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Molecular and craniological analysis of leopard, *Panthera pardus* (Carnivora: Felidae) in Iran: support for a monophyletic clade in Western Asia

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As the largest extant cat species in west Asia, the leopard (*Panthera pardus*) shows high morphological variation, which has led to the description of seven different subspecies in the region. Different investigations have tried to clarify its phylogenetic structure; however, sample size and spatial distribution insufficiently represent the Iranian population, the largest remaining bulk of the Persian leopard (*P. p. saxicolor*) in the Middle East that probably functioned as a source for the subspecies' range. We examined sequence variation in the mitochondrial *NADH-5* gene for 25 leopards from different parts of Iran. Also, we examined 49 adult male skulls to understand the morphological variation of the Iranian leopard population. Our craniometrical results revealed that while no differentiation is seen based on size or shape characteristics from different parts of Iran, larger individuals normally belong to the northern range. Time-calibrated Bayesian phylogenetic analysis suggested that the Iranian female lineage is a monophyletic group that diverged from a group of Asian leopards in the second half of the Pleistocene. Three closely related haplotypes were identified for the entire country: one commonly found haplotype throughout Iran, south Caucasus and Turkmenistan and two localized haplotypes were sequenced from southern Zagros and eastern Alborz. Accordingly, the Persian leopard population in Iran as well as in neighbouring countries can be protected as a large management unit through large-scale conservation planning. Moreover, the available captive stock of the Persian leopard represents an invaluable source for reintroduction for countries interested in restoring their locally extinct population. *Biological Journal of the Linnean Society*, 2015, **114**, 721–736.

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INTRODUCTION

As one of the most widely distributed cats of the world (Nowell & Jackson, 1996), the leopard (*Panthera pardus*) shows high polymorphism in morphologic and morphometric characteristics (Trimen, 1883; Pocock, 1930; Stuart & Stuart, 1991; Meijaard, 2004; Khorozyan, Baryshnikov & Abramov, 2006). Such polymorphism can be associated with a wide range of habitat conditions (Sunquist & Sunquist, 2002;

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Gavashelishvili & Lukarevskiy, 2008) and prey types (Norton, 1984; Hayward *et al*., 2006). Accordingly, taxonomists have described up to 30 subspecies based on the species' geographic range and phenotypic variation (Ellerman & Morrison-Scott, 1951; Herrington, 1986). Western Asia has been known to support seven putative subspecies (*P. p. dathei*, *P. p. sindica*, *P. p. saxicolor*, *P. p. ciscaucasica*, *P. p. jarvisi*, *P. p. nimr*, *P. p. tulliana*) (Zukowsky, 1964; Herrington, 1986) with continuous debate on their geographic extent (Khorozyan *et al*., 2006).

In order to address this taxonomic issue, the leopard has been studied genetically in the past 2 decades at the global (Miththapala, 1992; Miththapala, Seidensticker & O'Brien, 1996; Uphyrkina *et al*., 2001, 2002) and country-scale level (Miththapala, 1992; Spong, Johansson & Bjorklund, 2000; Uphyrkina *et al*., 2002; Dutta *et al*., 2013). All applied a wide range of molecular genetic markers to explore taxonomy, phylogeny, and genetic variation of the species, placing them among the most polymorphic of the big cats (Newman *et al*., 1985). It was revealed that the Western Asian population, ranging from Afghanistan through Iran to Iraq and the Caucasus is distinctive phylogenetically (Miththapala *et al*., 1996; Uphyrkina *et al*., 2001), which was further supported by subsequent craniometric analysis (Meijaard, 2004). It was therefore concluded that no significant geographical barriers are present that would lead to morphogenetic isolation of the subspecies in this region (Uphyrkina *et al*., 2001), with the exception of the population of the Arabian Peninsula whose isolated position from other Western Asian leopards was supported by both genetic and morphometric analysis (Uphyrkina *et al*., 2001; Khorozyan *et al*., 2006).

In addition to a small sample size, almost all samples used in both classic genetic studies (i.e. Miththapala *et al*., 1996; Uphyrkina *et al*., 2001) were obtained from captive-born individuals, raising some controversy about the certainty of the systematics and geographic origin of the leopards. Therefore, Khorozyan, Baryshnikov & Abramov (2006) addressed the issue using comparative analysis of leopard skulls from the Caucasus and adjacent areas and concluded that four putative subspecies are valid in the Middle East, namely *P. p. sindica* (Pakistan and southern Iran), *P. p. saxicolor* = *P. p. ciscaucasica* (northern Iran, Caucasus and Turkmenistan), *P. p. nimr* (Arabian peninsula), and *P. p. tulliana* (western Turkey). While the latter is considered to be extinct in the wild (Akin, 1991), it was concluded that except for the Arabian leopard, two extant subspecies exist in Western Asia, contradicting former genetic studies that indicate only a single subspecies (i.e. *P. p. saxicolor*; Uphyrkina *et al*., 2001). However, a recent genetic assessment using 24 samples from Western Asia supports the presence of a single subspecies in the region, except for western Turkey which needs further assessment (Rozhnov, Lukarevsky & Sorokin, 2011).

While the Persian leopard population has lost most of its global range outside of Iran (Lukarevsky *et al*., 2007; Stevens *et al*., 2011; Raza *et al*., 2012), the subspecies is known to occur within vast areas of the country (Kiabi *et al*., 2002) and makes up more than two-thirds of the wild population of the subspecies (Kiabi *et al*., 2002; Khorozyan, Baryshnikov & Abramov, 2006). Indeed, Iran's population nucleus is the largest single population likely to function as a source for natural re-colonization within the Persian leopard range, particularly the Caucasus (Breitenmoser *et al*., 2010). However, both recent craniometrical and genetic studies used a limited number of Iranian samples (five skulls, Khorozyan *et al*., 2006; three hair and claw samples from northern parts of the country, Rozhnov *et al*., 2011), which is unlikely to confidently represent the subspecies' existing main population. This lack of knowledge could have a direct impact on conservation measures, especially ex-situ breeding programs, hampering the function of captive stocks as potential conservation units.

Accordingly, the present study aims to more accurately evaluate the taxonomic status of west Asian leopards within their largest nucleus, i.e. Iran, therefore addressing issues of non-availability of appropriate samples in previous studies. We apply both molecular (using mtDNA) and craniometric approaches to clarify the phylogeny and genetic variation of the Persian leopard as well as to verify the taxonomic position of the Iranian population. We use the *NADH dehydrogenase subunit 5* (*NADH-5*) mitochondrial gene, which exhibits relatively high rates of mutation in carnivores (Lopez *et al*., 1997) and has been employed for analysis in the majority of felids, including the leopard (Miththapala *et al*., 1996; Uphyrkina *et al*., 2001). This region accounts for 44 of 50 variable sites found in mtDNA regions of the leopards studied by Uphyrkina *et al*. (2001). Also, Lopez *et al*. (1994; Lopez, Cevario & O'Brien 1996) concluded that these variable sites were not part of the transposed mtDNA in the felid nuclear genome (*Numt*), which can create *Numt* contaminations, and hence may confound population genetic and phylogenetic studies in Felidae (Antunes *et al*., 2007). *Numt*s have a decreased rate of evolution compared to that of mitochondrial sequencing and have been shown not to differentiate tiger subspecies (*P. tigris* spp) (Zhang *et al*., 2006). In addition to being of systematic interest, the present research will aid in setting conservation and research priorities for the

