



## First Data on DNA Barcoding of the Agamidae (Reptilia: Squamata) from Anatolia, Turkey

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**Abstract:** Species delimitation tests were applied to the Anatolian agamid species (*Stellagama stellio*, *Paralaudakia caucasica*, *Phrynocephalus horvathi* and *Trapelus ruderatus*) for the first time. Cytochrome c oxidase I (COI) gene region (564 bp) was used for DNA barcoding. Automatic Barcoding Gap Discovery (ABGD), Poisson-Tree-Processes (PTP), Generalized Mixed Yule Coalescence (GMYC) and Refined Single Linkage (RESL) analysis were used for determining the operational taxonomic units (OTU). The number of OTUs varied from 4 to 7 among these methods. The interspecific genetic distance within the Agamidae ranged from 16.60% to 20.8%. The COI sequences were also compared with the Barcode of Life (BOLD) data system. This initial attempt indicated the validity of COI barcodes for Anatolian agamids in delimiting species and contributing to the growing library of DNA barcodes of animal species of the world. Our results might be useful in tackling taxonomic problems, understanding species distribution and resolving nomenclature conflicts associated with the Anatolian agamids.

**Key words:** Species delimitation tests, Agamidae, Anatolia, COI

### Introduction

Agamidae is the fourth largest family among lacertilians and is distributed across Africa, Asia and Australia (BAIG et al. 2012). The Anatolian agama group consists of four species: *Stellagama stellio* (Linnaeus, 1758), *Paralaudakia caucasica* (Eichwald, 1831), *Phrynocephalus horvathi* (von Mehely, 1894) and *Trapelus ruderatus* (Olivier, 1804). According to RAS-TEGAR-POUYANI (2000), the holotype of *Agama lessonae* de Filippi, 1865 is a representative of populations previously referred to as *T. ruderatus* (Olivier, 1804) and proposed to use the combination *Trapelus lessonae* (de Filippi, 1865) for the latter species. In order to stabilise the nomenclature in this group, ANANJEVA et al. (2013) selected the holotype of *Agama lessonae* as the neotype of *Agama ruderata* Olivier, 1804; hence, *A. lessonae* became an objective junior synonym of *A. ruderata* and the proper combination for this species

is *Trapelus ruderatus* (Olivier, 1804). *Phrynocephalus helioscopus* (Pallas, 1771) has been recognised as a senior synonym of *P. horvathi* but, according to other opinions (SOLOVYEVA et al. 2011), *P. horvathi* was placed as a subspecies of *P. persicus*. On the basis of mtDNA phylogeny data, MELNIKOV et al. (2008) suggested that *P. persicus* and *P. horvathi* should be considered as separate species. They also indicated that *P. horvathi* can be easily distinguished from *P. helioscopus* based on their morphology. However, published studies largely disagree with this statement (ARAKELYAN et al. 2011, SOLOVYEVA et al. 2011, MILTO & BARABANOV 2012). In the present study, the species nomenclature is in accordance with the *International Union for Conservation of Nature Red List Database* (IUCN RED LIST). Globally, there is a pressing need for accurate taxonomic treatment of the Anatolian agamid species, as the population of *P. horvathi* is dwindling and it has been categorised as a critically

endangered species in the database of the International Union for Conservation of Nature Red List (IUCN).

DNA barcoding is a promising tool for the rapid and accurate identification of various species (HEBERT et al. 2003, NAGY et al. 2012). Although numerous molecular markers have been described for DNA barcoding, the mitochondrial cytochrome oxidase I (COI) gene region has proved to be most useful in the case of animals. As the amplification of COI sequences is problematic in reptiles due to their high variability, there is a scarcity of DNA barcode data for reptile groups. Till date, only one study has applied DNA barcoding to specimens of the family Agamidae (Russian population) (ANANJEVA et al. 2010). The present study is the first attempt to evaluate the effectiveness of species delimitation tests on the members of the family Agamidae from Turkey. It aims to reveal the success of the COI barcoding in ascertaining boundaries and showing the species richness for this group.

