Sequence variation in the mtDNA, ND4-tRNA<sub>LEU</sub>, segments of *Laudakia nupta* (De Filippi, 1843) in Iran

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*Laudakia nupta*, with numerous local populations through Iran, is one of the most widely distributed species of the Genus *Laudakia* in Iran. Eight hundred and fifty nine bp of mitochondrial ND4-tRNA<sub>LEU</sub> were sequenced and analyzed for 47 specimens of *L. nupta* and three specimens of *Laudakia melanura*, as an out-group taxon. All specimens were collected during field work in Iran. Based on branch pattern of the phylogenetic trees and the amounts of genetic distances within and between major clades recovered in the phylogenetic trees, *L. nupta*, as a species complex in Iran, should be fundamentally revised taxonomically. Based on our results, two clear geographically isolated clades could be distinguished; one nominate species (*L. nupta*) distributed through southwest to eastern Iran, and the other consisting of the populations of western foothills of the Zagros Mountains. The morphological analysis would enable us to describe the latter populations as a new species.

**Key words:** Agamidae, new entity, phylogeny, species complex, taxonomy.

**INTRODUCTION**

The genus *Laudakia* Gray, 1845, comprises about 20 species, mainly occurring in upland and mountainous regions of the central and southern Asia. Of these, at least five species occur in Iran (Šmíd et al., 2014). Based on a non-phylogenetic morphological analysis, *Laudakia* has recently been divided into three genera *Stellagama, Paralaudakia* and *Laudakia* (Baig et al., 2012). But, shortly after that, a robust molecular phylogenetic analysis, strongly supported monophyly of the genus *Laudakia* Gray, 1845 suggesting that the taxonomic revision of the genus is not necessitated (Pyron et al., 2013).

*Laudakia nupta*, with numerous local populations through Iran, is one of the most widely distributed species of the Genus *Laudakia* in Iran (Anderson, 1999). In 1843, DeFilippi described *L. nupta*, based on material collected from Persepolis, about 45 km NE of Shiraz, Fars Province in Iran. Ever since its description, taxonomic status of *L. nupta* has been the subject of controversial interpretations. Latter, two subspecies of this taxon were introduced: *L.nupta nupta* and *L. n. fusca* (Blanford, 1876). Subsequently, Boulenger (1885) supported this grouping furthermore, separated *fusca* from the nominate form by having more developed spinose scales on the sides of head and
FIGURE 1. Sampling locations along the distribution range of Laudakia nupta, in Iran (see Table 1 for individual sampling sites).

neck. Smith (1935), however, did not find any significant difference between L. n. nupta, L. n. fusca, and L. carinatus, and placed all three under the nominate form nupta. For many years, some authors (e.g. Anderson, 1999) have considered the Eastern populations in SE Iran and Pakistan as a subspecies, L. n. fusca. Despite that, other authors have considered this taxon as a full species (Cheatsazan et al., 2008; Khan, 2006; Rastegar-Pouyani et al., 2008). The latest taxonomic revision by Baig et al. (2012), however, resurrects subspecies status for L. n. fusca.

Considering the confusion surrounding the status of L. nupta, the main goal of this study is to elucidate the taxonomical position of the Iranian populations of L. nupta using mitochondrial ND4-tRNA sequences.

MATERIAL AND METHODS
Specimens used in the present study were collected during expeditions to different parts of Iran since 2010 to 2013. Description on localities, geographic coordinates, voucher numbers and NCBI accession numbers are presented in table 1 and localities on the Iranian map are shown in figure 1. The specimens and DNA materials are vouchered in the department of Biology, Hakim Sabzevari University, Iran. Specimens were identified according to the morphological keys as presented in Anderson (1999).

