and 30 parsimony informative sites that strongly indicated considerable variations in nucleotides (Figure 4.18) with high resolving power to unravel the genetic diversity of indigenous cattle in respect to global perspective. The sequence alignment showed considerable variations within HVR-I regions of D-loop regions among our 34 sequences when compared against global dataset.

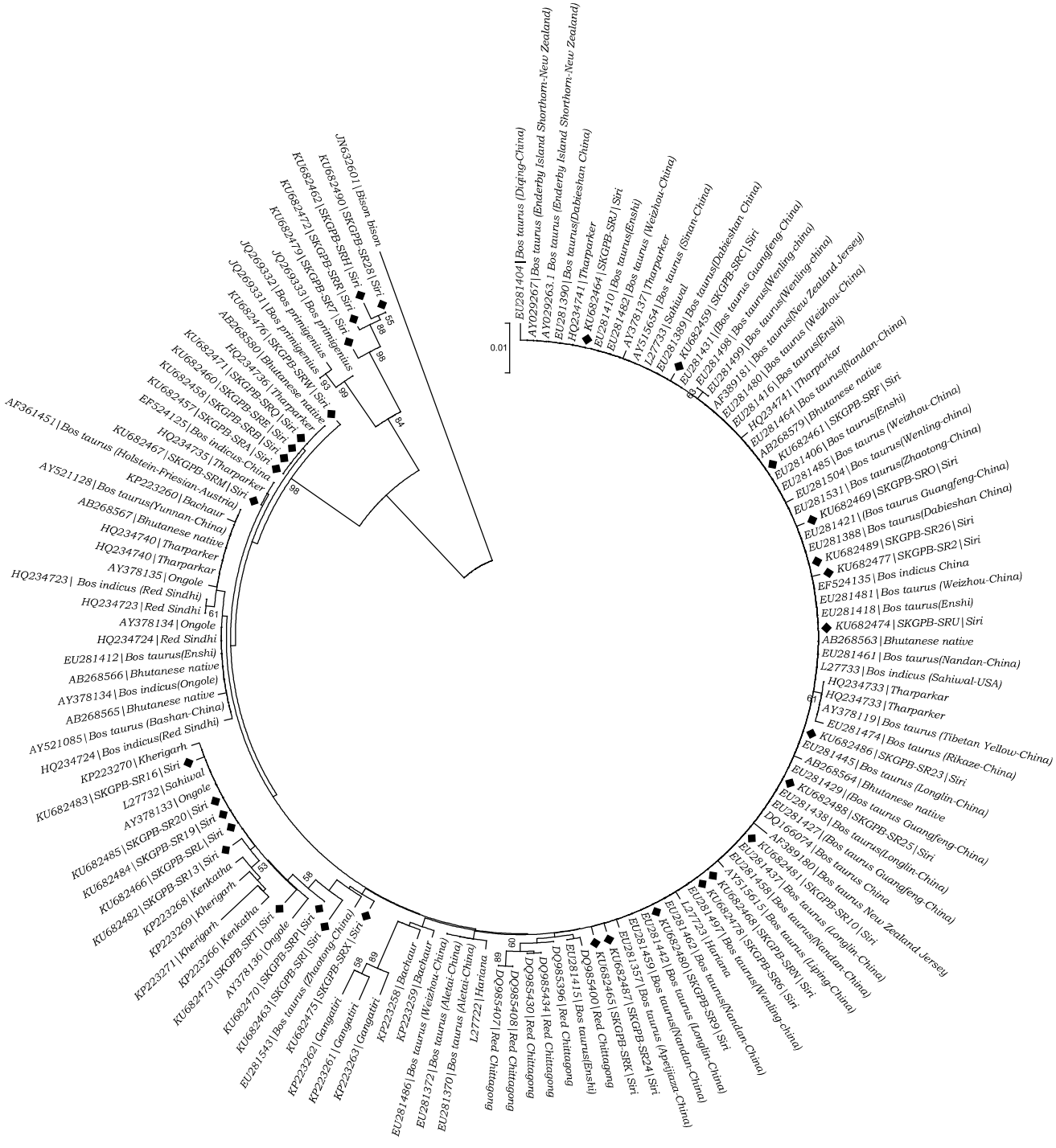

Figure 4.17: Neighbour-Joining unrooted tree with bootstrap support as well as interior branch test with CI (confidence interval) at 95% confidence (1000 replicates) showing molecular clustering of Siri cattle diversity based on D-loop sequences from Eastern India and different country of world. The black dots represent the generated sequences while rests are mined from NCBI.

