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Assessment of Environmental DNA for Detection of an Imperiled Amphibian, the Luristan Newt (*Neurergus kaiseri*, Schmidt 1952)

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***Abstract.*—The isolation and identification of DNA fragments from environmental samples (eDNA) provide a non-invasive and efficient technique for detection of a target species. The Luristan Newt (*Neurergus kaiseri*) is a critically endangered amphibian endemic to the southern Zagros Mountains of southwestern Iran. This study presents the results of a PCR-based eDNA assay capable of detecting the Luristan Newt. Environmental DNA had the cabability of detecting the presence of the newt in various water bodies where adults or larvae of the species were present. Results of occupancy modeling showed that detection probability of Luristan Newt eDNA was higher in lentic systems with lower water velocity and when the time lag between sampling and freezing was shorter. The stability of eDNA was estimated in water in an ambient environment at about 15 d and under exposure to direct sunlight up to 6 d.**

Key Words.—eDNA; endangered species; species detection; water samples; Zagros Mountains

Introduction

Environmental DNA (eDNA) analysis is a relatively new and low impact tool that has improved the detection of aquatic species by isolating DNA from environmental samples, such as water (Ficetola et al. 2008; Goldberg et al 2011; Jerde et al. 2011). One popular method includes collecting, extracting and amplification of short DNA fragments, such as mitochondrial DNA, specific to the species of interest (Ficetola et al. 2008). The total eDNA in an aquatic habitat originates from sloughed cellular materials, excreted or secreted from species occupying or visiting the water system. It can provide evidence for the recent presence of a particular species within that water system, without the need for direct observation (Ficetola et al. 2008; Jerde et al. 2011).

Visual survey techniques usually require physical capture or direct observation of individuals (Heyer et al. 1994) at several sites, which can be time-consuming and costly, with varying detection probability depending on the species. In addition, detection may be reduced at low density, which may also limit the detection of species, such as invasive species at early stages of invasion (Secondi et al. 2016; Klymus et al. 2017) or threatened and endangered species. The rarity of the latter species and legal restrictions limit visual observations and routine handling of such species through traditional methods (Thomsen et al. 2012). The use of eDNA methodology may help to sample a relatively large area in a short period of time and eliminate the potential harm to species during sampling (Rees et al. 2014b).

Research during the past few years has shown the ability of eDNA method to detect the presence of various aquatic species (Jerde et al. 2011: Thomsen et al. 2012; Wilcox et al. 2013; Boothroyd et al. 2016). Further, this method can be more sensitive compared to traditional sampling methods for determining the presence of rare, low density, or threatened species. For example, Sigsgaard et al. (2015) used eDNA method for detecting endangered fish and reported the increased effectiveness of eDNA over the traditional surveys. Wilcox et al. (2014) optimized eDNA technologies for detection of the endangered Bull Trout (*Salvelinus confluentus*). Environmental DNA analyses have been used to detect threatened amphibians, such as the Eastern Hellbender (*Cryptobranchus a. alleganiensis*; Olson et al. 2012) and the Great Crested Newt (*Triturus cristatus*; Rees et al 2014a). However, few conservation programs have taken advantage of this method, and the efficiency of such methodology has not been tested for many threatened species.

The Luristan Newt (*Neurergus kaiseri*, Fig. 1) is endemic to the southern Zagros Mountains of Iran. It is classified as Critical Endangered (CR) by the International Union for the Conservation of Nature (IUCN) Red List due to habitat loss, limited geographic range and illegal trading, coupled with the effects of severe droughts in recent years (IUCN 2009). The species is highly dependent on ponds and streams and is patchily distributed in mountainous areas. Visual surveys for this species require visiting several sites to establish reliable presence/absence data. This is time

Figure 1. The Luristan Newt (*Neurergus kaiseri*), endemic to the southern Zagros Mountains of Iran. (Photographed by Forough Goudarzi).

consuming and costly, and may be limited by weather and topographic conditions. Another important issue with field visual surveys for Luristan Newts is the relatively short survey period. The active period for this species in the southern Zagros Mountains starts in March and ends in July. During this time when temperature allows feeding and breeding, Luristan Newts can be found in ponds and springs. Therefore, additional survey methods, such as eDNA, can act as a complement to the visual surveys and facilitate the detection of this rare and endangered species.

