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Original article

ANALYSIS OF MOLECULAR GENETIC DIVERSITY OF ENDANGERED PUNJAB URIAL (*OVIS VIGNEI PUNJABIENSIS*) BASED ON INTERLEUKIN 2 GENE SEQUENCES

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Summary

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Punjab urial (*Ovis vignei punjabiensis*) is endemic to wild northern Punjab, Pakistan and also an endangered species according to red list categories of International Union of Conservation of Nature and Natural Resources. Better understanding of genetics of immune response of this species can be helpful to design effective conservation strategies. The objective of this study was to assess the molecular genetic diversity on interleukin 2 (IL-2) gene sequences of endangered Punjab urial (*Ovis vignei punjabiensis*) as a gene encoding a cytokine involved in some vital activities of immune response regulation. The IL-2 gene (492 bp) was amplified and sequenced in DNA samples collected from wild as well as captive *O. v. punjabiensis,* followed by alignment and phylogenetic analysis. The neighbour joining tree constructed from MEGA6 showed that *O. v. punjabiensisi* was closer to *Ovis ammon* (Argali) than the *Ovis aries* (sheep); the phylogenetic tree also depicted *O. v. punjabiensis* relationships with other mammalian species. The analysis of study results showed that *O. v. punjabiensis* is a unique isolated population found in Pakistan which is endemic as well as endangered. The species is closely related to Argali and sheep but has differences in the genetic makeup. Therefore, *in-situ* and *ex-situ* conservation techniques for Punjab urial present a good solution to preserve this endangered species from extinction.

Key words: endangered species, interleukin 2, Pakistan, phylogeny, Punjab urial, wildlife

INTRODUCTION

Biodiversity conservation and maintenance of resources for the future, better understanding and dynamics of the animal populations involved are major milestones that guide the complex domestication process (Bruford *et al.* 2003). Goats and sheep are the earliest animals domesticated at the end of Mesolithic period, around 10,000 years ago in Fertile Crescent (Zeder & Hesse, 2000; Zeder *et al.* 2006; Zeder, 2008), originating from at least three ancestral subspecies (Urial, Argali and Mouflon) (Bruford & Townsend, 1997; Pedrosa *et al.* 2005; Chessa *et al.* 2009). Furthermore, domesticated sheep and their wild ancestor, urial, are considered as two different species by some taxonomists, called *Ovis aries* and *Ovis orientalis* (scientific synonym: *Ovis vignei* Blyth, 1841), respectively (Shackelton, 1997; Grubb, 2005).

Researches on genetics of small populations that generally consist of limited number of individuals with breeding capacities suggest that these kinds of populations are highly vulnerable to the loss of genetic polymorphism. Vulnerability of small pockets of populations to extinction from stochastic events increases many times when their genetic diversity combined with inbreeding depression shows lower values (Lynch, 1996; Lacy, 1997). Lynch (1996) suggested that a minimum of 10,000 adults are required to protect adaptive genetic variation in a breeding population, whereas 1000 individuals are needed to protect against the fixation of deleterious genes in a population. Unfortunately, in an endangered or fragmented species, such population sizes are rarely encountered (Hedrick & Kalinowski, 2000; Larson, 2012). *O. v. punjabiensis* stands at a critical juncture for its survival because estimation number is decreasing in a rapid phase (Table 1); the continuous decline of its population necessitates an investigation of its genetic diversity as well as taxonomic identity. According to Table 1; *O. v. bocharensis* from Eastern Europe such as Tadjikistan, Turkmenistan and Uzbekistan in critical level of its survival.

