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Analysis of Mitochondrial *COI* Gene for Species/ Breed Level Identification of the Genus *Capra* (Linnaeus, 1758)

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BM conducted the research work and wrote the article. NT supervised the research work. NA helped in data analysis and lab work. HAA helped in sampling and data collection. AK helped in write up, review and editing of the manuscript. SKK, N Ali and N Ahmed helped in literature review.

Key words
DNA barcodes, Goat breeds, genetic distance, evolutionary analysis, Genus *Capra*, Pakistan

ABSTRACT

The current study mainly aimed to identify the goat breed at species level through *COI* gene sequencing. In this study the effectiveness of *COI* gene was examined in distinguishing the goat breeds which has not been inclusively investigated in mammals. To fill this gap, we determined the COI barcodes for six different breeds of goat and found them all as domestic goats (*Capra hircus* Thomas, 1911). Mean intraspecific genetic distance was 0.0016%, obtained from 528 COI positions, lower than the average interspecific genetic distance 0.03%, indicating the discrimination efficacy of *COI* gene. Using the *COI* gene sequences the phylogenetic analysis confirmed that the domestic goat breed has a very close kinship with Siberian goat breeds, *Capra sibirica* (Pallas, 1776), a wild goat species. It is concluded that molecular based identifications of animal species are more accurate, compared to morphological approaches for identification of the indigenous breeds. Furthermore, for future study, the *COI* gene inclusive exploration is suggested to find the genetic divergence in goat breeds of the country.

INTRODUCTION

The genus *Capra*, which include domestic goats and their wild relatives (bezoars, turs, markhors, and ibex) displays a uniquely old-world dispersion and exhibits some phenotypic differences (Hartl *et al*., 1999; Pidancier *et al*., 2006). Goats are known as livestock that produce valuable milk and meat products. In terms of nutritional value, goat meat is appreciated for low fat and high protein content (Webb *et al*., 2005). The global number of goats exceeds one billion (Aziz, 2010) and continues to increase (FAO, 2019). Asia has the largest proportion of the world population (52%), followed by Africa (39%), Europe (5%), the Americas (4%), and Oceania (<1%) (Wang *et al*., 2015; Miller and Lu, 2019). Fossil data suggest that the *Capra* species first appeared in Central Asia and that an adaptive species radiation occurred during the Plio-Pleistocene period (Pilgrim, 1947). Historically, the *Capra* species is poorly interpreted, making it difficult to assess the number of species and their phylogenetic relationships (Chen *et al*., 2008; Wang *et al*., 2017). The *Capra* taxa apparently occurred rapidly by the fact that there may be evidence from radiocarbon dating that goats are more commonly kept as supplier of meat, and the morphological changes that can be seen in bone and horn cores of the goats (Clutton-Brock, 1999).

The domestic goat, *Capra hircus* (Mammalia, Artiodactyla) is one of the first herbivores to be domesticated about 10,000 years ago (Zeder and Hesse, 2000) in the highlands of western Iran and is currently one of the most widely distributed domestic animals (Vahidi *et al*., 2014). Goat populations have been able to adapt to different and extreme environments over a long period which leads to some genetic changes, experiencing exponential growth and successful geographic expansion throughout the world (Wang and Yu, 2008; Wang *et al*., 2015). The preservation of local breeds is necessary to limit the loss of genetic

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resources (FAO, 2007), in particular for the species that are more important for food production, rural development, and environmental protection (Hersleth *et al*., 2012).

Pakistan is ranked the third biggest goat producing country in the world, containing the large volume of goat genetic resources (Ali *et al*., 2016). According to recent records, Pakistan constitutes 54 million goats and their production rate is increasing by more than 3% per annum (Group *et al*., 2009). Punjab, Khyber Pakhtunkhwa, Balochistan and Sindh comprises of 37, 18, 22 and 23 percent goat population. In Pakistan there are 25 pure goat breeds are present and two wild species of goat namely Markhor and Ibex are found (Khan *et al*., 2008).