Despite the effectiveness of eDNA technology, it should be noted that DNA in the environment can rapidly diffuse and disperse from its source (Deiner and Altermatt 2014). The loss and degradation of eDNA can be influenced by several factors, such as ultraviolet (UV) radiation, pH, and temperature Dejean et al. 2011; Piaggio et al. 2013; Pilliod et al. 2014). Higher temperatures, neutral pH, and moderately high UV-B have been shown to contribute to favorable environments for microbial growth (Strickler et al. 2015). Parameters that can influence eDNA survival and availability should, therefore, be considered. We used environmental DNA methodology to detect the endangered Luristan Newt in its known habitats and we evaluate the factors that may lead to eDNA degradation and reduce the reliability of the results.

Materials and Methods

Field survey and sampling.—We conducted field surveys across the distribution area of the Luristan Newt in south-western Iran during spring 2014 to locate ponds, springs, and streams with potential presence of the species. We conducted visual encounter surveys for larvae or adults of the target species, and we identified 11 Luristan Newt-positive and three Luristan Newt-negative sites that we then subjected to eDNA analysis. Because of the drought from 2010 to 2017, many of the ponds and springs in the region were dry, limiting our selection of sites. The Luristan Newt is the only newt species in the region and no overlap has been reported between the distribution range of it and other species of *Neurergus* (Barabanov and Litvinchuk 2015). The Common Frog (*Rana temporaria*) was the only other amphibian species observed at the sites where Luristan Newts were present, and no fish species were observed.

At each site, we recorded the stage of a newt at the time of sampling (adult and larvae), habitat type (lotic/lentic), water temperature, pH, and velocity (Table 1). We classified ponds with standing water or water velocity less than 1m/min as low water flow, streams with water velocity between 1 to 5m/min as moderate water flow, and streams with water flow greater than 5m/min as high water flow. In addition, we collected three 15 mL water samples from each site, and instantly added 1.5 mL of sodium acetate 3M and 33 ml absolute ethanol to the water samples (Ficetola et al. 2008). To obtain a better coverage of a site, we collected one water sample from each of three different points around a site. We protected samples from direct sunlight exposure in the field and stored at -20° C prior to DNA extraction. We included both negative and positive controls provide measures of contaminations. As positive procedural controls, we obtained three 15 mL water samples from an aquarium containing Luristan Newts. For negative controls, we used three 15 mL water samples from a pond with no newt population and tap water. Because of the lack of facilities in some remote areas, we were not able to freeze samples immediately after collecting

Table 1. Characteristics of sites sampled for eDNA analysis of the Luristan Newt (*Neurergus kaiseri*) in south-western Iran and the summary of the visual survey (positive or negative) and PCR results (the number of positive PCRs of the nine PCR replicates, which is based on three PCRs per sample and three water samples per sites).

Site number	Survey detection	PCR detection	Life stage	Habitat type	Water flow	pH	Temperature° C	Time in transit
1	Positive	5/9; 2/3	Adult	Lentic	Low	8.4	20	Short
2	Positive	3/9; 1/3	Larva	Lentic	Low	8.4	20	Intermediate
3	Positive	6/9; 3/3	Adult	Lentic	Low	8.4	20	Intermediate
4	Positive	2/9; 1/3	Adult	Stream	Moderate	7.5	17	Short
5	Negative	4/9; 2/3	Adult	Stream	Moderate	8.4	22	Short
6	Positive	4/9; 3/3	Adult	Stream	Moderate	7.5	22	Intermediate
7	Positive	5/9; 1/3	Adult	Lentic	Low	7.5	20	Short
8	Negative	0/9; 0/3	–	Stream	Moderate	8.4	25	Short
9	Negative	0/9; 0/3	–	Stream	Moderate	8.0	22	Short
10	Positive	1/9; 0/3	Adult	Lentic	Low	8.0	20	Long
11	Positive	5/9; 3/3	Larva	Lentic	Low	7.5	20	Short
12	Positive	4/9; 3/3	Adult	Stream	Moderate	8.4	17	Short
13	Positive	0/9; 0/3	Adult	Stream	High	8.0	17	Long
14	Positive	0/9; 0/3	Adult	Stream	High	8.4	17	Long

at each site, which could affect the detectability of a species (Rees et al. 2014a). Therefore, we recorded the time between sampling and storing at -20º C as time in transit into one of three categories: less than half a day (short), half a day to one day (intermediate), and more than one day (long; Table 1).

DNA persistence.—We prepared a water tank with a volume of 5 L tap water (pH = 7, about 20° C), and we transferred one adult Luristan Newt into the tank to provide water samples for an eDNA persistence experiment. We kept the newt in the tank for one week, kept the tank conditions (pH and temperature) constant, and fed the animal autoclaved fish food. After one week, we removed the newt from the tank. We kept half (2.5 L) of the tank water under conditions of ambient temperature with no direct light (treatment 1) and exposed the other half to direct sunlight (treatment 2). We extracted and amplified DNA (described below) from 15 ml of water sampled from each treatment daily until we did not detect any DNA/PCR product.