Effective immune system counteracting pathogenic viruses, microorganisms and parasites is a fundamental requirement for the survival of an organism (Turner *et al.* 2011). Interleukins are a group of cytokines (secreted proteins/ signalling molecules) expressed by leukocytes, where they are recognised as regulators of inflammatory and immune responses (Sotiriou & Makris, 2013). Interleukin-2 (IL-2) plays a very important role

Table 1. Subspecies of Urials, their global distribution and population estimates

*Ovis v.*subspecies	Global distribution	Estimated population
O. v. arkal	Iran, Kazakhstan, Turkmenistan and Uzbekistan	< 11,000
O. v. bocharensis	Tadjikistan, Turkmenistan and Uzbekistan	< 1,000
O. v. cycloceros	Afghanistan, Pakistan, Turkmenistan	< 12,000
O. v. gmelinii	Iran, Azerbaijan	< 2,000
O. v. vignei	India, Pakistan	< 1,500
O. v. punjabiensis	Pakistan	~ 1,500

in T-helper cell defense, especially in immune response to infectious diseases. T-cell growth factor, known as interleukin 2, is a lymphokine produced by mitogen activated T-cells (Gillis *et al.* 1978; Bressani *et al.* 2014). At present, limited published information is available in the relevant scientific literature based on this important gene diversity and polymorphism in animals (Turner *et al.,* 2011). In cattle, radioactive *in situ* hybridisation analyses showed that IL-2 gene was localised to the q22-->q23 bands of chromosome 17. Gene location homology and increasing evidence for chromosomal band formation within the Bovidae suggests that the IL-2 gene maps to chromosome 17 in goats, buffaloes and sheep (Chowdhary *et al.,* 1994). Since the IL-2 gene evolves at a rapid pace in ruminants, study of this gene could give more insight on adaptive selection over short evolutionary period (Zelus *et al.,* 2000). Therefore, this investigation was aimed to determine the origin and genetic diversity of endangered Punjab urial based on IL-2 gene.

MATERIALS AND METHODS

Samples collection

Eight unrelated (n=8) individuals of Punjab urial with typical phenotypic features were collected from the wild as well as from captive locations after rather extensive field search in their natural habitats. Three mL blood was collected aseptically from each animal from the jugular vein of *O. v. punjabiensis* confiscated by the Punjab Wildlife and Parks Department and brought to LoiBher Wildlife Park, Rawalpindi, and Lahore Zoo with 0.5M ethylenediamine tetra-acetic acid (EDTA) as an anti-coagulant. The blood samples were stored on ice immediately after collection. They were then brought to the laboratory and further stored temporarily at –20 ^{o}C prior to DNA extraction. Hair and skin samples of *O. v. punjabiensis* were collected from wild animals in Slat Range, Kala Chitta mountain range region of Punjab.

DNA extraction and quantification

The stored samples were thawed (at room temperature using water bath) for the genomic DNA isolation using DNA extraction kit (BioBasic, Canada) as per manufacturer's guidelines and stored at –20 ^{o}C for further use. Quantification of the extracted DNA samples was carried out with the help of agarose gel electrophoresis (0.8 %) as well as NanoDrop (Thermo scientific, USA). Standard DNA/DNA ladder was added. All samples were brought to same level of concentration of 50 ng/µL.

Primers and PCR amplification

Amplification of IL-2 specific primers – IL-2 Forward 5'CCCATCATATTTTTC CAGA3' and IL-2 Reverse 5'TGCTAT TAATCCAGTTAGTGTG3' (*Ovine* chromosome 17 ranging from 37994357 to 37994848) were designed from *Ovisaries* IL-2 precursor gene (AF287479) available at GenBank, National Centre for Biotechnology Information (NCBI) using Primer 3 software and Insilico PCR web facility **(**Rozan & Skaletsky, 2000). PCR was performed according to the protocol of primers set, DNA polymerase, polymerase chain reaction (PCR) buffer, dNTPs, $MgCl_2$, genomic DNA and nuclease-free water were used for the targeted (492 bp) regions amplification using thermocycler (IcyclerBioRad, USA). PCR was performed in reaction volume of 25 µL using cycling conditions: initial denaturation at 95 ^{o}C for 4 min followed by 35 cycles of 94 ^{o}C for 1 min; 54 ^{o}C for 1 min; 72 ^{o}C

for 1 min with final extension at 72 ^{o}C for 7 min.

