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MITOCHONDRIAL *ATP6* AND *ATP8* GENES BASED MOLECULAR DIVERSITY AND PHYLOGENETIC ANALYSIS IN PUNJAB URIAL (*Ovisvigneipunjabiensis*)

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ABSTRACT

The taxonomy of wild sheep has been a complex debate with different classifications and revisions suggested based on their morphology, geographical distribution and chromosome number. In Pakistan the Punjab Urial (*O. vigneipunjabiensis*), is an important sub-species of *O. vignei*, however a scarce molecular data is reported that urged us to investigate its genetic diversity and phylogeny using mitochondrial DNA, *ATPase6* and *ATPase8* genes. A total of 842 bp complete coding region of both genes were amplified by PCR followed by sequencing. The sequences were aligned and edited using CodonCode Aligner and single nucleotide polymorphisms (SNPs) were identified. The boot strapped Neighbor joining tree constructed from MEGA6.1 explained the genetic relationships of *O. v. punjabiensis*is with *Ovisammon* (Argali) than the *Ovisaries* (sheep). The phylogenetic analysis also showed the genetic positioning of Punjab Urial with respect to other 23 different reported mammalian species as well. The study gave us useful genomic information about genetic diversity in Punjab Urial and its phylogenetic relationships with related taxa, emphasizing on need of executionof conservation strategies to protect this unique genetic resource of Pakistan.

Key Words: Punjab Urial, Mitochondrial *ATPase6* and *ATPase8* genes, Polymorphisms, Molecular diversity, Phylogenetics

INTRODUCTION

Punjab Urial (*Ovisvigneipunjabiensis*) is an endemic and endangered sheep breed belonging to Pakistan which is facing serious threat of extinction due to poaching, grazing, exploitation of natural resources, pet trade, infrastructure construction projects, road vehicles, habitatsloss,fast urbanization and various agricultural practices (Hess *et al.,* 1997; Awan *et al.,* 2005; Awan, 2006; Valdez, 2008; Ayaz *et al.,* 2012). This medium-sized wild sheep belongs to large family Bovidae consisting of 140 species. There are six to nine species of *Ovisorientalis* present which have different color and size of their winter neck ruff of males, saddle patches and horns color. Urial closely resembles with the Marco Polo sheep in general body texture and colour. In Pakistan three sub species of Urial *Ovisorientalisvignei*, *Ovisorientalisblanfordi* and *Ovisorientalispunjabiensis* have been identified in Gilgit, Baluchistan and Punjab, respectively. These three sub species can be differentiated by color of ruff (Roberts, 1977).

The advent of Polymerase Chain Reaction (PCR) has revolutionized molecular marker based studies on population genetics (Sunnucks, 2000; Nagaraju*et al*., 2000). Molecular markers have been playing a significant role in unfolding the relatedness within closely related species (Loxdale and Lushai, 2007;Arif and Khan, 2009). Mitochondrial DNA (mtDNA), only genetic material found outside the nuclear DNA on cells has been widely used for assessing the genetic relationship in animals, plants and microbes (Stoneking and Soodyall, 1996; Chinnery and Schon, 2004; Knudsen *et al.,* 2006; Morin *et al.,* 2010; Storey *et al.,* 2013). In comparison to some of the other markers, they possess useful characters such asfast evolutionary rate (high copy rate per cell: normally 10^3 to 10^4 per cell), small molecular weight, simple structure, easy isolation, not showing tissue specific and low recombination rate (Castro *et al.,* 1998; Curole and Kocher, 1999;Shin *et al.,* 2004; Wan *et al.,* 2004; Arif and Khan, 2009; Patwardhan *et al*., 2014; Pilli *et al.,* 2014). Therefore, mitochondrial DNA sequences are preferred over other markers for the studies related to molecular diversity and phylogenetic studies(Avise, 2004; Abdel-Mowgood, 2012).Animal mitochondrial genes ATPase 6 and ATPase 8 typically possess features to accumulate nucleotide substitutions that allow assessing genetic polymorphism between species and as

well within populations of same species (Avise 2000; Barraclough and Nee, 2001; Perdices and Doadrio, 2001; Wong *et al.,* 2004). Even though it has been identified as a coding region, nucleotide substitution of this region can be comparable with the control region (D-loop) of these genes which is considered as the most variable segment of the mtDNA (Bernatchez and Danzmann 1993; Ray *et al.,* 2004; Shin *et al.,* 2004;Faulks *et al.,* 2008; Pilli *et al.,* 2014).