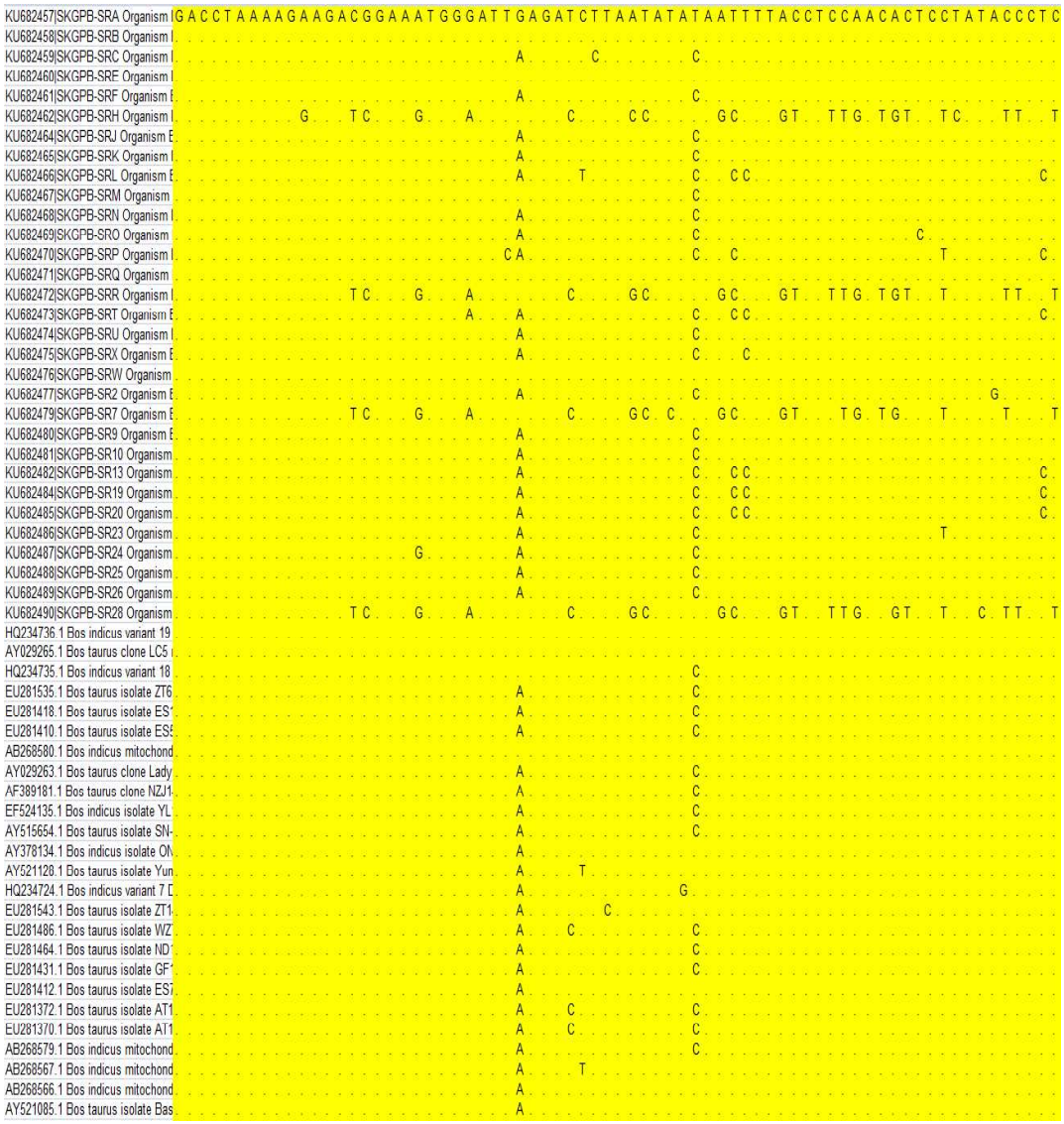

Figure 4.18: Representative image of variable sites within HVR-1 of D-loop fragment.