Sequencing

PCR amplifications were seen by running 6 µL of PCR product mixed with 2 µL of loading dye on 1.5% agarose gel at a constant voltage of 100 V for 50 min in 1× TAE buffer. The resulting bands were visualised under UV light using gel documentation system (BioRad, USA). The amplified PCR products were purified using DP203-TIANquick Mini Purification Kit (China) as per provided instructions. The quality of DNA was examined on 2% agarose gel. Purified PCR products were then sent to Singapore for Sanger's sequencing.

Bioinformatics analysis

The sequences were aligned by using NCBI BLAST tool and CodoncCode Aligner software was used for sequence editing, alignment and detection of variable sites. Finally, trimmed and edited sequences of 433 bp were used for further analyses. DnaSP was used to measure the nucleotide and haplotype diversity, while MEGA 6 programme was used for phylogenetic analysis using neighbor joining method with 1000 bootstrap value and amino acid analysis (Tamura *et al.,* 2011). The sequence analysis was compared with the available amino acid sequences of other *Ovis* species: *O. v. punjabiensisi* to *O. ammon* (Argali) and *O. aries* (sheep) as well as with other species, *Capra falconeri* (Makhor), *C. aegagrus* (wild goat), *C. hircus* (goat), *Bos gaurus* (gaur), *B. taurus* (cattle), *B. indicus* (Cattle), *Bison bison* (American bison), *Babalus bubalis* (water buffalo), and *Mus musculus* (house mouse), and *Homo sapiens* (human) as outer groups.

DNA sequences undergo rapid changes during the evolutionary process (Balhoff & Wray, 2005). The measure of pairwise distance is appropriate for DNA sequences undergoing a synchronous evolution that is -4 ln [det(P)], where "P" is the conditional probability matrix derived from evolutionary distance profile. Evolutionary changes between the sequences were estimated along the path by means of weighing each change by applying inverse of the probability of the nucleotide when it changed. Computed asynchronous distance as first proposed by Barry & Hartigan (1987) approximately agreed with the estimate of total substitutions when the marginal probability of each nucleotide was taken as I at all points in time.

Multi dimensional scaling (MDS) is a graphical representation that provides better understanding of the variation pattern present between biological sequences (Cox & Cox, 2010). These authors actually used pair-wise distance matrix generated among sequences and through scaling, the data set is transformed into n–1 ('n' number of sequences) number of dimensions (Gentleman *et al.,* 2004). The "R" package "stats" contains a built-in cmdscale (Gower, 1985) function for MDS analysis allowing comparison between two data sets having more related variables (species) placed in the same cluster on the basis of the shortest distance present between them. The typical setup explored these variables that were best correlated to sample similarities of the biological sequences (e.g., species polymorphisms). In this study, the similarity matrix of the population was fixed, while subsets of the environmental variables were used in the calculation of the environmental similarity matrix. Spearman's correlation coefficient) between the two matrices was calculated and the best sub-

set of environmental variables could then be identified while significance was implicitly achieved through permutation test, for instance IL-2 in that study. The phylogenetic tree, MDS and evolutionary distance analyses were carried out by using "R" software (Cox & Cox, 2001).