## Materials and Methods

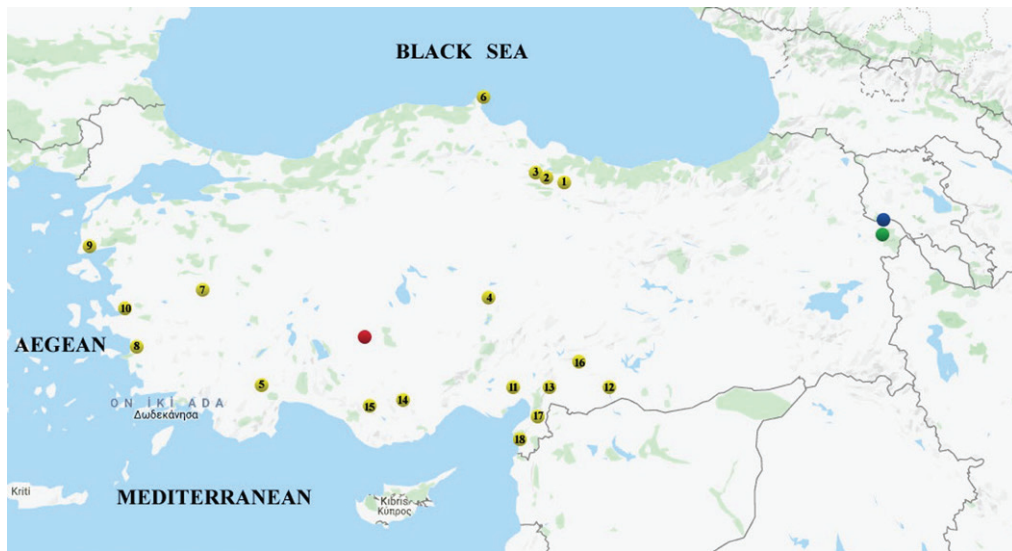
Muscle tissue samples of species under evaluation were obtained from the Çanakkale Onsekiz Mart University Zoology Museum (COMU-ZM). Preliminary identification of specimens was based on morphology and on previous literature on Anatolian agamids for clarification of species-level assignments. Species determination was made based on morphometric, pholidosis and coloration patterns of specimens. A total of 35 tissue samples from four species were analysed (for localities, see Table 1 and Fig. 1). Total DNA was extracted using the DNeasy® Blood & Tissue kit (Qiagen Inc., Hilden, Germany). PCR reactions were carried out using primer set mentioned by FOLMER et al. (1994) (LCO1490 5'-GGTCAACAAT-CATAAAGATATTGG-3' and HCO2198 5'-TAAACTTCAGGGTGACCAAAAATCA-3'). The following PCR protocol was used for amplification: initial denaturation at 95 °C for 2 min; followed by 38 cycles of denaturation at 95 °C for 30 s, primer annealing at 48 °C for 45 s and sequence elongation at 72 °C for 45 s; followed by final elongation at 72 °C for 7 min and storing the product at 4 °C. Sequence alignment was carried out with the BioEdit software (HALL 1999) and was trimmed to 564 bp. DNASP program (LIBRADO & ROZAS 2009) was used to construct the haplotype data and for estimating sub-populations division. Genetic differentiation (FST) and gene flow (NM) values were computed for inference of sub-species status of *S. stellio* according to HUDSON et al. (1992). Median-joining haplotype network were generated using the NETWORK soft-

ware (available at fluxus-engineering.com). The haplotype data were submitted to GenBank and Barcode of Life data system (BOLD). Accession and BIN numbers for the submitted sequences are given in Table 1. Intra- and inter-specific genetic distances and informative data of sequences were calculated with Mega X (KUMAR et al. 2018).

The determination of the total number of individuals in separate samples is crucial for the estimation of total species diversity. Species were compared using four different species delimitation methods (two tree-based and two distance-based): Poisson-Tree-Processes (PTP), Generalized Mixed Yule Coalescence (GMYC), Automatic Barcoding Gap Discovery (ABGD) and the Refined Single Linkage algorithm (RESL). The results of the different molecular species delimitation methods are summed up in terms of operational taxonomic units (OTUs). ABGD (PULLANDRE et al. 2012) was used for detecting the level of barcode gap (Relative gap=1, Pmin=0.001, Pmax=0.1, Steps=10, NBins=10, K2P). The maximum likelihood phylogenetic tree was constructed in PhyML (GUINDON et al. 2010) with 1000 bootstrap replications for species delimitation via mPTP (ZHANG et al. 2013). The analysis was done on the PTP and mPTP web servers (<http://species.h-its.org/> and <http://mptp.h-its.org/#/tree>). Before conducting GMYC analysis, the ultrametric tree was generated using BEAST 1.8.2 (DRUMMOND et al. 2012) statistical phylogenetics and coalescent-based population genetics are becoming increasingly central to the analysis and understanding of molecular sequence data. We present the Bayesian Evolutionary Analysis by Sampling Trees (BEAST). The analysis was carried out for 50 million generations with a sampling frequency of 10,000. All runs were checked with Tracer 1.6. The maximum clade credibility tree was arranged using TreeAnnotator 1.8.2 (DRUMMOND et al. 2012). The resulting ultrametric tree was imported into R 3.1.3 (R CORE TEAM 2013) and the single threshold ST-GMYC analysis was carried out using the R packages (Splits; EZARD et al. 2009 and Ape libraries; PARADIS 2004).