Punjab Urial, being an animal of special interest in the wild mammals of Pakistan, there is scarcity of genomic diversity studies and reports to explore the molecular genetic diversity and phylogeny in comparison of other mammals. In this context we explored the mitochondrial *ATPase6* and *ATPase 8* genes in Punjab Urial from different locations to have insight about its genetic architecture by measuring polymorphisms and phylogenetic relationships within Urial populations and related mammalian species.

MATERIALS AND METHODS

Sample collection and DNA isolation: Samples were collected from 21 Punjab Urial from different locations after the official permissions from the concerned authorities in the country. The samples were taken from captive as well as from wild animals(Table1). The DNA was extracted by method as used by Hussain *et al.*(2013). DNA samples were then quantified through Nano Drop 2000/2000c (Thermo scientific USA).

Table 1: Sampling details of Punjab Urial samples used in this study

Sample ID	Species	Sex	Location	Google coordinates
1U	Punjab Urial	Male	Khokhar Zair, Chakwal, Punjab	32°48'9.9"N 72°51'28.3"E
2U	Punjab Urial	Female	Khokhar Zair, Chakwal, Punjab	32°48'9.9"N 72°51'28.3"E
4U	Punjab Urial	Female	Bhalwal, Sargodha, Punjab	32°15'37.1"N 72°53'59.0"E
5U	Punjab Urial	Female	Bhalwal, Sargodha, Punjab	32°15'37.1"N 72°53'59.0"E
9U	Punjab Urial	Female	Thatha, Attock, Punjab	33°39'31.1"N 70°34'55.9"E
11U	Punjab Urial	Male	Kala Bagh, Mianwali, Punjab	33°52'53.0"N 72°15'02.7"E
12U	Punjab Urial	Male	Kala Bagh, Mianwali, Punjab	32°57'53.4"N 71°33'24.2"E
13U	Punjab Urial	Female	Kala Bagh, Mianwali, Punjab	32°58'31.0"N 71°33'30.6"E
14U	Punjab Urial	Female	Kala Bagh, Mianwali, Punjab	32°57'53.4"N 71°33'24.2"E
15U	Punjab Urial	Female	Pipplaan, Mianwali, Punjab	32°16'57.9"N 71°21'56.7"E
16U	Punjab Urial	Male	Nizampur, Attock, Punjab	33°52'21.4"N 72°12'46.5"E
18U	Punjab Urial	Male	Bhalwal, Sargodha, Punjab	32°15'37.1"N 72°53'59.0"E
19U	Punjab Urial	Male	LoiBher Wildlife Park, Rawalpindi, Punjab	33°57'49.5"N 73°11'93.1"E
20U	Punjab Urial	Male	LoiBher Wildlife Park, Rawalpindi, Punjab	33°57'49.5"N 73°11'93.1"E
22U	Punjab Urial	Female	Jehlum, Punjab	32°94'04.3"N 73°75'65.3"E
24U	Punjab Urial	Female	LoiBher Wildlife Park, Rawalpindi, Punjab	33°57'49.5"N 73°11'93.1"E
25U	Punjab Urial	Female	LoiBher Wildlife Park, Rawalpindi, Punjab	33°57'49.5"N 73°11'93.1"E
2UZ	Punjab Urial	Female	Lahore Zoo, Punjab	31°55'53.2"N 74°32'53.8"E
3UZ	Punjab Urial	Male	Lahore Zoo, Punjab	31°55'53.2"N 74°32'53.8"E
7UZ	Punjab Urial	Female	Lahore Zoo, Punjab	31°55'53.2"N 74°32'53.8"E
8UJ	Punjab Urial	Male	Jotana House, Lahore, Punjab	31°57'55.8"N 74°47'75.0"E

Specific Primer designing: A specific pair of primers(ATP8/6-Fw5'AGCCATGACCCCTCCTTAGT3' and ATP8/6-Rev 5'TGGTATGCGTGAGTCTGGTG3') was designed from NCBI, GenBank sequence database (www.ncbi.nlm.nih.gov) from Accession Number: JN632608 using Primer fox software (www.primerfox.com) that amplified 890 bp portion including 842 bp coding region of *ATPase6* and *ATPase8* mitochondrial genes of Punjab Urial.