DNA extraction and PCR.—To collect DNA, we centrifuged water samples (5,500 g, 35 min, 6° C; Ficetola et al. 2008), discarded the supernatant, and used the air-dried pellets for DNA extraction. We performed DNA extraction using IraiZol Kit (RNA Biotech, Isfahan, Iran), following the instructions provided by the manufacturer. To quantify DNA, we used a PicoDrop200 spectrophotometer (Saffron Walden, Uttlesford, UK) to measure DNA concentrations. To test for cross-contamination, we used extraction blanks with tap water. The designed primers were Forward (5´-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA -3´) and Reverse (5´- AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA -3´), which targeted a 307 bp fragment of the Cytochrome *b* gene in salamander species (e.g., Noël et al. 2008). The primers tested positive on a genomic DNA extracted from a Luristan Newt tissue sample available from another study on population genetics (Forough Goudarzi, unpubl. data). We used PrimerTree 1.0.3 (Cannon et al. 2016) to perform *in silico* primer search and did not detect DNA from other *Neurergus* species (e.g., the Yellow Spotted Newt, *Neurergus crocatus*, and the Kurdistan Spotted Newt, *Neurergus microspilotus*). To further investigate the possibility of nonspecific amplification, we collected three 15 mL water samples from a pond with no Luristan Newts on different occasions, and three15 mL water samples from an aquarium with only species of fish present. We extracted and amplified DNA from each of these samples (three replicates each).

Initially, we conducted our PCR reactions in a total volume of 25 µL, consisting of 5 µL of DNA extract, 1 µL of 10x PCR Buffer (Applied Biosystem), 1 µL dNTP (10 mM), 2.5 µL MgCl2 (25mM), 1 µL (10 pmol) of each primer, 0.5 µL Ampli*Taq* DNA polymerase (Applied Biosystems) and 14 µL ddH_2O. We performed thermocycling in a SensoQuest thermocycler (Biomedizinische Elektronik, Göttingen, Germany) using an initial denaturing at 94° C for 4 min, 45 cycles of denaturing at 94° C for 35 s, annealing at 53° C for 40 s, extension at 72° C for 90 s, followed by a final extension at 72° C for 10 min. We used two positive controls, including two separate DNA extracts from aquarium water holding Luristan Newts and two negative controls consisting of one

Table 2. Model ranking of occupancy models evaluating the probability of detecting eDNA of the Luristan Newt (*Neurergus kaiseri*) from water samples. The small sample sized-corrected AIC values (AIC_c), the AIC_c differences (delta AIC_c), the Akaike weight for each model, and deviance are reported for each model.

Model	AIC_c	Delta AIC_c	Akaike weight	Deviance
P(htype+velocity+transit)	202.562	0.000	0.99987	37.987
P(velocity+transit)	227.237	10.871	0.00013	89.189
P(htype+transit)	236.876	72.435	0.00000	145.681
P(htype+velocity)	241.672	86.982	0.00000	151.411
P(htype+pH+tm+velocity +transit)	249.234	98.445	0.00000	165.681
P(htype+tm+velocity)	252.218	122.557	0.00000	176.123
P(htype+tm+transit)	258.567	135.443	0.00000	182.353
P(transit)	269.236	145.556	0.00000	188.681
P(velocity)	278.342	157.987	0.00000	192.655
P(htype)	289.123	178.321	0.00000	208.342
All other models (39)	> 300	> 200	0.00000	211.664

extraction blank and a PCR control with ddH_2O in place of DNA template. The amplified DNA were then visualized and assessed on a 1.5% agarose gel. Because of the lack of detectable PCR products, we further increased the amount of DNA template in PCR reactions to 10 µl and amplifications were performed as described above. We amplified each water sample three times (nine repeats per site). Positive PCR Products were sequenced on an automated DNA sequencer (ABI-3700 Applied Biosystem). Finally, to check the specificity of obtained sequences and ensure that the amplicon was from the Luristan Newt, we conducted a Basic Local Alignment Search Tool (BLAST) of NCBI GenBank.

Statistical analyses.—We used an occupancy approach implemented in Program MARK (White and Burham 1999) to estimate the probability of detection (p) of the Luristan Newt eDNA and to account for imperfect eDNA detection/ (Schmidt et al. 2013). We developed 49 models representing relation between various combination of sample- and site-specific predictors. We included habitat type (lentic/ lotic), water temperature (17º, 20º, 22º, 25º C), pH (7.5, 8.0, 8.4) and velocity (low, moderate, high), newt life stage (larvae/ adult) and time in transit (short, intermediate, long). We used Akaike's Information Criterion (AIC) to assess support for different models. Models were ranked by their small sample sized-corrected AIC values (AICc; Schmidt et al. 2013) and estimates of the probability of detection reported here come from the top-ranked model.

