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Genomic Variation within *Laudakia stellio* (Linnaeus, 1758) (Sauria: Agamidae) in Turkey, Based on Analyses of Mitochondrial 12S rRNA Sequences

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Abstract: The starred agama, *Laudakia stellio* (Linnaeus, 1758) has a wide distribution from Northern Africa to Southeast Europe and Southwest Asia and was thought to form two subspecies, *Laudakia stellio-stellio* (Linnaeus, 1758) and *Laudakia stellio-daani* in Turkey. Almog examined specimens from Greece, Aegean Islands and Anatolia for exploring the systematic status of *L.s. daani* relative to *L.s. stellio*. They recognized the whole Anatolian populations as *L.s. daani* indicating that the question of the eastern boundary of the subspecies remains open, Delos and Mykonos populations as *L.s. stellio* and emphasized the ambiguity of the taxonomic status of South-east Anatolia populations. So partial sequences (404 bp) of the mitochondrial 12S ribosomal RNA gene of six populations were sequenced to examine the genomic variation of the species in Anatolia. The analyses clearly suggest that two distinct mtDNA lineages exist in Turkey separated by 2.28 and 4.51% for the 12S rRNA sequences and conclude that the South, Blacksea and Aegean regions may represent *L. stellio-daani* because morphologically they fit the subspecies *L.s. daani*, however the southeast region remains to be addressed.

Key words: Agamidae, *Laudakia stellio*, 12S rRNA, taxonomic status, RNA gene, Turkey

INTRODUCTION

The Agamidae are a family of lizards that includes 54 genera and over 330 species. Agamid lizards are widely distributed on Australia and through the old world and inhabit different habitats including arid, tropical and subtropical regions (Moody, 1980; Rastegar-Pouyani and Nilson, 2002). Phylogeny and classification of the family Agamidae were controversial (Moody, 1980, 1983; Frost and Etheridge, 1989; Joger, 1991; Lazell, 1992; Schwenk, 1994; Macey *et al*., 1997). Then, Honda *et al.* (2000) studied the phylogenetic relationships of the family Agamidae based on 860 base positions of a mitochondrial DNA sequence of 12S and 16S rRNA genes.

The results confirmed the monophyly of this family including Leiolepis and Uromastyx (Leiolepidinae) and indicated the sister relationship between Agamidae and Chamaeleonidae and also the presence of two major clades in Agamidae.

There are five genera (Calotes, Laudakia, Phrynocephalus, Trapelus, Uromastyx) including approximately 25 species in the Middle East, Northern Africa and Arabia (Rastegar-Pouyani and Nilson, 2002). Laudakia, Phrynocephalus and Trapelus genera are represented in Turkey by the species *Laudakia caucasia*, *L. stellio*, *Trapelus ruderatus* and *Phrynocephalus persicus*. The starred agama or hardun, *Laudakia stellio* (Linnaeus, 1758) has a wide distribution from Northern Africa to Southeast Europe and southwest Asia. Seven subspecies of *L. stellio* have been known for some time around the periphery of its distribution: *L.s. stellio* (Linnaeus, 1758) in the Cyclades, *L.s. daani* in the Aegaean, Anatolia and Vicinity, *L.s. cypriaca* (Daan, 1967) on Cyprus, *L.s. picea* in North-eastern Jordan, *L.s. brachydactyla* in the Negev and vicinity, *L.s. vulgaris* (Latreille, 1801) in lower Egypt and *L.s. salehi* in Southern Sinai (Werner, 2007).