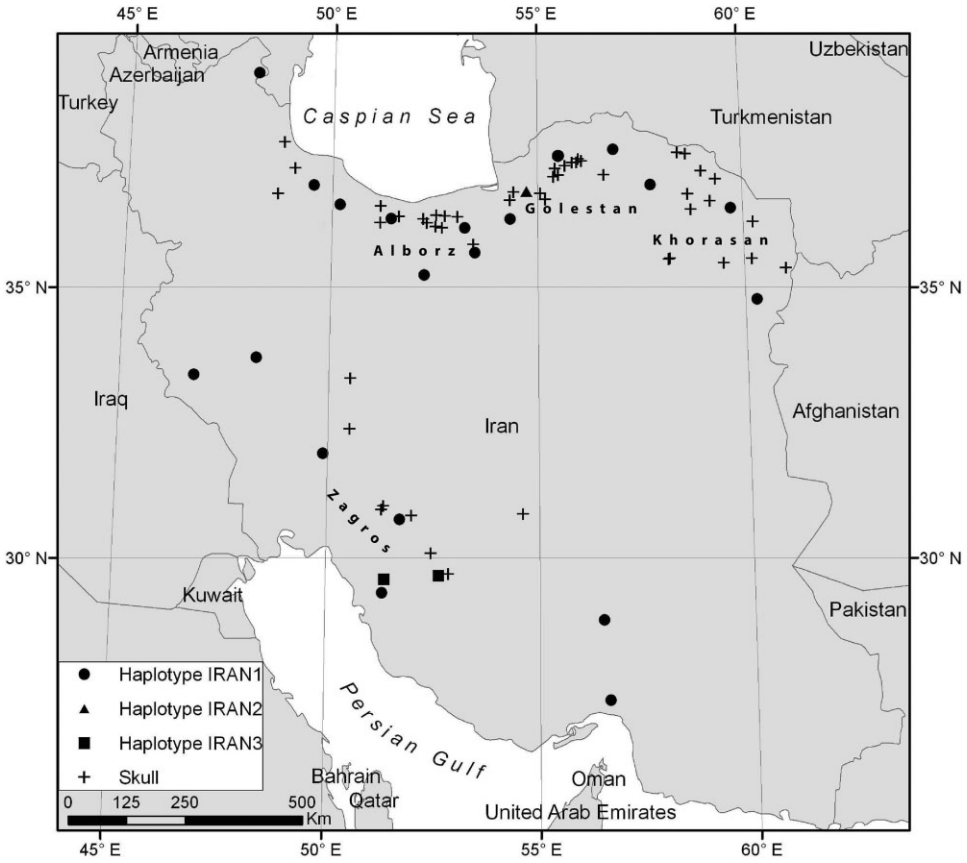

Figure 1. Locations of Persian leopard samples taken for genetic and craniometric analysis. The map shows the distribution of the three mtDNA haplotypes found in Iran: IRAN1 ($N = 22$), IRAN2 ($N = 1$) and IRAN3 ($N = 2$). Ten more individuals of haplotype az2/k1 and 2 more individuals of haplotype ir2 from the Alborz range and its immediate surroundings (Rozhnov *et al*., 2011) are identical to IRAN1 and IRAN2, respectively (Projection: Mollweide; False Easting: 0; False Northing: 0; Central Meridian: 53.682614; WGS 1984).

subspecies in Iran as well as Western Asia (Nowell & Jackson, 1996).

MATERIAL AND METHODS

Genetic analysis

Sample collection

In total, 38 tissue samples, from both muscle and skin were obtained from wild-born dead leopards with known geographic origin in Iran (permission number 31/12630 issued by the Iranian Department of Environment; hereafter, DoE). Of these 38, 33 were confiscated from poachers by the DoE between 2007 and 2010 while the remaining five were obtained from road incidents or mounted pelts in museums or zoological collections, dating back to the early 2000s (Fig. 1; Table 1).

DNA extraction and amplification

Total DNA was extracted from leopard tissue and pelt samples using a standard phenol/chloroform method

Table 1. Geographic distribution within Iran of skull and muscle/skin samples of leopards used in the analysis. Skull samples denote adult male skulls. Muscle/skin samples denote to successfully amplified individuals

	Number of samples	
Geographic regions	Skull	muscle/skin*
Zagros	8	8
Khorasan	14	4
Golestan	13	4
Alborz	14	6
Caucasus	0	2
Total	49	24†

*All muscle/skin samples used in this study are stored in Genetic Resources Bureau of Iran Department of Environment.

†There is one sample with unknown origin from Iran which was included in the analysis. Thus, the total number of muscle/skin samples is 25 individuals.

described in Sambrook, Fritsch & Maniatis (1989). No detectable DNA (as visualized on agarose gel) or polymerase chain reaction (PCR) product was obtained from any of the negative controls. Sample preparation and DNA extraction were conducted in a laminar flow hood in an area isolated from other samples to prevent any contamination.

DNA sequencing and genotyping

In order to investigate genetic diversity of the leopards from mtDNA, the fragment of 611 bp of the 5′ end of the *NADH-5* mitochondrial gene corresponding to positions 12632–13242 in the complete *Panthera pardus* mtDNA sequence (GenBank accession number EF551002.1, Wei *et al*., 2011), was amplified in two separate pieces that overlapped over approximately 140 bp, following Uphyrkina *et al*. (2001). Two sets of primer pairs were combined as follows: F/RL2 (F: 5′-GTGCAACTCCAAATAAAAG-3′ and RL2: 5′-TAAACAGTTGGAACAGGTT-3′) and FL2/RL4 (FL2: 5′-CGTTACATGATCGATCATAG-3′, and RL4: 5′-TTAGGTTTTCGTGTTGGGT-3′). Apart from the forward primer F (from Johnson *et al*. 1998), all primers were designed from leopard *(P. pardus)* sequences (Uphyrkina *et al*., 2001).

Polymerase chain reaction (PCR) experiments were carried out on an Astech thermal cycler (PC 320). A final volume of 50 μL containing 1.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleotide triphosphate (dNTP), 1× PCR buffer (Cinagene Co., Tehran, Iran), 2.5 unit μL^{-1} of *Taq*-Gold DNA polymerase, 70 ng of genomic DNA and 1 μM of each primer and 35 μL molecular grade water. For each reaction 32 cycles were performed with 8 min initial denaturation at 94 °C followed by denaturation at 94 °C for 1 min, 1 min annealing at 50 °C, and 1 min extension at 72 °C. Products were checked in 1.5% agarose gel in Tris–borate–EDTA (TBE) buffer. Purification was carried out using column-based purification kit (millipore) using a vacuum for filtering.

The *NADH-5* segment was sequenced in both forward and reverse directions by Macrogen Company (Seoul, Rep of Korea) and the results were visualized using an ABI-3730 XL genetic analyzer (Applied Biosystems; http://www.appliedbiosystems.com). Sequences have been deposited in the GenBank database (accession numbers KF768352 to KF768354).

