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Molecular Characterization of Heat Shock Protein 70-1 Gene of *Capra aegagrus blythi*

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Authors' Contribution
AN conceived and designed the study. AN and FF did sampling, genome extraction, amplified the markers and wrote the article. MJ analyzed the data.

Key words
Heat shock protein, Genetic diversity, Polymorphisms, Phylogenetic analysis, Sindh ibex.

ABSTRACT

Sindh ibex (*Capra aegagrus blythi*) is a vulnerable subspecies of wild goat (*Capra aegagrus*), which faced serious population threats. In the present study, HSP70.1 gene has been partially sequenced (1344 bp) to study genetic diversity and evolutionary characteristics of Sindh ibex. In this research, samples of Sindh ibex were collected from Kirthar National Park, Sindh, Pakistan, the gene of interest was amplified, PCR products were sequenced and data was analyzed. The results of genetic diversity indicated that Parsimony informative sites were found to be 11 while Singleton variable sites were not detected. The total number of haplotypes (h) was found to be 17 and haplotype diversity (Hd) was 0.862. Nucleotide diversity (pi) was calculated to be 0.00210 and Tajima's *D* neutrality test for neutral mutation indicated a negative value. Moreover, the results of homology analysis showed maximum similarity with sequences of goat and sheep sequences (partial) and were found to be 99.93% and 99.35%, respectively which was further confirmed by constructing phylogenetic tree which indicated that Sindh ibex, domestic goat, and sheep share a common ancestor.

INTRODUCTION

Sindh ibex (*Capra aegagrus blythi*) is a vulnerable subspecies of wild goat (*Capra aegagrus*) which is scattered among arid mountain ranges of Pakistan, including low Mekran coastal range, the Koh-i-Maran range south of Quetta (District Kalat), and also the Kirthar range (District Dadu and Las Bela) (Shackleton, 1997). According to Yamada *et al*. (2004), the population of Sindh ibex faced a serious decline due to massive hunting by humans and dropped to 200 goats in Kirthar National Park before the conservation plan was started in 1967. As the habitat suggests, Sindh ibex lives in a very harsh environment, where it experiences various stress situations including nutritional decline, physical and psychological stress, climate change, and thermal stress.

Heat Shock Proteins (HSP) are one of the most important biomolecules involved in thermoregulation during stress conditions (Ravaschiere *et al*., 2017; Datta *et al*., 2017). They are chaperones and are produced as a result of increased expression of mRNA of HSPs due to activation of heat shock transcription factor 1 in the cells (Sharma *et al*., 2013). They are found in every cell of all organisms and are involved in a variety of functions including folding/unfolding of protein, cell signaling, protein transport into subcellular compartment, inhibition of caspase activation to prevent apoptosis and regulator of immune response (Hendrick and Hartl, 1993; Parsell and Lindquist, 1993; Karlin and Brocchieri, 1998; Feder and Hofmann, 1999; Li and Srivastava, 2004; Schmitt *et al*., 2007; Zuo *et al*., 2016; Chatterjee and Burns, 2017; Edkins *et al*., 2018). They are highly conserved in almost all species (Karlin and Brocchieri, 1998; Li and Srivastava, 2004) with HSP 70 as the most conserved proteins in the evolutionary lineage (Gupta and Singh, 1994; Daugaard *et al*., 2007).

The HSP 70 kDa is one of the highly produced HSPs during thermal stress. It serves as an ideal marker in the animal studies for the quantification stress induced by environmental heat (Archana *et al*., 2017; Dang *et al*., 2018). This protein is encoded by a family of four genes in bovine. This family includes HSP70-1, HSP70-2, HSP70-3, and HSP70-4 genes which are expressed under different stress conditions. Expression of HSP70-1gene, however, plays a major role during heat stress in goats. HSP70-1 gene-which is located on chromosome 23 in bovine species-consists of single exon and has the size of 1926 nucleotide in goats (Gade *et al*., 2010). Due to its prime role in stress conditions, HSP70 has been studied widely in caprine species (Pawar *et al*., 2013; Romero *et al*., 2013; Banerjee *et al*., 2014). HSP70-1 has not been studied in Sindh ibex before. The molecular characterization of this

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gene and its comparison with that of the other animals will provide a road map for the better understanding of the gene pools of the wild goat to plan a better strategy for conservation and management.