PCR Amplification, Sequencing and Bioinformatics Analysis: For the amplification of mitochondrial *ATPase 6* and *ATPase8* genes the PCR was carried out using BioRad (USA) thermocycler in a reaction volume of 25 µL containing genomic DNA, PCR buffer, dNTPs, $MgCl_2$, forward and reverse primers, *Taq* DNA polymerase (Fermentase, Germany) and nuclease-free water. The conditions used were: initial denaturation 95˚C for 5 min, followed by 30 cycles of 94˚C for 45 sec; 54˚C for 45 sec; 72˚C for 1 min and final extension at 72˚C for 10 min. The PCR products (4 µL of PCR product and 2µL of loading dye mixed) were run on 1.5 % Agarose gel at 100 Voltages for 45 min in 1X TAE buffer and seen by gel documentation system (BioRad, USA) under UV light. The positive samples were decontaminated using DP203-TIANquick Mini

Purification Kit (China) and sent for sequencing to 1st Base Singapore.

The obtained sequences were aligned with the help of online NCBI BLAST (http/www.ncbi.nlm.nih.com) to see relevant reported sequences. The sequences were trimmed, aligned and editedthrough Codon Code Aligner version 3.7.1 software. The finally selected 842 bp was used for the identification of single nucleotide polymorphisms. DnaSP v. 5software (Librado and Rozas, 2009) was used to reconfirm SNPs and to observe haplotype, nucleotide diversity and neutrality tests. MEGA 6 program package (Tamura *et al.*, 2011) was used to construct UPGMA (Unweighted Pair Group Method using Arithmetic Mean) phylogenetic trees (1000 bootstrap value) for Punjab Urial and other GenBank, NCBI reported mammalian species.

RESULTS

Single Nucleotide Polymorphisms: SNPs were identified by using DnaSP (Librado and Rozas, 2009) in Punjab Urial 842 bp aligned fragment of *ATP6* and *ATP8* sequences. There were 829 invariable (monomorphic) sites while 13 variable (polymorphic) were observed out of which 2 were Singleton variable sites and 11 were Parsimony informative sites with two variants (Table 2).

Haplotype/Nucleotide Diversity: In Punjab Urial 21 samples we identified 6 haplotypes (h) with 0.552 haplotype (gene) diversity (hd), the variance of Haplotype diversity was found 0.01477, with standard deviation of 0.122, and per site nucleotide diversity (P_i) 0.00334. The Sampling variance of P_i was calculated as 0.0000018 and Standard deviation of P_i was 0.00133.

Based on DnaSP results the value for Tajima's D was calculated as -0.79706 (not significant, P > 0.10), Fu and Li's D* test 0.70158 (not significant, P > 0.10), Fu and Li's F* test statistic 0.30524 (not significant, P > 0.10), Fu's Fs statistic 0.806 and Strobeck's S statistic 0.520.

Table 2: Single Nucleotide Polymorphisms (SNPs) Identified in 842 bp fragment of ATP6 and ATP8 sequences in 21 Punjab Urial samples.

Nucleotide Position	Variation
45	Parsimony informative site
47	Parsimony informative site
57	Parsimony informative site
100	Parsimony informative site
254	Parsimony informative site
257	Parsimony informative site
275	Parsimony informative site
366	Parsimony informative site
390	Parsimony informative site
449	Parsimony informative site
494	Parsimony informative site
634	Singleton variable site
692	Singleton variable site

Distance Analyses and Visualization: The matrix constructed using bioconductor an open source softwares for bioinformatics using"R"statistical package containing entries in the case of 3UZ (Lahore Zoo), 8UJ (Jotana House, Lahore) and 15U (Wild) while referring them a phylogenetic distance was plotted with respect to other selected species (Figure 1).

