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Research Article

DNA barcoding of the Luristan newt (*Neurergus kaiseri*) in south-western Iran

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Abstract

The Luristan newt (*Neurergus kaiseri*) is an endemic and vulnerable species to the southern Zagros Mountains of Iran, inhabiting streams and ponds in open woodlands, dominated by oak tree (*Quercus brantii*). In the current study, 20 specimens of the Luristan newt were collected and sequenced for a 651bp fragment of the mitochondrial cytochrome c oxidase subunit 1 (CO1). Sequence divergence values varied from 0.0% to 2.8%. The phylogenetic analysis revealed the existence of two distinct clades and a clear divergence between Northern and Southern populations. The genetic variation among the two clades was significant (F_{ST} = 0.95, $P < 0.001$). Five haplotypes were observed among *N. kaiseri* sequences, of which two haplotypes were found in the Northern populations and the other three haplotypes in the Southern populations. None of the haplotypes was shared between the two clades. The distinction of Northern and Southern populations may be due to the rough topography of its habitats and the low dispersal ability of the Luristan newt.

Keywords: Amphibian, COI, habitat features, phylogeny.

Introduction

Three genera of salamanders including, *Triturus*, *Salamandra* and *Neurergus* can be found in Iran freshwater basins, all belonged to Salamandridae family. The Luristan newt (*Neurergus kaiseri*, Schmidt 1952), also known as Kaiser`s spotted newt, is endemic to the southern Zagros Mountains of Iran, inhabiting streams and ponds in open woodlands, dominated by oak tree (*Quercus brantii*). This newt is classified as vulnerable (VU) by the IUCN Red List (Mobaraki *et al.* 2016) due to limited geographic range, habitat loss, and illegal trading (IUCN 2018).

Current knowledge on *N. kaiseri* is restricted to some aspects of the ecology such as distribution, including reports of new localities for the species (Sharifi *et al.* 2013, Mobaraki *et al.* 2014), and demography (age structure, longevity and growth patterns) of a local population (Farasat and Sharifi 2015). A recent molecular study reported the presence of two genetically distinct clades within the Luristan newt populations in the species type locality (Farasat *et al.* 2016).

DNA barcoding has emerged (Hebert and Gregory 2005) as rapid and effective method for species identification, which has accelerated biodiversity assessments (Hebert and Gregory 2005, Valentini *et al.* 2008, Hebert *et al.* 2010, Friedheim 2016). This method employs sequence diversity in standardized gene regions (e.g. cytochrome c oxidase subunit I, (COI)) to identify species (Hebert *et al.* 2003). The effectiveness of CO1 DNA barcodes for identification of intra- and interspecific variations has been shown in various taxa including amphibians (Smith *et al.* 2008, Xia *et al.* 2012, Chambers and Hebert 2016) and this technology can greatly contribute to the taxonomy science (Vences *et al.* 2005, Estupiñán *et al.* 2016, Pereyra *et al.*

2016). For example, DNA barcoding provided evidence for numerous cryptic species in amphibians (Stuart *et al.* 2006, Fouquet *et al.* 2007).

In this context, we used DNA barcoding to investigate intraspecific diversity within the Luristan newt. The current study aimed to employ a standardized region of COI gene to provide sequence diversity and genetic barcodes for *N. kaiseri*.

Material and methods

Sampling was conducted in April 2015, and tail tip biopsies (~ 1cm) of individuals were collected from six sites (Fig. 1). Due to the drought in the past few years, many of the ponds and streams in the region were dried out, limiting the number of sampling sites. Six sites were sampled, covering both Northern (Kerser, Daregoand, Vegenab) and Southern (Tove, Bozorgab and Monagre) populations (Table 1). Samples were preserved in 70% ethanol and stored at -4°C, prior to DNA extraction. The permit for collecting tail clips from live *N. Kaiseri* was issued by the Iranian Department of Environment (permission number: 94/4469).

Table 1. Sampling sites and the number of samples obtained for DNA barcoding of N. kaiseri in south-western Iran.

Site	acronyms	Altitude (m)	coordinates	N
Daregol	DRGL	1050	32˚55′N, 48˚10′E	
Kerser	KRSR	1100	32˚06′N, 48˚01′E	
Vegenab	VOGN	950	33˚00′N, 48˚4′E	
Tove	TOVE	940	32˚06′N, 48˚45′E	
Bozorgab	BOZG	1080	32˚90′N, 48˚51′E	
Mongare	MNGR	960	32˚00′N, 48˚02′E	

DNA extraction and barcoding

We extracted DNA from 20 samples using CinnaPure DNA extraction kits (Cinnagen Co. Iran), following the manufacturer's instruction.