RESULTS

PCR and phylogenetic analysis

The amplification of all the samples at DNA encoding region of Punjab Urial IL-2, 492 bp target region was successful sequenced. It had confirmed the localisation of IL-2 gene at the q22-->q23 bands of chromosome 17 in Punjab Urial as previously suggested by Chowdhary *et al.* (1994), Echard *et al.* (1994) and Hediger *et al.* (1991). The neighbour joining tree showed that *O. v. punjabiensisi* is closer to *O. ammon* (Argali) than the other species, *Ovis aries* (sheep), *Capra falconeri* (Makhor), *Capra aegagrus* (wild goat), *Capra hircus* (goat), *Bos gaurus* (gaur), *Bos taurus* (cattle), *Bos indicus* (cattle), *Bison bison* (American bison), *Babalus bubalis* (water buffalo), and *Mus musculus* (house mouse) and *Homo sapiens* (human) used as outer group (Fig. 1). Moreover, the phylogenetic tree of *O. v. punjabiensis* displayed that *O. ammon* (Argali) and *O. v. punjabiensis* have a common ancestor and the two species are sister organisms. Phylogenetic analysis revealed that the IL-2 sequences of different tested ruminants here are form a single cluster. Punjab Urial's IL-2 is evolutionarily more related to the Argali, and it might have diverged recently from the same ancestor.

Pairwise evolutionary distance

The matrix containing entries in the case of *O. v. punjabiensis* related to evolutionary changes compared with other selected organisms was screened out and plotted on Fig. 2. Punjab Urial was found to be the closest relative of *O. ammon* and *O. aries* as indicated with a straight line on zero level on horizontal axis which means

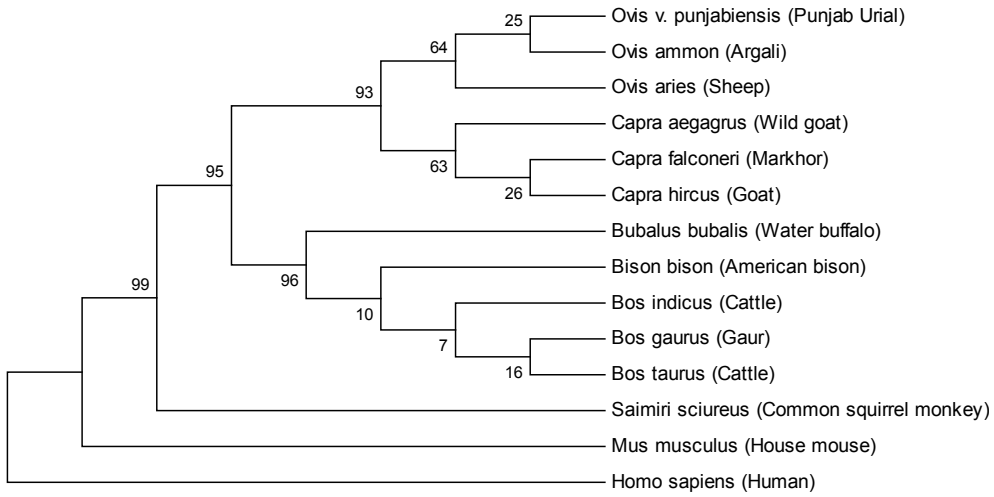

Fig. 1. Neighbour joining tree constructed with MEGA6 using IL-2 sequences from Punjab Urial and other different mammalian species.

