Molecular Phylogeny and Characterization of Mundri Sheep (*Ovis aries*) of Pakistan through Sequencing of Mitochondrial Cytochrome b and Cytochrome Oxidase Subunit I

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Abstract

The main focus of this research is to determine the molecular phylogeny and characterization of Mundri Sheep (*Ovis aries*) through sequencing of mitochondrial Cytochrome b and Cytochrome Oxidase Subunit I (COI). This sheep breed appears morphologically different from other local sheep breeds of Pakistan. The current research is carried out to appraise the status of Mundri sheep whether it is a different breed from other breeds or not. Blood samples of Mundri sheep were collected from Livestock Experiment Station, (LES) Fazilpur in district Rajanpur (Punjab). DNA was isolated and subjected to Polymerase Chain Reaction (PCR) for amplification of Cytochrome b and COI genes using appropriate primers. PCR products were sequenced and analyzed by MEGA X software. The phylogeny analysis categorized *Ovis aries* including Mundri sheep into three and two groups for Cytochrome b and COI genes respectively. It showed Mundri sheep as a separate group and thus as a separate breed from all other local sheep breeds. Hence the study validates based on Cytochrome b and COI that Mundri sheep is a distinctive breed from the rest of the local sheep breed.

Key words: *Mundri Sheep; Ovis aries; Molecular Phylogeny; Characterization; Cytochrome b; Cytochrome Oxidase Subunit I; COI; Pakistan*

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Introduction

Although there exists controversy and uncertainty regarding the origin of sheep (*Ovis aries*) evidence shows that, sheep were probably first domesticated in the Fertile Crescent region of the Near East about 11,000 years ago. Sheep (*Ovis aries*), is a well-known quadrupedal livestock animal. The ancestor of household sheep is the Asian mouflon (*O. orientalis*). Urial (*Ovis vignei*) is one of the closest related species of all modern sheep, found primarily in the mountainous area of Central Asia. Mouflon, Urial, and Argali are wild sheep and thought to be ancestors of domestic sheep (Aljumaah et al., 2014; Atavliyeva and Tarlykov, 2018). Human beings domesticated sheep around 10,000 BC (Taberlet et al., 2011). The classification of sheep is Domain: *Eukaryota* (Whittaker and Margulis, 1978) Kingdom: *Animalia* (Cuvier, 1812) Phylum: *Chordata* (Bateson, 1885) Subphylum: *Vertebrata* (Cuvier, 1812) Class: *Mammalia* (Cuvier, 1812) Subclass: *Theria* (Bateson, 1885) Order: *Artiodactyla* (Owen, 1848) Suborder: *Ruminantia* (Owen, 1848) Family: *Bovidae* (Owen, 1848) Subfamily: *Caprinae* (Owen, 1848) Genus: *Ovis* (Owen, 1848) Species: *Aries* (Owen, 1848) (Özgen, 2008).

Countries that have large areas of grassland are the major producers of sheep. Most of the population of sheep lives in areas where the source of feeding includes the residues of crops, natural shrubs, and different types of grasses. New Zealand, Australia, India, China, South Africa, the United States, Argentina, and Turkey are the major national producers of sheep. Pakistan ranks 10th in the world in terms of sheep population (Khan et al., 2008). Pakistan is at 3rd position in Asia as far as the population of small ruminants is concerned, with a yearly growth rate of 4%, the most elevated in Asia (Khan and Ashfaq, 2010). The current population of farm animals in Pakistan consists of 31.2 million sheep, 78.2 million goats, 41.2 million buffaloes, 1.1 million camels, and 49.6 million cattle (Khan et al., 2007). Sheep cultivation or sheep farming is the raising and rearing of household sheep. Commonly people from everywhere throughout the world, keep sheep for an agricultural reason so as to acquire their meat, milk, and wool. They also yield sheepskin and parchment.

There are approximately 200 sheep breeds in Asia. Local breeds of sheep in Pakistan are 30 (Afzal and Naqvi, 2004). The classification has been made on the basis of tail morphology. These breeds are classified into thin tail and fat tail sheep. In the irrigated areas the thin tail sheep breeds are generally found whereas fat tail sheep breeds are found in arid lands and mountainous areas of Sindh, KPK, and Azad Kashmir. Thin tail sheep consist of Buchi, Baltistani, Damani, Cholistani, Kaghani, Kail, Kajli, Kooka, Hissardale, Kali, Lohi, Kachhi, Pahari, Poonchi, Sipli, and Thalli. Fat tail sheep consist of Balochi, Bibrik, Balkhi, Gojal, Dumbi, Hashtnagri, Harnai, Khijloo, Kohai-Ghizar, Michni, Latti/Salt Range, Rakhshani, Tirahi, and Waziri (Khan et al., 2007). Sheep breeds Buchi, Lohi, Thalli, Latti/Salt Range, Cholistani, Sipli, Khijloo, Hissardale, and Kajli are found in Punjab; Dumbi, Kachhi, and Kooka are found in Sindh; Balkhi, Damani, Kaghani, Hashtnagri, Michni, Tirahi, and Waziri are found in KPK; Balochi, Bibrik, Harnai, and Rakhshani are found in Balochistan; Baltistani, Gojal, Kail, Kali, Kohai-Ghizar, Pahari, and Poonchi are found in the Northern area, and Azad Jammu and Kashmir AJK (Khan and Ashfaq, 2010). There may be still other sheep breeds available in the country which awaits documentation. In the Rajanpur district of Punjab province, a breed of

sheep exists which is not still recognized as a separate breed, called the Mundri sheep by the local people. The sheep is found in other parts of the country too but exists in large numbers in this region. Mundri sheep appear morphologically different from other local breeds of sheep. This sheep has different physical features from other local Pakistani breeds therefore, there is also a need to study genetically that this sheep is different from other breeds or part of some already existing breed.

Mundri sheep are fairly large, a thin tail breed found in district Rajanpur province of Punjab with white color extremely short ears and sharp roman nose. Other characteristic of Mundi sheep is listed in the Table 1. Mundri sheep breed habitats the region between the bed of Koh-e-Suleman range to the delta region of river Indus in District Rajanpur and also few

Table 1. Other major characteristics of Mundri Sheep

Sr. No	Characteristic	Male	Female
1	Color	White	White
2	Head Color	White	White
3	Body Color	White	White
4	Ear type	Short ears	Short ears
5	Ear size	8 cm	8.5 cm
6	Nose type	Sharp roman nose	Sharp roman nose
7	Legs	55 to 65 cm	62 to 70 cm
8	Tail type	Short thin tailed	Short thin tailed
9	Tail	23 cm	23cm
10	Height	92 to 96 cm	84 to 90 cm
11	Length from tip of nose to tip of tail	165 to 170 cm	155 to 160 cm
12	Chest girth	110 to 112 cm	107 to 110 cm
13	Belly girth	125 to 130 cm	120 to 135 cm
14	Wool yield per annum	2.5 kg	2 kg
15	Wool fiber size	23 to 26 cm	22 to 27
16	Hip height	92 to 96 cm	84 to 90 cm
17	Head length	26 cm	23 cm
18	Head width	17 cm	15.5 cm
19	Eye to eye length	16 cm	14.5 cm
20	Testicular length	23 cm	-
21	Scrotal circumference	30 cm	-
22	Birth weight	5 kg	4.5 kg
23	Adult weight	85 to 95 kg	75 to 85 kg
24	Weaning weight	23.5 kg	18.5 kg
25	Twining percentage	-	45 to 55%

Source: Livestock Experiment Station, (LES) Fazilpur, Rajanpur

flocks were reported in Koh-e-Suleman and across the river bed in Muzaffargarh especially in Alipur and Zahir Pir.