MATERIALS AND METHODS

Sample collection and DNA isolation

Blood/meat samples (n=25) of wild goats (*Capra aegagrus blythi*) were collected from Kirthar National Park, Sindh, Pakistan. Meat samples were also collected and stored in 70% ethanol. Blood of hunted animals was collected into vacutainers containing ethylenediamine tetra acetic acid (EDTA) as an anticoagulant and was stored at -20ºC before extraction. DNA extraction was done through standard organic extraction method for genomic DNA and then was checked for quality and quantity by agarose gel electrophoresis and Thermo scientific 2000 nano spectrophotometer. The samples were dilute dup to 50 ng/µL for polymerase chain reaction (PCR).

PCR amplification of HSP70-1 gene

For partial amplification of HSP70-1 gene in Sindh ibex, two sets of overlapping primers were designed using primer 3 software. The HSP70-1 partial gene sequence (1344 bp) of the domestic goat was aligned with chromosome 23 sequence of goat available on NCBI (GenBank: CM001732.2) and then chromosomal sequence was used for primer designing. The primers used include: HSPF1 5'GTGACGTTCAGGATGCCATT3', HSPR1 5'GGCATCTTCGAGGTGAAGG3', HSPF2 5'TGTTCTGGCTGATGTCCTTCT3', and HSPR2 5'ATCATCGCCAACGACCAG3'. PCR conditions were optimized for both primer sets. The 25µL reaction mixture for PCR contained 50ng/µL DNA, 10X Taq polymerase assay buffer (750 mM Tris-HCl with pH 8.8 at 25°C, 200 mM$(NH_4)_2SO_4$, 0.1% (v/v) Tween 20), 25mM/µL $MgCl_2$, 2.5mM/µL dNTPs, 10 mM/µL of each primer, and 3U/µL Taq Polymerase. The conditions for thermocycler were as: initial denaturing at 950ºC for 5 min followed by 35 cycles with denaturation at 94ºC for 30 seconds, annealing of primers at 64ºC-52ºC for 30 seconds and extension for one minute at 72ºC and then followed by final extension for 10 min at 72ºC. After PCR, the amplicons were checked for specificity by resolving the products on 1.2% agarose gel along with molecular weight marker.

Sequencing and analysis of PCR products

PCR products were purified for any non-specification by precipitating them with 100 µl of 80% ethanol. After precipitation, amplicons were sequenced through chain termination method. The sequencing was done bi-directionally by Big Dye™ Terminator on ABI 3130XL Genetic analyzer. The sequences were then analyzed using BioEdit software version (V.7.0) (Hall, 1999) and Nucleotide BLAST program available at NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) for seeking similarity with the domestic goat (Altschul *et al*., 1990). Any change in the DNA sequence was confirmed by sequencing both sense and antisense strands. Percentage similarity was calculated using MUSCLE (Edgar, 2004). To evaluate the polymorphism in the sequences, DNASP v.5 (Librado and Rozas, 2009) was used. Molecular Evolutionary Genetics Analysis (MEGA V.6.0) (Tamura *et al*., 2013) was used to estimate the evolutionary distances between sequences by computing the of nucleotide differences between each pair of sequences and to construct the phylogenetic trees.

RESULTS

PCR amplification and sequencing

PCR amplification was carried out for both the primer sets (IbF1, IbR1; IbF2, IbR2) and the resulting products were sequenced. The results of sequencing were analyzed by performing multiple sequence alignment and BLAST. Nine variations were identified in the partial sequence of HSP70-1 gene (Table I). The analysis showed that all individuals were found to contain polymorphism. Two of all variations were transversion type while the remaining were transition types.

Table I.- Nucleotide sequence variations found in HSP70.1 partial gene of Sindh ibex.