Generation of haplotypes using combined dataset (global + generated sequences) by DnaSP 4.10.9 for analysis of cattle biodiversity resulted in 17 haplotypes (Table 4.18). Specifically, indigenous cattle breed of Siri Hap_1-17 were found to be at the hilly region of Sikkim and Darjeeling of Eastern part of India. Of this, Hap_ 1, 3, 6 and 16 were also found in cattle breeds of Bhutan and China. Among the different breeds, many sequences from China and India shared haplotypes with our Siri breeds. The result indicated 0.784 haplotype diversity among the HVR-I of D-loop.

Table-4.18: Haplotype variations of the indigenous cattle breed of Siri along with other breed sequences from Genbank.

Sl No	Haplotypes	No. of sequences of each haplotype	Generated Sequences	Database Sequences
1	Hap_1	8	KU682457, KU682458, KU682460, KU682471, KU682476,	HQ234736,AY029265, AB268580
2	Hap_2	1	KU682459	
3	Hap_3	47	KU682461, KU682464, KU682465, KU682468, KU682474, KU682480, KU682489, KU682481, KU682488,	EU281535, EU281418, EU281410, AY029263, AF389181, EF524135, AY515654, EU281464, EU281431, AB268579, EU281531, EU281497, EU281482, EU281462, EU281461, EU281459, EU281442, EU281437, EU281429, EU281427, EU281416, EU281406, EU281389, EU281388, L27733, HQ234741, EU281484, EU281481, EU281480, EU281357, AB268564, AB268563, DQ166074, AY029267, EU281499, EU281498, EU281438.1, EU281421.1
4	Hap_4	4	KU682462, KU682463, KU682478, KU682483,	
5	Hap_5	1	KU682466	
6	Hap_6	3	KU682467	HQ234735, EF524125
7	Hap_7	1	KU682469	
8	Hap_8	1	KU682470	
9	Hap_9	1	KU682472	
10	Hap_10	1	KU682473	
11	Hap_11	1	KU682475	
12	Hap_12	1	KU682477	
13	Hap_13	1	KU682479	
14	Hap_14	3	KU682482, KU682484, KU682485	
15	Hap_15	1	KU682486	
16	Hap_16	2	KU682487	EU281458
17	Hap_17	1	KU682490	

4.3.4 Phylogenetic analysis:

As already established in the various cases, D-loop gives better resolution of breed delineation. It is also demonstrated with the cattle breeds in previous sections. Therefore, mtDNA D-loop sequences for breed identification and phylogenetic analysis was employed.

4.3.4.1 Maximum Likelihood approach:

4.3.4.2 Model test for Maximum Likelihood analysis:

The optimal model that best explain the evolution of the sequences or in other words the model that fits for the sequences to be used for phylogenetic analysis for ML analysis were filtered through goodness-of-fit test of each model measured through Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC) with and without assuming the existence of evolutionary rates among sites modeled by discrete gamma distribution (+G) and allowance of the presence of invariant sites (+I). This result is an evaluation of 24 models for nucleotide substitutions. For each of these models, MEGA6 provided the estimated values of shape parameter of the Gamma distribution, the proportion of invariant sites, and the substitution rates between bases or residues, as applicable and presented in Appendix- 4. Depending on the model, the assumed or observed values of the base frequencies used in the analysis were also provided. Both BIC and AIC selected substitutions models that was more complex than the true model. By rules, the true model was among the top three when BIC was used and among the top five when AIC was used. However, the models with the lowest BIC scores are considered to describe the substitution pattern the best and as barcodes sequences showed extreme variable sites. In this study for ML based phylogenetic interpretation, the model Hasegawa-Kishino-Yano (HKY) along with discrete gamma rate categories (+G) was favored.