Genetic characterization helps in the detection of variations as a result of differences either in modifying factors, DNA sequences, or in specific genes. For phylogenetic examination, mitochondrial DNA is used (Rubinoff and Holland, 2005). Phylogenetic can be defined as the process in which the relationships that are related to evolution can be estimated and investigated effectively. In order to find out the evolutionary relationships between different organisms, the nucleic acid sequences are explored and then compared with the sequences on nucleic acid of the other organism. The results of the study may be interpreted in such a way that those organisms that will be having fewer differences in these components will be considered to be having a close relationship with the other organism while the organisms in which there will be many differences in the sequence of the components of nucleic acids will be having a less close relationship between them (Chenna et al., 2003).

In the past, various old methods were used for the similar purpose of finding out the evolutionary relationships between the organisms, and these included the approaches based on the morphology or the traits reading life cycle. However, in this era, with the advancement of technology, more advanced ways are used in order to find out the evolutionary relationships between the organisms. One of the most important advantages of the recent methods or approaches is that the results or the differences among the sequences of different organisms can be quantified effectively. This quantification is based on the number of differences that are observed in the sequences of the components of nucleic acids of different organisms. The results that are obtained by phylogeny are generally represented in the form of a 'tree'. This tree is having various branches that are actually the indications of the relationships of that organism with other organisms (Saitou and Imanishi, 1989).

According to the past studies, in order to determine the phylogeny of the organisms and to find out the level at which those organisms are related to each other in one way or the other, the most commonly used and effective tool is the mitochondrial DNA (mtDNA) (Brown et al., 1979). The reason behind the use of mtDNA in order to find out the phylogeny of different organisms is that there are various molecular markers at different levels of the evolutionary history of those organisms. These molecular markers are actually based on the distinct and special properties and characteristics that have been inherited from the ancestors of those organisms (Galtier et al., 2009).

There is no certain information regarding the origin of the domestic sheep also called *Ovis aries*. In this regard, studies have shown that some wild species of sheep are the actual source or origin of the domestic sheep and the various breeds of domestic sheep have been the results of breeding of those wild species (Mason and Mason, 1984). Some of the examples of wild sheep breeds that have been discussed in the literature include Urial, Mouflon, and Argali (Zeuner, 1963). All these species or breeds are supposed to have a contribution in the domestic breeds of sheep.

Cytochrome c oxidase I (COI), a mitochondrial gene, could fill in as the center of a worldwide bio identification framework for animals. Sometimes, the success rate is 100% in the accurate identification of cases. Hence, the COI is a dependable accessible solution and cost-effective identification system for the existing issue of identification of species (Jiang et al., 2015).

Abbas et al. (2017) investigated the usefulness of mitochondrial cytochrome b, cytochrome c, and d-loop region to find out the molecular phylogeny and diversity of hog deer. PCR method was employed and only nucleotide polymorphisms were found. A phylogenetic tree was constructed by using bioinformatics apparatus. The results revealed a minor genetic variation.

Savar Sofla et al. (2017) examined genetic relationships and differences between two Iranian breeds of sheep by exploiting the cytochrome b (cyt-b) gene sequence. The findings of the study revealed a separate group of Iranian sheep. From this study, future researchers can have help and information for the determination of the genetic structure of different breeds.

Genetic characterization of Mundri sheep and its comparison with other sheep breeds is still an unexplored area within Pakistan. Therefore, this current study has been conducted to fill this niche through exploratory research. A large number of Mundri sheep are present in Rajanpur District. No study has so far been conducted to establish whether Mundri sheep is a separate breed or it is a part of some existing breed. The purpose of this study is to investigate the genetic characteristics of Mundri sheep in order to determine the molecular phylogeny of this sheep. As a pioneer work, this study will contribute significantly to determine the evolutionary relationship of Mundri sheep among various breeds of sheep based on the similarities and differences in its genetic characteristics. The scope of this study is to understand the genetic diversity of Mundri sheep of District Rajanpur and to evaluate the genetic structure/make-up of this breed, using mitochondrial Cytochrome b and Cytochrome Oxidase Subunit I (COI) gene sequences.

Materials and methods

Blood collection and Ethical clearance

The research work was performed at the Animal Genomics Laboratory of the Department of Molecular Biology of the Virtual University of Pakistan located at its 1-Davis Road, Lahore campus. Blood samples of the Mundri sheep breed were collected from Livestock Experiment Station, (LES) Fazilpur, Rajanpur. By using venipuncture procedure 10 mL blood samples were collected from 30 sheep (male and female) and transferred into ethylenediaminetetraacetic acid (EDTA) containing tubes/Vacutainer immediately (Sambrook and Russell, 2001). The tubes were shaken to ensure mixing of blood with EDTA, to avoid coagulation. The blood samples were placed in an Ice chilled container and transferred to a lab and stored at -20°C until further use. Sample collection was performed according to standard procedures without any stress or harm to animals (European Union, 2010). All laboratory works were conducted at the Virtual University of Pakistan. Institutional Animal Care and Use Committee (IACUC) from Virtual University of Pakistan was obtained the Ethical Clearance before the experiment.

DNA Extraction

DNA was extracted from the blood samples by using this protocol (Sambrook and Russell, 2006). Blood sample of 200 µL of Mundri Sheep was taken in an Eppendorf tube. Lysis buffer 1000 µL was added to it. It was vortexed for a few seconds. It was centrifuged at 10,000 rpm for 10 minutes. Pellet formation was checked and the supernatant was discarded. Lysis buffer 1000 µL was added and centrifuged at 10,000 rpm for 10 minutes and this step was repeated 3 times. 250 µL buffer A1, 80 µL 10% SDS, and 20 µl

proteinase K were added. It was incubated at 58°C overnight for degradation of the protein. On the next day, 300 µL PCI was added to each sample. It was vortexed for a few seconds. It was centrifuged at 13,000 rpm for 15 minutes. Three layers were formed. The upper aqueous layer was carefully transferred into a separate Eppendorf tube. Isopropanol 600 µL was added and it was mixed gently with a pipette. It was centrifuged at 13,000 rpm for 15 minutes. The upper layer was discarded. Ethanol 1000 µL was added. It was centrifuged at 13,000 rpm for 10 minutes. The upper layer was discarded and the pellet was dried. Injection water 150 µL was added and the pellet was dissolved in it. DNA was stored at -20°C for further use. Inorganic method (Sambrook and Russell, 2001) was used for genomic DNA extraction. The final concentration of DNA was brought to 50 ng/uL and stored at -80°C before further use.