## Results

In total, there were 186 variable sites of data, of which 160 were parsimony informative. The sequences had an average nucleotide composition of 38.0% T, 17.1% C, 34.4% A and 10.5% G. Most variable sites occurred in the first codon-position. Within- and between-group pairwise genetic distance was measured with Mega X (Table 2). Also, the pairwise genetic distance of subspecies of *Stellagama* was



**Fig. 1.** Distribution map of the studied species (numbers as in Table 1). Colours indicate species of Agamidae: in red *T. ruderatus*, in green *P. caucasia*, in blue *P. horvathi*, in yellow *S. stellio*.

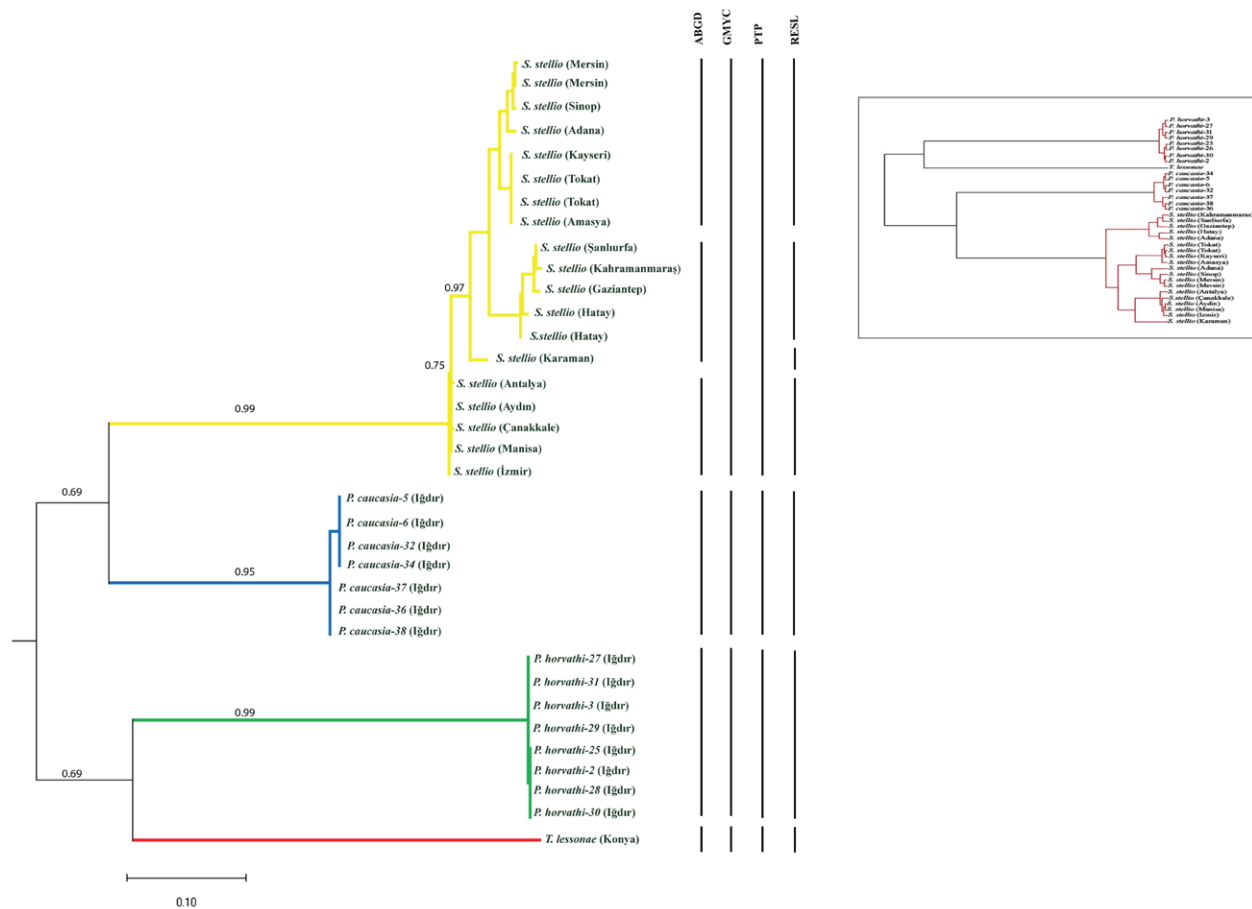
**Table 1.** Locality information for the studied agamid specimens, haplotype data, BIN numbers and GenBank numbers.