Phylogenetic analysis & spatial genetic variability

From the total 38 leopard samples, 25 were taken to final analysis (only those successfully amplified and sequenced for total length of both mtDNA segments). Sample localities are provided in Appendix S1, dated 2001–2009.

Chromatograms were visualized with Chromas software (version 2.33) (Technelysium Pty., Ltd, Mt. Gravatt Plaza, Queensland, Australia) and the sequences aligned using BioEdit software package (version 7.0.0) (Hall, 1999).

Phylogenetic analysis was established by comparison with published segments of *NADH-5* of various subspecies of leopards available in GenBank using BLAST 2.2 software (Altschul *et al*., 1990), which have been reported by Miththapala *et al*. (1996), Uphyrkina *et al*. (2001), Rozhnov *et al*. (2011) and Wei *et al*. (2011).

To reconstruct phylogenetic trees, we combined our leopard sequences with those downloaded from the GenBank database ($N = 41$), using tiger (*Panthera tigris*, $N = 4$), lion (*Panthera leo*, $N = 2$), and snow leopard (*Panthera uncia*, $N = 1$) as the outgroup taxa (Appendix S2).

We used the software MEGA 5 (Tamura *et al*., 2011) and Bayesian Information Criterion (BIC) to identify the best model of nucleotide substitution. This analysis suggested that the HKY + G model of nucleotide substitution (a gamma distribution with five rate categories) best described the substitution pattern.

Network analysis for the mtDNA sequence data was carried out using the software package NETWORK v4.5.0.1 (Bandelt, Forster & Röhl, 1999). We applied a median-joining algorithm under the default parameters of weights = 10 and epsilon = 0, for reconstruction of all possible evolutionary pathways among the haplotypes. This procedure also helped to identify haplotype diversity.

Additionally, a Bayesian phylogenetic analysis was performed using the software BEAST v1.5.1 (Drummond & Rambaut, 2007) to build phylogenetic trees from unique haplotypes and estimate the timings of node divergence. Divergence times were estimated by calibrating time against the number of substitutions based on feline-specific mtDNA *NADH-5* gene substitution rates of 1.22% per million years (Lopez *et al*., 1997). Bayesian analysis was initiated from random starting trees, employing the HKY + G model of nucleotide substitution with five category discrete gamma, assuming the uncorrelated log-normal relaxed clock model. Posterior distributions of parameters were approximated using Markov chain Monte-Carlo (MCMC). The initial runs were used to examine MCMC performance, and operators were adjusted as suggested by the output analysis. The final run was performed with the MCMC chain length = 100 000 000 and the sample frequency = 10 000, discarding the first 10% as burn-in. This procedure provided a sufficient sample size for each parameter (i.e. effective sample size (ESS) > > 100, as suggested by the BEAST manual).

We grouped leopards into geographic subpopulations in order to analyze genetic intra and inter-variability across them. In doing so, we calcu-

lated molecular diversity indices and performed the analysis of molecular variance (AMOVA) as well as pairwise F_{ST} tests based on Kimura 2-parameter distances (Kimura, 1980), using ARLEQUIN v 3.5.1.2 (Excoffier, Laval & Schneider, 2005).

Craniometric analysis

In total, 73 Persian leopard skulls from all over Iran, except for the Caucasian part, were measured in this investigation. Ten specimens belonging to non-adult individuals were omitted from the analysis. Specimens of adult individuals were identified based on skull characteristics (Meijaard, 2004) and dental features (Stander, 1997). Due to the small sample size of female skulls ($N = 14$) as well as a high degree of inter-sexual dimorphism among the leopards (Christiansen & Harris, 2012; Farhadinia, Kaboli, Karami & Farahmand, 2014), we only analyzed those of adult males. The 49 male skulls represented a majority of the leopard's range in Iran (Fig. 1; Table 1 and Appendix S3).

Examined specimens were from the following collections: Iranian Museum of Natural History (MNH), Provincial Offices of MNH in Iran, British Museum of Natural History (BMNH), regional zoological collections, and several private collections in Iran (see Supplementary Material for a complete list of craniometric samples).

For consistency, all measurements were conducted by only one investigator with two repeats. Adapted from Khorozyan *et al*. (2006), 24 variables of the cranium were measured by vernier calipers to the nearest 0.05 mm, abbreviated in Figure 2. However, we excluded vertical diameter of the infra-orbital foramina (VDI) and horizontal diameter of the infra-orbital foramina (HDI) from our analyses due to their inaccessibility in some samples. Also, with respect to the different growth rates of bones and teeth (C. Groves personal communication), the greatest length of carnassial P4 (LP4) was left out to concentrate data exploration only on bone variables. In order to carry out shape analysis, we developed six indices to assess shape between different regions as below:

(1) ZYG/BAL ratio: (zygomatic breadth/basal length) × 100;
(2) BPC/BAL ratio: (postorbital constriction breadth/basal length) × 100;
(3) BPC/IOB ratio: (postorbital constriction to minimum inter-orbital breadth);
(4) IOB/CBL ratio: (minimum inter-orbital breadth/condylobasal length) × 100;
(5) ROB/CBL ratio: (rostrum breadth/condylobasal length) × 100; and
(6) SSP/CBL ratio: (skull height at the supraorbital processes/condylobasal length) × 100.