The mitochondrial genome which codes the cytochrome oxidase subunit I (COI) is used for the identification of various biological specimens (Hebert *et al*., 2003). This modern technique requires the set of primer for the amplification of 648 base pairs (bp) region. In addition, to establish the DNA barcoding barcode of life has been introduced as a global norm based on eukaryotic sequence identification (Hebert *et al*., 2003; Jinbo *et al*., 2011). Species level identification of specimens through DNA Barcoding is correspond to create the phylogenetic trees by compiling the various dimensions of barcodes, showing ancestral distances and relationships among species (Kerr *et al*., 2007; Kress and Erickson, 2008; Savolainen *et al*., 2005).

Newly proposed phylogeny by biologists explains the evolutionary relationships of organisms and evaluates the genetic distances among *COI* gene barcodes develops the evolutionary basis and taxonomy (Stoeckle *et al*., 2004). Mitochondrial DNA has an effective discrimination power because it is maternally inherited (Sultana *et al*., 2003; Ivanova *et al*., 2006). Mitochondrial genome is dominated over nuclear genome due to the absence of introns. Generally, they have a haploid number of genes and have a reduced recombination (Hajibabaei *et al*., 2006).

Pietro *et al*. (2003) investigated complete mtDNA of *Capra hircus* revealed that mtDNA have the potential of producing furnished phylogenetics and DNA barcodes from *COI* gene that facilitates species identification (Avise *et al*., 1987). Although GenBank has many goat sequences derived from various gene locations whereas recent studies suggest 400-800bp region of mtDNA as a standard barcode region. The main purpose of the present study was to identify goat at breed/species level by *COI* gene, and to develop the barcodes and comparing these with the barcode of life data system and NCBI, then producing evolutionary relationships with other closely related species and identifying the complete organism from a single tissue (Bush *et al*., 2017).

MATERIALS AND METHODS

Field sampling

In this study, six breeds of goat were examined. Among six breeds five were pure breeds and one was hybrid. The samples were collected during 2022 from three different farm locations in Balochistan (Table I). DNA was extracted from blood and tissue and then processed for *COI* gene amplification and sequencing.

Table I. Sample collection from different farm locations.

Animal	Breed name	Synonym	Farm location
Domestic goat (*Capra hircus*)	Angora	Pak- Angora	Shalla
	Barbari	Bari	Quetta
	Lehri		Maslakh
	Pahari	Kajlee	Quetta
	Sindhi	Kamori	Shalla
	Angora cross		Shalla

DNA isolation, COI amplification and sequencing

The DNA extraction was carried out in the laboratory of Biotechnology, Balochistan University of Information Technology Engineering and Management Sciences (BUITEMS) using inorganic method following the optimized protocol proposed by Grimberg (1989). DNA was quantified on agarose gel, and a 528 bp region of the *COI* gene was amplified by using the forward and reverse primers (DBGB1 5' GCCTGAGCTGGCATAGTAGG 3'and DBGB2 5' CTCCTGCTGGGTCAAAGAAG 3'), designed from the default settings of Primer-3 plus, version 0.40, then registered on BOLD. The 20µl PCR reaction mixture included 10 µl Master Mix, (Thermo Fisher Scientific, Catalogue no. K0171) 0.5 µl reverse primer (10pm/ µl), 0.5 µl forward primer (10pm/ µl), 8 µl PCR water and 1 µl DNA sample. The *COI* gene amplification was performed on DNA thermal cycler (T100 -Thermocycler -BIO – RAD; Singapore). The optimized thermal cycler condition consisted of an initial denaturation step of 3 min at 94°C followed by 5 cycles consisting of denaturation at 94°C for 40 s, annealing at 60°C for 40 s and extension at 72 °C for 1 min, followed in turn by 30 cycles of 40 s at 94°C, 1 min at 51 °C and 1 min at 72°C and final extension was carried out at 72°C for 10min. PCR products were visualized on 2% gel and were sent for sequencing by using DBGB1, forward primer.

