Morphological and Molecular identification of some *Uromastyx species* (Reptilia; Agamidae) in Makkah, Saudi Arabia by forensically informative nucleotide sequencing (FINS) of 16S rRNA gene and electrophoretic protein patterns

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Abstract: The morphological examination, of the collected Spiny-tailed lizards, revealed three *Uromastyx* subspecies (*U. a. aegyptia; U. a. microlepis;* and *U. ornate ornate* or *U. ornate philbyi*) to be inhabited in the holy land of Makkah of Saudi Arabia . FINS (Forensically Informative Nucleotide Sequencing) approach of 16SrRNA gene could confirm the morphologically identified first two subspecies to be *U. a. aegyptia* and *U. a. microlepis,* and identified the third subspecies to be *U. ornate philbyi*. This is the first successful typing of mitochondrial 16S rRNA gene with FINS approach carried out to identify the Spiny-tailed lizard, *Uromastyx sp.* in Saudi Arabia. The electrophoretic protein pattern analysis on SDS-PAGE showed the protein band of the molecular weight 275 kDa to be a characteristic protein marker for *U. aegyptia microlepis;* the protein band of 150 kDa to be a characteristic protein marker for *U. aegyptia aegyptia;* and the three protein bands of 200, 15, and 5 kDa to be characteristic protein markers for *U. ornate philbyi.* The obtained results suggested that protein electrophoresis is not only powerful tool in targeting the genetic variability within species but also in identifying them.
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1. Introduction

Uromastyx species are small to medium-sized ground or rock-dwelling agamid lizards. Their habitats are generally characterized by high temperature, low precipitation, sparse vegetation and marked seasonal changes. Many systematic studies of agamid lizards have been carried out on the Morphological and Biochemical levels in KSA (Amr and Kumazawa, 2005; 2009; Harris *et al*., 2007; Wilms and Schmitz, 2007; Al-Seeni, 2009; Amr, 2011; Amr*et al.,* 2012). However, little is known about those inhabited the holy land of Makkah. Therefore, the current work aimed to identify *U. species* in Makkah.

Molecular identification has two important advantages over the conventional techniques of microscopic examination. At first identification can be made using a very small amount of material, and is much more accurate than the previous traditional morphological methods. In addition, species identification based on DNA analysis is more sensitive and reliable, since it is independent of the tissue being compared. Generally, the identification of species is extensively carried out by the polymerase chain reaction (PCR) to generate species specific DNA products of different size (Barvi*et al.,* 2004; Pepe *et al.,* 2005; Guha and Kashyap, 2006). While, random amplified polymorphic DNA (RAPD) analysis is a technique for rapid detection of genomic DNA utilizing a single short oligo-nucleotide primer of arbitrary sequences in a PCR that can generate several species specific products. However, the several species specific products make the results difficult to interpret, particularly when samples incorporate material from more than one species. Even though, the cost of RAPD was found to be intermediate between enzyme electrophoresis and restriction fragment length polymorphism (RFLP) and repetitive DNA analysis for detecting genetic variation (Guha and Kashyap, 2006).

Recently, another approach has been deriving the DNA sequence of a target gene and uses such information for species identification. This approach is called forensically informative nucleotide sequence or FINS (Bartlett and Davidson, 1992) and successfully applied with several genes, including the P1 protamine gene, the mitochondrial cytochrome b, and ribosomal RNA genes (Forrast and Carnegia, 1994). In particular, due to the high mutation rate of mitochondria DNA (mtDNA), 10 times greater than nuclear DNA, point mutations accumulate very quickly allowing the discrimination of closely related species (Jord*et al*., 1998). Most of these methods make use of PCR amplification and direct sequencing of a conserved gene from sample such as mt-12S

rRNA (Rastogi*et al*., 2004), mt-16S rRNA (Borgo*et al*., 1996), and cytochrome b gene (Cytb) containing a species specific information.

In the present work, FINS with mitochondrial 16s rRNA gene, as a cost effective approach, was applied for the first time, for the molecular identification of *U.* species in Makkah. In addition, the electrophoretic protein patterns for the identified species were also analyzed to address each species-specific marker.

2. Materials and Methods

Samples

Uromastyx specimens "34 individuals" were randomly collected from five different localities in Makkah region (Al-Sharai, Muzdalifah, Arafat, Al-Shishah, Al-Shumaysi). Animals were subjected to morphological examination using the external characters (16 meristic, 6 metric, 2 qualitative) as described by (Wilms and Bohme, 2007). At least, three individuals from each preliminary grouped sample were dissected in Ringer's solution (130 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$) and limb muscles were stored at –80 °C or subjected directly to protein extraction.