Results

The concentrations of eDNA obtained from water samples ranged from 1.2 to 5.1 ng/µl. We were able to successfully amplify the 307 bp fragment of Cytochrome *b* gene when we used 10 µL of DNA template. The amount of template DNA used in eDNA PCR reactions varies in the literature and increasing the amount of template DNA has been shown to improve the eDNA successful amplification (Rees et al. 2014a). The BLAST search confirmed the identity of the sequences as the Luristan Newt. A 100% match existed between our sequences and a GenBank cytochrome *b* sequence (Accession no. DQ092233.1). Two haplotypes could be identified among our sequences (see discussion below), therefore, sequences of these two haplotypes were deposited in GenBank (Accession numbers: KY404094–KY404095). None of the negative controls resulted in a positive reaction and all extraction blanks were negative. To assess the presence of inhibitors, 3µL of Luristan Newt DNA was added into samples with negative amplification. All reactions were positive, confirming that PCR amplification was possible under the condition of our experiment.

We were able to detect the target species using eDNA at nine sites out of 11 survey-positive sites. The average Luristan Newt DNA amplification success in survey-positive sites was 0.35 ± 0.09 (min = 0/9; max = 6/9, n = 11) and for survey-negative sites, it was 0.15 ± 0.06 (min = 0/9; max = 4/9, n = 3; Table 1). Using eDNA, we detected the Luristan Newt at one site (# 5) that was survey negative (in 2015) with 4/9 reactions being positive (Table 1). A further field survey in April 2016, however, confirmed the presence of the species at this site. Three survey positive sites (10, 13 and 14) gave 0/9 to 1/9 positive amplification, possibly due to the longer time lag between sampling and freezing the water samples (see below). We detected Luristan Newt in both lentic (5/6 sites) and lotic (4/8 sites) systems where adults or larvae of the species were present.

We used site occupancy modeling to analyze the detection probability and quantify the effect of various parameters. Model with the highest rank included habitat type, water velocity and time in transit (Akaike weight = 0.9999; Table 2). The next model supported by the analysis by AIC = 0.0001 included water velocity and transit time (Table 2). The highest probability of detection (0.74, Table 3) was obtained for the combination of lentic system, low water velocity and short time in transit. We did not detect positive amplification from water samples taken after 15 d of storage at ambient temperature or six days for the water exposed to direct sunlight, presumably because of increasing temperature and the effect of UV radiation on DNA degradation (Strickler et al. 2015).

Discussion

Detection of Luristan Newts.—In the current study, we evaluated the use of eDNA analysis to detect the presence of Luristan Newt in various water bodies in

Table 3. The probability of detection of eDNA of the Luristan Newt (*Neurergus kaiseri*) estimated based on the best model (Table 2) of habitat type (lentic/lotic), water velocity (low, moderate, high) and transit time (short, intermediate, long). The highest probability of detection is in bold.

Models	Probability of Detection (p)
Lotic+low+short	0.22
Lotic+low+intermediate	0.19
Lotic+low+long	0.05
Lotic+moderate+short	0.05
Lotic+moderate+intermediate	0.00
Lotic+moderate+long	0.00
Lotic+high +short	0.01
Lotic+high+intermediate	0.00
Lotic+high+long	0.00
Lentic+low+short	**0.74**
Lentic+low+intermediate	0.56
Lentic +low+long	0.22
Lentic +moderate+short	0.22
Lentic+moderate+intermediate	0.12
Lentic +moderate+long	0.12
Lentic +high +short	0.00
Lentic +high+intermediate	0.00
Lentic +high+long	0.00

southwestern Iran. We were able to detect the target species using eDNA at nine sites out of 11 survey-positive sites, which gave a per-site detection rate of 82% and the number of positive PCR replications varies from 1/9 to 6/9. None of the negative controls resulted in a positive reaction and all extraction blanks were negative. Using eDNA, we detected the Luristan Newt at one survey-negative site (no. 5). Further field surveys, however, confirmed the presence of the species at this site, supporting the value of environmental DNA for use in detecting rare and secretive species with less time and effort compared to traditional surveys. This could suggest that multiple visits may be required to improve eDNA results.