Mitochondrial Genome analysis

To amplify the complete mitochondrial Cyt b gene (1609 bp), three pairs were of primers was designed from *Bos indicus* (NCBI accession number AF492350) using software Primer3 (Steve and Skaletsky, 2000). Primer set : MtCCF1 (5′- GTCATCATCATTCTCAC ATGGAATC -3′) and MtCCR1 (5′- CTCCTTCTCTGGTTTACAAGACCAG-3′). A fragment of the mitochondrial gene cox1 was amplified using a set of forward primer set: MtCCF2 (5′- GCAGAGTTTGAAGCTGCT 3′) and MtCCR2 (5′- AGCTGACGTGAAGTAAGC-3′). PCR was performed in a 25 µL reaction mixture containing 1 µL of template (the genomic DNA of each sample was used as a template for PCR), 1 µL of each primer (10 pmol/µl), 12.5 µL of 2× Taq PCR MasterMix and 9.5 µL of ddH2O. Negative controls were always included in PCR reactions to assess possible contamination.

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a technique to amplify the target DNA segment, also for the detection of bacteria, viruses, and other scientific researches. In this research, we have used the touchdown PCR method for the amplification of Cytochrome b and Cytochrome Oxidase Subunit I (COI) genes. In the touchdown method, the temperature is reduced in every cycle by the specified value. The touchdown value for Cytochrome b is 50-48°C (-0.3 per cycle) 30 seconds and for COI is 54-50°C (-0.5 per cycle) 1 minute. The standard PCR conditions for Cyt b were followed: initial denaturation temperature 4 min at 95 °C, 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for primer sets 1, for 30 s and extension at 72 °C for 45 s followed by final extension at 72 °C for 10 min. PCR was performed in 25 µL reaction mixture using about 50 ng DNA as template with 2 units Taq DNA polymerase (Fermentas, Thermo Fisher Scientific Inc. USA). For COI, PCR was performed using initial denaturation at 95 °C for 4 min, and then 35 cycles of denaturation at 94 °C of for 30 s, annealing at 54 °C for 30 sand extension at 72 °C for 45 s following 10 min of final extension at 72 °C. 25 µL reaction mixtures were used for PCR using 50ng DNA as template and 1 unit Taq DNA polymerase. PCR product was purified by ethanol precipitation and sequenced using an automated 300 DNA sequencer ABI PRISM® 3130Xl Genetic Analyzer (Applied Biosystem Inc, Foster City, CA).

Gel Electrophoresis, DNA Sequencing and Analysis

The PCR product of both Cytochrome b and COI genes were run on 1.2% Agarose gel for 45 minutes at 120 volts. 5:3 composition of the sample is loaded on

the gel *i.e.* 5 µL DNA is mixed with 3 µL Bromophenol dye. The target product size of the Cytochrome b gene is 1140 bp and the product size of the Cytochrome Oxidase Subunit I (COI) is 1035 bp. Thermo Scientific 1Kb ladder is used for this purpose. 3 µL of the ladder is loaded on the gel. The results were observed on the gel doc system. PCR products were purified with 80% ethanol and sent for sequencing. 19 PCR products of Cytochrome b gene and 19 PCR products of Cytochrome Oxidase Subunit I (COI) were sequenced from the Lab Genetix G-3 AL-Hafeez Business Center, 89, Block B 3 Gulberg III, Lahore, Pakistan. 35 µL of PCR product of each sample with 100 µL of Cytochrome b-F and COI-F primer were sent for sequencing. The products from Sanger sequencing were analyzed by using Bioedit 7.2.5 (alignment editor for biological sequences) software. For phylogenetic analysis, various bioinformatics tools will be used, such as MEGA 6 software (Tamura et al., 2013). The resultant sequences were analyzed for the nucleotide analysis by using the software MEGA X (Kumar et al., 2018). These alignments were used to make the phylogenetic tree of Cytochrome b and COI.

Multiple Sequence Analysis

The newly determined complete mitochondrial Cyt b gene and COI sequences from Pakistani Mundri Sheep were submitted in GenBank for Cyt b gene and for COI. 667 nucleotides of Cytochrome b were used for the molecular charac-

Table 2. Shows Cytochrome *b* GenBank Accession Numbers used for *Ovis aries*

Serial no	Accession Number	Species	Isolate	Locality
1	JX546108	Ganjam sheep *Ovis aries*	GAN145	India
2	KP229295	Tibetan sheep *Ovis aries*	TS7	China
3	KP229228	Tianjun White Tibetan sheep *Ovis aries*	TJ6	China
4	KP229117	Qiaoke sheep *Ovis aries*	QK59	China
5	KP228991	Minxian Black Fur sheep *Ovis aries*	MX33	China
6	KP228961	Langkazi sheep *Ovis aries*	LKZ6	China
7	KP228950	Jiangzi sheep *Ovis aries*	JZ5	China
8	KP228897	Huoba sheep *Ovis aries*	HB3	China
9	KP228874	Gannan Oula sheep *Ovis aries*	GN8	China
10	JX546084	Nellore sheep *Ovis aries*	NEL155	India
11	JX546051	Deccani sheep *Ovis aries*	DEC19	India
12	JX546022	Patanwadi sheep *Ovis aries*	PAT19	India
13	JX545998	Sonadi sheep *Ovis aries*	SON116	India
14	JX545969	Jaisalmeri sheep *Ovis aries*	JAS149	India
15	JX545944	Marwari sheep *Ovis aries*	MAR18	India
16	JX545912	Kheri sheep *Ovis aries*	KHE136	India
17	JX545896	Chokla sheep *Ovis aries*	CHO157	India
18	JX545863	Nali sheep *Ovis aries*	NAL18	India
19	JX545831	Muzzafarnagri sheep *Ovis aries*	MUJ151	India
20	MG407528	*Ovis aries*	29	Egypt