Polymorphism type (wild>mutant)	Nucleotide position
Transition (G>A)	27516397
Transition (G>A)	27516490
Transversion (C>A)	27517282
Transition (G>A)	27517273
Transition (G>A)	27517255
Transversion (C>A)	27517249
Transition (G>A)	27517285
Transition (G>A)	27517228
Transition (C >T)	27517225

Polymorphism analysis

Polymorphism analysis of the sequences was carries out and variable (polymorphic/segregating) sites (S) and total number of mutations (Eta) were found to be 11 (Table II). Parsimony informative sites were found to be 11 while Singleton variable sites were not detected in the sequences. The G+C content was found to be

0.646 (Table IV). The average number of nucleotide differences (k) was calculated to be 2.82154 (Table III). Considering the variance of k (for both recombination and non-recombination), the stochastic errors were found to be more dominant towards total variance than were the sampling variance (Tajima, 1983). This shows that various factors are involved in the difference of k values among the individuals. Moreover, sequence conservation value was found to be 0.992 (Table IV).

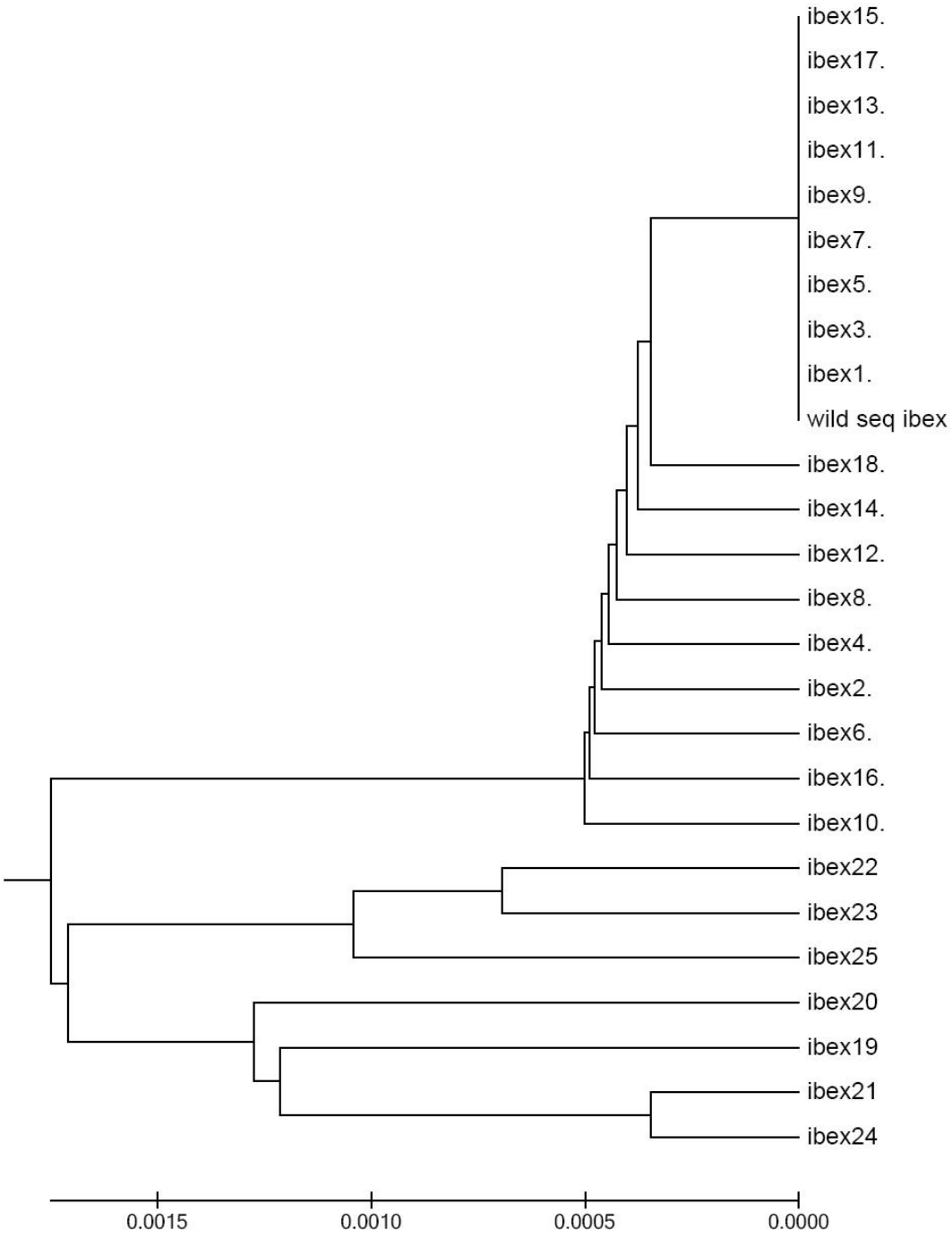

Fig. 1. UPGMA phylogenetic tree made on the basis of HSP70.1 gene sequences of Sindh ibex after alignment using clastal W program.