The BLAST search confirmed the identity of the sequences as the Luristan Newt, and two sequences belonged to two identical haplotypes and were deposited in GenBank (Accession numbers KY404094-KY404095). A genetic study based on the variation of mtDNA (D-loop) in southwestern Iran (Farasat et al. 2016), revealed the presence of two genetically distinct clades (Northern and Southern clades). The sequence KY404094 was obtained from northern part of the species range (Northern clade) and the sequence KY404095 was from southern populations (Southern clade).

We detected Luristan Newts in both lentic and lotic environments, where adults or larvae were present. Water temperature and pH did not affect the probability of detection. Experimantal studies have shown that degradation rates are lowest under cold temperatures and in alkaline conditions (Barnes et al. 2014; Strickler et al. 2015). Aquatic environments located in colder regions, more protected from sunlight, and more alkaline are likely to hold eDNA longer compared to habitats that are warmer, sunnier, and neutral or acidic (Strickler et al. 2015). The pH value of the water at the sampling sites was slightly alkaline (7.5–8.4). These sites are mainly located on karstic-carbonate formations (Raeisi 2004). The persistency of eDNA is related to water qualities and seems to be more permanent in calcareous and gypsum water (Barnes et al. 2014; Eichmiller et al. 2016).

Water velocity and time in transit seems to affect the eDNA detection probability. In flowing water, DNA can rapidly diffuse and disperse from its source (Deiner and Altermatt 2014). Experimental research has shown that the amount of eDNA quantified in a low water flow tank was higher than tanks with faster flow rates, potentially, due to less DNA being flushed out of a low water flow tank (Klymus et al. 2015). Because of the rapid dispersal of eDNA within river systems, filtering large volumes of water for eDNA species detection might be helpful (Dejean et al. 2011; Thomsen et al. 2012; Wilcox et al. 2013; Deiner and Altermatt 2014; Pilliod et al. 2014).

Transport and degradation of eDNA.—We found that the time lag between collecting and freezing the water samples (transit time) can affect eDNA detction probability. For the three sites (false negatives) transit time was about 2 d before storing samples at -20º C. These results indicate that the stabilization of eDNA and storage at -20º C prior to extraction plays important role in the success of eDNA amplification. In the Great Crested Newt, for example, a lower success in amplification was attributed to increased time between sample collection and storage at -20° C, which was varied between immediate freezing of samples and up to 4 d before storage (Rees et al. 2014a). Therefore, long transit times are not ideal, and we recommend adhering to a strict sampling regime where water samples are collected, ethanol is immediately added to minimize DNA degradation, and samples are stored at -20° C prior to extraction analysis. To further investigate the effect of time lag, it is required to take replicates from the same site at the same time and given different transit times.

Persistence of eDNA.—Degradation is the main mechanism by which environmental DNA detection is limited, and several factors such as temperature, pH, and UV radiation affect the degradation rate. Previous studies have shown that eDNA becomes undetectable between two weeks and one month after the removal of its animal

source (Dejean et al. 2011; Thomsen et al. 2012; Piaggio et al. 2013; Pilliod et al. 2014). In the present study, no positive amplification was obtained from water samples taken after 15 d of storage at ambient temperature or 6 d for the water exposed to direct sunlight, presumably due to increasing temperature and the effect of UV radiation on DNA degradation (Strickler et al. 2015). In a previous study on the Great Crested Newt (Rees et al. 2014a), the authors could not amplify eDNA from the water samples taken after six days of water storage in an ambient environment.

Conclusion

Determining species presence and absence and distribution accurately are crucial to developing conservation and management strategies for endangered species, but a challenging task for small populations. This is the first study to evaluate the efficiency of eDNA analysis in detecting the presence of the Luristan Newt in Iran. Our results show that our eDNA assay is able to detect Luristan Newt presence in various water bodies (lemtics/lotics) and in different life stages (adult/larvae), though, water velocity and transit time can affect the eDNA detection probability. For the flowing water, filtering water may be more effecient than taking 15 mL of water and instant freezing of samples after collection can reduce eDNA degradation.

Analysis of eDNA can save time compared to traditional visual surveys (Thomsen et al. 2012, Biggs et al. 2015, Stoeckle et al. 2016). A field survey for the Luristan Newt may take between 8–12 h over several weeks of site visits; whereas, eDNA analysis take about 20 min to collect the sample and few hours to extract DNA and conduct PCR. Therefore, eDNA analysis provides a relatively quick tool for collecting Luristan Newt distribution data to monitor the presence of the species in Western Iran.

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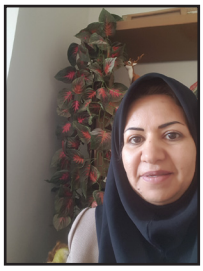

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