4.3.4.3 Phylogenetic analyses based on Maximum Likelihood approach with other breeds of India and Bangladesh:

The ML analysis in MEGA6 started with an initial tree that was automatically generated as default tree by MEGA6 by the Neighbor-Joining and BioNJ algorithm using a matrix of pair wise distances estimated under Tamura and Nei model for nucleotide sequences. Based on the automatically generated initial tree a final ML tree with the highest log likelihood value of -779.9209 with well supported bootstrap support proportion was produced and shown in Fig-4.19 (here sequences of generated D-loop and enlisted sequence of indigenous cattle breed of Table 4.16 were considered). The entire ML tree was constructed based on 69 sequences belonging to different breeds. The highlighted green colour box is the sequences of *Bos primigenius* which is considered to be the ancestor of the *B. indicus* and *B. taurus* cattle. From the tree it is cleared that the Siri is distinct breed of cattle from rest of the cattle breeds of India, as it distinctly clustered with respect to other cattle breeds. However, there are many other Siri cattle sequences which clustered near to other breeds. Such clustering may occur due to the presence of cross-breeds sequences.

4.3.4.4 Phylogenetic analyses based on Maximum Likelihood approach with other breeds (Global) of cattle:

ML tree was also constructed by aligning 34 generated sequences with the database sequences of different global breeds of cattle (Table-4.17).

Similar to the ML tree of Indian cattle, here also we could find the consensus result. From the tree it is clear that the Siri is distinct breed of cattle from rest of the cattle breeds of the world comprising *Bos indicus* and *Bos taurus*, as it distinctly clustered with respect to other cattle breeds (Fig-4.20). Moreover, it appears that Siri might be one of the oldest breed in comparison to other cattle breeds as many of its sequences clustered nearest to the extinct ancestor *(B. primigenius)* of the modern cattle.