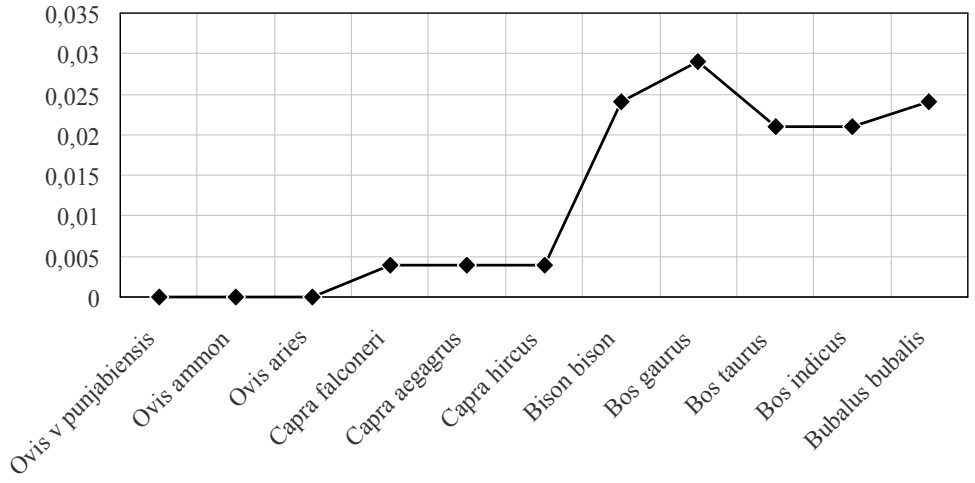

Fig. 2. Evolutionary distance was calculated between selected species by making pairwise comparisons among them. Species were plotted around horizontal axis, while phylogenetic distance achieved by them with the passage of time was placed as "Evolutionary Distance" along vertical axis. The graph shows the distance pattern for *O. v. punjabiensis* lineage with respect to selected individuals.

negligible difference between their IL-2 sequences. The large deviations were observed when it was compared for pairwise analyses with *Bos gaurus, Bos taurus* and *Bos indicus, Bison bison* and *Bubalus bubalis*. The larger peaks revealed their farther evolutionary relationship.

MDS analysis of variation pattern

The different species present at the same spot were given identical colour. For example, lower left compartment contained top most data point representing three organisms. Thus, their respective colours were kept similar, whereas each data entry in the rest of the plot was given unique colour as all entries were representing their respective organisms.

In order to cluster the data, a suitable transformation was achieved by scaling it with a suitable eigen value that results into its linear combinations. During MD scaling here 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 1st eigenvalue while 2nd dimension transformation vector was computed with the help of 2nd value. In this way, '*n*-1' eigenvalues could be achieved to get 'n-1' number of data shapes/transformations. After obtaining data in two new shapes, they were clustered based on small difference between them. In total, 13 sequences were previously analysed to get evolutionary distance matrix. The computed MDS profile with the help of distance matrix is shown below on Fig. 3 (Cox & Cox, 2001).

All data points and their corresponding labels were given same colour for the sake of proper interpretation. In the lower left compartment, the data point is representing three organisms. *O. v. punjabiensis*, *O. ammon* and *O. aries* are represented by lowest data point drawn as a small circle, while above it, in the same compartment,