DNA extraction

DNA was extracted from the different frozen *Uromastyx* muscle tissues for overnight incubation at 56°C with lysis buffer and proteinase K (20 mg/ml) followed by phenol-chloroform organic extraction according to the method described by (Sambrook*et al*., 1989). All samples were purified by Centricon-100 (Amicon, Inc., USA), then spectrophotometrically quantified at 260/280 nm.

DNA amplification with mitochondrial 16s rRNA universal primer pair

Amplification of ~ 500 bp 16s rRNA gene regions were performed with ~30 - 50 ng of extracted DNA for all samples and with universal primer pair for 16s rRNA gene (16sFwd: 5`-CGCCTGTTTATCAAAAACAT-3` and 16sRev: 5`-CTCCGGTTTGAACTCAGATC-3`). The PCR reaction mixture was made up of 0.2 µM of each primer and the amplification was carried out in a PE 2700 thermo-cycler as follows; an initial denaturation step of 94°C for 90 s followed by 35 cycles of denaturation at 94°C for 45s, annealing at 47°C for 30 s and extension at 72°C for 90 s. PCR products were purified using Qiaquick purification kit (Qiagen). Sequencing was performed using an ABI prism Big-Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA, USA) and a DNA sequencer (ABI, Prism 3100 Genetic Analyzer, PE, Applied Biosystems).

Sequence analysis

The obtained 16S rRNA sequences of unidentified samples (individuals) along with all reference sequences *U. aegyptia microlepis* (GenBank accession number: FJ639620.1); *U. aegyptia aegyptia* (GenBank accession number: FJ639619.1); *U. ornate philbyi* (GenBank accession number: EF081046.1); *U. ornate ornate* (GenBank accession number: AB116951); of *U. ocellata* (GenBank accession number: EF081044.1); and *U. leptieni* (GenBank accession number:FJ639622.1)] were entered unaligned into BioEdit program for CLUSTALW multiple alignments. The Genbank database (National Center for Biotechnological Information, USA: NCBI Home page http://www.ncbi.nlm.nih.gov) was search for homologous sequences for 16s rRNA gene within closely related species. The retrieved sequences were aligned together with those sequenced. The automated alignment allows the sequences for gap and slid alignment to generate a general pattern of variability in data. The phylogenetic tree was constructed based upon the Kimura-2-paramater distance matrix using the neighbor-joining method for all the aligned sequences in the software MEGA v5.0.

Protein electrophoresis

Muscle tissues were homogenized manually in homogenization buffer (5 mM KH_2PO_4, 100 mM KCl, 4 mM NaCl, 15 mM $MgCl_2$, 2 mM $CaCl_2$, pH 6.2) supplemented with protease inhibitor cocktail (one tablet/50 ml of the buffer solution) (Complete, Roche Diagnosis GmbH, Mannheim, Germany). Each homogenate was centrifuged at 5000 *g* for 10 min. Protein content in the supernatant was measured using a DC protein assay Kit (Bio-Rad, Hercules, CA, USA) and a plate reader at 595 nm. Bovine serum albumin was used as a standard protein. A total of 20 µg form each sample was used for electrophoresis.

SDS-PAG of 12% was performed according to (Laemmli, 1970). Briefly, the extracted protein for each sample was mixed with an equal volume of 2× sample buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, 50% glycerol, 0.4 ml 2-mercaptoethanol 0.05% (w/v) bromophenol blue), denatured by heating in boiling water for 10 min, followed by immediate cooling on ice for 2 min and loaded on the gel.

The *Rm* (Rate of mobility), molecular weights, and the similarity coefficient were calculated according to (Lynch, 1990) by using Gel Doc-2001 Bio-Rad system, CA, USA.

3. Results and Discussion

Morphological examination

The morphological examination of the randomly collected specimens from different localities of Makkah (Fig.1A and B) revealed two different *Uromastyx* (*U.*) species: *U. aegyptia* and *U. ornata.* The main characters used to distinguish these two species, as described in details by (Wilms and Bohme, 2007), are the large body size combined with very small body scales, as a characteristic feature for the

aegyptia species and the ratio of tail length to maximum tail width at the 5th whorl, as a characteristic feature for *ornate* species. The external features used to distinguish the members of *U. aegyptia* species are the lack of enlarged tubercular scales on the flanks and the presence of skin folds at the sides of the neck covered with tubercles. The number of scales on belly, the presence of pre-anal pores, and femoral pores are used to identify the 2 subspecies (*U. aegyptia aegyptia* and *U. aegyptia microlepis*) within the collected samples. Regarding the second species (*ornata*), the measurements for the ration of tail length to maximum tail width at the 5th whorl for all individuals in this group show the ratio of 3.51. According to (Wilms and Bohme, 2007), the ratio 3.61–5.3 is referred to the subspecies *ornate* .however*;* the ratio between 3.03-3.96is referred to the subspecies *philbyi.* As a result, these 2 subspecies may be belong to ornate species.