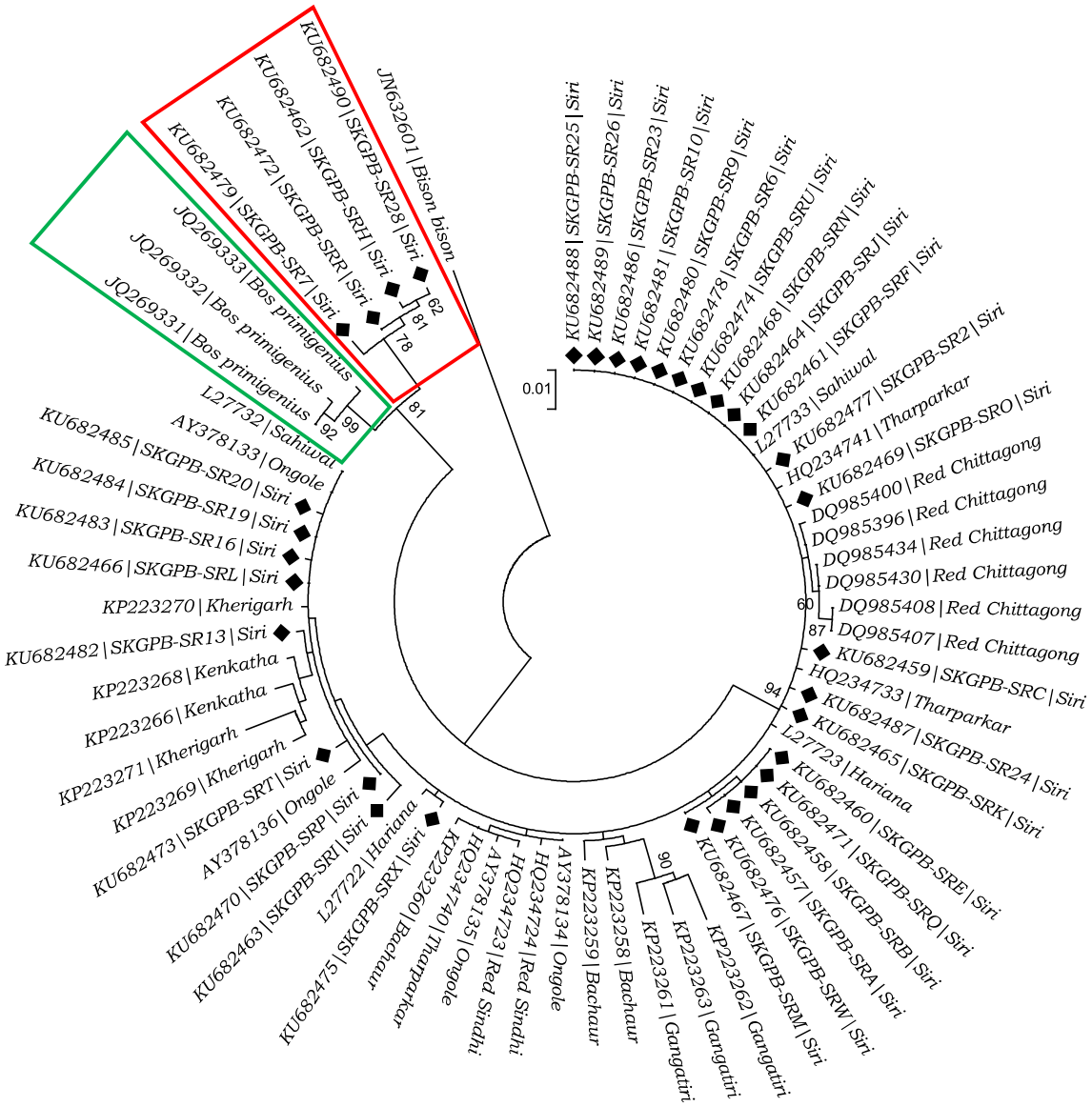

Fig 4.19: ML Tree showing clustering of Siri breed based on D-loop sequence from different parts of India and Bangladesh. The black dots represent the generated sequences while rests are mined from NCBI.