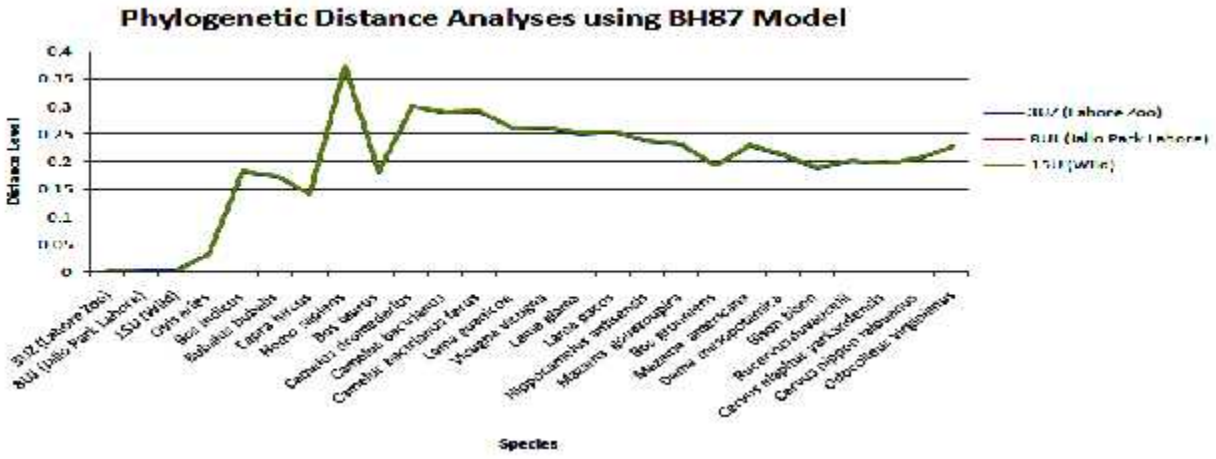

Figure 1: The distance plot constructed by taking 3UZ (Lahore Zoo), 8UJ (Jotana House, Lahore) and 15U (Wild) as reference species and compared with selected mammalian species.

In the above figure, the horizontal axis was calibrated for species and phylogenetic distance was placed on vertical side by using *ATPase 6* and*8*gene sequences against species. Any two sequences, which are capable to undergo less number of variations corresponding to each other are provided with small index value with respect to the other member sequences of different organisms. Similarly, sequences with larger number of substitutions present between them are regarded as distant and assigned a higher phylogenetic distance value.

Here, it is interesting to see that *Ovisaries* was found to be the closest relative of 3UZ, 8UJ and 15U, as indicated with a steep line between *Ovisaries* and Urials on the basis of *ATPase 6* and *8*gene sequences. There was a regular trend seen in all species might be due to their own lineage specific variation except *Ovisaries* and *Homo sapiens*, out of these two, human underwent the largest number of variations in the region under observation,that is why it isvisible in the form of highest peak while the previous was found to be the closest relative of Urial given a spot steeper with respect to other species (Figure 2).

Multidimensional Scaling Plot: Multidimensional scaling is sophisticated tool to get symmetrical variations in a dataset. MDS was performed using mathematical transformation using R statistical package. Furthermore, the analysis was designed in such a way that each data point (species) would be spotted and shown its unique color. During MD scaling here in two eigenvalues were implicitly computed and scaled to get two linear combinations of the phylogenetic distance variation data. Linear combination in 1st dimension was obtained by using 1^{st}eigen value while 2^{nd} dimension transformation vector was computed with the help of 2^{nd} value. In the similar way up to 'n-1' eigen values could be achieved to get 'n-1' number of shape linear transformations. After obtaining data in two new shapes, they were clustered based on small values between them. In the plot, codes were also elaborated on its right hand side (Figure 3).