Species	Localities	Haplotype	BIN	GenBank Accession number
<i>S. stellio</i>	Tokat (Gülbayır village) (1), Kayseri (İncesu) (2), Amasya (Tasova) (3), Tokat (Erbaa) (4)	H1	AAF1316	MN639676
	Antalya (Korkuteli) (5)	H2	ADZ8502	MN639677
	Sinop (6)	H3	AAF1316	MN639678
	Manisa (Selendi) (7), Aydın (Söke) (8)	H4	ADZ8502	MN639679
	Çanakkale (Asos) (9)	H5	ADZ8502	MN639680
	İzmir (Karagöl) (10)	H6	ADZ8502	MN639681
	Adana (Ceyhan) (11)	H7	AAF1316	MN639682
	Şanlıurfa (Birecik) (12)	H8	AAF1314	MN639683
	Gaziantep (Islahiye) (13)	H9	AAF1314	MN639684
	Karaman (Sariveliler) (14)	H10	ADZ9926	MN639685
	Mersin (Mut) (15)	H11	AAF1316	MN639686
	Mersin (Mut) (16)	H12	AAF1316	MN639687
	Kahramanmaraş (Pazarcık) (17)	H13	AAF1314	MN639688
	Hatay (Kırıkhan) (18)	H14	AAF1314	MN639689
	Hatay (Samandağ) (19)	H15	AAF1314	MN639690
<i>P. caucasia</i>	Iğdır (Doğubeyazıt) (20)	H16	AEA2689	MN639691
		H17	AEA2689	MN639692
<i>P. horvathi</i>	Iğdır (Melekli village) (21)	H18	AAE1476,	MN639693,
		H19	AAE1476	MN639694
<i>T. ruderatus</i>	Konya (Sultanbeyli) (22)	H20	AAC9968	MN6396955

identified (Table 3; subspecies identified based on literature data). The  $F_{ST}$  value between the western and southern groups and the NM value calculated from this  $F_{ST}$  were 0.39 and 0.39, respectively.

The number of OTUs in a DNA barcode dataset generally depends upon the method used. ABGD analysis produced more OTUs as compared to other

methods and concluded 6 OTUs for the Agamidae (Fig. 2). Three clusters were recognised for *S. stellio* in the ABGD analysis. The specimens from Tokat, Kayseri, Amasya, Sinop, Adana and Mersin represented the group-1; those from Antalya, Manisa, Aydın, Çanakkale, İzmir and Karaman constituted the group-2; samples from Şanlıurfa, Gaziantep,



**Fig. 2.** ML tree of the agamid species from Anatolia based on COI gene sequences. Bootstrap values are indicated in left side of the tree (1000 replications). Candidate species determined by the species delimitation test are stated with black boxes. GMYC species delimitation tree represented in the box. Red clusters and black lines (singletons) indicate putative species calculated by the model.

