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Sequence variation in the mtDNA, ND4-tRNA^{LEU}, 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^{LEU}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, 1845suggesting 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. naupta* 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

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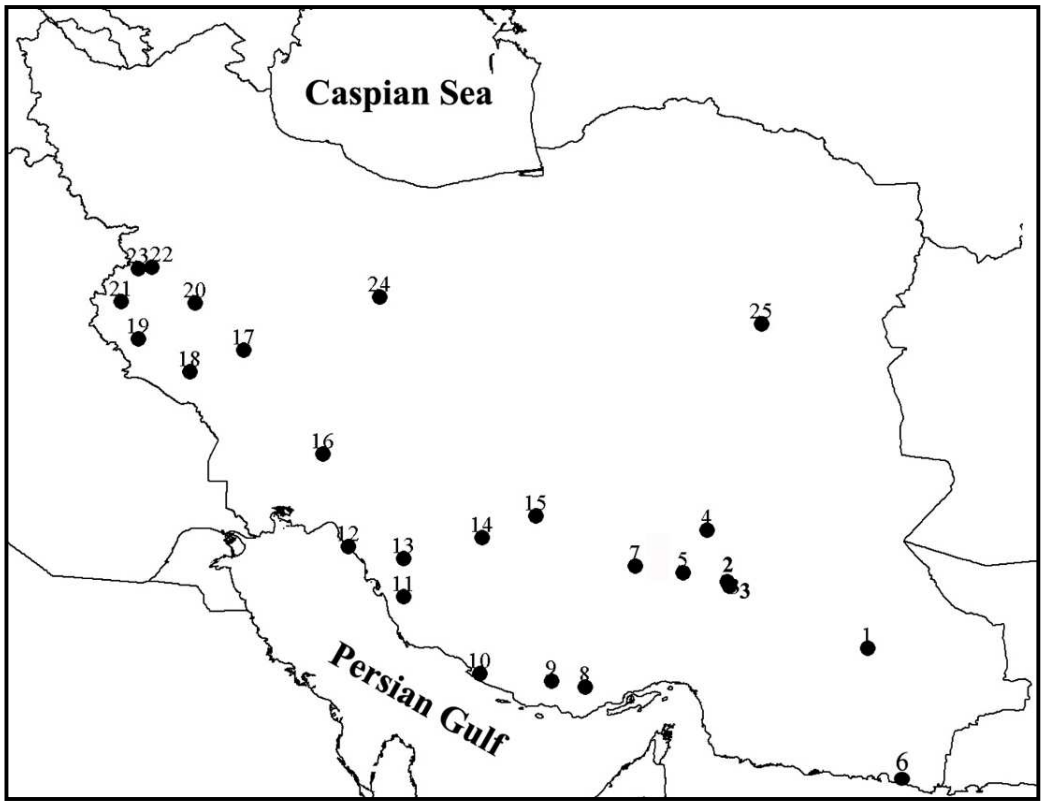

FIGURE 1. Sampling locations along the distribution range of *Laudakia nupta,* in Iran (see Table 1 for individual sampling sites).

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 accretion 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 H_2O, and finally DNA concentration was determined using spectrophotometer; that ranged from 50-900 ng/ml. Mitochondrial gene encoding

TABLE 1- List of the specimens and their localities (see Fig. 1).