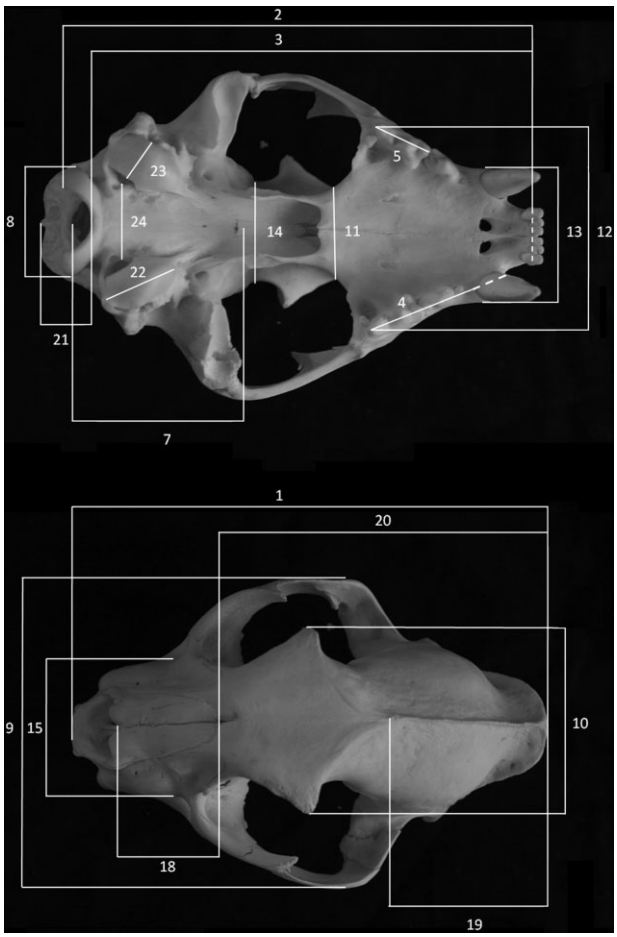

Figure 2. Measured cranial variables. The numbers correspond to the following variables: (1) GRL: greatest length; (2) CBL: condylobasal length; (3) BAL: basal length; (4) LUT: length of upper tooth row C-P4; (5) LP4: greatest length of carnassial P4; (6) SSP: skull height at the supraorbital processes; (7) LBR: length of the braincase from opisthion to postorbital constriction; (8) BOC: greatest breadth of the occipital condyles; (9) ZYB: zygomatic breadth; (10) FRB: frontal breadth; (11) IOB: minimal inter-orbital breadth; (12) GPB: greatest palatal breadth posterior to P4, alveoli included; (13) ROB: rostrum breadth, alveoli included; (14) BPC: minimal breadth of the postorbital constriction; (15) BIF: breadth between the infra-orbital foramina; (16) VDI: vertical diameter of the infra-orbital foramina; (17) HDI: horizontal diameter of the infra-orbital foramina; (18) MIN: minimum length of the nasals (suture length); (19) LOB: length between opisthocranion and bregma; (20) LON: length between opisthocranion and nasion; (21) LOP: length between opisthocranion and basion; (22) LBO: length of bulla ossea; (23) HBO: width of bulla ossea; (24) BBB: breadth between bulla ossea. Measurements 6, 16 and 17 are not displayed on the figure.

Skull variables were transformed to their natural logarithm to meet a normality assumption.

We analyzed two types of data in the present study: First, we used log-transformed data to evaluate geographic polymorphism. Then, with respect to significant correlations between skull length and the other morphometric characters, transformation of absolute measurements to size-independent shape variables was done to remove the effect of size. Size-dependent variation for morphometric characters was removed using geometric mean (Darroch & Mosimann, 1985). Using log-transformed data, geometric mean was computed as the average of the logged variables.

All data were subjected to Multivariate Analysis of Variance (MANOVA) and Principal Component Analysis (PCA) in order to determine whether the differences between geographic regions are significant and select which characters can be effective for differentiation between regions. Additionally, we performed crossvalidation using discriminant analysis (DA) to determine whether the phenotypic traits could be reliably used to assign individuals to their respective regions. Statistical analysis was done using SPSS 16 software (SPSS 16 for Windows) and ADE-4 software (Thioulouse *et al*., 1997).

RESULTS

Genetic analysis

We extracted and sequenced DNA successfully from 25 individuals (two additional samples failed in genotyping) representing most of the leopard's range in Iran (Appendix S1). Successful DNA extraction was not possible for the remaining samples using the adopted method (after a mean of three efforts/sample), as most of these were not collected and stored for the purpose of genetic analysis. In total, 550 bp was recovered from the *NADH-5* gene, corresponding to position numbers 12781–13330 (nucleotide numbers from the reference *Panthera pardus* sequence – GenBank accession number EF551002.1, Wei *et al*., 2011).

Our Iranian samples were grouped into three closely related haplotypes: IRAN1 ($N = 22$), IRAN2 ($N = 1$), and IRAN3 ($N = 2$), differing from one another only in one substitution (Fig. 3, Table 2 and Appendix S1). IRAN1 and IRAN2 were identical to the published haplotypes az2 ($N = 9$) and ir2 ($N = 2$), respectively, from the Alborz range and its immediate surroundings (Rozhnov *et al*., 2011). The haplotype IRAN1 was dominant in our sample, occurring throughout Iran. One individual belonging to Haplotype IRAN2 was from the Alborz while two individuals assigned to Haplotype IRAN3 were from the southernmost Zagros (Fig. 1). For simplicity we refer to both haplotypes of az2 and ir2 as Iranian leopard haplotypes because they were genetically identical to our Iranian leopards and most of their distribution range fell within Iran. The Iranian leopard haplotypes differed by one synonymous substitution (nucleotide position 13232; Table 2) from all the other leopard haplotypes. The pairwise substitutions among the Iranian leopards were non-synonymous altering amino acid coding from leucine (L) in IRAN1 and IRAN2 to proline (P) in IRAN3 at nucleotide position 12995, and from threonine (T) in IRAN1 and IRAN3 to methionine (M) in IRAN2 at position 13043. The Bayesian phylogenetic analysis showed that all Iranian leopards together with leopards from Central Asia, East Asia, Indonesia and the Indian Subcontinent form a monophyletic clade in relation to an African and South Arabian clade (Fig. 4). Within this clade, leopards from Iran represent a monophyletic subclade (with posterior probability 0.86). A median-joining network of mtDNA haplotypes further illustrated the patterns observed in the Bayesian phylogenetic tree, suggesting the divergence of the Iranian female lineage from Asian leopards and the polyphyletic kinship between the Asian and Afro-Arabian leopards (Fig. 3). The time-calibrated Bayesian phylogenetic analysis indicated that the Iranian female lineage is a monophyletic group that diverged from a group of Asian leopards between 16 393-270 492 years BP (95% highest posterior density), that is, in the Middle and Upper Pleistocene. The median divergence time was estimated to be 122 951 years BP that coincides with the end of the last interglacial and the beginning of the last glacial period.

Table 3 summarizes the molecular diversity of the mtDNA gene segment sequences that we used in our analyses. To measure the extent of intra- and inter-population differentiations in leopards we compared three geographic groupings: Iran, Asia, and Africa. AMOVA suggested 63.68% of the variation between the groupings and 36.32% within the groupings ($P < 0.0001$ at 1000 permutations). Each grouping was significantly different from the others by pairwise Fst for mtDNA data (Table 4). The greatest differentiation was between Iranian and African leopards while Iranian and Asian leopards were least differentiated.

Craniometric analysis

In the multivariate comparisons, the F-value derived from Wilk's Lambda, showed significant difference between geographic groups based on log-transformed data ($F < 1.578$, $P < 0.029$) whereas standardized data indicated no significant differences ($F < 1.298$, $P < 0.139$). Therefore, it can be concluded that when