Sequence analysis

Molecular identification of six goat breeds was determined by alignment tool BLAST (Basic Local Alignment Search Tool) used by National Center for

Biotechnology Information (NCBI) and BOLD. All goat breed's *COI* gene sequences were exercised manually and didn't comprise interpolation and obliteration then aligned by using CLUSTAL W, as part of BioEdit (Thompson *et al*., 1994). Chromatograms were visualized on Finch TV software. Sequence divergences were computed by using the K2P distance model Kimura (1980). A Neighbour-Joining (NJ) tree was constructed for the graphical representation of interspecific and intraspecific divergences of species (Kumar *et al*., 2018). The NJ method was used to infer the evolutionary history followed Saitou and Nei (1987). The nearest-neighbour distance, the minimum genetic distance between the goat breeds and its closest relative was examined to test the discriminatory power of COI barcodes. A bootstrap consensus NJ tree of K2P distances was inferred from 1000 replicates (Felsenstein, 1985).

Specimen details, sequence information, and trace files are available within the DNA barcoding of goat breeds project in the published projects section of the barcode of life data systems (Bold, www.barcodinglife.org).

RESULTS

All the six breeds of goat showed 99% to 100% identity with *Capra hircus* (domestic goat) vouchered on Barcode library from different countries. The sequenced fragment of *COI* gene containing 528 nucleotides. Alignment results are presented in Table II. Multiple sequence alignment of goat breeds assessed the discrimination efficiency of *COI* gene (Sukontason *et al*., 2007). Two nucleotide variations were found in pure Angora and Angora Cross. In Angora and Angora cross at position 136 and 139 cytosine and thymine are replaced by thymine and cytosine, respectively. To determine the breed level efficiency of *COI* gene the multiple sequence alignment was performed between the same representatives of Angora. In which two variations were found. Prior nucleotide change was appeared in Angora 02 at nucleotide position 141 in which thymine was substituted while the new nucleotide change was observed in Angora 03 at nucleotide position 366 where tytosine is replaced with thymine. The rest of the species have a same *COI* gene sequence (Table III). The low genetic divergence suggested the close relationship among breeds (Table IV). An average K2P divergence of *COI* gene was 0.01% (Table V). In phylogenetic analysis (Table VI) Siberian ibex and Nubian ibex appeared as an outgroup and considered as the ancestor for two famous wild goats, ibex (*C. ibex*) and markhor (*C. falconeri*) while the bezoar was located in the cluster of domestic goat. Spanish ibex and the West Caucasian tur was appeared in the cluster of ibex and markhor, respectively. The average interspecific Kimura-

Table II. Molecular identification of goat breeds using BLAST tool on BOLD and NCBI.

Name of breeds	Similarity on NCBI	Identity %	Similarity on bold	Identity %
Angora	Capra hircus	99%	Capra hircus	99.81%
Angora cross	Capra hircus	100%	Capra hircus	100%
Barbari	Capra hircus	99%	Capra hircus	100%
Lehri	Capra hircus	99%	Capra hircus	100%
Pahari	Capra hircus	100%	Capra hircus	100%
Sindhi	Capra hircus	100%	Capra hircus	100%

Table III. MSA performed on BioEdit by using the tool CLUSTAL W. Multiple sequence alignment expressed the change of one nucleotide in Angora 02, Angora 03 and in Angora cross.

Breeds and accession number	No. of SNPs	Variable nucleotides and their positions		
		136	139	361
Angora 02	1	T	T	C
Angora 03	1	C	T	T
Angora cross	1	C	C	C
Barbari	0	C	T	C
Lehri	0	C	T	C
Pahari	0	C	T	C
Sindhi	0	C	T	C

Table IV. Intraspecific genetic distance: The pairwise distances were computed using the Kimura-2-parameter model and are in the units of the number of base substitution per site. An average divergence of 0.0016% was obtained. This analysis involves 7 nucleotide sequences.