Species	Field Number	Accession Number	location Number	Geographic Coordinates	Locality
L. nupta	ERP 1151	KX131008	16	31.52 N 49.85 E	Khuzestan Province, 40 Km east of Haftgel
L. nupta	ERP 1400	KX130985	25	34.08 N 58.06 E	South Khorasan province, Around Ferdows
L. nupta	ERP 1401	KX130986	25	34.08 N 58.06 E	South Khorasan province, Around Ferdows
L. nupta	ERP 1404	KX130987	25	34.08 N 58.06 E	South Khorasan province, Around Ferdows
L. nupta	ERP 892	KX130982	8	27.5 N 54.50 E	Hormozgan province, BandareLengeh
L. nupta	ERP 893	KX130983	8	27.5 N 54.50 E	Hormozgan province, BandareLengeh
L. nupta	ERP 459	KX130984	24	34.30N 50.56E	Qom Province, around the city of Qom
L. nupta	ERP 1239	KX130967	10	27.36 N 52.62 E	Bushehr Province, Asalooye- Nayband region
L. nupta	ERP 1199	KX130965	11	28.80 N 51.39 E	Bushehr Province, Around Ahram
L. nupta	ERP 1200	KX130966	11	28.80 N 51.39 E	Bushehr Province, Around Ahram
L. nupta	ERP 1181	KX130963	13	28.53 N 51.39 E	Fars Province, Konartakhte
L. nupta	ERP 1182	KX130962	13	28.53 N 51.39 E	Fars Province, Konartakhte
L. nupta	ERP 1184	KX130964	13	29.53 N 51.39 E	Fars Province, Konartakhte
L. nupta	ZUTCREP.1857	KX130988	1	27.50N 60.12E	Sistan and Baluchistan Province, Bazman
L. nupta	ZUTCREP.1854	KX130979	2	29.5N 57.32E	Kerman Province, Jiroft
L. nupta	ZUTCREP.1855	KX130980	2	29.5N 57.32E	Kerman Province, Jiroft
L.nupta	ZUTCREP.1853	KX130978	3	29.0N 57.35E	Kerman Province, Delfard
L.nupta	ZUTCREP.1856	KX130977	4	30.4N 57.9E	Kerman Province, Jupar
L.nupta	ZUTCREP.1858	KX130976	5	29.16N 56.42E	Kerman Province, Baft
L.nupta	ZUTCREP.1845	KX130968	9	27.12N 54.12E	Hormozgan province, Bastak
L.nupta	ERP437	KX130981	9	27.12N 54.12E	Hormozgan province, Bastak
L.nupta	ZUTCREP.1846	KX130969	12	29.45N 50.9E	Bushehr Province, Genaveh
L.nupta	ZUTCREP.1851	KX130972	14	29.56N 52.53E	Fars Province, Perspolis
L.nupta	ZUTCREP.1850	KX130971	14	29.56N 52.53E	Fars Province, Perspolis
L.nupta	ZUTCREP.1849	KX130970	14	29.56N 52.53E	Fars Province, Perspolis
L.nupta	ZUTCREP.1847	KX130973	15	30.20N 53.54E	Yazd Province, Harat
L.nupta	ZUTCREP.1848	KX130974	15	30.20N 53.54E	Yazd Province, Harat
L.nupta	ZUTCREP.1872	KX131005	17	33.29N 48.21E	Lorestan Province, Khoramabad
L.nupta	ZUTCREP.1876	KX131006	17	33.29N 48.21E	Lorestan Province, Khoramabad
L.nupta	ZUTCREP.1877	KX131007	17	33.29N 48.21E	LorestanProvince, around Khoramabad
L.nupta	ZUTCREP.1875	KX131004	18	33.05N 47.20E	Ilam Province, Dareshahr
L.nupta	ZUTCREP.1873	KX131002	18	33.05N 47.20E	Ilam Province, Dareshahr
L.nupta	ZUTCREP.1871	KX131001	18	33.05N 47.20E	Ilam Province, Dareshahr
L.nupta	ZUTCREP.1874	KX131003	18	33.05N 47.20E	Ilam Province, Dareshahr
L.nupta	ZUTCREP.1864	KX131000	19	33.42N 46.21E	Ilam Province, Around Ilam
L.nupta	ZUTCREP.1862	KX130999	19	33.42N 46.21E	Ilam Province, Around Ilam
L.nupta	ZUTCREP.1867	KX130998	19	33.42N 46.21E	Ilam Province, Around Ilam
L.nupta	ZUTCREP.1859	KX130989	20	34.23N 47.26E	Kermanshah Province, Bisoton
L.nupta	ZUTCREP.1863	KX130991	20	34.23N 47.26E	Kermanshah Province, Bisoton
L.nupta	ZUTCREP.1861	KX130990	20	34.23N 47.26E	Kermanshah Province, Bisoton
L.nupta	ZUTCREP.1865	KX130997	21	34.25N 46.01E	Kermanshah Province, Rijab
L.nupta	ZUTCREP.1860	KX130992	22	35.04N 46.36E	Kermanshah Province, Palangan
L.nupta	ZUTCREP.1866	KX130993	22	35.04N 46.36E	Kermanshah Province, Palangan
L.nupta	ZUTCREP.1868	KX130994	22	35.04N 46.36E	Kermanshah Province, Palangan
L.nupta	ZUTCREP.1870	KX130996	23	35.02N 46.21E	Kermanshah Province, Paveh
L.nupta	ZUTCREP.1869	KX130995	23	35.02N 46.21E	Kermanshah Province, Paveh
L.nupta	ZUTCREP.1852	KX130975	7	29.23N 55.47E	Kerman Province-Sirjan
L. melanura	ZUTCREP.1879	KX131010	6	25.24N 60.77E	Sistan and Baluchistan Province, Near Chabahar
L. melanura	ZUTCREP.1880	KX131011	6	25.24N 60.77E	Sistan and Baluchistan Province, Near Chabahar
L. melanura	ZUTCREP.1878	KX131009	6	25.24N 60.77E	Sistan and Baluchistan Province, Near Chabahar