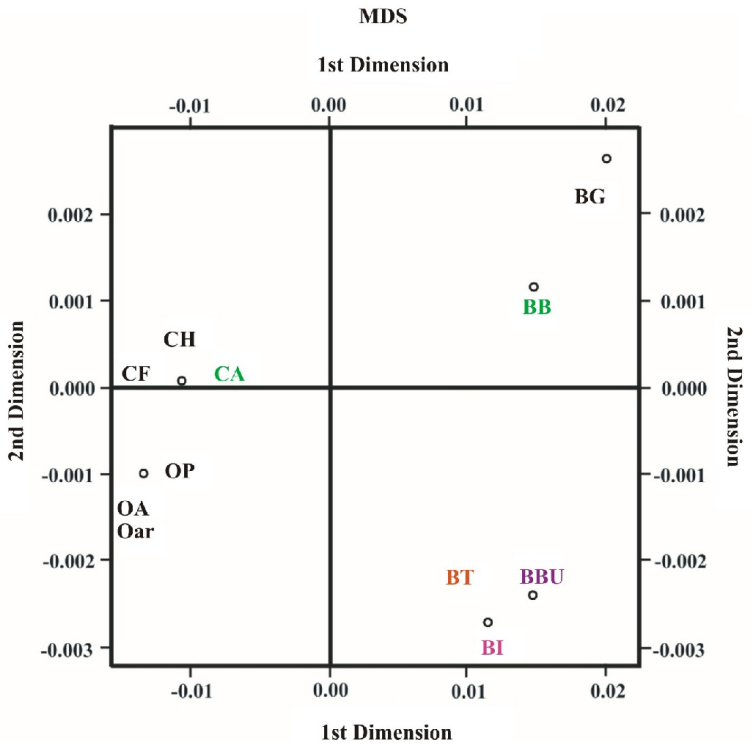

Fig. 3. Multidimensional scaling for IL-2 gene was plotted to see within-species pattern variations; for instance, *Ovis v. punjabiensis* (Punjab Urial) was abbreviated to OP; *O. ammon* (Argali) to OA; *O. aries* (sheep) to OAr; *Capra falconeri* (Markhor) to CF; *C. aegagrus* (Wild goat) to CA; *C. hircus* (goat) to CH; *Homo sapiens* (human) to HS; *Bison bison* (American bison) to BB; *Bos gaurus* (gaur) to BG; *Bos taurus* (cattle) to BT; *Bos indicus* (cattle) to BI; *Bubalus bubalis* (water buffalo) to BB.

the other data point coloured green are pointing to *Capra falconeri* (Markhor), *Capra aegagrus* (wild goat) and *Capra hircus* (goat).

DISCUSSION

Due to highly fragmented world, where wildlife fauna and flora have rather limited opportunity to maintain gene-flow and thereby overall effective population size, it becomes imperative for management policies to encourage genetic diversity. The ultimate goal should be the maintenance of maximum diversity within wildlife pristine populations to ensure maximum potential to respond to environmental perturbations; population management decisions need to be based on maintaining genetic diversity rather than maintaining unique populations, such as subspecies (Larson, 2012). As an endangered species, the Punjab Urial, is going through several pressures for its survival and it is high time to consider conservation of this animal from extinction. Even though the Urial closely resembles some

of its ancestral forms of domestic sheep, it has a genetically distinct population. For many domestic animal populations, uniqueness is broadly defined and differences between populations may be the functions of few different genes, often closely related with a lone physical character or small group of specific characters (Henson, 1992; Groenveld *et al.,* 2010). Any population(s) that had been isolated historically, biogeographically or reproductively, might be considered to be a unique population (Henson, 1992) such as the Punjab Urial, where it represents an important source of uncharacterised genetic diversity, and adaptations restricted to less intensively managed highland populations. Perseverance of feral populations that are highly valuable but vulnerable sources of genetic diversity for domestic relatives has been highlighted in other species (Tapio *et al.* 2005; Taberlet *et al.* 2008; Medugorac *et al.* 2009). Since the reported population estimate of Punjab Urial is merely around 1,500, this number is much lower than the suggested number of individuals required for protecting adaptive genetic variation in a breeding population. Therefore, *in situ* and *ex situ* conservation techniques for this animal will be a good solution to preserve this endangered animal species from extinction. Furthermore, as suggested by Awan (2006) community participation bordering the wildlife sanctuaries and game reserves could improve the conservation of Punjab urial. Since this vital biodiversity component of Pakistan face threats from different fronts, the recovery system should also include all possible mechanisms to protect them.

Furthermore, according to several analyses presented in the present study, the Punjab Urial has a unique isolated population in Pakistan that is endemic and endangered as well. Although the Punjab Urial is closely related to Argali and sheep, it is different in genetic makeup from them. Despite the negligence of good management practices for conservation of the Urial, it maintains a distinct genetic signature that should be conserved immediately by the relevant stakeholders. In light of the above-mentioned rather limited number and highly restricted geographical distribution of the Punjab urial, this animal should be classified as rare in terms of its population with extinction status for the provision of appropriate conservation measures. This study illustrates the genetic diversity and taxonomic relevance (with related animals) in feral and poorly managed populations to unravel genetic structure and relatedness.

In conclusion, with regard to the studied gene encoding IL-2 production, the study provides new information and knowledge in support of conservation strategies of endangered breeds. Further studies on urials on these lines should enhance our understanding of their genetic diversity patterns, genetic finger-printing and bottleneck tests.

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