In total, 26 *ATPase 6* and *8* sequences were previously analyzed to get evolutionary distance matrix are employed to get MDS profile and plotted in figure 2 (Cox and Cox, 2000). Among those, *Homo sapiens* could be spotted as a very much different data point because it did not cluster with any of the other data point entries. Three Punjab Urial samples were implicitly placed nearly the same spot with *Ovisaries* and *Capra hericus* also lying there in the same group (Figure 4).

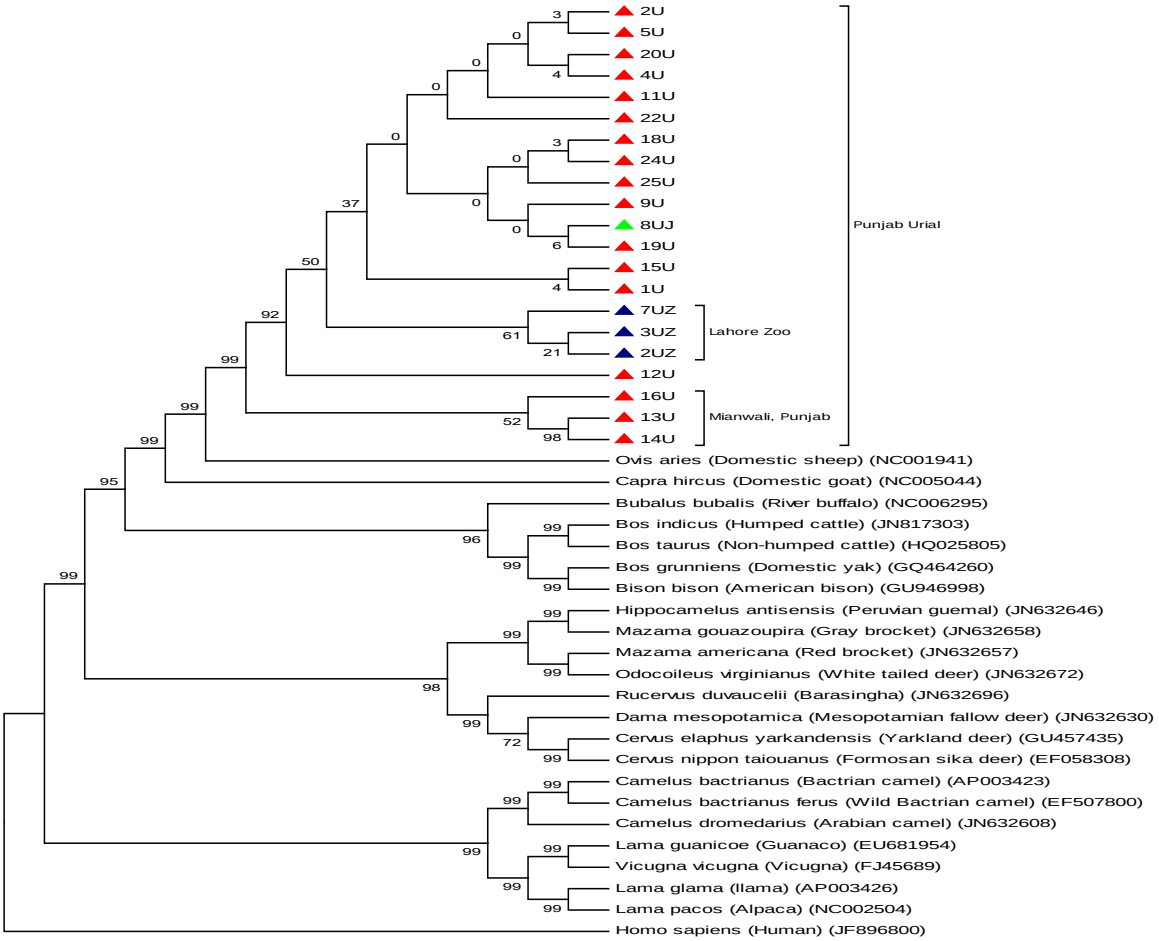

Figure 2:UPGMA Tree constructed with MEGA6 using mitochondrial *ATPase6*and*ATPase8* genes sequences from Punjab Urial and NCBI reported mammalian sequences.