FINS approach

The forensically informative nucleotide sequencing analysis has been carried out on sequences obtained from each of the morphologically identified Uromastyx samples: individual 1 (expected to be: *U. aegyptia microlepis*); Individual 2 (expected to be: *U. aegyptia aegyptia*); and Individual 3 (expected to be: *U. ornate ornata* or *U. ornate philbyi*).About ~500 bp fragments were PCR amplified, from each sample using the universal primer pair for 16S rRNA gene and directly sequenced. The obtained sequences were subjected to *ClustalW* multiple alignments together with the reference sequences for *Uromastyx* species found in the Genbank database. FINS analysis revealed relatively high inter-specific variability, while maintaining a low intra-specific variability. These high variable sites have enough information to enable the differentiation of specimens on species and subspecies levels. Using the multiple alignments data, the Neighbor-Joining tree was drawn (Fig.2) using the Kimura-2-parametere distance matrices in the software MEGA5 (Tamura *et al*., 2011). The optimal tree with the sum of branch length = 5.02868223 is shown. The analysis involved 9 nucleotide sequences. Codon positions included were1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 417 positions in the final dataset.

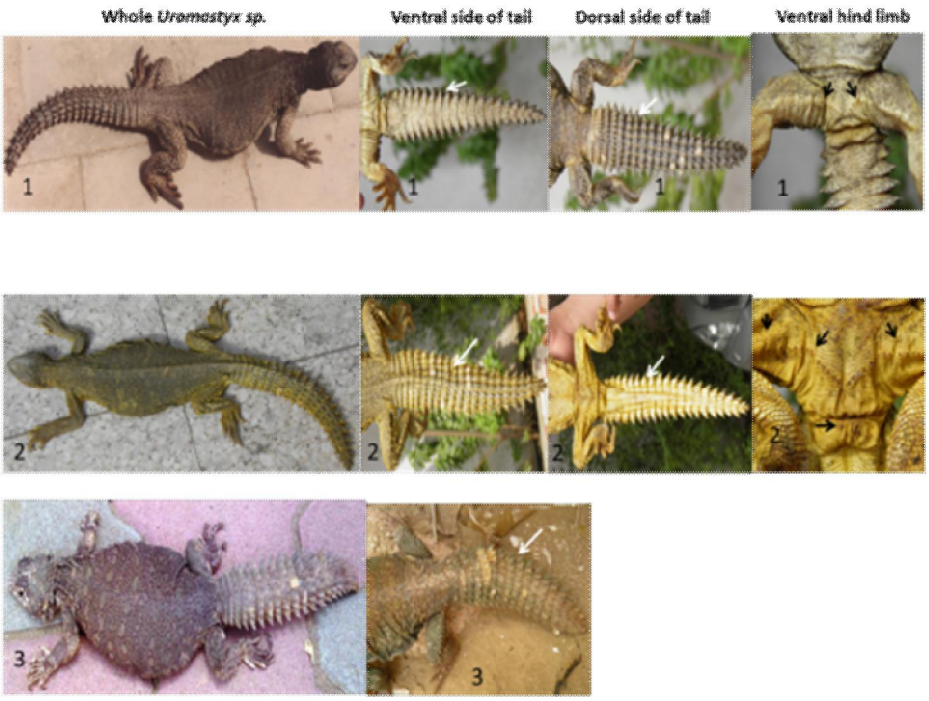
From the tree, the unknown individula1 clustered closest to the reference sequence of *U. aegyptia microlepis* (Genbank accession number: FJ639620.1) with 100% bootstrap support; individual 2 was shown at the same cluster group but close to the reference sequence of *U. aegyptia aegyptia* (Genbank accession number: FJ639619.1)*;* The unknown individual 3 was grouped close to the reference sequence of *U. ornate philbyi* (Genbank accession number: EF081046.1) and not with that of *U. ornataornata* (Genbank accession number: AB116951)in the second cluster of the tree (Fig.2). Together, the *U. species* collected from Makkah could be identified by FINS analysis to be *U. aegyptia microlepis, U. aegyptia aegyptia* and *U. ornate philbyi.*

Interestingly*, U. aegyptia aegyptia* is found to be the most abundant species of 88% of the collected samples of *U.* species from Makkah. This approach has been previously used to identify *Calotes versicolor* (Agamid Garden lizard) in India by Guha and Kashyap (2006). The present work is the first successful typing of mitochondrial 16S rRNA with FINS approach to identify *Uromastyx species* in Makkah.