DNA was extracted using non-organic DNA Extraction Procedure (Proteinase K and Salting out Rastegar-Pouyani et al., 2014). After washing the pellet in ice-cold 70% EtOH once, the air-dried DNA was dissolved in 100µl of ultrapure, sterile H₂O, and finally DNA concentration was determined using spectrophotometer; that ranged from 50-900 ng/ml. Mitochondrial gene encoding
the fourth subunit of NADH dehydrogenase (plus downstream Serine, Histidine, and Leucine tRNAs; hereafter collectively referred to as ND4 was amplified using standard PCR procedures with the following primers, ND4F, 5'-CACCTATGACTACAAAAGCTCATGTAGAAGC-3' (Thaug et al., 2009) and Leu R, 5'-CATTACTTGTACTGGATTGCAACCA-3'(Arevalo et al., 1994). PCR reactions performed in 20μl with the following conditions: Initial denaturation stage of 95°C (05:00) followed by the 36 cycles with denaturation at 95°C (00:40), annealing at 50°C (00:40) and extension at 72°C (01:40) then single extension cycle at 72°C (05:00).
ALIGNMENT AND PHYLOGENETIC ANALYSIS
Following Baiget al., (2012), *Laudakia melanura* was designated as the out-group taxon. Sequences were aligned using Clustal W, as implemented in Bioedit version 7.0.5.3 (Hall, 1999). Prior to analysis, sequences of the ND4 gene were translated into amino acids using vertebrate mitochondrial translation code implemented in the program Mega 6 (Tamura et al., 2013) to check if there were any inspected stop codons and to ensure that all the sequences were protein coding and functional instead of pseudo genes. Genetic distances among the major clades were also calculated by Mega 6 (Tamura et al., 2013).

Three methods of phylogenetic analysis were used: The software PAUP* 4.0b10 (Swofford, 2001) for maximum parsimony, MrBayes v3.2.0 (Huelsenbeck & Ronquist, 2001) for Bayesian inference, and RaxML GUI v. 0.95 (Silvestro & Michalak, 2012) for Maximum likelihood. Because of the negligible effects of saturation in our data set, the MP analysis was performed with all sites weighted equally. For ML and BI analyses J Modeltest 2.1.4 (Darriba et al., 2012) was used, to select the most appropriate model of sequence evolution. Nonparametric bootstrapping (Felsenstein, 1985) performed with 1000 replicates to estimate stoutness of the branches of the shortest MP and ML trees.

RESULTS
A total of 859 characters of mtDNA ND4 were clearly aligned and analyzed in 50 specimens (including three out-group and 47 in-group taxa). No premature stop codons were observed in ND4, indicating that the obtained sequences were mitochondrial in origin and not nuclear pseudo copies. Of these characters, 603 characters were invariable and 238 sites (27.7%) were variable; just 224 sites (26.0%) were parsimony informative. A+T proportion (58.1%) was much higher than the C+G (41.9%) proportion. Uncorrected genetic divergence and Kimura-2-parameter genetic distance (Table 2) among the major groups of the tree indicated a considerable distance among the major clades. The selected models under Akaike information criterion, was TrN+I with the following parameter settings: $-\ln L = 2467.895$; base frequencies: $A = 0.3729$, $C = 0.2866$, $G = 0.1249$, $T = 0.2157$; six substitution types: $A$–$C = 1.0000$, $A$–$G = 18.7567$, $A$–$T = 4.0405$, $C$–$G = 1.0000$, $C$–$T = 11.7774$, $G$–$T = 1.0000$; $P_{i}$var$= 0.6110$. The trees generated using different methods of phylogenetic reconstruction resulted in same general topology, insofar only the Bayesian tree is shown in figure 2. Two major clades were revealed in the phylogenetic tree (Fig. 2) with clade one

| Table 2. Uncorrected genetic divergence ($p$-distance) for major clades and sub-clades recovered in this study and the outgroup taxon. |
|-----------------|-----------------|-----------------|
| **Outgroup**    | **Sub-clade 1A**| **Sub-clade 1B**|
| Sub-clade 1A    | 0.223           |                 |
| Sub-clade 1B    | 0.226           | 0.013           |
| Clade 2         | 0.220           | 0.097           | 0.096           |