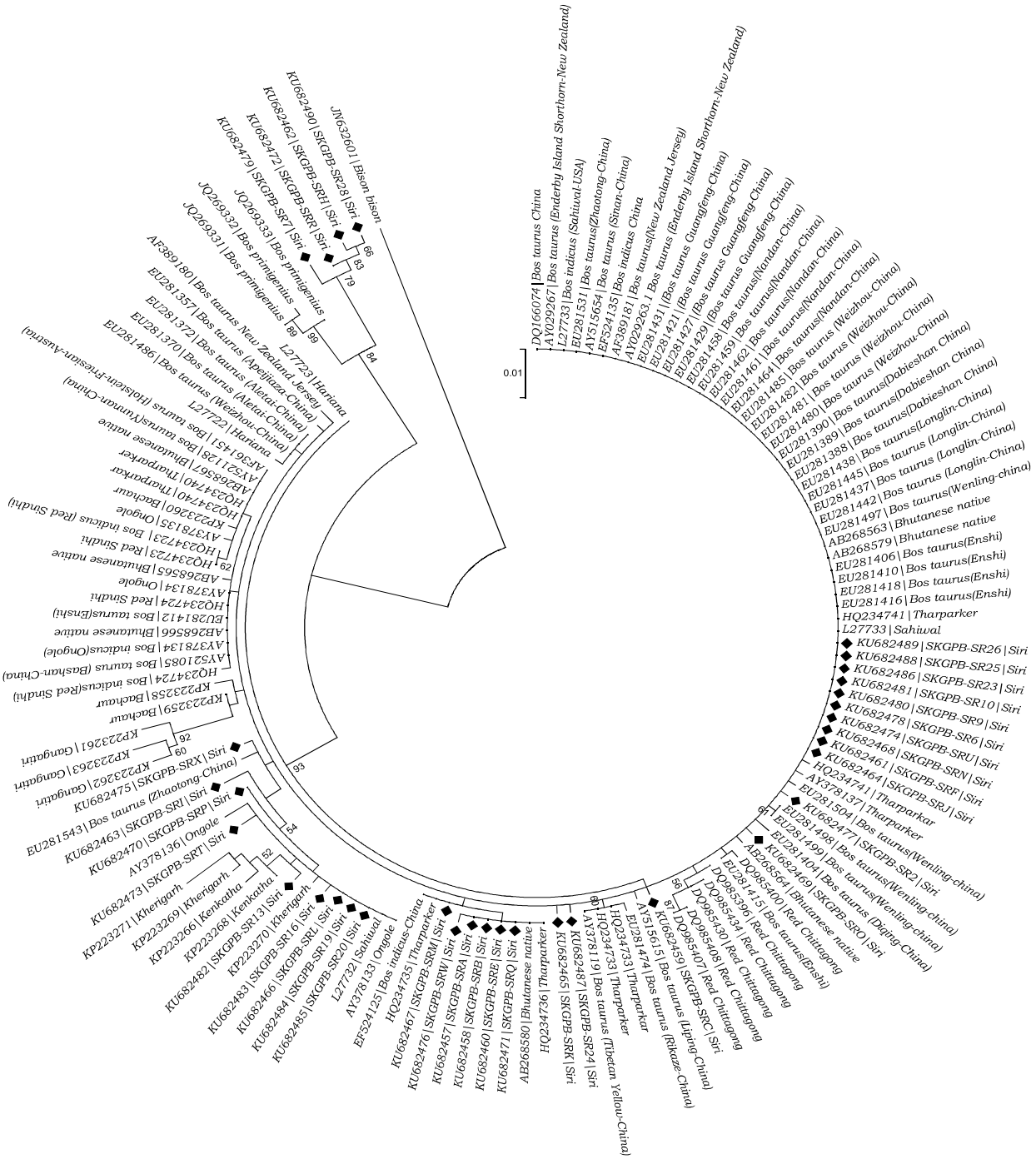

Fig- 4.20: ML Tree showing clustering of Siri breed based on D-loop sequence from different countries of the world. The black dots represent the generated sequences while rests are mined from NCBI.

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4.3.4.5 Molecular clock in estimating time of evolution of Siri breed: ML approach:

For estimation of divergence time, the molecular clock uses the mutation rate of DNA/amino acid sequence of organism to deduce the time of divergence or evolution of two organisms from a common ancestor. This is achieved by performing a Maximum Likelihood test of the molecular clock hypothesis for a given tree topology and sequence alignment. The molecular clock must first be calibrated against independent evidence about dates, such as the fossil records.

4.3.4.6 Testing the molecular clock hypothesis:

The molecular clock test was performed by taking into consideration the COI gene of mtDNA by comparing the ML value for the given topology with and without the molecular clock constraints under HKY model (+G) from model test (Appendix-4). Differences in evolutionary rates among sites were modeled using a discrete Gamma (G) distribution (shape parameter shown) and allowed for invariant (I) sites to exist (estimate of percent invariant sites shown). The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level ($P<0.05$). The Likelihood ratio of the test is summarized in Table 4.19. All positions containing gaps and missing data were eliminated. There were a total of 230 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Table 4.19: Results from a test of molecular clocks using the Maximum Likelihood method.

	***ln*L**	**Parameters**	**(+G))**	**(+I)**
With Clock	-148860.792	107	0.133	n/a
Without Clock	-779.552	208	0.20	n/a

***ln*L** = Log likelihood ratio.

4.3.4.7 Estimation of divergence time:

The time of divergence of different cattle was estimated by generating a ML tree using representative cattle D-loop of mtDNA sequences under study using HKY (+G) substitution model following Baysian Scores. A strict calibration method was applied at the MRCA (Most Recent Common Ancestor) of the *B. primigenius*

and another alternate calibration at *Bos-Bison* split. The average substitution rate across the tree was estimated at $5.89X10^{-7}$ substitutions per site per year (95% Highest Posterior Density: $4.66X10^{-7}$ – $7.10X10^{-7}$ substitutions per site per year), equivalent to 58.9% per Myr (Million Year) (Ho et al. 2008).

The time tree was generated by putting the calibrated time on ML tree of different Indian cattle breed (Fig-4.19). From our analysis it is clear that Siri originated around 6510 YBP (year before present) earlier than other breed of Indian origin and can be considered to be the most primitive than the other breeds of *B. indicus* (Fig- 4.21A). When calibrated time was put on the ML tree of different *B. Taurus* and *B. indicus* from global database along with generated D-loop sequences of Siri, it was evident that the Siri breed (*B. indicus*) originated in an around 10,884 YPB (Fig-4.21B). Hence, it can be considered that the Siri breed of cattle is the most primitive breed cattle, which has originated in Himalayan foothills of Eastern India during the course of domestication process.