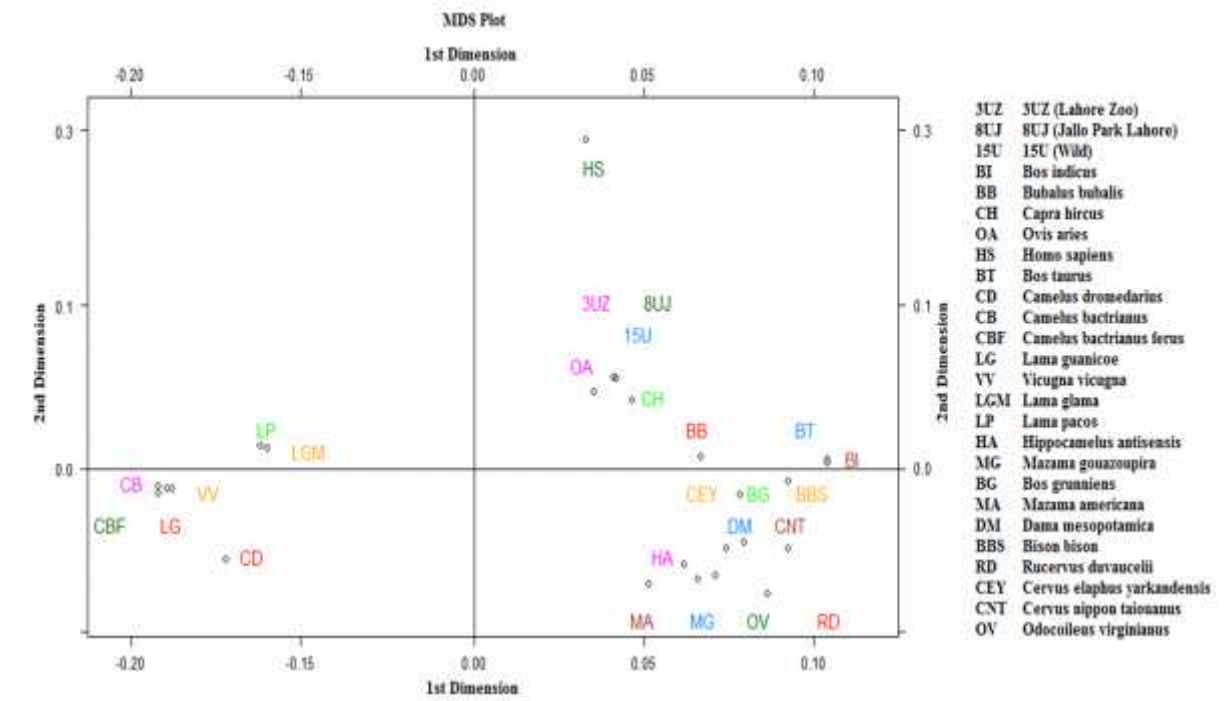

Figure 3: Multidimensional scaling plot is shown in the figure to elaborate the things related to association of species with respect to increasing number of variations in *ATPase6* and *ATPase8* genes of various mammals. The figure is generated using the 1st and 2nd dimensional linear combinations achieved through MDS analyses.