Table II.- Polymorphic sites in HSP70.1 gene of Sindh ibex.

Type of sites	Number of sites
Sites with alignment gaps or missing data	0
Invariable (monomorphic) sites	1333
Variable (polymorphic/segregating) sites (S)	11
Total number of mutations (Eta)	11
Singleton variable sites	0
Parsimony informative sites	11
Singleton variable sites (two variants)	0
Parsimony informative sites (two variants)	11
Site positions	6 10, 342, 345, 354, 372, 378, 399, 402, 1137, 1230
Variable sites (three variants)	0
Variable sites (four variants)	0

The total number of haplotypes (h) was found to be 17 and haplotype diversity (Hd) was estimated to be 0.862 (Table III). Nucleotide diversity (pi) was calculated to be 0.00210. Tajima's *D* neutrality test for neutral mutation indicated the negative value.

Homology analysis and phylogenetics

Results of homology analysis indicated that HSP 70.1 gene partial sequence of Sindh ibex showed maximum similarity with that of goat and sheep sequences (partial) and was found to be 99.93% and 99.35% (Fig. 2). The phylogenetic tree showed a close evolutionary relationship of Sindh ibex with goat and sheep (Fig. 3).

Table III.- Haplotype and nucleotide diversity.

Character	Value
Number of Haplotypes (h)	17
Haplotype (gene) diversity (Hd)	0.862
Variance of Haplotype diversity	0.00463
Standard Deviation of Haplotype diversity	0.068
Nucleotide diversity (per site) (Pi)	0.00210
Sampling variance of Pi	0.0000001
Standard deviation of Pi	0.00036
Average number of nucleotide differences (k)	2.82154
Stochastic variance of k (no recombination), Vst(k)	2.184
Sampling variance of k (no recombination), Vs(k)	0.180
Total variance of k (no recombination), V(k)	2.365
Stochastic variance of k (free recombination), Vst(k)	0.941
Sampling variance of k (free recombination), Vs(k)	0.075
Total variance of k (free recombination), V(k)	1.016
Tajima's D	-0.07043 (not significant, P > 0.10)

Table IV.- Sequence conservation.

Character	Value
Net number of analyzed sites (L)	1344
Number of variable/polymorphic sites (S)	11
Sequence conservation (C)	0.992
G+C content	0.646 (1344.00 sites)

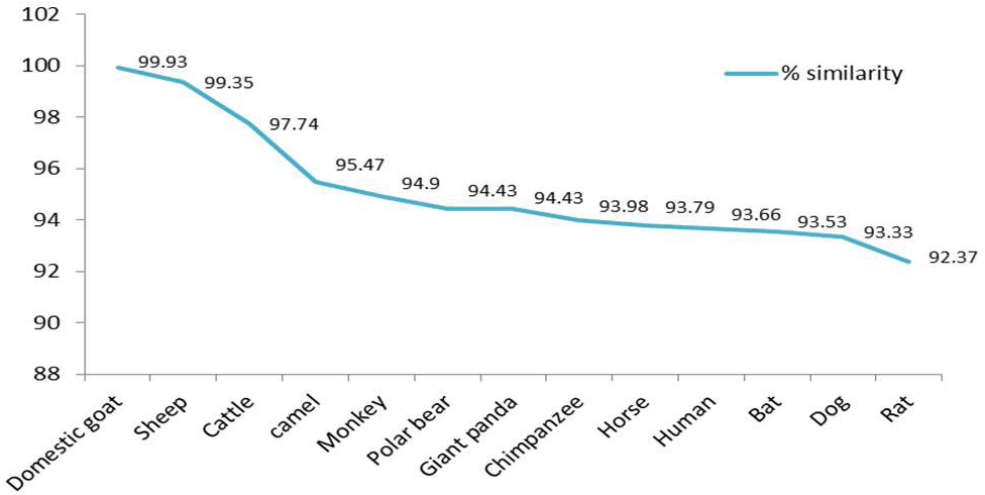

Fig. 2. Homology analysis of HSP70.1 gene of Sindh ibex with that of other animals by using MUSCLE alignment tool.