In Turkey, the distribution covers the West, South, Central and South-East regions (Baran and Atatur, 1998). Daan (1967) considered the Anatolian populations to represent the nominate subspecies, *Laudakia stellio-stellio*. Then, Baran and Oz stated that the Western and Southern Anatolian populations are rather similar to *L.s. daani* and they classified specimens from South-eastern Anatolia (Gaziantep and Sanliurfa) as *L.s. stellio*. They also emphasised the complicated taxonomic status of the Hatay population and suggested

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that this region should be subjected to a detailed morphological examination. In later studies (Baran and Atatur, 1998; Gocmen *et al*., 2003), it was also stated that *L.s. daani* populations occur in the west and *L.s. stellio* populations in Southeast Anatolia. Almog *et al*. (2005) morphologically examined museum specimens from Greece, Aegean Islands and Anatolia for exploring the systematic status of *L.s. daani* relative to *L.s. stellio*. They used conventional mensural, meristic, qualitative pholidosis and coloration characters and recognized the whole Anatolian populations as *L.s. daani* indicating that the question of the eastern boundary of the subspecies remains open, Delos and Mykonos populations as *L.s. stellio* and emphasized the ambiguity of the taxonomic status of South-East Anatolia populations.

Three specimens from this area (Adana, Antakya, Iskenderun) participated in their cluster analyses; one (Antakya) clustered within the *L.s. stellio* branch and the other two clustered as *L.s. daani* (Iskenderun, Adana). The status of Antakya specimen in their study raises the question if there is only one or there are two lineages in Anatolia. So, we sequenced 404 nucleotides of the mitochondrial 12S ribosomal RNA gene of 6 populations from Turkey (including Hatay region) to solve this problem by examining the genomic variation among the populations.

MATERIALS AND METHODS

Twelve specimens were collected from Adana, Antakya, Diyarbakir, Mersin and Izmir by hand during the field researches in May to August (2006-2008). All specimens used were deposited in the collection of the Biology Department in Canakkale University connected to ZDEU (Department of Zoology, Ege University) collection (Table 1).

DNA extraction and sequencing: Total genomic DNA was extracted from 12 Agama stellio from Turkey (Fig. 1) using a Nucleospin kit for tissue following the manufacturer's directions. Then partial sequence of mitochondrial 12S rRNA gene was sequenced. Primers used in both amplification and sequencing were L1091 (5'-AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC ACT AT-3') and H1478 (5'-TGA CTG CAG AGG GTG ACG GGC GGT GTG T-3') (Kocher *et al*., 1989) for the (5'-AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC ACT AT-3') and H1478 (5'-TGA CTG CAG AGG GTG ACG GGC GGT GTG T-3') (Kocher *et al*., 1989) for the partial mitochondrial 12S rRNA gene. PCR amplification was performed in a final volume of 50 μL containing 10 x PCR buffer, 1.5 mM $MgCl_2$, each dNTP at 2 mM each primer at 1 uM, 1 μL genomic DNA, 1 unit Taq polymerase. All amplifications began with an initial 2 min denaturation at 94°C.

Thermocycling parameters were; 94°C for 15 sec, 50°C for 15 sec and 72°C for 5 min for 35 cycles. Purification of PCR products and sequencing was performed by Macrogen Inc. (Seoul, Korea). The sequences have been deposited in GenBank (Accession numbers GU952112-GU952123).

Phylogenetic analyses: Alignments were performed using Clustal X (Thompson *et al*., 1997) with some adjustments made by eye and obtained a final alignment of 404 bp for 12S rRNA for phylogenetic analyses. We used a *Laudakia caucasia* (AY053643) sequence as the outgroup for phylogenetic reconstruction.

Modeltest 3.6 (Posada and Crandall, 1998) was used to select the most appropriate model of sequence evolution for the Maximum Likelihood (ML) analysis of the dataset under the AIC (Akaike Information Criterion). The GTR+G (General Time Reversible plus Gamma) model had the best likelihood score (-lnL = 886.5657). Parameter estimates were base frequencies: 0.3624 (A), 0.2749 (C), 0.1707 (G), 0.1920 (T). Pairwise distances were also

Table 1: Samples used in this study with mitochondrial haplotypes. Specimen codes identify each sample sequenced and its locality (Fig. 1)

