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Abstract: We have characterized the endangered Punjab urial via its interferon alpha-A gene. DNA extraction was done from blood, followed by PCR and sequencing using a 3130xl genetic analyzer. Single nucleotide polymorphism and phylogenetic analyses were performed with BioEdit 7.0.9.0 and MEGA 6.1. The highly polymorphic nature revealed will lead to identifying molecular markers for breeding purposes.

Key words: Punjab urial, interferon, polymorphic, phylogenetic tree, endangered, Pakistan

The Punjab urial (a type of wild sheep; *Ovis vignei punjabiensis*) is facing a serious threat of extinction in Pakistan. This medium-sized wild sheep belongs to the large family Bovidae, which consists of 140 species (Glazko et al., 2011). It is distributed in the regions of western central Asia from northeastern Iran and western Kazakhstan to Pakistan's Balochistan and the Ladakh region of northern India. The urial possesses interesting physical features, such as reddish-brown long fur that fades during the winter, gregarious behavior, and sexual dimorphism (Aleem, 1977; Schaller, 1977). Males weigh about 40 kg and have large curly horns up to 80 to 100 cm long, whereas adult females weigh about 25 kg and have horns that are straight and only 12 cm long. Females give birth to 1 or 2 lambs in early April (Awan, 2001). Males are characterized by a black ruff stretching from the neck to the chest and the significantly larger horns. There are 6 to 9 species of *Ovis orientalis*, which have different coloration and sizes of the winter neck ruff of males and saddle patches, and varied horn color. Urials closely resemble Marco Polo sheep in general body texture and color. In Pakistan, 3 subspecies of urial (*Ovis orientalis vigeni*, *Ovis orientalis blanfordi*, and *Ovis orientalis punjabiensis*)

have been identified in Gilget, Baluchistan, and Punjab, respectively. These 3 subspecies can be differentiated by the color of the ruff (Roberts, 1977). Unfortunately, the study of these fascinating animals has been very limited, and according to the Red List of the International Union for Conservation of Nature (IUCN), they are endangered. Therefore, studies related to these endangered but taxonomically impressive animals require more attention from the scientific community (Valdez, 2008).

Interferons (IFNs) belong to the large family of multifunctional proteins known as cytokines, which play critical roles in mediating antiproliferative, antiangiogenic, gene-modulatory, and immune-regulatory responses as the first defensive line against viral infections, among other immune-relevant functions (Pereiro et al., 2008). IFNs can be categorized into 2 broad groups, type I (INF or IFN) and type II (IFN), which act on independent cell-surface receptors, activating the transcription of distinct but interrelated sets of genes. Additionally, IFNs play a role in improving the defenses of the host by upregulating antigen presentation by major histocompatibility complex (MHC) antigens (Mantegazza et al., 2013). It is suggested by the evidence that genetic diversity is very important in

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vertebrates at the level of the MHC; hence, genes of the MHC are considered to be the best candidate genes for studies of molecular mechanisms in vertebrates (Sommer, 2005). The present study on the IFN α -A gene in the Punjab urial of Pakistan will be significant for further studies on immunity genes to explore the genetic makeup of not only sheep, but also other mammals.

Animals with typical phenotypic features of the Punjab urial were selected from several respective breeding areas such as the Salt Range (District Chakwal: Choa Saidan Shah, Kallar Kahar, and Lillah; District Jehlum: Pind Dadan Khan) and the Kala Chitta Mountains (District Attock), as well as zoos (Islamabad, Lahore, and Bahawalpur). Aseptic blood samples were collected from the jugular veins of the urials; these 3-mL blood samples were kept in 15-mL Falcon tubes containing anticoagulant (200 μ L) with ethylenediamine tetraacetic acid (0.5 M EDTA). Samples of blood were placed on ice just after their collection and then brought to the laboratory. Before extracting DNA, these samples were stored in a freezer at -20°C . All selected and sampled urials were handled with care, fulfilling the pertinent guidelines. Animal ID, sex, breed, location of animal, and age were recorded. The authors abided by the policies of the ethical committee of the Virtual University (VU) of Pakistan for vertebrate animal research. The reference number of approval of the VU ethical committee is VUEC-19, dated on 25-09-2014.