the fourth subunit of NADH dehydrogenase (plus downstream Serine, Histidine, and Leucine tRNAs; hereafter collectively referred to as ND4was amplified using standard PCR procedures with the following primers,ND4F, 5'-CACCTATGACTACCAAAAGCTCATGTAGAAGC-3' (Thaung *et al.,* 2009) and Leu R, 5'- CATTACTTTTACTTGGATTTGCACCA-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%) wasmuch 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: -lnL= 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; Pinvar= 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
Outgroup			
Sub-clade1A	0.223		
Sub-clade1B	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
Outgroup			
Sub-clade1A	0.270		
Sub-clade1B	0.273	0.013	
Clade 2	0.264	0.107	0.106

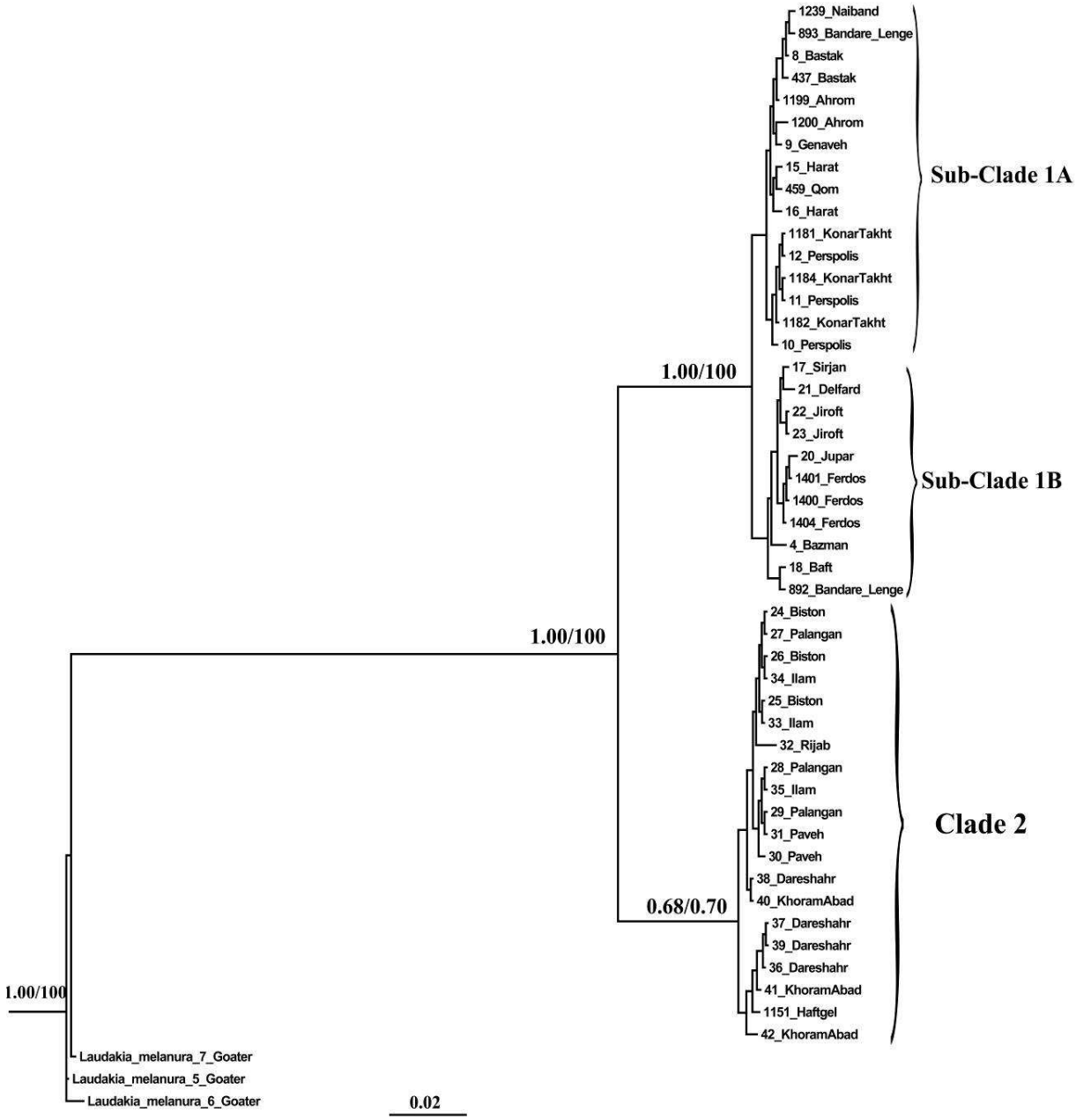

FIGURE 2. Phylogenetic relationships between different populations of *Luadakia nupta* (Bayesian inference) based on the 859 bp of ND4 (tRNAsHis+Ser+Leu). *L. melanura* was designated as out-group taxon. Numbers next to the nodes indicate clade credibility (Posterior probability) followed by bootstrap 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-clade1B 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 *g*eographically 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

Anderson, S.C., 1999. The lizards of Iran. Society for the Study of Amphibians and Reptiles, Ohio**,** Oxford.

Arevalo, E., Davis, S., Sites, J., 1994. Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosome races of *Sceloporus grammicus* complex (Phrynosomatidae) in central Mexico. *Systematic Biology* 43, 387–418.