21	KU569710	Kail sheep *Ovis aries*	6	Pakistan
22	MH938654	Iranian Lori sheep *Ovis aries*		Iran
23	KY366508	*Ovis aries*	1030o	Georgia
24	KY662385	Afshari sheep *Ovis aries*		Iran
25	KU246233	Birbhum sheep *Ovis aries*		India
26	JX235878	Shenwari sheep *Ovis aries*	4608	Pakistan
27	JX235854	Salt range sheep *Ovis aries*	4809	Pakistan
28	KP228780	Guide Black Fur sheep *Ovis aries*	GD5	China
29	KF677302	Hemsin sheep *Ovis aries*		Turkey
30	KF677301	Norduz sheep *Ovis aries*		Turkey
31	AY879584	Mongolian sheep *Ovis aries*	Mongolian1	Mongolia
32	JX546109	Ganjam sheep *Ovis aries*	GAN149	India
33	JX546133	Garole sheep *Ovis aries*		India
34	KP710135	Racka sheep *Ovis aries*	Racka39	Romania
35	AY879571	Romney sheep *Ovis aries*	R359	Australia
36	JX235868	Lohi sheep *Ovis aries*	4423	Pakistan
37	JX235866	Thalli sheep *Ovis aries*	2564	Pakistan
38	JX235861	Awassi sheep *Ovis aries*	2656	Pakistan
39	JX235851	Kaghani sheep *Ovis aries*	6820	Pakistan
40	JX235876	Pak-Karakul sheep *Ovis aries*	4210	Pakistan
41	JX235844	Kachi sheep *Ovis aries*	3817	Pakistan
42	JX235842	Dumari sheep *Ovis aries*	7016	Pakistan
43	JX235837	Bulkhi sheep *Ovis aries*	6079	Pakistan
44	FR873153	*Ovis aries*	haplotype H6	Cyprus
45	KF977846	Sahelian sheep *Ovis aries*		Benin
46	KF938358	Kulunda sheep *Ovis aries*		Russia
47	KF938342	Aland sheep *Ovis aries*		Finland
48	KF938327	Hulun Buir sheep *Ovis aries*		China
49	KF938317	Sunite sheep *Ovis aries*		China
50	KF302461	Comisana sheep *Ovis aries*	CO027	Italy
51	NC 001941	European type *Ovis aries*	haplotype B	
52	KF977845	Djallonke sheep *Ovis aries*		Benin
53	MF004246	Karadi sheep *Ovis aries*	KarM	
54	KF938356	*Ovis aries* breed Viena *Ovis aries*		Russia
55	KF938357	Mountain Carpathian Sheep *Ovis aries*		Ukraine
56	KF938355	Finn sheep *Ovis aries*		Finland
57	KF938353	Kainuu grey sheep *Ovis aries*		Finland
58	KF938351	Karachai sheep*Ovis aries*		Russia
59	KF938350	Wrzosowka sheep		Poland
60	KF938349	Swiniarka sheep *Ovis aries*		Poland

Table 3. Shows Cytochrome Oxidase Subunit I (COI) GenBank accession number for *Ovis aries*

Serial no.	Accession Number	Species	Isolate	Locality
1	KF385961	*Ovis aries*	PSU<THA_:0A2_	Thailand
2	JX218081	*Ovis aries*	SUF	Philippines
3	KP761778	Awassi sheep *Ovis aries*	C	Iraq
4	HM102308	*Ovis aries*	strain ATCC CRL-1700	USA
5	HQ603178	*Ovis aries*	Sheep2	Tanzania
6	JF443355	*Ovis aries*	HBL008387	Canada
7	JN245995	*Ovis aries*	AUS-EAS1	India
8	JQ735465	*Ovis aries*	OACHM1	India
9	KX233971	*Ovis aries*	Hap_56	Turkey
10	MG571551	Barki sheep *Ovis aries*		Egypt
11	JF444380	Musimon sheep *Ovis aries*	ROM:PM13074	
12	KX859288	*Ovis aries*	MGL-152_	Mongolia
13	JX567087	*Ovis aries*	clone 82e	South Africa
14	FJ958344	Merino Polish *Ovis aries*	PL-IGAB-ZBM27	Poland
15	KX233916	*Ovis aries* haplotype	Hap_1	Turkey
16	KT750038	*Ovis aries*	3s	Saudi Arabia
17	EF490464	Black Welsh Mountain sheep *Ovis aries*	Clone CB1 (F1BLW3F1)	Scotland
18	EF490469	Poll Dorset sheep *Ovis aries*	FM2 (No.9E782)	Scotland
19	EF490470	Scottish Blackface sheep *Ovis aries*	FM3 (No.9E213)	Scotland
20	MN 124247	*Ovis aries*	Lamb OD	Kenya
21	EF490457	Black Welsh Mountain sheep *Ovis aries*	Cell line BLWF1	Scotland

terization of Mundri sheep (*Ovis aries*). Sequences from our samples of Mundri sheep were aligned with other sheep sequences through MEGA X software by using CLUSTAL O (1.2.4). To discover the evolutionary relationships between genes and to find out shared patterns amongst functionally or a structurally related gene, multiple sequence alignment is a tool used in research to find out about closely related genes or proteins. The multiple alignments were saved in FASTA format for further analysis. These Mundri sheep sequences were aligned with the downloaded FASTA files of other *Ovis aries* sequences on MEGA X along which showed the highest similarity ratio with our query sequences from GenBank. All the accession numbers (when a biological polymer sequence (DNA, protein) submitted to a sequence database a unique identifier assigned to it) of the sequences used in this analysis are given below (Table 2 and Table 3).

Phylogenetic analysis

Phylogenetic trees were constructed through MEGA X software by using multiple Cytochromes b and COI sequences alignments of Mundri sheep, and other sheep breeds. The Maximum likelihood (ML) statistical method was used for the construction of a Phylogenetic tree using bootstrap methods. Further, we used the

Figure 1. Cytochrome *b* (P-distance) Genetic Distance Table showing the; evolutionary divergence between sequences

1	Mundri_sheep_P9_Pakistan
2	Mundri_sheep_P7_Pakistan	0.00
3	Mundri_sheep_P20_Pakistan	0.01	0.00
4	Mundri_sheep_P2_Pakistan	0.01	0.00	0.00
5	Mundri_sheep_P16_Pakistan	0.01	0.01	0.01	0.01
6	Mundri_sheep_P15_Pakistan	0.01	0.01	0.00	0.00	0.01
7	Mundri_sheep_P14_Pakistan	0.01	0.01	0.01	0.01	0.01	0.01
8	Mundri_sheep_P11_Pakistan	0.01	0.01	0.01	0.01	0.01	0.01	0.01
9	Mundri_sheep_P1_Pakistan	0.01	0.00	0.01	0.00	0.01	0.01	0.01	0.01
10	Ovis_aries_Tibetan_sheep_China	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01
11	Ovis_aries_Tianjun_White_Tibetan_sheep_China	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00
12	Ovis_aries_Qiaoke_sheep_China	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.00
13	Ovis_aries_Minxian_Black_Fur_sheep_China	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00
14	Ovis_aries_Langkazi_sheep_China	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.01	0.00
15	Ovis_aries_Jiangzi_sheep_China	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.01	0.00	0.00
16	Ovis_aries_Huoba_sheep_China	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00
17	Ovis_aries_Gannan_Oula_sheep_China	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
18	Ovis_aries_Guide_Black_Fur_sheep_China	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
19	Ovis_aries_Hemsin_sheep_Turkey	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
20	Ovis_aries_Norduz_sheep_Turkey	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
21	Ovis_aries_Mongolia	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
22	Ovis_aries_Romney_sheep_Austrailia	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
23	Ovis_aries_Racka_sheep_Romania	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
24	Ovis_aries_Garole_sheep_India	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
25	Ovis_aries_Ganjam_sheep_India	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
26	Ovis_aries_Nellore_sheep_India	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
27	Ovis_aries_Deccani_sheep_India	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
28	Ovis_aries_Patanwadi_sheep_India	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
29	Ovis_aries_Sonadi_sheep_India	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	Ovis_aries_Jaisalmeri_sheep_India	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
31	Ovis_aries_Marwari_sheep_India	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	Ovis_aries_Kheri_sheep_India	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
33	Ovis_aries_Chokla_sheep_India	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
34	Ovis_aries_Nali_sheep_India	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
35	Ovis_aries_Muzzafarnagri_sheep_India	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
36	Ovis_aries_isolate_29_Egypt	0.01	0.01	0.01	0.02	0.02	0.01	0.02	0.02	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
37	Ovis_aries_Kail_sheep_Pakistan	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
38	Ovis_aries_Lori_sheep_Iran	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
39	Ovis_aries_Georgia	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
40	Ovis_aries_Afshari_sheep_Iran	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
41	Ovis_aries_Birbhum_sheep_India	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
42	Ovis_aries_Shenwari_sheep_Pakistan	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
43	Ovis_aries_Salt_range_sheep_Pakistan	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
44	Ovis_aries_Lohi_sheep_Pakistan	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
45	Ovis_aries_Pak-Karakul_sheep_Pakistan	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
46	Ovis_aries_Thalli_sheep_Pakistan	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
47	Ovis_aries_Awassi_sheep_Pakistan	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
48	Ovis_aries_Kaghani_sheep_Pakistan	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
49	Ovis_aries_Kachi_sheep_Pakistan	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
50	Ovis_aries_Dumari_sheep_Pakistan	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
51	Ovis_aries_Bulkhi_sheep_Pakistan	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
52	Ovis_aries_H6_Cyprus	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
53	Ovis_aries_Sahelian_sheep_Benin	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
54	Ovis_aries_Kulunda_sheep_Russia	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
55	Ovis_aries_Aland_sheep_Finland	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
56	Ovis_aries_Hulun_Buir_sheep_China	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
57	Ovis_aries_Sunite_sheep_China	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
58	Ovis_aries_Comisana_sheep_Italy	0.01	0.01	0.01	0.02	0.02	0.01	0.02	0.02	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
59	Ovis_aries_haplotype_B_(European_type)	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
60	Ovis_aries_Djallonke_sheep_Benin	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