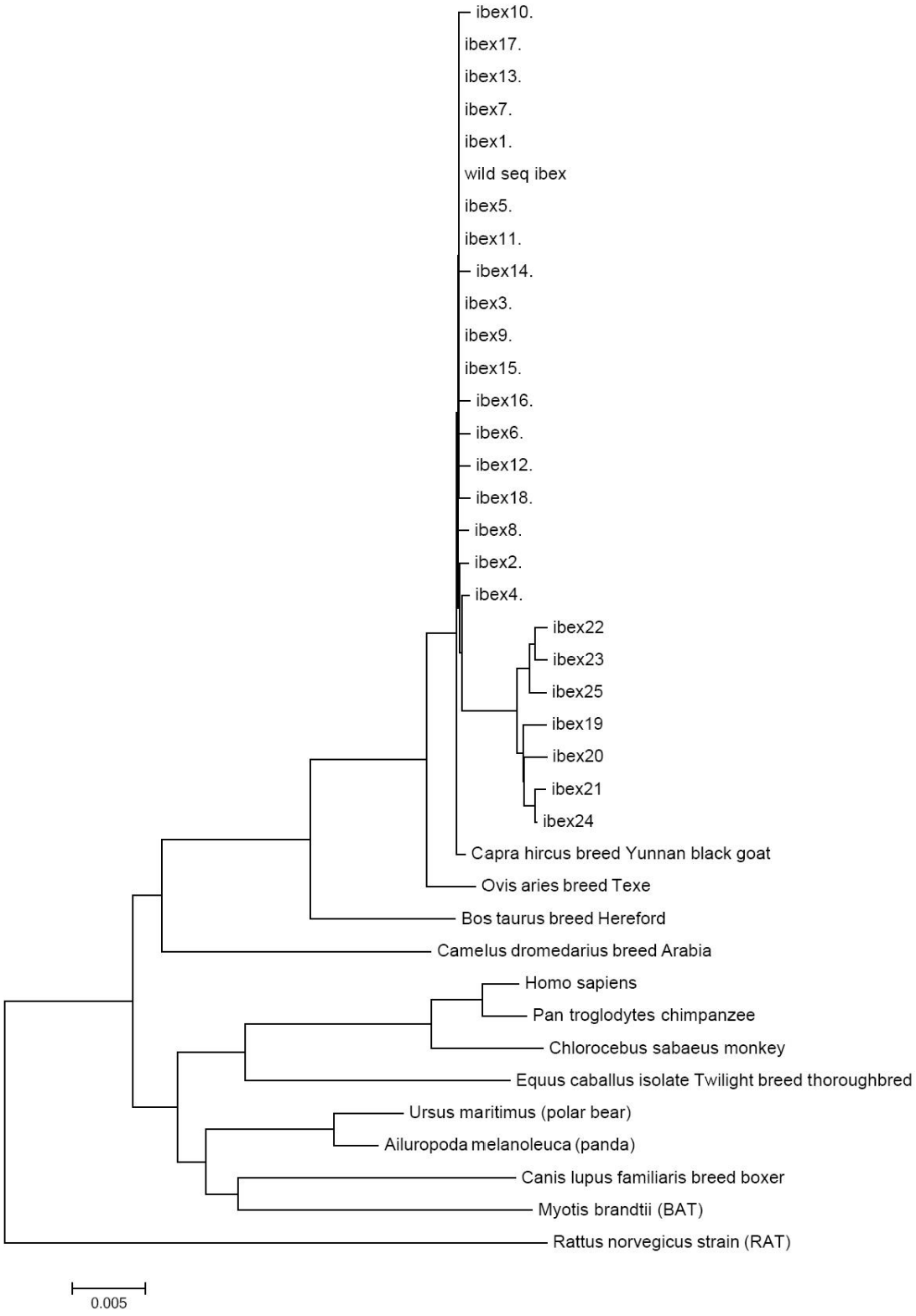

Fig. 3. Neighbor joining phylogenetic tree made on the basis of HSP70.1 gene sequences of Sindh ibex and other species after alignment using clastal W program.