| Table 3. Kimura-2-parameter genetic distance for major clades and sub-clades recovered in this study and the outgroup taxon. |
|-----------------|-----------------|-----------------|
| **Outgroup**    | **Sub-clade 1A**| **Sub-clade 1B**|
| Sub-clade 1A    | 0.270           |                 |
| Sub-clade 1B    | 0.273           | 0.013           |
| Clade 2         | 0.264           | 0.107           | 0.106           |
**Figure 2.** Phylogenetic relationships between different populations of *Laudakia nupta* (Bayesian inference) based on the 859 bp of ND4 (tRNAsHis+Ser+Leu). *L. melanura* was designated as out-group taxon. Numbers next to the nodes indicate clade credibility (Posterior probability) followed by bootstrap values obtained under ML Tree with 1000 replicates.
being subdivided into two distinct sub-clades. Clade one consists of specimens distributed in SW and Central Iran, through eastern Iran, and also along the coastal regions of the Persian Gulf (localities 1-15, 24, and 25; Fig. 1), whilst clade 2 consists of specimens restricted to western foothills of the Zagros Mountains (localities 16-23; Fig. 1). Although the sub-clade 1B is geographically distributed in eastern part of Iran, but due to the low genetic distance between the sub-clades 1A and 1B (Table 2 and 3), we consider them both as members of the same major clade.

**DISCUSSION**

We have produced the first detailed and well-supported molecular phylogeny pattern for the Iranian populations of *L. nupta*. The results clearly showed that the Iranian populations of *L. nupta* are composed of two major monophyletic clades. These clades are correlated well with the geographic distribution of the species. Despite various debates about species tree and gene tree (Goodman et al., 1979), one mitochondrial genetic distance reflects taxonomic status of reptiles (Johns & Avise, 1998). Based on the results presented here, we propose that two major clades of *L. nupta* in Iran could be signed as distinct taxa at species level. Based on our proposal, the clade 1 that contains specimens from Perspolis (the type locality) should be named as traditional *L. nupta* and Clade 2, containing populations from western Iran (Fig. 1 and 2), should be described as a new species. Considering topology of the tree and the amounts of genetic distances between the sub-clades 1A and 1B, they together constitute the same major clade (Table 2, Fig. 2). Samples from type locality of *L. n. fusca* were not available for our study (mostly because of security considerations), therefore we are not able to make decision about taxonomic status of *L. n. fusca* in our phylogenetic analysis. However, specimens of sub-clade 1B are morphologically close to description of this subspecies (unpublished data), in addition these are geographically close to the terra typical for *L. n. fusca* and It has been found only at its type localities, near (Kalagan area Jalq (34°02’N, 64°42’E) in Baluchistan, close to the Iran-Pakistan border line) (Rastegar-Pouyani & Nilson, 2002). According to Anderson (1999) and Mahjoorazad et al. (2005) the range of *L. n. fusca* extends westwards along the coast of the Persian Gulf in Southwestern Iran. However, our tree does not support the occurrence of *L. nupta fusca* along coastal regions of the Persian Gulf, because populations of this area are all grouped within the sub-clade 1A (*L. n. nupta*). Based on the results and distribution pattern of *L. nupta* in Iran, it could be concluded that possibly the Zagros Mountains uplifting has played an important role in genetic divergence among clade 1 and 2. With this hypothesis, divergent time of two major clades probably goes back to the Late Miocene, around 10–12.4 MYA (Mouthereau, 2011; Sborshchikov et al., 1981). Influence of the geological event of the uplifting of Zagros Mountains on the Iranian herpetofauna has been proposed in a couple of studies (Macey et al., 1998, 2000; Rastegar-Pouyani et al., 2009).

In conclusion, it sounds that more field samplings as well as supplementary ecological and morphological studies, and further molecular data are necessary to shed light on the taxonomic status and historical biogeography of *L. nupta* in Iran. However, this preliminary study suggests that the taxonomic status of populations traditionally attributed to *L. nupta* in Iran should be fundamentally revised.

**LITERATURE CITED**


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