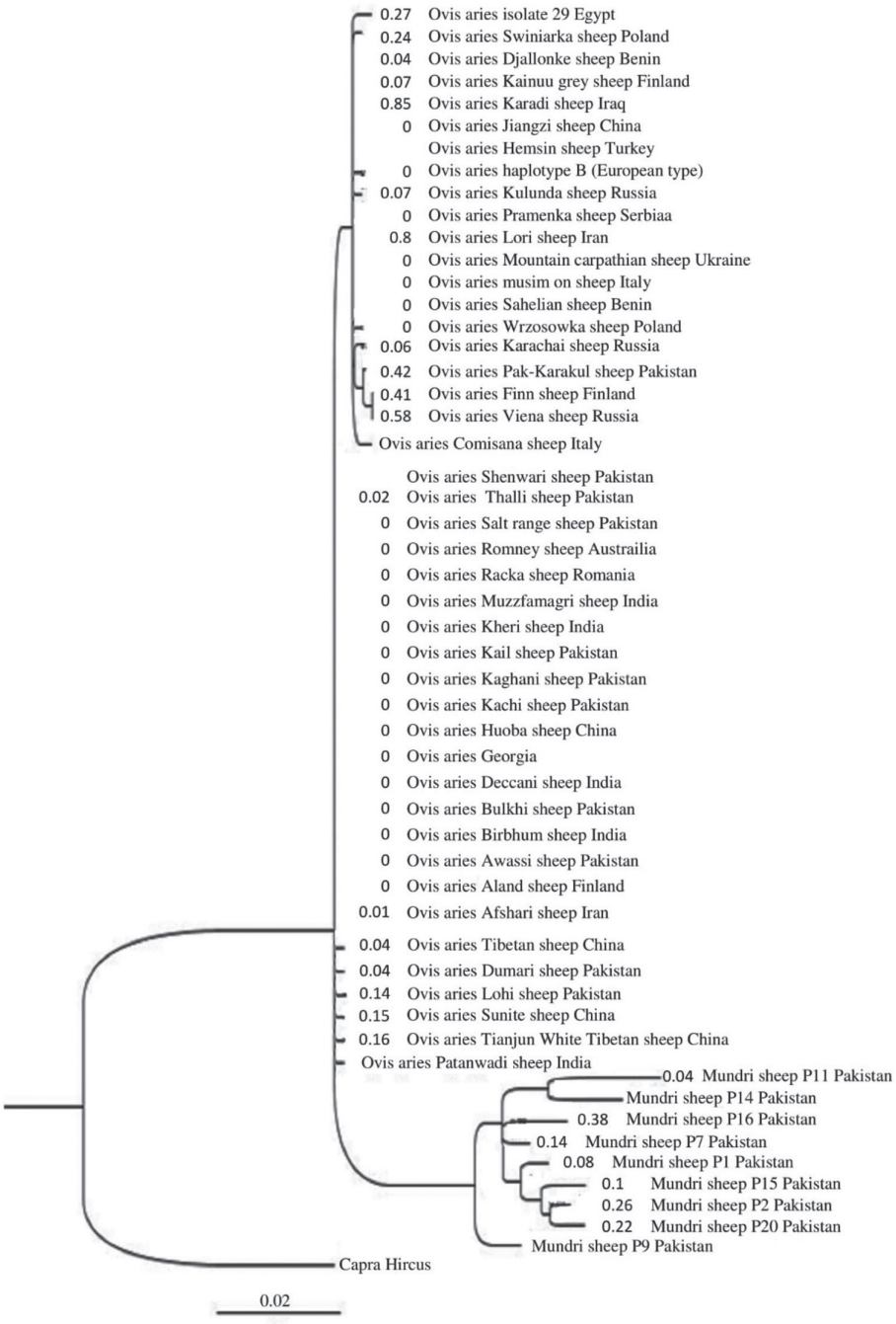

Figure 2. The Maximum likelihood phylogenetic tree of Cytochrome *b* gene represents the evolutionary relationship of Mundri sheep with other *Ovis aries* breeds. The pattern of branching reflects how sheep breeds evolved from common ancestor *Capra hircus*

Jukes-Cantor model for this phylogenetic analysis. The bootstrap test method was used by adjusting 500 replicates values. In this study, goat (*Capra hircus*) genes (Cytochrome b and COI) were used as an out-group. The length of the branches shows genetic distance. Scale 0.02 (2%) shown at the bottom of phylogenetic tree is the amount of genetic change/variation. The Bootstrap value of >0.6 shows strong support for tree topology.

Results

Cytochrome b and Cytochrome Oxidase Subunit I (COI) are the main players for the determination of the molecular Phylogeny of animals. To evaluate the genetic phylogeny of Mundri sheep, DNA isolation was done by extraction protocol as described by Sambrook and Russell (2006). The quantity of DNA was measured by NanoDrop (Thermo Fisher Scientific). Cytochrome b and COI genes were amplified by touchdown Polymerase chain reaction (PCR) was done. PCR products were confirmed by Agarose gel (1.2%) electrophoresis with 1Kb DNA ladder (Thermo Fisher Scientific). For the sequencing of Cytochrome b and COI, PCR products were sent to Lab Genetix G-3 AL-Hafeez Business Center, 89, Block B 3 Gulberg III, Lahore, Pakistan. The sequencing of the Cytochrome b gene was analyzed by using Bioedit 7.2.5 (alignment editor for biological sequences) software. We trimmed some sequences from the 5` end and 3` ends of all our sequence samples because the quality of the sequencing was not so good. 09 query sequences of Cytochrome b whose sequence result was very clear were chosen for further analysis.