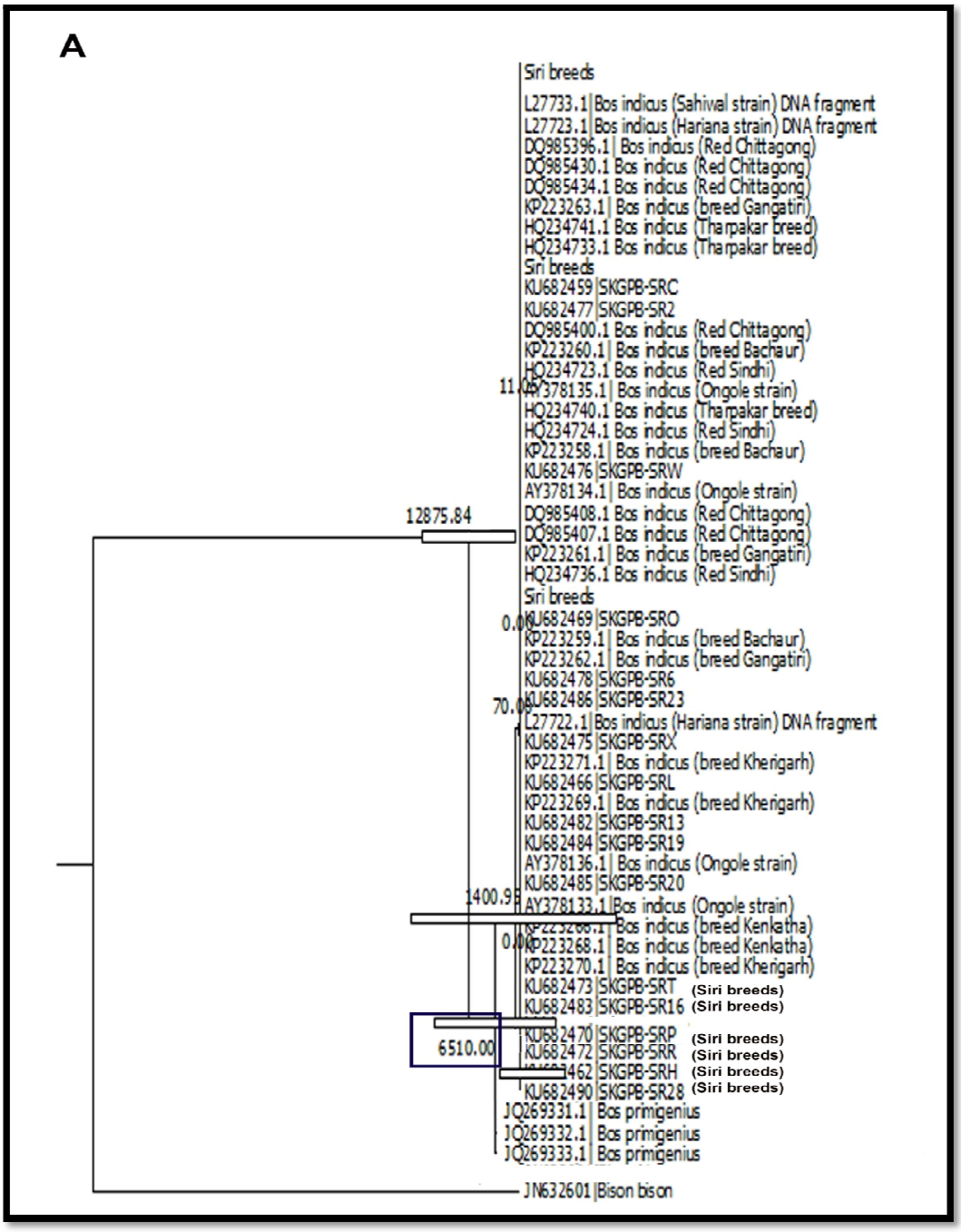

Figure 4.21A: Time tree of the *Bos indicus* breeds of cattle.

(Time to common ancestors for Siri cattle as compared with the other established cattle breeds in India).