Protein markers

In order to detect possible protein markers for the identified *Uromastyx*, in the present work, an electrophoretic analysis on 12% SDS-PAGE for the protein extracts from muscle tissues was carried out (Fig.3). The analysis revealed 15 protein bands to be shared in all samples, while the total number of protein bands detected (Major to moderate) were 18 bands for *U. aegyptia microlepis, U. aegyptia aegyptia* and 19 bands for *U. ornate philbyi.* The molecular weights of these bands showed a wide range between a maximum of 275 kDa and a minimum of 5 kDa (Table.1). The number of bands shared between *U. aegyptia microlepis,* and *U. aegyptia aegyptia* were 17 with similarity coefficient (SC) of 94%; between *U. aegyptia microlepis* and *U. ornate philbyi* were 15 bands with SC of 81%; and between *U. aegyptia aegyptia* and *U. ornate philbyi* were 16 bands with SC of 86.4%. As shown in table.1, the protein band of molecular weight 275 kDa is detected to be a characteristic protein marker for *U. aegyptia microlepis;* the protein band of 150 kDa is detected to be a characteristic protein marker for *U. aegyptia aegyptia;* the three protein bands of 200, 15, and 5kDa are detected to be characteristic protein markers for *U. ornate philbyi.* This is the first report on detecting protein markers that demarcate the investigated *U. species*. Similarly, protein electrophoresis technique was conducted to investigate the genetic variations within *U. aegyptia microlepis* inhabiting different localities of Saudi Arabia (Amr, 2011). The obtained results, in the present work, suggested that protein electrophoresis is not only powerful tool in targeting the genetic variability within species but also in identifying them.

1A



1B

Fig. 1. (A) The map of Makkah of Saudi Arabia showing the sites of samples collection indicated by circled numbers: 1, Alsharai; 2, Alshishah; 3, Arafat; 4. Muzdalifah; 5, Ashshumaysi. (B) The three individual preliminary identified by morphological characters: 1, *U. aegyptia microlepis;* 2, *U. aegyptia aegyptia;* 3, *U. ornate ornata or U. ornate philbyi.* Black arrows point to femoral and pre-anal pores; white arrows point to scales.

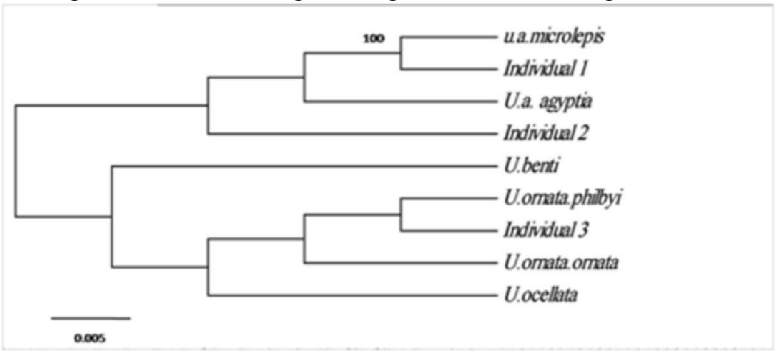


Fig. 2. Phylogenetic tree for identification of unknown *Uromastyx* samples using 16S rRNA gene variable fragment by FINS (Forensically Informative Nucleotide Sequencing) with Kimura-2-parameter distances. The reference sequences are *U. aegyptia microlepis* (GenBank accession number: FJ639620.1); *U. aegyptia aegyptia* (GenBank accession number: FJ639619.1); *U. ornate philbyi* (GenBank accession number: EF081046.1); *U. ornate ornate* (GenBank accession number: AB116951); of *U. ocellata* (GenBank accession number: EF081044.1); and of *U. leptieni* (GenBank accession number:FJ639622.1).

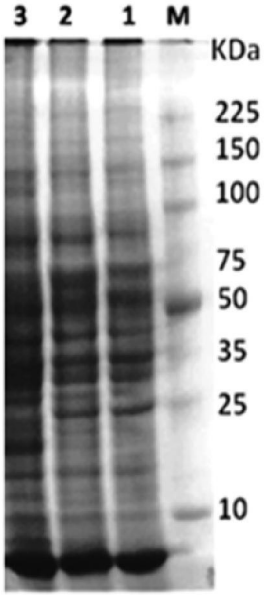


Fig. 3. Electrophoretic separation pattern of protein extracts (20 µg of each per lane) prepared from muscle tissues of *U. aegyptia microlepis* (lane 1); *U. aegyptia aegyptia* (lane 2); *U. ornate philbyi* (lane 3) separated on 12% SDS-PAGE. Molecular weight protein marker (M).

Table 1. Protein bands molecular weights scored and measured in kDa for the different identified *U.* species in Mahhah. *U. aegyptiamicrolepis* (lane 1); *U. aegyptia aegyptia* (lane 2); *U. ornata philbyi* (lane 3). All are separated on 12% SDS-PAGE. Protein molecular weight in kDa (MW). The bands detected as specific protein markers are shown by gray rectangles.

MW	**275**		**200**		**150**											**15**					**5**
Lane1	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-
Lane2	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-
Lane3	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+

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