Breeds	Pairwise distance						
	Angora 02	Angora 03	Angora cross	Barbari	Lehri	Pahari	Sindhi
Angora 2	-						
Angora 3	0.0038						
Angora cross	0.0038	0.0038					
Barbari	0.0019	0.0019	0.0019				
Lehri	0.0019	0.0019	0.0019	0.000			
Pahari	0.0019	0.0019	0.0019	0.000	0.000		
Sindhi	0.0019	0.0019	0.0019	0.000	0.000	0.00	-

2-parameter divergence was 0.03% (Table VII).

Table V. The pairwise distance was conducted using the Kimura-2-parameter model. This analysis involved 8 nucleotide sequences. The average genetic distance was calculated as 0.01%. There were total 528 positions in the dataset. Evolutionary analysis was conducted in MEGAX.

Breeds	Pairwise distance							
	sibirica	Angora 02	Angora 03	Angora cross	Barbari	Lehri	Pahari	Sindhi
Capra sibirica	-							
Angora02	0.045							
Angora03	0.050	0.003						
Angora cross	0.045	0.003	0.003					
Barbari	0.047	0.001	0.001	0.001				
Lehri	0.047	0.001	0.001	0.001	0.00			
Pahari	0.047	0.001	0.001	0.001	0.00	0.00		
Sindhi	0.047	0.001	0.001	0.001	0.00	0.00	0.00	-

DISCUSSION

The simplest test of species identification by DNA barcode is whether any of the species are found in Database library, all the species were in this study. All the six breeds of goat were identified as a *Capra hircus* the domesticated goat. None of the sequence identity was below 99%. For more vertible identification two genome browsers were used in comparison. In the second step, we found, that all the COI sequences within breeds were shared. The COI sequences in the six breeds of goat represented by four breeds were identical include Barbari, Lehri, Pahari and Sindhi breed. The one nucleotide difference was found in Angora 02 at nucleotide position 136 where cytosine is replaced with thymine and in Angora Cross at nucleotide position 140 the cytosine is replaced with thymine (Fig. 1).

Table VI. Interspecific breeds; For interspecific analysis the COI gene sequence of vouchered goat breeds was adopted from BOLD. These sequences were analyzed on BioEdit for multiple sequence alignment and on MEGA X to determine the evolution.

Scientific name	Common name	GenBank accession number
Capra hircus	Domestic goat/Goat	KT750040
Capra falconeri	Markhor	NC_020626
Capra ibex	Ibex	AB743816
Capra sibirica	Siberian ibex	NC_020626
Capra nubiana	Nubian ibex	NC_020624
Capra caucasica	West Caucasian tur	NC_020683
Capra pyrenaica	Spanish ibex, Iberian ibex	NC_020625
Capra aegagrus	**Bezoar**	NC_028161.1

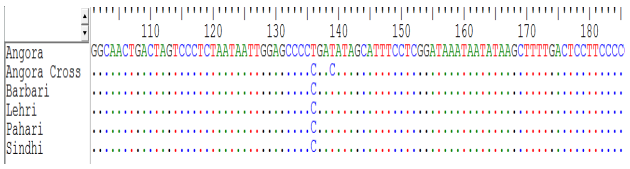

Fig. 1. Multiple sequence alignment of goat breeds represented one SNP.

Table VII. Interspecific Genetic Distance: The pairwise distance was analyzed by using Kimura-2-Parameter model and is in the number of base substitutions per site. This analysis involved 8 nucleotide sequences. The average genetic distance was 0.03%. *Capra sibrica* showed the basis of evolution with 0.05% divergence level. There were a total of 1542 positions in the final dataset, conducted on MEGAX.