Genomic DNA was extracted from blood samples using standard protocol, which involved lysis of white blood cells, digestion of protein, and finally precipitation after isolation of DNA and purification. Dissolved DNA samples in TE buffer (pH 8.0) were stored at -20°C for use in the future. DNA samples were quantified with the help of agarose gel electrophoresis (0.8%); for comparison, a standard DNA/DNA ladder was used. All samples were brought to the same concentration level of 50 ng/ μ L.

Specific primers were designed using Primer 3 software and the Insilico PCR web facility (Rozaan and Skaletsky, 2000) for the IFN α -A gene from a previously reported sequence (Accession No. NM_001017411.1) available from GenBank, National Center for Biotechnology Information (NCBI). For the amplification of the IFN α -A gene (401 bp) through a thermocycler (Bio-Rad, USA), genomic DNA, a set of primers, dNTPs, PCR buffer, MgCl_2 , nuclease-free water, and DNA polymerase were used according to standard protocol. The PCR product was analyzed through 1.5% agarose gel electrophoresis, and the amplified bands were visualized under UV light using a gel documentation system (Bio-Rad).

The amplified PCR products were precipitated with 80% ethanol and were dissolved in a final volume of 10–15 μ L of deionized water. The quality of the DNA was checked

on 2% agarose gel before sequencing using an ABI PRISM 3130 XL genetic analyzer (Applied Biosystems, USA).

Almost invariably as a result of human activity, a total of 11,046 species of animals and plants are threatened and face a high risk of extinction in the near future. Although the potential use of reproductive biotechnologies for safeguarding endangered wildlife species is indisputable, practical efforts have met with limited success to date (Ptak et al., 2002). The Punjab urial is one of the endangered and endemic animals found in Pakistan with some unique physical features.

Comparison of urial sequences was done with interferon alpha-A sequences of other species. It was observed that the sequences of 2 species (i.e. Indo-Pacific humpbacked dolphin and bottlenose dolphin) were entirely different from all other sequences at various positions, i.e. nucleotide positions 96, 101, 103, 105, 106, 107, 113, 115, 116, 117, 118, 119, 120, 121, 123, 124, 125, 126, 127, 128, 129, 130, 132, 134, 136, 140, 141, 142, 146, 150, 151, and 152 (Table).

To better understand the evolutionary relationship among urials of Pakistan, a phylogenetic tree (Figure) was constructed using the neighbor-joining method implemented in MEGA 6.1 (Tamura et al., 2011). The reliability of the branches was assessed by bootstrap analysis. Comparison was done with *Bubalus bubalis*, *Sousa chinensis*, *Delphinapterus leucas*, *Orcinus orca*, *Odocoileus virginianus*, *Bos grunniens*, *Capra hircus*, *Ovis aries*, *Bos taurus*, and *Tursiops truncatus* as the outer group. It is clear from the analysis of the phylogenetic tree that the domestic sheep is closer to the urial than other species that were in the outer group.

However, a mutation at position 121 was also observed in beluga whale, killer whale, white-tailed deer, water buffalo, and domestic yak, as well as in Indo-Pacific humpbacked dolphin and bottlenose dolphin; the SNP at nucleotide position 140 was also observed in some species such as domestic yak, domestic sheep, Indo-Pacific humpbacked dolphin, and bottlenose dolphin. Among these mutations, some were transitional mutations, while others were of transversional nature, as described in detail in the Table. This study clearly described the need for the conservation of this endangered and highly valuable fauna. The relevant authorities should consider conservation strategies, as it's a "now or never" situation.

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Table. Polymorphic sites detected in the IFN α -A region of Punjab urial sequences.

Name of breed	SNPs position	SNP nucleotide	Changes with	Transition or transversion
Indo-Pacific humpbacked dolphin	121	A	T	Transversion
	140	G	C	Transversion
Bottlenose dolphin	121	A	T	Transversion
	140	G	C	Transversion
Beluga whale	121	A	T	Transversion
Killer whale	121	A	T	Transversion
White-tailed deer	121	A	T	Transversion
Water buffalo	121	A	T	Transversion
Domestic yak	121	A	T	Transversion
	140	G	A	Transition
Domestic goat	-	-	-	-
Cattle	121	A	T	Transversion
Domestic sheep	140	G	A	Transition
22U	-	-	-	-
5UZ	-	-	-	-
21U	-	-	-	-
6UZ	-	-	-	-
2U	-	-	-	-
24U	-	-	-	-
25U	-	-	-	-
20U	-	-	-	-
3U	-	-	-	-
11U	-	-	-	-
19U	-	-	-	-
1U	-	-	-	-
18U	-	-	-	-
12U	-	-	-	-
3UZ	-	-	-	-