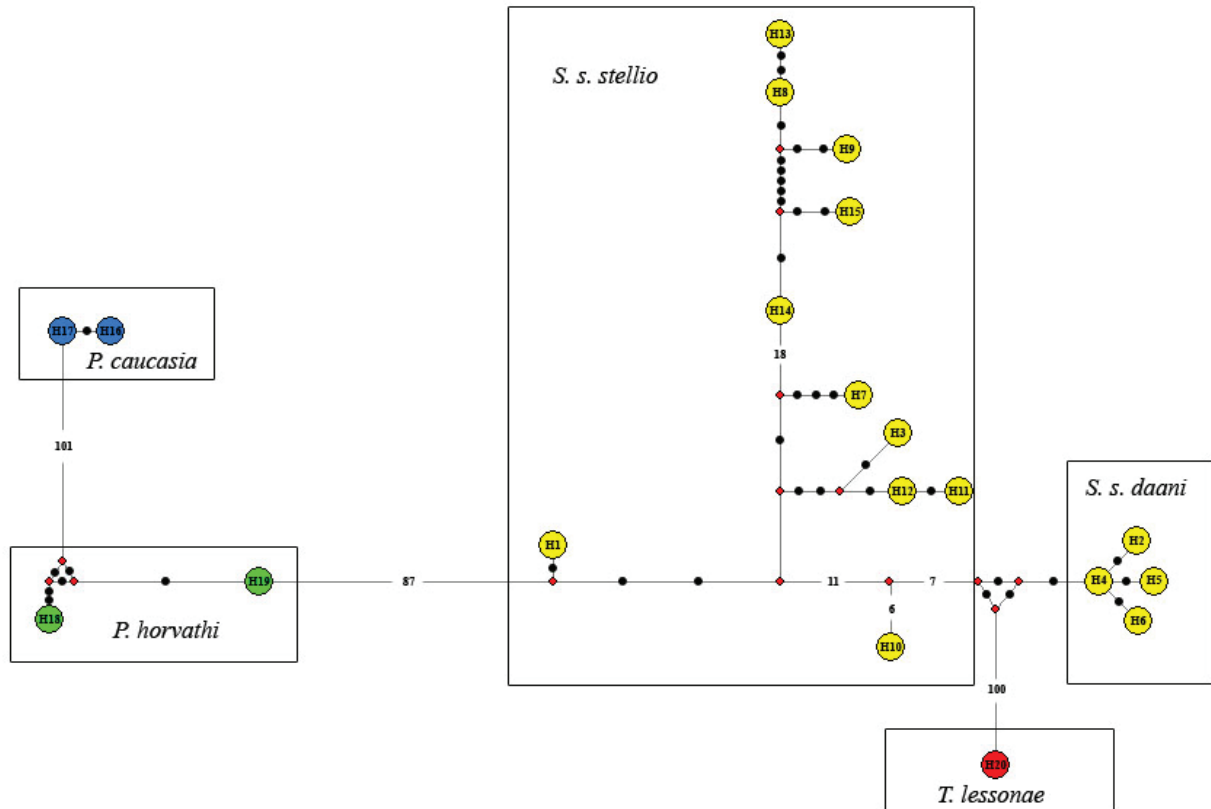
Kahramanmaraş and Hatay formed the group-3. GMYC and PTP methods yielded the same number of clusters. The single threshold GMYC (sGMYC) model resulted in the identification of four clusters of species of Agamidae (*S. stellio*, *P. caucasia*, *P. horvathi* and *T. ruderatus*), with high probabilities (confidence interval [CI] = 2–8, lnL of null model = 222.983, ML of GMYC model = 226.9927,  $p = 0.018^{**}$ ), resulting into a total of four entities. The trees resulting from PTP analysis gave similar results as those obtained by GMYC. PTP recovered by maximum likelihood search resulted into four species (estimated number of species is between 4 and 17), with relatively high support values (Species 1, *T. ruderatus* = 1.000; Species 2, *P. horvathi* = 0.560; Species 3, *P. caucasia* = 0.336; Species 4, *S. stellio* = 0.903).

Haplotype network also revealed three clusters for *S. stellio* (Fig. 3). Congruently, cluster sequence analysis results, which were run into BOLD, presented similar genetic structuring. Single-linkage clustering of OTU boundaries based on the uncorrected pairwise distances (p-distance) were generated through RESL

in the BOLD system (RATNASINGHAM & HEBERT 2013). Totally, seven OTUs were defined by the RESL analysis and four clusters were defined for *S. stellio*. The specimens from Tokat, Kayseri, Amasya, Sinop, Adana and Mersin constituted the group-1; Antalya, Manisa, Aydın, Çanakkale and İzmir specimens represented the group-2; those from Şanlıurfa, Gaziantep, Kahramanmaraş and Hatay were the group-3; the samples from Karaman were the group-4.

## Discussion

Anatolian agama complex is a taxonomically controversial group, which is frequently disputed. The present study is the first attempt for testing species delimitation methods in members of the Agamidae from Anatolia. However, the primary aim of DNA barcoding is not phylogenetic analysis, since the species delimitation test has been designed for species identification and species discovery. However, results, in most cases, can be compared with multi-gene-based phylogenetic studies.