Figure 1 shows genetic distance (P-distance) of Mundri sheep with other closely related local sheep breeds and from the rest of the world taken from the NCBI Genbank. Analyses were carried out using the Maximum Composite Likelihood model. 60 nucleotide sequences were involved in the analysis. There were a total of 667 positions in the final dataset. Maximum evolutionary divergence between sequences shown by the Cytochrome b gene was 0.06; which implies that there is a little variation amongst Mundri Sheep and other sheep breeds compared with it.

Figure 2 is a phylogenetic tree for Cytochrome b dividing the sheep breeds into 3 clades. The phylogenetic tree shows Mundri sheep (*Ovis aries*) from Pakistan as a separate group thus indicating as a different breed. In this clade, the tree shows that Mundri Sheep P11 and P 14 shared evolutionary history with Mundri sheep P1 which further shared history with Mundri sheep, P2, and P 15 but this is away from P16, P7, P 20, and P9. This also shows that the parents of Mundri P11, P14, P1, P2, and P15 are the same and all Mundri sheep having a common ancestor. The divergence between goat (*Capra hircus*) which has been used as out-group and Mundri sheep breeds query sequence varies up to 0.06 whereas divergence between other local, and other sheep breeds varies up to 0.04. This study shows that more morphological and genetic data can produce more resolution in the relationship of sheep breeds.

The analysis of the Cytochrome Oxidase Subunit I (COI) gene was made through Bioedit 7.2.5 software. Some sequences were terminated from the 5` end and 3` ends of all our sequence samples having not good quality. Very clear 14 query sequences of COI results were chosen for further analysis. 747 nucleotides of Cytochrome Oxidase Subunit I (COI) were used for the molecular characterization of Mundri sheep (*Ovis aries*). Sequences from our

Figure 3. Cytochrome Oxidase Subunit I (COI) Genetic Distance (P-distance) Table showing the evolutionary divergence between sequences

1	Ovis_aries_Poll_Desert_sheep_Scotland																															
2	Ovis_aries_Black_Welsh_Mountain_sheep_Scotland	0.00																														
3	Ovis_aries_Nairobi_Kenya	0.00	0.00																													
4	Ovis_aries_Merino_Polish_sheep_Poland	0.00	0.00	0.00																												
5	Ovis_aries_India	0.00	0.00	0.00	0.00																											
6	Ovis_aries_Canada	0.00	0.00	0.00	0.00	0.00																										
7	Mundri_sheep_PC1_Pakistan	0.00	0.00	0.00	0.00	0.00	0.00																									
8	Mundri_sheep_PC9_Pakistan	0.01	0.01	0.01	0.01	0.01	0.01	0.01																								
9	Mundri_sheep_PC18_Pakistan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01																							
10	Mundri_sheep_PC2_Pakistan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00																						
11	Mundri_sheep_PC10_Pakistan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00																					
12	Mundri_sheep_PC19_Pakistan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00																				
13	Mundri_sheep_PC11_Pakistan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00																			
14	Mundri_sheep_PC4_Pakistan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01																		
15	Mundri_sheep_PC5_Pakistan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00																	
16	Mundri_sheep_PC13_Pakistan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00																
17	Mundri_sheep_PC6_Pakistan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00															
18	Mundri_sheep_PC7_Pakistan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00														
19	Mundri_sheep_PC15_Pakistan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00													
20	Mundri_sheep_PC8_Pakistan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00												
21	Ovis_aries_Jialuo_sheep_China	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00											
22	Ovis_aries_Comisana_sheep_Italy	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00										
23	Ovis_aries_Sopravissana_sheep_Italy	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00									
24	Ovis_aries_Shandong_large-tailed_sheep_China	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00									
25	Ovis_aries_Morkaraman_sheep_Turkey	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01								
26	Ovis_aries_United_Kingdom_Scotland	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00							
27	Ovis_aries_Tibetan_sheep_China	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00						
28	Ovis_aries_Texel_sheep_China	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00					
29	Ovis_aries_Oula_sheep_China	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00				
30	Ovis_aries_Kazakh_sheep_Kazakhstan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00			
31	Capra_hircus_(Goat)	0.03	0.03	0.03	0.03	0.02	0.03	0.02	0.03	0.02	0.02	0.02	0.02	0.03	0.03	0.02	0.02	0.02	0.02	0.02	0.03	0.03	0.03	0.02	0.03	0.03	0.03	0.02	0.03			
32	Mundri_sheep_PC16_Pakistan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02