Taxa	Locality	Locality on Fig.1 (museum no.)	GenBank accession no.	Haplotype
Laudakia stellio	35°55 N; 36°96 E	Adana (126/2007)	GU952112	Adana
Laudakia stellio	35°55 N; 36°96 E	Adana (126/2007)	GU952113	Adana
Laudakia stellio	36°32 N; 40°75 E	Amasya (62/2008)	GU952114	Amasya
Laudakia stellio	36°32 N; 40°75 E	Amasya (62/2008)	GU952115	Amasya
Laudakia stellio	36°62 N; 36°99 E	Gaziantep (130/2007)	GU952116	Gaziantep
Laudakia stellio	36°62 N; 36°99 E	Gaziantep (130/2007)	GU952117	Gaziantep
Laudakia stellio	35°95 N; 36°08 E	Hatay (113/2006)	GU952118	Hatay
Laudakia stellio	35°95 N; 36°08 E	Hatay (113/2006)	GU952119	Hatay
Laudakia stellio	28°57 N; 36°87 E	Mugla (101/2008)	GU952120	Mugla
Laudakia stellio	28°57 N; 36°87 E	Mugla (101/2008)	GU952121	Mugla
Laudakia stellio	37°97 N; 36°99 E	Sanliurfa (90/2008)	GU952122	Sanliurfa-1
Laudakia stellio	37°97 N; 36°99 E	Sanliurfa (90/2008)	GU952123	Sanliurfa-2
Laudakia caucasia		Iran, Mazandaran province	AY053643	

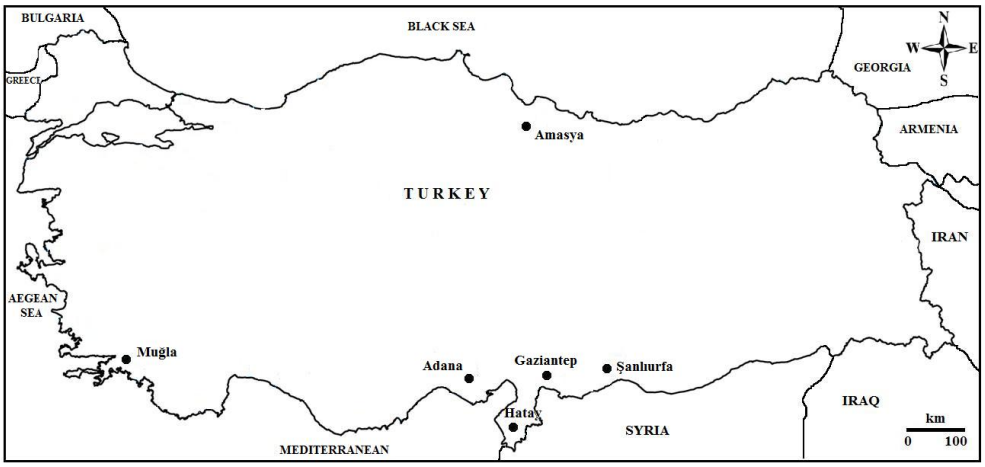

Fig. 1: Sampling sites of *Laudakia stellio* in this study

estimated using GTR model. Both Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses were performed using PAUP*4.0 b10 software (Swofford, 1998) and included heuristic searches involving Tree Bisection and Reconnection (TBR) branch swapping with 100 random stepwise additions of taxa. Reliability of the MP and ML trees was assessed by bootstrap analysis involving 1000 replications.

In addition, sequences were connected under the 95% probability of parsimony criterion using the software TCS (version 1.18) (Clement *et al*., 2000). The resulting network represents reconstructed gene genealogy of the haplotypes.

RESULTS AND DISCUSSION

A total of 404 bp of the 12S rRNA was obtained from each *Laudakia stellio* (n = 12) and 7 haplotypes were identified (Table 1). A total of 404 bp were included in the phylogenetic analysis. The ML and MP analyses produced approximately the same trees so the 50% majority-rule consensus tree is shown in Fig. 2 with bootstrap supports that the samples of Adana, Amasya and Mugla represent an independent lineage separated from the samples of Hatay, Gaziantep and Sanliurfa. The sequence divergences between haplotypes are given in Table 2. Among seven haplotypes of *L. stellio* sequence divergence ranged between 2.28 and 4.51% for this study; the haplotypes are far from the sequence of the outgroup *L. caucasia* (18.78-20.34%).