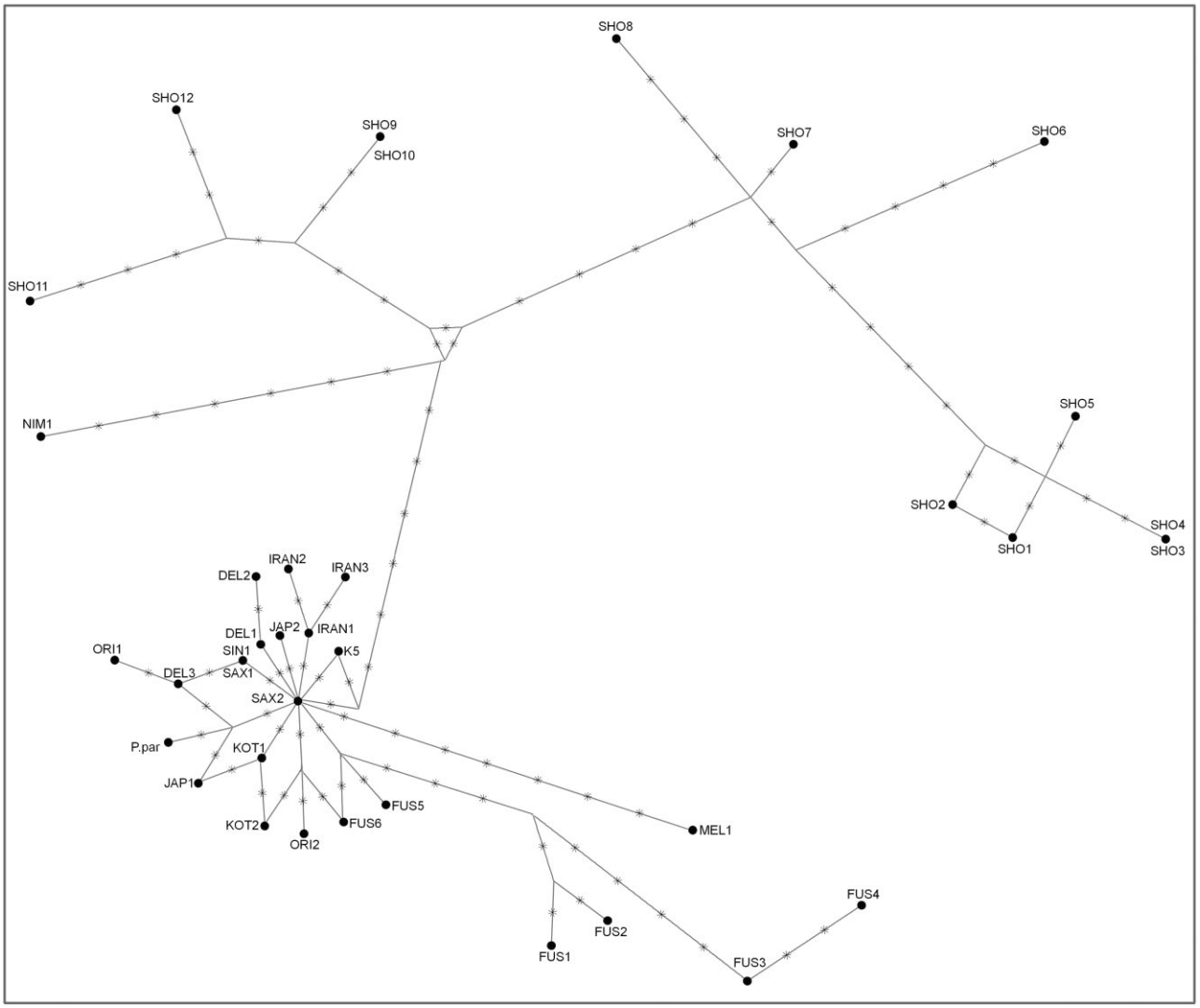

Figure 3. Median-joining network showing the relationships among leopard mtDNA haplotypes, based on 550-bp sequence of the mtDNA *NADH-5* gene. Lines connecting the mtDNA haplotype groups are proportional to the number of mutation positions. Line intersections indicate hypothetical nodes. Haplotypes ir2, az2/k1, af1, dv14265 and dv3 from Rozhnov *et al*. (2011) are not shown because they are identical to IRAN2, IRAN1, SAX2, ORI2 and FUS5, respectively. The number of individuals sharing the Iranian haplotypes is 32 for IRAN1 (incl. az2/k1), 3 for IRAN2 (incl. ir2), and two for IRAN3.

size-independent data are produced, no significant effect of regions on all of the skull characteristics as a group, is seen.

Results of the PCA analysis are provided in Figure 5 and Table 5. Based on log-transformed data, two main components were extracted which accounted for a cumulative variance of 76.95% (Table 5). Most skulls from different regions failed to become separated on both first and second principal components, whereas some slight differentiations were detected on the first component, indicating larger size of skull samples from Golestan and Alborz in contrast with Zagros individuals, which were smaller in size. The first factor represented mostly skull size measurements, as most correlations between skull size variables and the first two factors were ≥ 0.60, except FRB, BPC, BBB, SSP, LBR, IOB, LBO and HBO (Table 5).

After removing the effect of size from skull variables, PCA was computed again using standardized data in which as a result, the cumulative variance achieved by the first two components does not exceed 32.7%. The first axis mostly represents body size, although, because of a reduction in effect of size on

Table 2. Variable site alignment across 550 bp of the mitochondrial NADH dehydrogenase subunit five genes for leopards (*Panthera pardus*) from Iran and elsewhere available from GenBank, with nucleotide positions assigned from the complete mitochondrial genome of *Panthera pardus* EF551002.1. A = adenine; C = cytosine; G = guanine; T = thymine; R = G or A; Y = T or C; K = G or T; M = A or C; S = G or C

	12784	12791	12797	12809	12845	12859	12869	12872	12876	12884	12887	12890	12903	12909	12932	12935	12941	12962	12971	12974	12982	12986	12992	12995	12998	13001	13016	13025	13040	13043	13049	13073	13103	13127	13157	13160	13169	13172	13181	13193	13199	13220	13232	13253	13259	13265	13268	13273	13293	13295
P. par	T	C	A	T	T	T	C	G	A	C	T	C	A	G	A	T	C	T	T	C	T	C	C	T	A	G	A	A	A	C	C	T	T	C	A	A	C	A	G	A	T	C	G	C	T	C	A	T	C	G	C
ORI1	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A	.
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SHO1	.	.	.	C	.	.	T	A	.	.	M	.	G	.	T	C	.	.	T	.	.	.	.	.	.	A	G	.	G	.	.	C	C	T	.	.	.	.	A	G	.	T	.	T	.	.	.	.	T
SHO2	.	.	.	C	.	.	T	R	.	.	M	.	G	.	T	C	.	.	T	.	.	.	.	.	.	A	G	.	G	.	.	G	C	T	.	.	.	.	A	G	.	T	.	T	.	.	.	.	T
SHO3	.	.	.	C	.	.	T	.	.	.	C	.	G	.	T	C	.	.	T	.	.	.	.	.	.	A	G	.	G	.	.	S	C	T	.	.	.	.	A	G	C	T	.	T	C	.	.	.	T
SHO4	.	.	.	C	.	.	T	.	.	.	C	.	G	.	T	C	.	.	T	.	.	.	.	.	.	A	G	.	G	.	.	C	C	T	.	.	.	.	A	G	C	T	.	T	C	.	.	.	T
SHO5	.	.	.	C	C	.	T	.	.	.	C	.	G	.	T	C	.	.	T	.	.	.	.	.	.	A	G	.	R	.	.	C	C	T	.	.	.	.	A	G	.	T	.	T	.	.	.	.	T
SHO6	.	.	.	C	.	C	T	.	.	T	C	.	G	.	T	C	.	.	T	.	.	.	.	.	.	A	G	.	G	.	.	C	C	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	T
SHO7	.	.	.	C	.	.	T	.	.	.	C	.	G	.	T	C	.	.	T	.	.	.	.	.	.	A	G	.	G	.	.	G	.	.	.	.	.	.	A	.	.	.	.	.	.	.	T	.	T
SHO8	.	.	.	C	.	.	T	.	G	.	C	.	G	.	T	C	.	.	T	.	T	.	.	.	.	A	G	.	R	.	.	K	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	A	T
SHO9	C	.	.	C	.	.	T	.	.	.	A	.	.	.	.	.	.	.	T	.	.	T	.	.	.	A	G	.	G	.	.	C	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	A	.
SHO10	C	.	.	C	.	.	T	.	.	.	A	.	.	.	.	.	.	.	T	.	.	T	.	.	.	A	G	.	G	.	.	C	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	A	.
SHO11	C	.	.	C	.	.	T	.	.	.	A	.	.	.	.	.	.	.	T	.	.	T	.	.	.	A	G	.	G	.	.	C	C	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T
SHO12	C	.	.	C	.	.	T	.	.	.	A	.	.	.	.	.	.	.	T	.	.	T	.	.	.	A	G	.	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	T	.	.	T
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IRAN1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.	T
IRAN2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.	T
IRAN3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.	T
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DV3	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	T	.	T