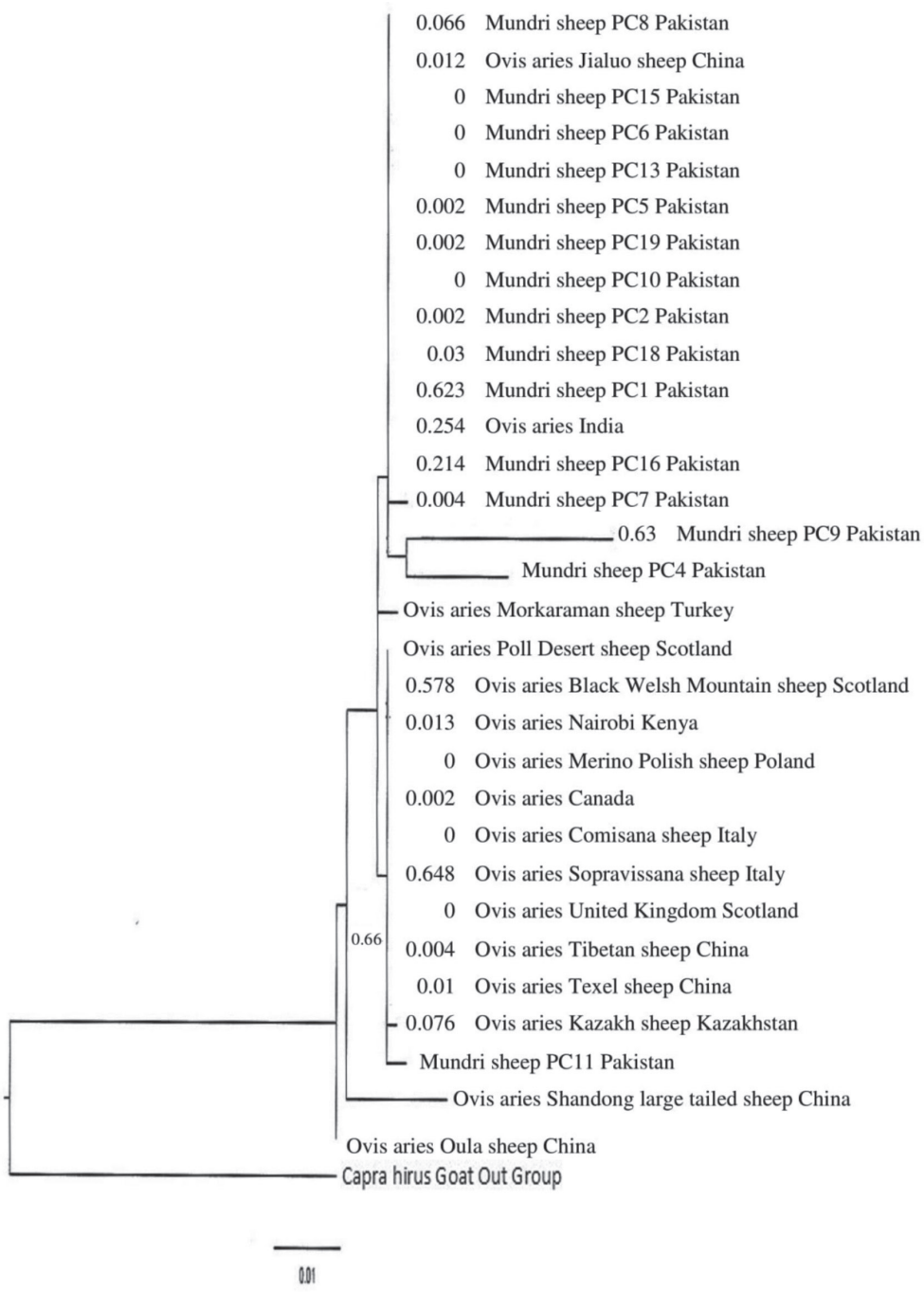

Figure 4. Maximum likelihood phylogenetic tree of Cytochrome Oxidase Subunit I (COI) represents evolutionary relationship of Mundri sheep with other *Ovis aries* breeds. The pattern of branching reflects how sheep breeds evolved from common ancestor *Capra hircus.*

samples of Mundri sheep were aligned with other sheep sequences through MEGA X software by using CLUSTAL O (1.2.4). To discover the evolutionary relationships between genes and to find out shared patterns amongst functionally or a structurally related gene, multiple sequence alignment is a tool used in research to find out about closely related genes or proteins. The multiple alignments were saved in FASTA format for further analysis.

The analysis of the Cytochrome Oxidase Subunit I (COI) gene was made through Bioedit 7.2.5 software. Some sequences were terminated from the 5` end and 3` ends of all our sequence samples having not good quality. Very clear 14 query sequences of COI results were chosen for further analysis. 747 nucleotides of Cytochrome Oxidase Subunit I (COI) were used for the molecular characterization of Mundri sheep (*Ovis aries*). Sequences from our samples of Mundri sheep were aligned with other sheep sequences through MEGA X software by using CLUSTAL O (1.2.4). To discover the evolutionary relationships between genes and to find out shared patterns amongst functionally or a structurally related gene, multiple sequence alignment is a tool used in research to find out about closely related genes or proteins. The multiple alignments were saved in FASTA format for further analysis.

Figure 3 shows genetic distance (P-distance) of Mundri sheep with other closely related local sheep breeds and from rest of the world taken from the NCBI Genbank. Analyses were carried out using the Maximum Composite Likelihood model. The analysis involved 32 nucleotide sequences. There were a total of 655 positions in the final dataset. The maximum evolutionary divergence between sequences shown by Cytochrome oxidase subunit I (COI) gene was 0.03; which implies that there is a little variation amongst Mundri Sheep and other sheep breeds compared with it.

Figure 4 is the second maximum likelihood phylogenetic tree dividing the sheep breeds into two clades. Goat (*Capra hircus*) COI was used as an out-group. Clade first shows Mundri sheep from Pakistan showed a very close relationship with *Ovis aries* from India and China (Jialuo sheep). Clade 2 of the tree shows that sheep breeds from China are closely related to sheep breeds from Turkey, Scotland, Italy, Poland, Canada, Kazakhstan, and Kenya.

Genetic distance graphs (P-distance) and Phylogenetic trees of both genes indicate that mitochondrial Cytochrome b gene is the best gene for Phylogenetic studies rather than mitochondrial Cytochrome Oxidase Subunit I (COI) gene.

Discussion

This study on molecular phylogeny and characterization through sequencing of mitochondrial Cytochrome b and Cytochrome Oxidase Subunit I (COI) genes of Mundri sheep was conducted. Mundri sheep breed is fairly large, thin tailed with white color extremely short ears and sharp roman nose, habitats in the Rajanpur district of Punjab province, appears physiologically different from other local sheep breeds present in the country.

The exploration of the phylogenetic relationship between species is one of the major areas of interest since the conception of the theory of evolution. It is found that variations occur between species, whether in physiology, morphology, ecology, in ways of behavior, or in geographical distribution, during the course of phylogenesis. With the advancement

in such fields of study Linnaean system has stepped forward to the modern system of biological classification such as systematics, cladistics, and phylogenetics based upon the evolutionary relationships between organisms. Genetic characterization of different breeds of particular species has been made possible with advances in science. For phylogenetic examination in animals, mitochondrial DNA is one of the most commonly used molecular markers. In the past, various studies have been conducted to determine the genetic characteristics and evolutionary relationship of various sheep breeds as well as goats, buffalo camel, etc. by using mitochondrial Cytochrome b and Cytochrome Oxidase Subunit I (COI). However, this study is conducted to explore the genetic characterization of Mundri sheep and its comparison with other sheep breeds which is still an unexplored area within Pakistan.

Phylogenetic analysis was conducted to find out the relationship of Mundri sheep with other breeds of sheep using mitochondrial Cytochrome b and Cytochrome Oxidase Subunit I (COI) gene sequencing. These genes were selected due to their unique properties of specie's identification and for determining a phylogenetic association between organisms. The evolutionary relationship of Mundri sheep with local sheep breeds as well as other sheep breeds was studied by constructing phylogenetic trees.

Cytochrome b and COI genes of Mundri sheep were amplified by PCR and sequenced to confirm the separate breed of Mundri sheep A total of 1,414 number of nucleotides (Cytochrome b= 667 and Cytochrome Oxidase Subunit I (COI) =747) were used for the molecular characterization of Mundri sheep (*Ovis aries*). We aligned our query sequences (Mundri sheep) along with the FASTA files of other *Ovis aries* sequences on MEGA X. Our findings showed high similarity of Mundri sheep sequences from Pakistan and other parts of the world whose genetic characterization and evolutionary relations were determined using either Cytochrome b gene sequence or Cytochrome Oxidase Subunit I (COI) genes sequence. The alignments were saved for further analysis.

The phylogenetic tree of Cytochrome b was constructed by using our query sequences and other *Ovis aries* sequences of local breeds and from different countries which showed the highest similarity. This analysis groups the Mundri sheep in a separate clad due to evolutionary divergence. Moreover, our results divide the sheep into three groups. Most of the sheep breeds from Pakistan, India, Iran, and China showed close relationship with Australian, Georgian and Russian sheep breeds. Some of the sheep breeds from Pakistan China, Iran also showed close relationship with sheep breeds from Poland, Italy, China, Finland, Russia, Iraq, Ukraine, and Turkey. Mundri sheep totally grouped separately in the phylogenetic analysis of the Cytochrome b gene thus appearing as a different breed from all other sheep breeds. The separate breed of Mundri sheep is also validated by the genetic distance (P-distance) table deduced from the Cytochrome b gene. The 0.06 evolutionary divergence value of the P-distance table with other sheep breeds is ample proof of its separate clad.