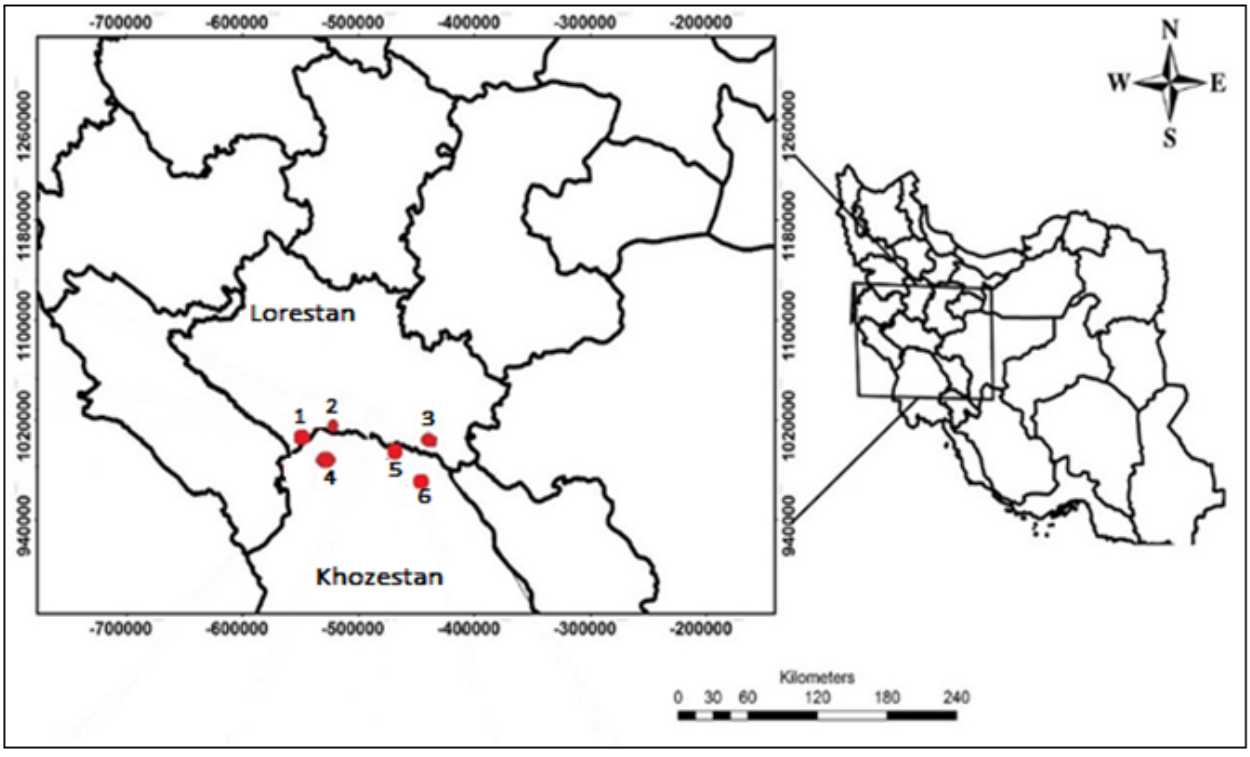

Figure 1. Sampling localities of *N. kaiseri* populations in the south-western Iran: 1) Kerser, 2) Daregol, 3) Bozorgab, 4) Tove, 5) Vegenab and 6) Mongare.

For DNA barcoding, a 651 bp fragment of the mitochondrial COI region was amplified using

primers LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198:5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Jing *et al.* 2012). Amplification was performed in a final volume of 25 μL reaction containing 1 μL of genomic DNA (~100 ng), 1μm of each primer, 3 mM MgCl, 0.5 mM dNTP, 2.5 μL of 1x PCR buffer (containing 100 mm Tris-HCl, pH 8.3, 500 ml KCl) and and 0.5 μL (5 U/μL) of Taq polymerase. Thermocycling was performed in a *SensoQuest* thermocycler (Biomedizinische Elektronik, Germany), using an initial denaturing at 94° C for 4 min, 35 cycles of denaturing at 94° C for 60 s, annealing at 53° C for 60 s, extension at 72° C for 90 s, followed by a final extension at 72° C for 7 min. Purification and sequencing of PCR products were commercially performed by Macrogen Inc, South Korea.