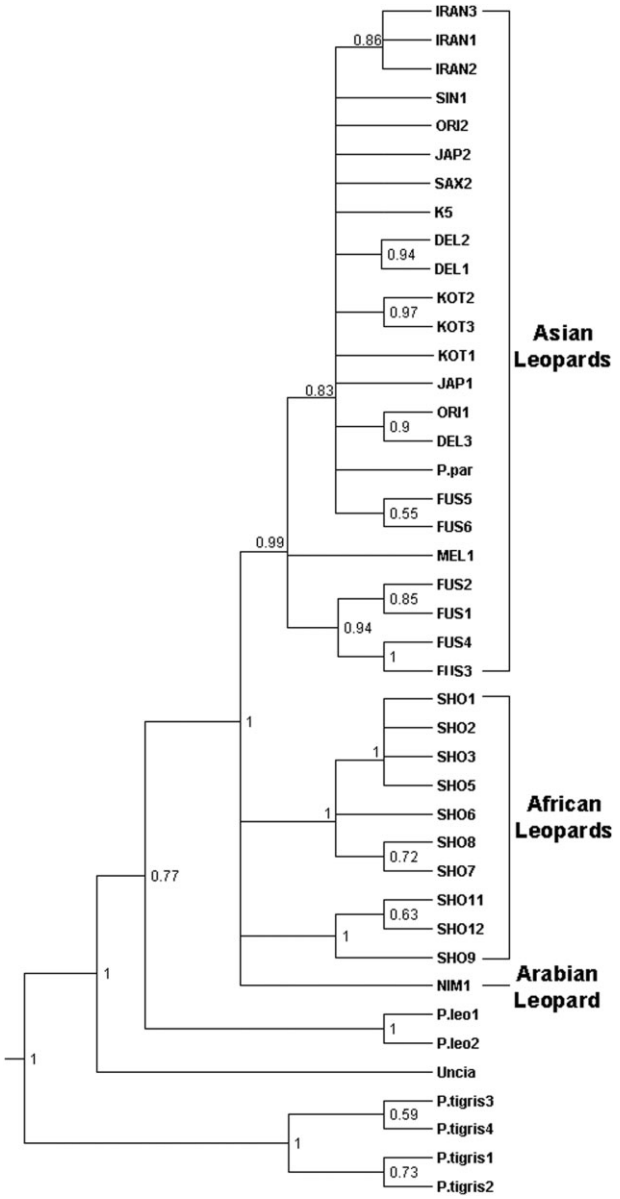

Figure 4. Maximum clade credibility tree based on Bayesian phylogenetic analysis of leopard mtDNA haplotypes from all over the world and several individuals of outgroup species. Values at nodes are posterior probabilities (only those with > 0.5 are shown). IRAN1, IRAN2 and IRAN3 are haplotypes from all over Iran, from the Alborz and from the southernmost Zagros, respectively. The names of the other individuals correspond to those in Table 1. Haplotypes ir2, az2/k1, af1, dv14265 and dv3 from Rozhnov *et al*. (2011) are not shown because they are identical to IRAN2, IRAN1, SAX2, ORI2 and FUS5, respectively.

measurements, it is mostly affected by shape of the skull. Figure 5 indicates that according to the variables used in this study, there is no significant discrimination between different regions. Zagros samples, which are from a more spatially distinctive region compared with other regions, showed surprisingly high overlap with others' convex hull (Fig. 5).

Discriminant analysis was not successful to classify correctly more than 40% of cross-validated samples regardless of data used. However, it was slightly higher for size data (40.8%) compared with shape and size data (30.6%). All geographic groups failed to obtain an accuracy of more than 50% for correct classification (Table 6).

DISCUSSION

Phylogenetic analysis

Our phylogenetic data reveal that Iran's leopards are comprised of a monophyletic clade. Both phylogenetic Bayesian tree and F_{ST} support this distinctiveness. Based on a single predominant haplotype occurring throughout most of the animal's range in Iran as well as two closely related haplotypes, it can be inferred that Iran's leopards are of the same maternal origin. This is consistent with the craniometrical analysis. Our analysis estimated Iranian maternal lineages to have split from the Asian group of maternal lineages sometime in the second half of the Pleistocene with the median divergence time coinciding with the beginning of the last glacial period (as defined in Petit *et al*. (1999) and EPICA Community Members (2004)). A possible scenario of the split is that dramatic global glaciation and aridification events typical of this period, restricted an ancestral group of Iranian leopards to a refugium where their genetic differentiation from other leopards was facilitated by limited gene flow.

Distinctive haplotypes found in southern and northern parts of the country might indicate localized evolutionary units in the Alborz and Zagros mountains, two main ranges for the leopards in Iran (Kiabi *et al*., 2002). However, occurrence of a common haplotype across Iran, including the southern part of the country, is not consistent with south Iran's leopards being a distinctive subspecies. The IRAN1 common haplotype (identical to az2; Rozhnov *et al*., 2011) has also been found in Turkmenistan and Azerbaijan, both bordering Iran (Rozhnov *et al*., 2011), indicating the widest range among the explored Persian leopard haplotypes in west Asia. According to the principle of priority belonging to the International Code of Zoological Nomenclature, taxa should be referred to by the older names given to them (ICZN, 1999), and therefore both IRAN1 and IRAN2 are cited as haplotypes az2 and ir2, respectively (Rozhnov *et al*., 2011). However, none of the two regional haplotypes, i.e. IRAN2 and IRAN3 (synonymous to ir2) have been reported from outside Iran

Table 3. Molecular diversity indices of mtDNA gene segment (*NADH-5*, 550 bp) in *Panthera pardus* (*P. p. nimr* was excluded from subdivision analysis due to a limited sample size of one individual)

Number of	Iran	Asia	Africa	All individuals
Gene copies	36	64	15	116
Unique haplotypes	3	19	11	34
Substitution sites	2	25	26	50
Private substitution sites	1	12	14	50
Transition sites	2	25	23	48
Transversion sites	0	0	3	4
Substitutions	2	25	26	52
Transitions	2	25	23	48
Transversions	0	0	3	4
Indels	0	0	0	0

Table 4. Genetic differentiation between geographical subdivisions in *Panthera pardus* based on pairwise F_{ST} tests with mtDNA *NADH-5* data (F_{ST} values are calculated with Kimura 2-Parameter distances; *P. p. nimr* was excluded from subdivision analysis due to a limited sample size of one individual)

Populations	Fst	*P*-values (Permutations: 100)
Iran vs. Asia	0.372	< 0.0001
Iran vs. Africa	0.821	< 0.0001
Asia vs. Africa	0.717	< 0.0001

(Miththapala *et al*., 1996; Uphyrkina *et al*., 2001; Rozhnov *et al*., 2011). Unique haplotype K5 of unknown geographic origin from Russian zoos (Rozhnov *et al*., 2011) might be a mutant of a single captive bred SAX2/af1 female. While further sampling and nuclear markers are essential, the yielded haplotype network of the leopards in west Asia generates a hypothesis that the leopards within the majority of their west Asian extant range, exhibit genetic closeness.