Baig, K.J., Wagner, P., Ananjeva, N., Böhme, W., 2012. A morphology-based taxonomic revision of *Laudakia* Gray, 1845 (Squamata: Agamidae). *Vertebrate Zoology* 62, 213–260.

Blanford, W.T., 1876.Eastern Persia. An account of the journeys of the Persian boundary commission 1870-71-72. Vol. II. The zoology and geology. Macmillan and Co., London.

Boulenger, G.A., 1885. Catalogue of the lizards in the British Museum (Natural History). Vol. I. Geckonidae, Eublepharidae, Uroplatidae, Pygopodidae, Agamidae. Trustees of the British Museum, London.

Cheatsazan, H., Rabani, V., Mahjoorazad, A., Kami, H.G., 2008. Taxonomic status of the yellow-headed agama, *L. nupta fusca* (Blanford, 1876) (Sauria: Agamidae). *Zoology in the Middle East* 44, 41–50.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. JModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* 9, 772.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791.

Goodman, M., Czelusniak, J., Moore, G.W., Romero-Herrera, A.E., Matsuda, G., 1979. Fitting the gene lineage into its species lineage, a parsimony strategy illustrated by cladograms constructed from globin sequences. *Systematic Zoology* 28, 132–163.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41, 95-98.

Huelsenbeck, J.P., Ronquist, F., 2001. Mr. Bayes: Bayesian inference of phylogeny. *Bioinformatics* 17, 754–755.

Johns G.C., Avise J.C, 1998. Comparative Summary of Genetic Distances in the Vertebrates from the Mitochondrial Cytochrome b Gene. *Molecular Biology and Evolution* 15:1481–1490.