These results are also supported by earlier studies conducted by various researchers on sheep phylogenetic relationship and taxonomic status. Although "Thalli" and "Lohi" sheep breed are very close to Indian sheep breed but these findings were derived by using the cytochrome b gene and other conserved markers. It was reported that

"Thalli" and "Lohi" sheep were very close to the Indian breed as we inferred that Mundri sheep showed its close resemblance with other sheep breeds of the world. Genetic relationship and differences between two Iranian breeds of sheep by exploiting Cytochrome b (cyt-b) gene sequence were examined. The findings of the study revealed a separate group of Iranian sheep (Savar Sofla et al., 2017) as we reported about Mundri sheep.

The second maximum likelihood phylogenetic tree was constructed using Cytochrome Oxidase Subunit I gene (COI) gene sequences. In the case of the COI gene, the phylogenetic analysis was done between query sequences of Mundri sheep with other *Ovis aries* local sheep breeds and from different countries. All sheep were divided into 2 groups. Mundri sheep from Pakistan showed a very close relationship with *Ovis aries* from India and China (Jialuo sheep). The findings of the study revealed that some sheep breeds from China showed a close relationship with sheep breeds from Scotland, Italy, Poland, Canada, Kazakhstan, and Kenya. P-distance table with COI gene showed a maximum evolutionary divergence of 0.03.

Earlier Sharifi et al. (2017) differentiated the genetic features of various breeds of goats in the Iranian context through sequence analysis and Polymerase Chain Reaction (PCR). The finding of the study revealed that Iranian goats possessed 1286 (bp) cytochrome Oxidase Subunit I (COI) gene in partial sequence and had four sites of variables and three haplotypes. The analysis showed that Iranian goats were clustered in a separate lineage in reference to the combination of GenBank.

In conclusion, the current study determined the molecular phylogeny of Mundri sheep in comparison to other local sheep breeds of Pakistan as well as the rest of the world using two mitochondrial molecular markers i.e. Cytochrome b and Cytochrome Oxidase Subunit I (COI). Based on Cytochrome b sequencing, Mundri sheep (*Ovis aries*) appeared as a separate group whereas this study also revealed a close association of Mundri sheep with *Ovis aries* from India and China (Jialuo sheep).

Phylogenetic relationship and characterization of Mundri sheep and its comparison with other breeds is an unexplored area within Pakistan. No study has so far been conducted to establish whether Mundri sheep is a separate breed or it is a part of some existing breed. The purpose of this study was to analyze the genetic characteristics of Mundri sheep in order to determine the molecular phylogeny of this sheep by Mitochondrial Cytochrome b and Cytochrome Oxidase Subunit I (COI) genes. Blood samples of Mundri sheep were collected from Livestock Experiment Station, (LES) Fazilpur district Rajanpur for this study. DNA was isolated from these blood samples using the inorganic method and subsequently quantified. Mitochondrial Cytochrome b and COI were amplified by using designed primers through PCR. PCR products were sequenced and analyzed through MEGA X software. For phylogenetic analysis, the maximum likelihood (ML) statistical method was used with the bootstrapping of 500 replications. Evolutionary divergence of Mundri sheep was observed by genetic distance (P- distance) tables of Cytochrome b and COI genes. Two different Maximum Likelihood phylogenies were obtained from Cytochrome b and COI gene. Goat (*Capra hircus*) was used as an out-group.

The phylogenetic analysis categorized *Ovis aries* including Mundri sheep into three and two groups for Cytochrome

b and COI genes respectively. Further, it showed that that Mundri sheep (*Ovis aries*) from Pakistan appeared as a separate group and thus grouped as a distinctive breed from local and rest of the world breed. Furthermost of the sheep breeds from Pakistan, India, Iran, and China showed a close relationship with Australian, Georgian and Russian sheep breeds. Some of the breeds from Pakistan China, Iran also showed a close relationship with sheep breeds from Poland, Italy, China, Finland, Russia, Iraq, Ukraine, and Turkey in this phylogenetic analysis.

There found a close relationship of Mundri sheep with the *Ovis aries* from India and China (Jialuo sheep). Moreover, the potential of Cytochrome b and COI for the identification of species and subspecies can be explored. The present study will help to evaluate the diversity of mammals, birds, reptiles, fish, and insects.

Conclusion

In the past, the classification of organisms was based upon morphological characters. Mundri sheep from Pakistan is already accepted as a separate breed because the physical appearance of this sheep is different from other local breeds of sheep. This study expressed the potential of mitochondrial Cytochrome b and Cytochrome Oxidase Subunit I (COI) genes to help in determining the molecular phylogeny and characterization of this sheep. Mundri sheep from Pakistan appeared as a different breed from all other local sheep breeds as derived from phylogenetic tree. This study will also be helpful in the genetic conservation of Mundri Sheep.

Recommendations

This study recommended that mitochondrial Cytochrome b and Cytochrome Oxidase Subunit I (COI) genes are effective tools in determining genetic characteristics and also are helpful for species identification and phylogeny. A substantial number of local sheep (n=30) breeds have so far been recognized in Pakistan. Most of the work on sheep breeds awaits documentation even there may still be other sheep breeds available in country which are not recognized till now. So by using these genes other sheep breeds and diversity of other fauna (birds, mammals, reptiles, fishes, and insects) should be studied. Further, the present molecular techniques should be applied on already recognized species/subspecies to get more insight about them.

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Molekularna filogenija i karakterizacija mundri ovce (*Ovis aries*) u Pakistanu sekvenciranjem mitohondrijskog citokroma b i podjedinice I citokrom oksidaze

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Glavni je cilj ovog istraživanja bio odrediti molekularnu filogeniju i karakterizaciju mundri ovce (*Ovis aries*) sekvenciranjem mitohondrijskog citokroma b i podjedinice I citokrom oksidaze (COI). Ova se pasmina ovaca morfološki čini drugačijom od ostalih lokalnih pasmina ovaca u Pakistanu. Ovo je istraživanje provedeno da bi se procijenio status mundri ovce, da bismo mogli odrediti radi li se o pasmini drugačijoj od ostalih pasmina. Uzorci krvi mundri ovce prikupljeni su iz Stanice za eksperimente na stoci (engl. *Livestock Experiment Station – LES*) Fazilpur u okrugu Rajanpur (Punjab). DNK je izolirana i podvrgnuta lančanoj reakciji polimerazom (PCR) zbog pojačanja citokrom B i COI gena uporabom prikladnih primera. PCR proizvodi su sekvencirani i analizirani pomoću MEGA X softvera. Filogenetska analiza kategorizirala je *Ovis aries* uključujući mundri ovcu, u tri i dvije skupine za citokrom b, odnosno COI gene. Istraživanje je pokazalo da je mundri ovca posebna skupina i time zasebna pasmina ovaca u odnosu na ostale lokalne pasmine. Na temelju citokroma b i COI, naša je studija potvrdila da je mundri ovca zasebna pasmina i da se razlikuje od ostalih lokalne pasmine ovaca.

Ključne riječi: *mundri ovca, Ovis aries, molekularna filogenija, karakterizacija, citokrom b, podjedinica I citokrom oksidaze, COI, Pakistan*