Morphological analysis

Unisex craniometric data used in this research showed that no significant differentiation in shape and size is seen between sampled regions of the leopard range in Iran. The leopards from Zagros region failed to be discriminated from other regions based on both craniometrical scenarios. Their least overlap on PC1, based on size-dependent data, indicates their relatively smaller size which, besides biological interpretations, might indicate that the leopards in these areas normally do not live long enough to have larger body sizes, due to a lower level of law enforcement and smaller number of established reserves. None of our leopard samples originating from Zagros belonged to old individuals, based on Stander (1997) and Balme, Hunter & Braczkowski (2012), in contrast with other regions. Conversely, exceptionally large skull sizes were found in the north of the country, particularly Alborz and Golestan. Slightly larger skull size of the leopards in northern Iran might be argued based on Bergmann's rule, stating that within warm-blooded vertebrate species, individuals in races from colder climates or higher latitudes are generally larger than those from races living in warmer regions or lower latitudes (Rensch, 1938). Significant positive correlation between skull length and latitude was found in 50% of carnivore species (Meiri, Dayan & Simberloff, 2004); However, Klein (1986) has shown that the leopards in southern Africa do not follow this rule. In future studies, more samples from southern Iran are necessary to be included for more robust conclusions because non-random sampling can affect the conditions of this rule (Palmer, 1999).

Prey body mass, habitat productivity and better energy accessibility have been known to be correlated with predator mass (Rosenzweig, 1968; Gittleman, 1985; Iriarte *et al*., 1990; Carbone, Teacher & Rowcliffe, 2007). In northern forested areas of Iran, the leopards partially feed on Maral red deer *(Cervus elaphus maral)* (Ziaie, 2008; Sharbafi *et al*., Unpublished data), the leopard's largest prey species in west Asia, which is absent from the Zagros region. Moreover, wild boars of larger body mass have been recorded in northern Iran (Tajbakhsh & Jamali, 1995). Therefore, the leopard's larger body size in northern Iran might be due to larger prey individuals, while in southern Iran energetics may place physiological constraints on body size due to the range of smaller prey items. Within southern Africa, 'bushveld' leopards that have regular access to impala-sized

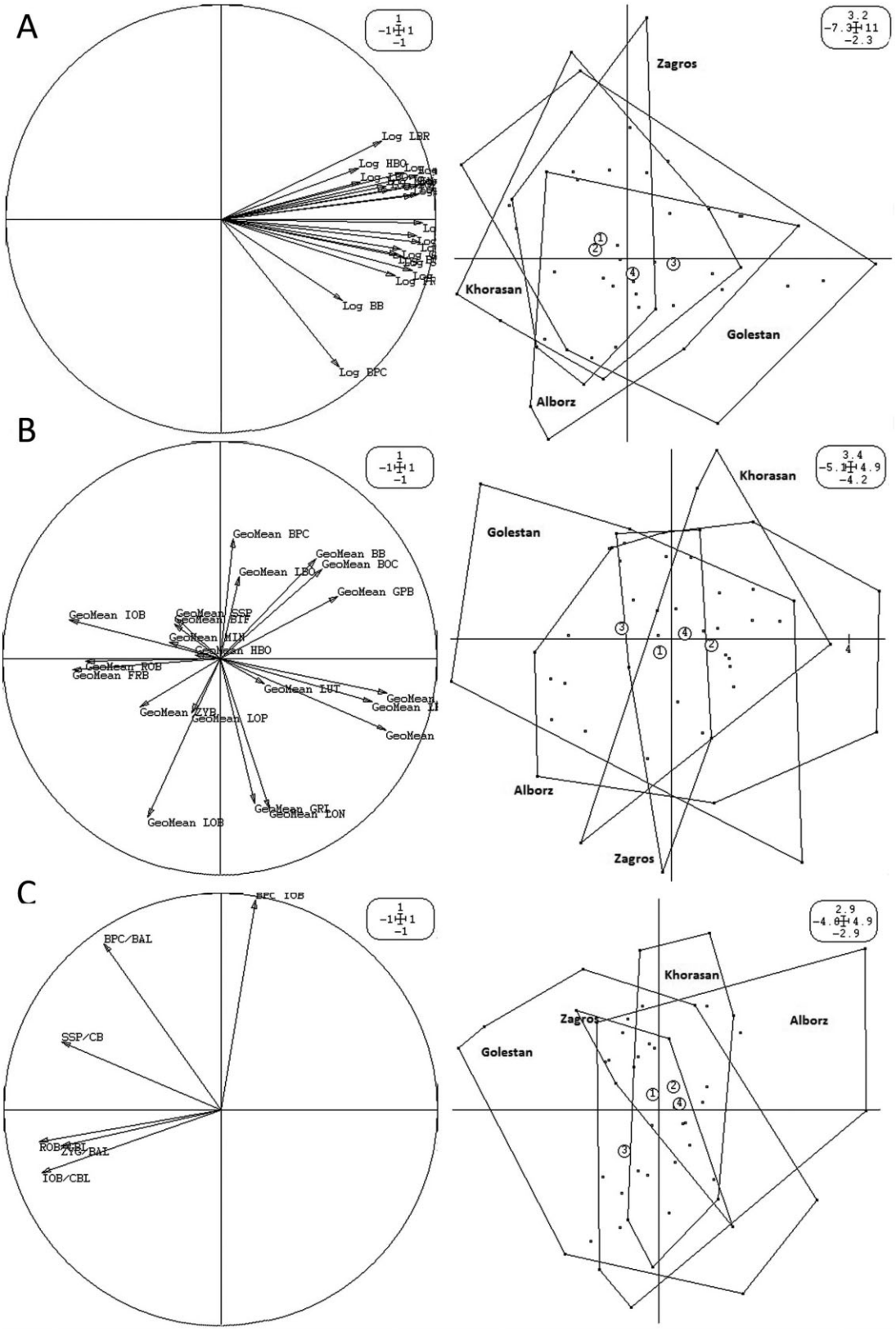

Figure 5. Plot of factors 1 and 2 from the principal component analysis of *P. p. saxicolor*, based on skull morphometric parameters with associated correlation matrix. (A) size-dependent 'log-transformed' data and (B) shape, size-independent 'standardized' data and (C) ratio data.