Khan, M.S., 2006. Amphibians and Reptiles of Pakistan. Krieger Publishing Company, Malabar, Florida.

Macey, J.R., Schulte, J.A., Kami, H.G., Ananjeva, N.B., Larson, A., Papenfuss, T.J.,2000. Testing hypotheses for vicariant separation in the agamid lizard *Laudakia caucasia* from mountain ranges of the Northern Iranian Plateau. *Molecular Phylogenetics and Evolution* 14, 479–483.

Macey, J., Robert, J.A., Schulte, J.A., Ananjeva, N.B., Larson, A., Rastegar-Pouyani, N., Sakhat, M.S.,Papenfuss, T.J., 1998. Phylogenetic Relationships among Agamid Lizards of the *Laudakia caucasia* Species Group: Testing Hypotheses of Biogeographic Fragmentation and an Area Cladogram for the Iranian Plateau. *Molecular Phylogenetics and Evolution* 10, 118–131.

Mahjoorazad, A., Cheatsazan, H., Kami, H.G.,Rabani, V., 2005. Distribution of the yellow-headed agama, *L. nupta fusca* (Blanford 1872), in Iran. *Zoology in the Middle East* 36, 21–26.

Mouthereau, F., 2011. Timing of uplift in the Zagros belt/Iranian plateau and accommodation of late Cenozoic Arabia-Eurasia convergence. *Geological Magazine* 148, 726–738.

Pyron, R., Burbrink, F.,Wiens, J., 2013. A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. BMC Evolutionary Biology 13(93), 1-53.

Rastegar-Pouyani, E., Eskandarzadeh, N., Darvish, J., 2014. Re-evaluation of the taxonomic status of sand boas of the genus *Eryx* (Daudin, 1803) (Serpentes: Boidae) in north-eastern Iran using sequences of the mitochondrial genome. *Zoology in the Middle East* 60, 320–326.

Rastegar-Pouyani, E., Rastegar-Pouyani, N., Kazemi-Noureini, S., Joger, U., Wink, M., 2009. Molecular phylogeny of the *Eremias persica* complex of the Iranian Pateau (Reptilia: Lacertidae), based on mtDNA sequences. *Zoological Journal of the Linnean Society* 158, 641–660.

Rastegar-Pouyani, N.,Nilson, G., 2002. Taxonomy and biogeography of the Iranian species of *Laudakia*. *Zoology in the Middle East* 26, 93–122.

Rastegar-Pouyani, N., Kami, H.G., Rajabzadeh, M., Shafiei, S., Anderson, S.C., 2008. Annotated checklist of Amphibians and Reptiles of Iran. *Iranian Journal of Animal Biosystematics* 4, 43–66.

Sborshchikov, I.M., Savostin, L.A.,Zonenshan, L.P., 1981. Present plate tectonics between Turkey and Tibet. *Tectonophysics* 79, 45–73.

Silvestro, D., Michalak, I., 2012.RaxML GUI: a graphical front-end for RAxML. *Organisms, Diversity and Evolution* 12, 335-337.

Šmíd, J., Moravec, J., Kodym, P., Kratochvíl, L., Hosseinian-Yousefkhani, S., Rastegar-Pouyani, E., Frynta, D., 2014.Annotated Checklist and Distribution of the Lizards of Iran. *Zootaxa* 3855, 001–097.

Smith, M.A., 1935. The fauna of British India, including Ceylon and Burma. Reptilia and Amphibia. Vol. II. Sauria.Taylor and Francis, Ltd. [Reprint Edition 1974, Today and Tomorrow's Printers and Publishers], London.

Swofford, D.L., 2001. PAUP*, Phylogenetic Analysis Using Parsimony (*and other Methods), Version 4.0b10. Sinauer Associates, Sunderland.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* 30, 2725-2729.

Thaung, H., Willoughby, T., Pradya, S., Duong, S., Setha, T., Sein, M., Chang, M., Catherine, W., 2009. Mitochondrial pseudogenes in the nuclear genome of *Aedes aegypti* mosquitoes: implications for past and future population genetic studies. *BMC Genetics* 10, 11.