For 404 bp sequences, the haplotypes joined in two networks (Fig. 3) using TCS (Clement *et al*., 2000) with a 95% connection limit and the results were in line with the other phylogenetic analyses reported earlier. There was no substitution between the samples in each population

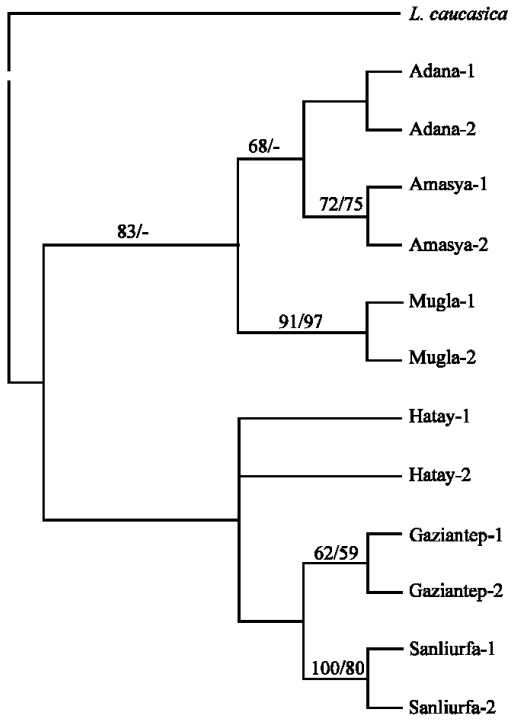

Fig. 2: The 50% majority-rule consesnsus tree of *Laudakia stellio* samples. Bootstrap values of ML and MP analysis are shown on each node of the tree (ML/MP, respectively)

except Sanliurfa samples with 3 substitutions. As can be shown in Fig. 3, Adana, Amasya and Mugla haplotypes are connected by ≤8 substitutions and Hatay, Gaziantep and Sanliurfa haplotypes are connected by ≤7 substitutions. The analyses clearly suggest that at least two distinct mtDNA lineages exist in Turkey separated by 2.28-4.51% for the 12S rRNA sequences. The first lineage

Table 2: Pairwise genetic distances between the haplotypes described in Table 1 for the mitochondrial 12S rRNA gene under the General Time Reversible (GTR) model of evolution

Haplotypes	Adana	Amasya	Mugla	Gaziantep	Hatay	Sanliurfa-1	Sanliurfa-2	*L. caucasica*
Adana	-							
Amasya	0.0050	-						
Mugla	0.0177	0.0177	-					
Gaziantep	0.0282	0.0336	0.0311	-				
Hatay	0.0228	0.0282	0.0257	0.0050	-			
Sanliurfa-1	0.0333	0.0388	0.0419	0.0152	0.0152	-		
Sanliurfa-2	0.0362	0.0418	0.0451	0.0179	0.0179	0.0075	-	
L. caucasica	0.1992	0.2034	0.2028	0.1878	0.1878	0.1879	0.1883	-