Breeds	Pairwise distance							
	C. hircus	*C. ibex*	*C. falconeri*	*C. pyrenaica*	*C. nubiana*	*C. aegagrus*	*C. caucasica*	*C. sibirica*
Capra hircus	-							
Capra ibex	0.031							
Capra falconeri	0.020	0.030						
Capra pyrenaica	0.030	0.009	0.028					
Capra nubiana	0.032	0.037	0.032	0.036				
Capra aegagrus	0.001	0.032	0.021	0.030	0.033			
Capra caucasica	0.024	0.030	0.023	0.029	0.034	0.023		
Capra sibirica	0.054	0.054	0.055	0.052	0.058	0.057	0.059	-

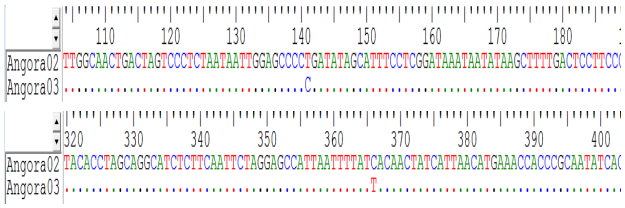

Fig. 2. Multiple sequence alignment between the different representatives of Angora breed (Intergeneric).

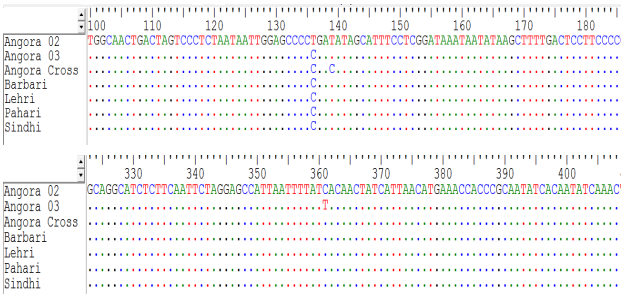

Fig. 3. Total two SNPs were found in the intraspecific Multiple sequence alignment of goat breeds.

In order to conservatively test the discrimination efficiency of mitochondrial *COI* gene, we established the intergeneric alignment. The representative of the study Angora 02 was used to examine the Angora03, and a variation of two nucleotides were found (Figs. 2, 3), and in this respect Figure 2 differ from Figure 3 by the multiple sequence alignment between the various representatives of only Angora breed (Intergeneric) rather intraspecific multiple sequence alignment of goat breeds. The measuring of differences between the multiple representatives of Angora cross was not assessed in the present study due to statute of limitation of time. However, this issue requires further investigation. Based on these results, 0.003% genetic distance was found among variant samples and the most congeneric species include Barbari, Lehri, Pahari and Sindhi showed no (0.00%) divergence. An average intraspecific distance was 0.0016%. The obtained data of our study are consistent with the results of studies by other scientists who demonstrated the great genetic distance of the most common dairy breeds (Saanen, Alpine, and Toggenburg) from the wool and milk-meat-wool goats grown in their breeding countries, such as Brazil (Araujo *et al*., 2006), Thailand (Seilsuth *et al*., 2016), China (Wang *et al*., 2017) and Russia (Kha and al-Sgfe, 2018). Phylogenetic analysis based on genetic distances between domestic goats and their wild relatives inveterate the origin of domestic goats from bezoar goats (*Capra aegagrus*).

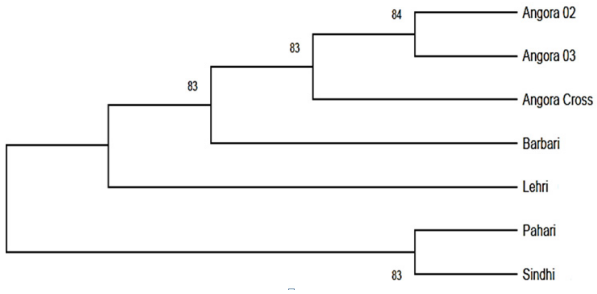

Fig. 4. Evolutionary analysis was conducted on MEGAX. The evolutionary history was inferred using the Neighbor joining tree method and the evolutionary distances were computed by using the p-distance method (d). The percentage of replicating trees in which the associated taxa clustered together in the bootstrap test of 1000 replicates. There were a total of 528 positions in the final dataset.