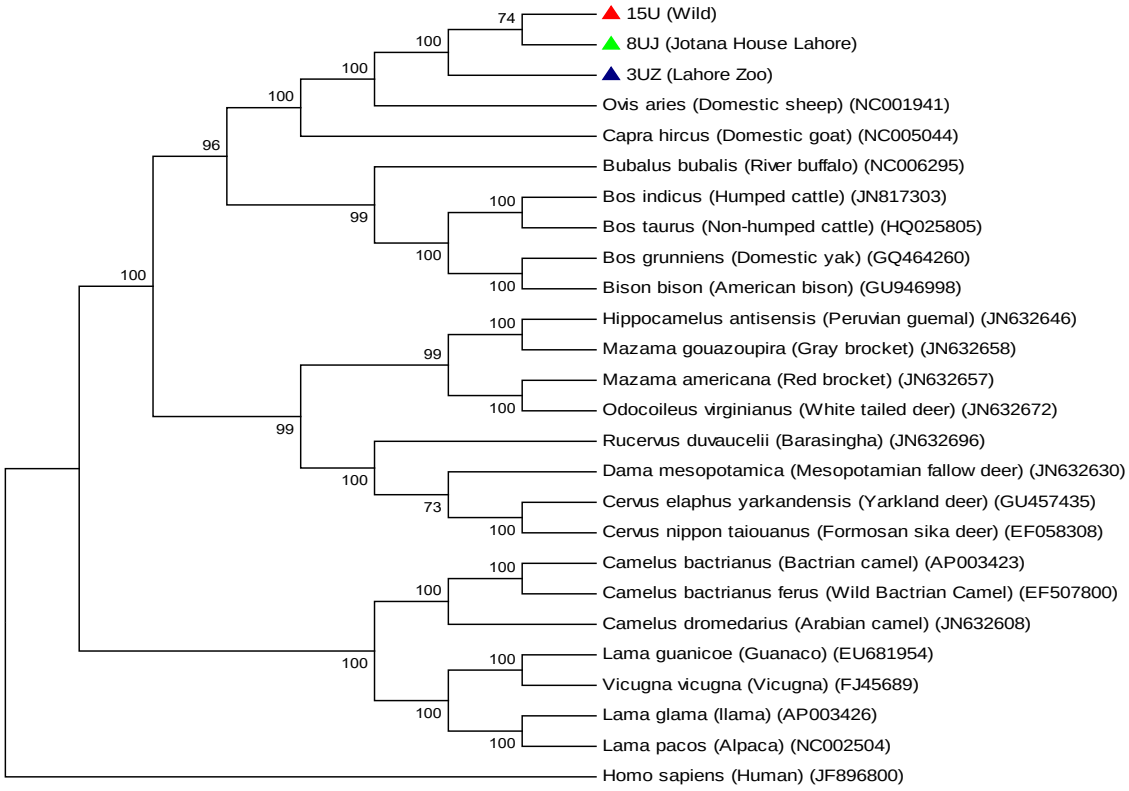

Figure 4: UPGMA Tree constructed with MEGA v.6 using mitochondrial *ATPase6*and*ATPase8* genes sequences of representative Punjab Urial from wild, Jotana house and Lahore zoo with NCBI reported mammalian sequences.

DISCUSSION

On planet, a total of 11046 species of animals and plants are threatened and havingdanger of extinction in near future mainly because of human activities in spite of use of modern reproductive biotechnologiesto conserve endangered species with marginal success (Ptak*et al.*, 2002). Among these species the Punjab Urial is an endangered, endemic and unique mammal found in Pakistan needing considerable attention for its protection.

In the past, this important animal has not been considered for its characterization and evaluation level at which this study has been conducted. Previously mtDNA has been widely used to investigate closely related domestic animals (Achilli *et al.*, 2009; Meadows *et al.*, 2011; Achilli *et al.*, 2012).Here, DNA samples of several species were isolated and the molecular diversity and phylogenetics were explored using*ATPase*6 and *8* mitochondrial genes in Punjab Urial of Pakistan. The phylogenetic analysis (Figure-4) showed the genetic positioning of Punjab Urial with respect to other 23 different reported mammalian species as well.

The phylogenetic tree along with the constructed MDS plot (Figure-3) showed that *Ovisaries* is the closest relative of Urial. This might be due to lack of significant number of variations between them which indicate that they probably share their common ancestorin this investigation which is evident from other related studies as well.

Variation pattern among other species revealed that they have adapted to the environment independently according to their unique capacity which represents that they have achieved lineage specific variations in that particular genetic region as they are distant equally but unique from Urial. There is need to exploremore from this important genetic resourceas it is disappearing from its community habitat.

Conclusion: Owing to small population size and remote habitat distribution of the Punjab Urial it should be considered as a rare animal to get attention for suitable conservation actions. This study demonstrates the genomic diversity and taxonomic connections of the Punjab Urial with other mammals. Our study provided useful material for supporting conservation plans for this important animal, however further genomic investigations should be carried out at larger scale.

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