DISCUSSION

DNA polymorphism analysis

DNA polymorphism data provides important information about the structure and evolutionary history of a population and species and about the relationship between various populations and species. It plays a vital role in the fields of conservation genetics, animal and plant breeding and epidemiology genetics (Rozas *et al*., 2003). Phylogenetic analysis of Pakistani livestock breeds such as buffalo, goat, sheep, camel (Babar *et al*., 2014, 2015; Hussain *et al*., 2009, 2013a, b, 2015; Ahmed *et al*., 2014) have previously been reported but wildlife species data is limited. Therefore our study describes, for the first time, polymorphism data on Sindh ibex, which is a vulnerable species, on the basis of HSP70.1 gene. HSP70 has been studied before for the genetic analysis of chicken (Gan *et al*., 2015). The results of our study showed high haplotype diversity in the population of Sindh ibex at Kirthar National Park. However, the nucleotide diversity was found to be low. Similar results have been in found in the wild populations of many species (Jiang *et al*., 2005; Wu and Fang, 2005; de Jong *et al*., 2011; Langille *et al*., 2014). Low nucleotide diversity and high haplotype diversity indicates that population has expanded rapidly and has survived a recent bottleneck (de Jong *et al*., 2011).

Further, the neutrality tests were performed to test the neutral theory of evolution (Kimura, 1984). According to this theory, most of the variations at the molecular level are neutral and random drift of selectively neutral or nearly neutral mutants are the cause of most of the evolutionary occasions. To test this theory various tests statistics are applied. Tajima's D test is one of the most popular tests. It assumes that mutation and random genetic drift are the major cause of evolutionary change. The results indicated negative value. This suggests that the population of Sindh ibex experienced severe bottleneck with successive expansion phase at present which is consistent with polymorphism data. The negative value of a Tajima's D test, however, was insignificant which specifies that the population contains neutral mutations and highlights the genetic drift as the mode of evolution rather than positive or direct selection (Tajima, 1989) as compared to results found in domestic goat which indicated the positive selection as the mode of evolution (Gade *et al*., 2010; Pawar *et al*., 2013). However, the main factor influencing the evolutionary difference was found to be the population status of Sindh ibex as compared with that of the domestic goat which has larger and better-maintained populations. Neutral mutations were also confirmed when deduced amino sequence of HSP70-1 protein of Sindh ibex samples was compared with that of the wild sample. All of the nucleotide variations were found to be synonymous.

Homology analysis and phylogenetics

The sequences of HSP70-1 gene of Sindh ibex were aligned and compared for phylogenetic analysis. The pairwise genetic distance was computed and the average was found to be 0.002. The phylogenetic tree was constructed which showed less diversity among the population and high similarity between individuals shown in Figure 1.

Results of homology analysis indicated that HSP70-1 gene of Sindh ibex is highly homologous to that of other organisms (Fig. 2) as studied by Gade *et al*. (2010), Banerjee *et al*. (2014), Gupta *et al*. (2015) and Kumar *et al*. (2015). The similarity with cattle and camel was found to be 97% and 95.4%, respectively. Moreover, Sindh ibex HSP70-1 sequence showed 95% similarity with that of human, dog, horse, monkey, chimpanzee, giant panda, polar bear, rat, and bat. Further, the phylogenetic tree showed a close evolutionary relationship of Sindh ibex with goat and sheep (Fig. 3). Similar results were found by Gade *et al*. (2010), who indicated higher homology of goat HSP 70.1gene with that of cattle, buffalo, yak, sheep (partial), pig, horse, and humans. This indicates that Sindh ibex, goat, cattle, buffalo and sheep evolved from a common ancestor.

CONCLUSION

This study was carried out in an effort of exploring the effects of stress factors on survival and diversity of Sindh ibex. As this animal faced serious population threats, our results will provide the first step to study its population structure and diversity and the evolutionary pathways. Moreover, large sample size and the use of various molecular markers is required to fully explore the genetic pool of Sindh ibex to better understand and plan more effective conservation strategies for Sindh ibex and other wildlife of Pakistan.

Statement of conflict of interest

Authors have declared no conflict of interest.

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