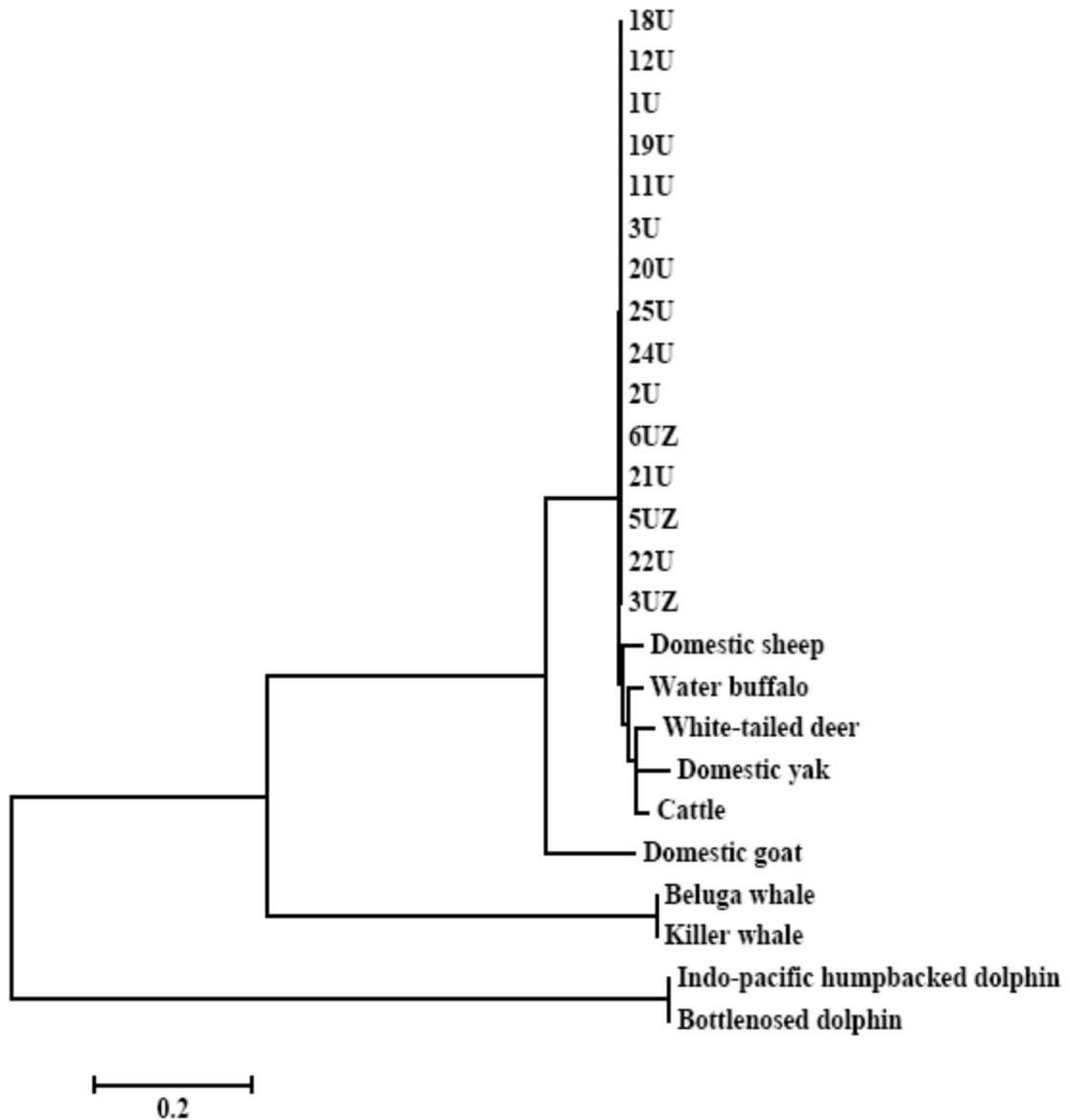


Figure. Interferon alpha-A based phylogenetic tree (neighbor joining method) constructed by MEGA 6.1 for Punjab urial in comparison with other mammalian species sequences available from GenBank (NCBI).

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