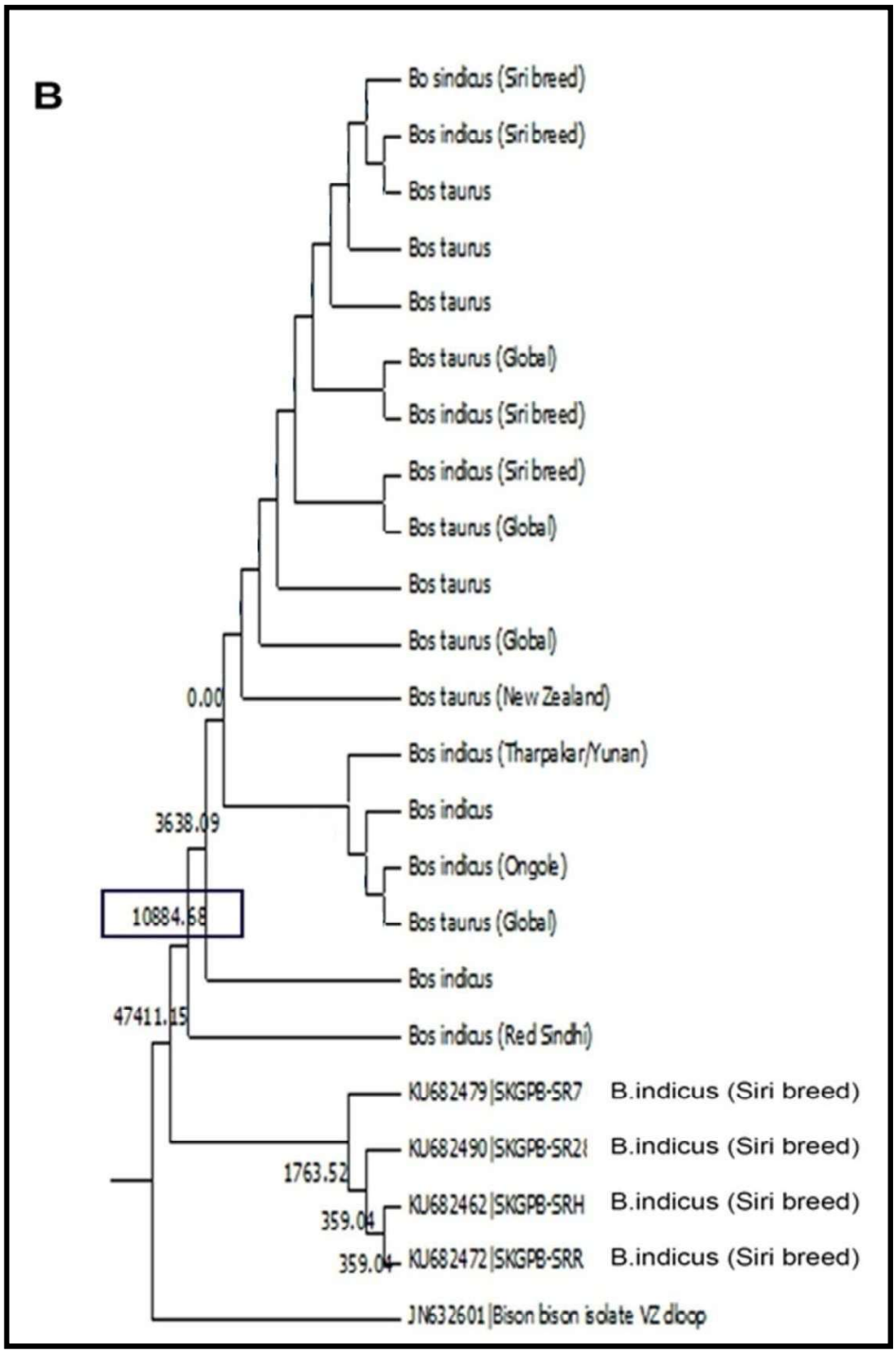

Figure 4.21B: Time tree of the *Bos indicus* and *Bos taurus* breeds of cattle. Time to common ancestors for Siri cattle as compared with the other cattle breeds (Global seque