Raw sequences were edited using SeqScape v2.6 (Applied Biosystems), aligned using the Clustal W algorithm as implemented in MEGA6 (Tamura *et al.* 2013), and checked visually. Sequences have been submitted to GenBank accession nos. MK334018-MK334037. Kimura 2-parameter model (K2-P) was selected as the best model of nucleotide substitution, using jModeltest 0.1.1(Posada 2008).

Phylogenetic analysis was implemented in MEGA6 (Tamura *et al.* 2013), using maximum likelihood (ML) method with 10000 bootstrap replicates. A sequence of *Triturus karelinii* (GenBank accession no. EF526007) was used as outgroup for the phylogenetic analysis. ARLEQUIN v3.5.2.2 (Excoffier and Lischer 2010) was used to estimate pairwise F_{ST} between populations and identify haplotypes and their frequency in populations based on a maximum likelihood approach. In addition, a median-joining (MJ) network was constructed using NETWORK v4.1 (Bandelt *et al.* 1999).

Results

Sequences for the 651 bp fragment of the COI gene were obtained for 20 samples. On average, the COI sequence was found to be A–T rich (A = 30%, T = 32%, G = 13% and C = 25%). The transition: transversion ratios ranged from 0.5 to 0.7. Transitions were more frequent than transversions with a majority of changes between A and G. A total of 17 variable sites and 17 parsimony informative sites were observed.

The COI sequences had open reading frames in all sequences, suggesting they are functional genes and unlikely to be nuclear copies of mtDNA. Further, no evidence for double PCR-amplification peaks and ambiguities in the sequence data was found to suggest the presence of nuclear copies in the mtDNA data set.

Five haplotypes were observed among *N. kaiseri* sequences (n = 20). Sequence divergence values obtained by applying the K2-P model to the dataset varied from 0.0% to 2.8%. The maximum value was observed between Daregol and Bozorgab, Mongare and Tova populations (Table 2). The ML phylogenetic tree revealed the existence of two distinct clades and a clear divergence between Northern and Southern populations (Fig. 2A). The haplotype network (Fig 2B) was in agreement with the topology of the ML tree. From the five haplotypes found in these populations, two haplotypes (Hap1and Hap5) were found in the Northern populations (Kerser, Daregol and Bozorgab) and the other three haplotypes (Hap2, Hap3 and Hap4) in the Southern populations (Vegenab, Mongare and Tove). Haplotype 1 was shared between Bozorgab and Kerser, however, Hap2, Hap3, Hap4 and Hap5 were unique and found in Tove, Vegenab, Mongare and Daregol respectively. None of the haplotypes was shared between the two clades. The haplotype network suggested little or no gene flow between the Southern and Northern clades. The AMOVA analyses showed significant differences among populations in each clade, when the Northern (F_{ST} = 0.76, P < 0.001) and Southern (F_{ST} = 0.31, P < 0.001) clades were analysed separately. The genetic variation between the two clades was also significant (F_{ST} = 0.95, P <

0.001).

Table 2. Sequence divergence values among the six populations of *N. kaiseri* obtained by applying the K2-P model to the dataset. Significant values ($P < 0.05$) are in bold.

		Bozorgab	Mongare	Tove	Daregol	Kerser
Mongare	2	0.009				
Tove	3	0.006	0.006			
Daregol	4	**0.028**	**0.028**	**0.028**		
Kerser	5	0.000	0.000	0.000	0.008	
Vegenab	6	0.008	0.008	0.008	0.008	0.002