Bondoc and Cerbito (2013) reported the pairwise distances (d) between domestic goat breeds of Philipines, which ranged from d = 0.77 to > 1 unit based on 589 nucleotide positions support our findings. The N-J tree of intraspecific species fell into two cluster (Fig. 4), and the variant samples showed a substantial divergence from their sister cluster. All the seven samples were aligned with each other, but there were clear differences when compared to the wild species, *Capra sibirica* (Fig. 4). This observation is consistent with the fact that environment and genotype can interact to influence the degree of diversification among populations, as different ancestral genotypes show the greatest diversification across the two surface-type environments.

All goat breeds in the current study showed 0.04% divergence with the wild species except the one representative of Angora breed which showed 0.05% divergence and separated from the cluster, thus, appeared in the next cluster due to its change in nucleotide position 361. An average K2P divergence of *COI* gene was 0.01%. Similar study by Piras *et al*. (2012) reported 166 variable positions, and the allelic states at these positions were visually summarized in a file also reporting the agreement with the alignments obtained in previous works (Naderi *et al*., 2007; Pereira *et al*., 2009).

Our finding has shown the effectivity of *COI* gene sequence in vouchered goat species. All the 8 species of the goat that were examined possessed a *COI* gene barcode array which enables their identification. Our results were in general agreement with the pattern described in previous studies (Khan *et al*., 2024) where phylogenetic analysis through CO1 nucleotide sequences within nucleotide range 1-767 showed nine polymorphic sites segregating into eight haplotypes. The mean intraspecific diversity

and mean interspecific diversity were calculated as 0.23 and 2.36%, respectively. Phylogenetic tree revealed that *Capra ibex* and native surguli goat have common ancestors. The *COI* gene has successfully proven to distinguish between various ruminant species and could be consider as an efficient tool for the ruminants identification and their classification into different taxonomic categories (Nagarajan *et al*., 2020). Ali *et al*. (2016) also suggested that *COI* gene sequences can be used as a possible tool to differentiate local Pakistani goat breeds from the exotic ones.

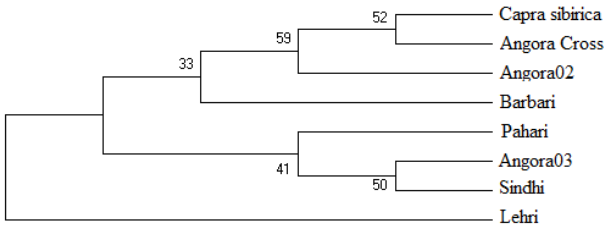

Fig. 5. Neighbor joining tree was constructed between the wild species and domestic goats and confirmed the lowest genetic distance (0.01%) as the *Capra sibirica* appeared in the same cluster with the domestic breeds of goat.

The evolutionary history was inferred using the neighbor-joining method. Out of 8 species, 6 showed a different pattern of variation, sequences fell into two clusters (Fig. 5). The results revealed that the genus *Capra* evolved from *Capra sibirica* which showed 0.05% divergence compare with other species. The Siberian ibex (*C. sibirica*) has been considered to be the strongest candidate for the ancestor of the domestic goat (*C. hircus*) (Fig. 5). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Fig. 6). These findings are in agreement with Piras *et al*. (2012) where they created a neighbour joining (NJ) tree based on the Kimura 2-parameters distance, using a gamma correction with a value of 0.17, identified 12 major clades and determined the robustness of haplotype clustering to produce a more manageable number of groups. A well-resolved phylogenetic tree can help to define the species taxonomy, population history, and evolutionary processes (Rumanta *et al*., 2020). In addition, Chen *et al*. (2008) reported that the domestic goat breeds, *Capra hircus* most likely descended from *Capra aegagrus*, which are still existing in Tibet and Inner Mongolia (Zeder and Hesse, 2000) and then spread rapidly due to human migration and trade activities (Harris, 1962; Luikart *et al*., 2001). This hypothesis not supports our results, which showed that *C. hircus* might be the patriarch ancestor of the *C. sibirica* (Siberian goat breeds), a wild goat species studied in this research, which has departed through adaption in diverse environment at different periods.