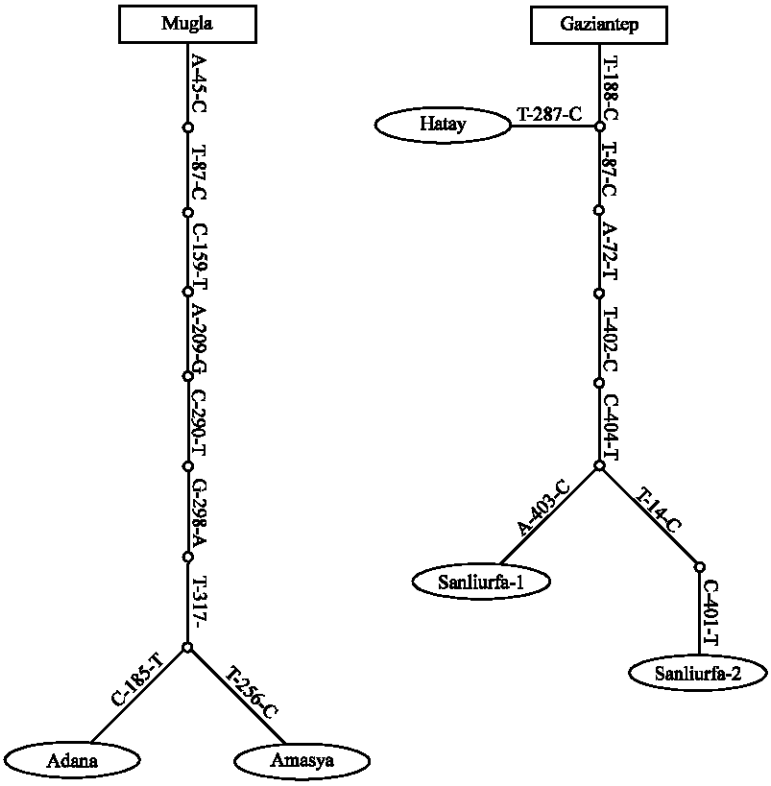

Fig. 3: The network of relationships among the partial 12S rRNA gene sequences for *Laudakia stellio* and the substitutions among the haplotypes. Circles along the branches indicate nucleotide changes, the rectangular on top indicates the presumed ancestral haplotype

includes Adana, Amasya and Mugla samples representing South, Blacksea and Aegean region. The second lineage includes samples from Sanliurfa, Gaziantep and Hatay representing southeast region of Turkey. Cigdem Gul also applied the key of Almog *et al.* (2005) to the Anatolian specimens and found that all the specimens generally fit the subspecies *L.s. daani* in terms of morphometric, pholidosis and coloration characters. However, these characters are slightly separating the samples from southeast region (Sanliurfa, Gaziantep and Hatay) from the western ones as in line with the molecular analyses that at least two distinct lineages exist in Turkey. Baran and Oz stated that the Western Anatolian populations are rather similar to *L.s. daani* and they classified specimens from South-eastern Anatolia (Gaziantep and Sanliurfa) including Hatay as *L.s. stellio*. Then, Gocmen *et al.* (2003) also concluded that the Hatay population should be included in the subspecies *L.s. stellio*. Compared with the results of these researchers (Gocmen *et al*., 2003), the molecular data at least confirmed the existance of two lineages of *Laudakia stellio* in Anatolia. The data are also in line with the study of Almog *et al.* (2005) that one Antakya (Hatay) specimen clustered within the *L.s. stellio* branch and two from Iskenderun and Adana clustered as *L.s. daani*. As the specimens fit the subspecies *L.s. daani* according to the

morphotypes of Almog *et al*. (2005), Cigdem Gul, we can conclude that the eastern boundary of *L.s. daani* may be Adana in Southeast and Amasya in Northeast. As explained before, 7 subspecies of *Laudakia stellio* have been known for some time around the periphery of its distribution: *L.s. stellio* in the Cyclades, *L.s. daani* in the Aegaean, Anatolia and Vicinity, *L.s. cypriaca* on Cyprus, *L.s. picea* in North-eastern Jordan, *L.s. brachydactyla* in the Negev and Vicinity, *L.s. vulgaris* in lower Egypt and *L.s. salehi* in Southern Sinai (Werner, 2007). However, Werner (2007) stated that all populations in the centre of the range from Northern Syria to the Northern Negev, remain taxonomically unstudied and undefined. As a result, the populations from southeast of Anatolia may be similar to the populations from Syria or may be different from both.

CONCLUSION

In this study, morphologic differences overlap with the genomic data. Therefore, molecular analyses based on mitochondrial 12S rRNA gene were supporting the existance of two morphologically distinct populations of *Laudakia stellio* in Anatolia. However, southeastern populations (Gaziantep, Sanliurfa and Hatay) were not similar to the subspecies *L.s. stellio* as understood by Almog *et al*. (2005).

This study provides additional data about the taxonomic status of *L. stellio* in Turkey. We can conclude that the South, Blacksea and Aegean regions may represent *L. stellio-daani* because morphologically they fit the subspecies *L.s. daani*, however the southeast region remains to be addressed. This study has also demonstrated the need for a phylogenetic study accross the lizard's whole distribution including Greece (*L. stellio-stellio*) and North Syria populations to verify the genetic variation, taxonomic subdivisions within the species.

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