Table 5. Eigenvalues, percentages of explained variance, cumulative percentage of explained variance, and contribution of the variables to the first two principal components in different geographic regions in Persian leopards *(Panthera pardus saxicolor)*

	Log-transformed data Principal component		Standard data Principal component	
Character	I	II	I	II
Eigenvalues	15.757	1.172	4.519	2.696
Percentages of explained variance	71.624	5.326	20.542	12.255
Cumulative percentages of variance	71.624	76.950	20.542	32.797
Contribution of the variables				
Greatest length (GRL)	.737	.605	.133	.152
Condylobasal length (CBL)	.777	.557	.648	.369
Basal length (BAL)	.741	.597	.749	.180
Length of upper tooth row C-P4 (LUT)	.800	.498	–.104	.709
Skull height at the supraorbital processes (SSP)	.579	.687	–.426	.064
Length of braincase from opisthion to postorbital constriction (LBR)	.606	.467	.685	.056
Greatest breadth of the occipital condyles (BOC)	.604	.626	.295	.106
Zygomatic breadth (ZYB)	.673	.681	–.423	–.146
Frontal breadth (FRB)	.276	.849	–.330	–.698
Minimal inter-orbital breadth (IOB)	.522	.787	–.464	–.571
Greatest palatal breadth posterior to P4, alveoli included (GPB)	.655	.604	.248	.202
Rostrum breadth, alveoli included (ROB)	.703	.607	–.703	.137
Minimal breadth of the postorbital constriction (BPC)	.066	.850	.010	–.270
Breadth between the infra-orbital foramina (BIF)	.698	.629	–.301	–.017
Minimum length of the nasals (suture length) (MIN)	.622	.565	–.062	–.057
Length between opisthocranion and bregma (LOB)	.611	.611	.001	–.422
Length between opisthocranion and nasion (LON)	.715	.586	.210	.138
Length between opisthocranion and basion (LOP)	.700	.481	.027	.001
Length of bulla ossea (LBO)	.569	.458	.117	.115
Width of bulla ossea (HBO)	.533	.490	–.059	–.082
Breadth between bulla ossea (BBB)	.477	.444	.279	.118

Table 6. Classification results of male leopards belonging to different regions based on discriminant analysis

			Predicted geographic group membership				Percentage of correct classification of cross-validated samples
			Zagros	Khorasan	Golestan	Alborz	
Cross-validated classification	Log-transformed data	Zagros	50.0%	12.5%	0.0%	37.5%	40.8%
		Khorasan	14.3%	50.0%	21.4%	14.3%	
		Golestan	15.4%	38.5%	30.8%	15.4%	
		Alborz	28.6%	21.4%	14.3%	35.7%	
	Standard data	Zagros	12.5%	12.5%	37.5%	37.5%	30.6%
		Khorasan	14.3%	42.9%	21.4%	21.4%	
		Golestan	23.1%	38.5%	23.1%	15.4%	
		Alborz	28.6%	21.4%	14.3%	35.7%	

prey, commonly weigh at least 20% more than 'mountain' leopards which must subsist mainly on smaller hyrax-sized animals (Norton, 1984).

Previous studies concluded that with the exception of the Arabian leopards, west Asian leopards should be considered as a single subspecies, i.e. *P. p. saxicolor* (Miththapala, 1992; Miththapala *et al*., 1996; Uphyrkina *et al*., 2001; Meijaard, 2004). The present research addressed the gap in sampling from Iran as the largest bulk of the west Asian leopards, and our craniomorphological and phylogenetical analysis further confirm the former finding.

Furthermore, our data do not support Khorozyan *et al*. (2006) who proposed the populations from southern Iran and Pakistan as one subspecies *P. p. sindica*, to be a different evolutionary linage from *P. p. saxicolor* in northern Iran as well as south Caucasus. Ignoring sexual dimorphism as well as a small sample size has been shown to result in unreliable conclusions in morphometric analysis among the leopard in contrast to genetic findings (Meijaard, 2004; Farhadinia *et al*., 2014).

All available Persian leopards within American and European zoos originate from several north Iranian and Afghan founders (Shoemaker, 1985), which still resemble each other, based on comparison between captive-born samples used by Uphyrkina *et al*. (2001) and wild-caught samples scattered across the northern range of the subspecies (Rozhnov *et al*., 2011). With respect to a small population size of the free-ranging leopards in most of the Iranian reserves (predominantly fewer than ten mature individuals; Ghoddousi *et al*., 2010; Farhadinia, Moqanaki & Hosseini-Zavarei, 2014), the existing captive population of Persian leopards could be potentially an invaluable source for any possible restoration program within the historical range of the Persian leopards, particularly within Asia Minor. Nevertheless, nuclear genetic assessment is encouraged to reconstruct the genetic structure of wild Persian leopards and to assure the purity of zoo individuals. Meanwhile, the mtDNA diversity of captive Persian leopards analyzed by Uphyrkina *et al*. (2001) was somewhat lower compared with most other leopard subspecies. Therefore, any Persian leopard individual which is subject to removal from the wild, particularly in Iran due to severe injury, being orphaned, etc. with no chance of re-wilding, is recommended to be taken into consideration as part of a properly organized international breeding program for Persian leopard for genetic enforcement of the existing captive bulk within zoos.

Conversely, our genetic findings together with previous studies (Uphyrkina *et al*., 2001; Rozhnov *et al*., 2011), indicate that the present captive source of Persian leopards within zoos do not maintain all available genetic variation of the subspecies (i.e. rare haplotypes). Most of the recent attempts to rescue the leopards from human casualties in Iran resulted in death (Farhadinia & Baradarani, unpublished data). Thus, while enhancing protection measures in associated areas of rare haplotypes is of high priority, it is critical to provide relevant training for local veterinarians and experts to ensure safe live capturing of injured/trapped leopards, particularly in the south of the country.

In order to understand management unit(s) across the subspecies' range, nuclear DNA data will be an invaluable tool. Nevertheless, the present investigation together with previous scientific efforts (Rozhnov *et al*., 2011) suggest a common cladistics phylogeny based on NADH-5 marker for most of Iran as well as for adjacent countries which could be considered to initiate large-scale conservation planning and prioritization. For larger species such as jaguars, Weber & Rabinowitz (1996) have noted that due to the species' broad distribution across many different nations and habitat types, small-scale conservation efforts selected ad hoc and focused on narrowly defined areas have not succeeded in stemming the tide of jaguar extirpation. Therefore, in order to safeguard wide-ranging cats, while considering the species' ecological roles in various habitat types, their entire range through political boundaries should be explicitly planned (Sanderson *et al*., 2002). The Persian leopard has been subject to different conservation planning (Breitenmoser *et al*., 2007; Zazanashvili *et al*., 2007) and mapping efforts (Zimmermann *et al*., 2007; Gavashelishvili & Lukarevskiy, 2008) within the Caucasian eco-region. However, most of the subspecies' range still suffers from a comprehensive prioritization to outline management units.

Future genetic studies are recommended to include additional individuals, locations, and nuclear recombinant markers, such as microsatellites in order to provide better information about population genetics and conservation actions. The leopards have been known to show moderate to high genetic variability at the country-wide scale (i.e. Tanzania: Spong *et al*., 2000; India: Dutta *et al*., 2013), but they can become genetically differentiated with increased habitat fragmentation (Dutta *et al*., 2013). Therefore, landscape genetic models are essential to guide protection measures based on source-sink spatial configuration of the leopards' ranges in west Asia.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Leopard sample localities used in the genetic study.
Appendix S2. Additional mitochondrial haplotypes (611 bp) downloaded from GenBank to construct phylogenetic trees.
Appendix S3. Characteristics of male leopard skulls from Iran (Abbreviations: DoE, Department of Environment; MNH, Museum of Natural History; and NP, National Park).