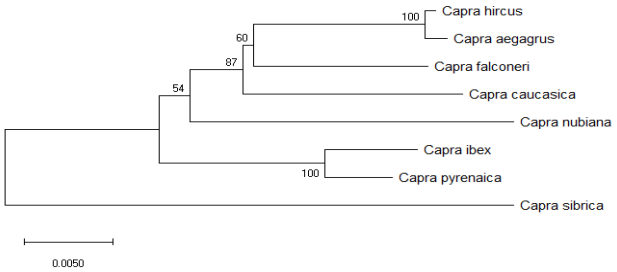

Fig. 6. The evolutionary history was inferred using the neighbor-joining method. The percentage of replicating trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the p-distance method (d). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. There were a total of 1542 positions in the final dataset.

However, to further investigate, compare *COI* gene sequence within species to geographic distances between the collection points for their specimens and find the relationships based on their differences and calculate the number of base substitutions. With just over 5000 species, the global mammal fauna represents a smaller challenge than the campaigns that seek to barcode all birds and fishes by 2011. As a consequence, despite a delayed start, it seems likely that a comprehensive barcode inventory for all mammals can reach closure by the same timeline.

In summary, we have established the potency of the *COI* gene for discrimination of the genus *Capra*. By extension, our results suggested that DNA barcode libraries will create a highly effective identification system for any regional goat fauna. We further conclude that the assembly of these local libraries will generate a substantial number of hypotheses regarding overlooked species.

DNA-based species identification depends on distinguishing intraspecific from interspecific genetic variation. The ranges of these types of variation are unknown and may differ between taxa. It seems difficult to resolve recently diverged species or new species that have arisen through hybridization. This point of view is supported by Cardoso *et al*. (2021) while valuating intraspecific admixture and interspecific hybridization levels in Iberian wild goats (*Capra pyrenaica*). The genotypic data showed strongly varied populations and low diversity of *C. pyrenaica*. Only 3/118 goats showed genomic evidence of mixed ancestry, although

rare, hybridization with domestic goats could become a potential threat to the genetic integrity of Iberian wild goats suggests to deter the presence of rampant herds of domestic or feral goats in mountainous areas inhabited by this iconic wild ungulate.

DNA barcoding does not work for hybrid species. Because mitochondrial DNA is maternally inherited. Thus, the paternal lineage is masked. There is no universal gene for DNA barcoding, no single gene that is conserved in all domains of life and exhibits enough sequence divergence for species discrimination. The validity of DNA barcoding, therefore, depends on establishing reference sequences from taxonomically confirmed specimens. That is likely to be a complex process involving co-operation between a variety of scientists and institutions.

A short DNA sequences about 500-1000bp from a standardized region of the genome provide a DNA barcode for identifying species. This essential basic of DNA barcodes limits their efficacy in resolving deep branches in phylogenies. Some controversy exists over the value of DNA barcoding, largely because of the perception that this new identification method would diminish rather than to enhance the traditional taxonomy based on morphology. Species determination is primarily based on the genetic divergence that could lead to incorrect recognition of species. However, we must keep open the possibility that the barcode sequences and their ever increasing taxonomic coverage could become extremely significant.

CONCLUSION

From the result of this study, it was concluded that less COI sequence divergence indicates that all the six breeds are similar to each other, and a shallow divergence of intraspecific species revealed that *Capra sibirica* formed the basis of genus *Capra*. In summary, we investigated the genetic distance among the various goat breeds of Balochistan, and the evolutionary basis of the genus *Capra* using the mitochondrial DNA fragment. A biological barcode library was made used DNA barcoding revealed six goat breeds and a phylogeny was generated for particular phyla that enlighten the animal kingdom.

DECLARATIONS

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Ethical statement

The protocol of the present re-search was approved by the committee for Advanced Studies & Research Board (AS-ARB) of SBK Women University, Quetta (SBK/Reg/GSO/ 297).

Ethics statement

The study followed ethical principles on the background of the current code of ethics that may apply to genetic diversity researchers.

Statement of conflict of interest

The authors have declared no conflict of interest.

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