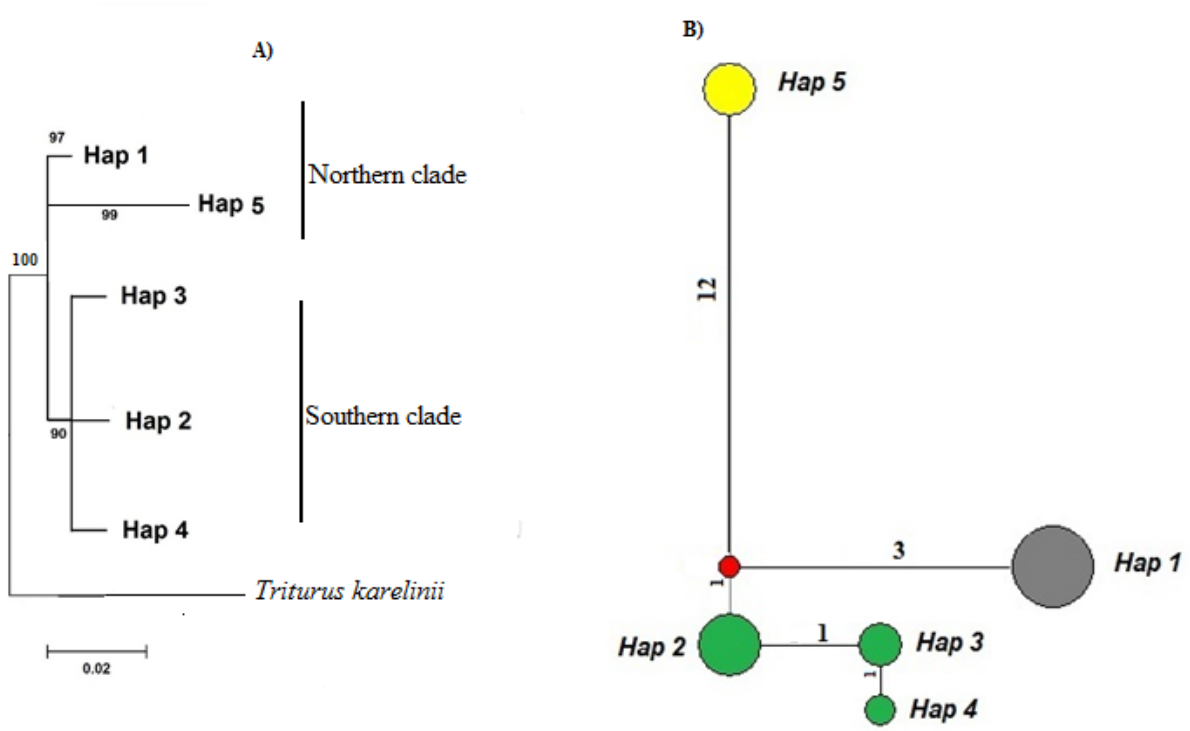

Figure 2. A: Maximum likelihood tree of the COI gene from *N. kaiseri* haplotypes. A sequence of *Triturus karelinii* was used as outgroup. Numbers on branches represent percentage of bootstrap values (10000 replicates). B: A median-joining network based on the COI gene depicting the relationships between the main two clades described for *N. kaiseri* (the Northern and Southern clades). The red dot represents a hypothetical haplotype. Numbers on the lines connecting haplotypes represent the number of mutations. Hap1 = Bozorgab and Kerser; Hap2 = Tove; Hap3 = Vegenab; Hap4 = Mongare; Hap5 = Daregol.

Discussion

Patterns of population structure and intraspecific variation within a species reflect both historical and current levels of gene flow (Sork and Waits 2010). Identifying patterns of genetic variation within a species can help determine the relative contribution of factors involved in evolutionary diversification and speciation (Simons 2002). This study examined sequences of the mitochondrial COI gene to enhance our understanding of the newt diversity in the region and hence to aid conservation planning.

The DNA barcoding analysis based on 651 bp of COI sequences clearly separated the Northern and Southern populations, confirming the presence of the two genetic clades (Farasat *et al*. 2016) in the species. No shared haplotypes were found in the two clades, which may partly be due to the rough topography of its habitats (Sharifi *et al.* 2013). In addition, the low dispersal ability and limited mobility of the Luristan newt may have also contributed.

Limited gene flow in many amphibians, including *N. microspilotus*, has been linked to the low dispersal ability (Sharifi and Afroosheh 2014).

Revealing genetic diversity within species and populations can direct conservation priorities (Arbogast and Kenagy 2001). DNA barcodes can aid conservation by assisting field workers in identifying species, by helping taxonomists determine species groups needing more detailed analysis, and by facilitating the recognition of appropriate scales for conservation planning (Smith *et al*. 2008). Results of this study provide more insights into the intraspecific diversity within *N. kaiseri*, and possible co-existence of two species in its distribution range. These findings have important conservation implications. Although the Luristan newt is included in the appendix I of the convention to the international trade to endangered species (CITES 2010, Mobaraki *et al.* 2016), illegal collection of adults and larvae of the newt for pet trade threaten the survival of its wild populations (Mobaraki *et al.* 2014). When smuggled newts are retrieved from illegal hunters, releasing them into the wild should be given special care, because of its significant intraspecific diversity. Ecological differences also exist between the two clades, as the Northern populations are located at higher elevations and in a more humid climate than the Southern ones (Farasat *et al.* 2016). Due to different ecological conditions between the Northern and Southern clades, the introduced newts may not survive, and if they survive, there is a concern about the loss of genetic diversity due to genetic erosion (Frankham *et al.* 2004).

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