**Fig. 3.** Median-joining haplotype network showing the mutational distance among haplotypes within the Agamidae from Anatolia. Colours indicate Agamidae species: in red *T. ruderatus*, in green *P. caucasia*, in blue *P. horvathi*, in yellow *S. stellio*.

**Table 2.** Inter- and intra-specific genetic distance of the members of the family Agamidae from Anatolia.

	<i>S. s. stellio</i>	<i>S. s. daani</i>	<i>P. horvathi</i>	<i>P. caucasia</i>	Intraspecific Mean Distance
<i>S. s. stellio</i>					0.0299
<i>S. s. daani</i>	0.0444				0.0021
<i>P. horvathi</i>	0.2090	0.2051			0.0010
<i>P. caucasia</i>	0.1662	0.1658	0.1845		0.0040
<i>T. ruderatus</i>	0.2005	0.1819	0.2066	0.1971	-

Among the species delimitation methods, tree-based delimitation tests (PTP and GMYC) gave more conservative results compared to distance-based ones (RESL through BOLD and ABGD analyses). Because of the tangled phylogenetic systematics of *S. stellio*, a high number of candidate species were observed in this taxon. The number of OTUs recognised by the ABGD analysis was concordant with the grouping inference based on haplotype network (Fig. 3, Table 1). In several studies, western and south-eastern Anatolia have been reported to harbour *S. s. daani* and *S. s. stellio*, respectively (BARAN & ÖZ 1985, BARAN & ATATUR 1998, GOÇMEN et al. 2003, ALMOG et al. 2005). According to ABGD analysis, haplotype network and ML tree, the most southern

boundary of *S. s. daani* was Karaman. Likewise, for *S. s. stellio* the boundary was Şanlıurfa, Hatay, Gaziantep and Kahramanmaraş. The distribution boundaries and the taxonomical status of *S. stellio* is debated by many taxonomist (ÖZDEMİR et al. 2011, BAIG et al. 2012, KUMLUTAŞ et al 2015). According to the species delimitation test and BIN cluster analysis, there are two subspecies in Anatolia and also two mitochondrial lineages belong to *S. stellio stellio*. The eastern boundary of *S. stellio daani* is questionable. As a result of the genetic clustering in this study, the most eastern boundary of *S. stellio daani* was found to be Antalya. BARAN & ÖZ (1985) stated that western and southern populations are rather similar to *S. stellio daani* but the Hatay population remained

**Table 3.** Pairwise genetic distance of *Trapelus ruderatus* specimens from BOLD data system. The type locality (Iran) and the nearest neighbouring localities in Azerbaijan were included for comparison.

Locality (BOLD Process ID)	1	2	3	4	5	6	7
1. Iran (NPLRP319-08)							
2. Iran (NPLRP321-08)	0.0163						
3. Iran (NPLRP707-10)	0.0127	0.01627					
4. Iran (ZISPA019-10)	0.0127	0.01627	0.00723				
5. Iran (ZISPA028-10)	0.0018	0.01447	0.01085	0.01085			
6. Azerbaijan (ZISPA035-10)	0.0108	0.01447	0.00542	0.00181	0.00904		
7. Azerbaijan (ZISPA036-10)	0.0108	0.01447	0.00542	0.00181	0.00904	0.00000	
8. Konya	0.1555	0.14828	0.15371	0.14828	0.15371	0.15009	0.15009

**Table 4.** Pairwise genetic distance of specimens of *Phrynocephalus horvathi* from BOLD data system (only specimens named *P. persicus horvathi* were considered)

Locality (BOLD Process ID)	1	2	3	4	5
1. Armenia (ABLRP327-07)					
2. Armenia (ABLRP496-09)	0.000				
3. Azerbaijan (NPLRP431-08)	0.091	0.091			
4. Azerbaijan (NPLRP475-08)	0.060	0.060	0.094		
5. Iğdır (AGMTR016-19)	0.006	0.006	0.094	0.067	
6. Iğdır (AGMTR017-19)	0.004	0.004	0.091	0.064	0.002

unsettled. According to that study, the Hatay population of *S. stellio daani* is grouped together with *S. stellio stellio*. In addition, they share the same BIN with the Syrian specimens and individuals from north-west and inner Anatolia are genetically different from these two subspecies. However, as the ABGD approach has many flaws, e.g. it is based solely upon nucleotide divergence data, there is no any information on the evolutionary processes (FUJISAWA & BARRACLOUGH 2013). Moreover, moderate  $F_{ST}$  and  $NM$  values were found between the western and southern groups. According to the phylogenetic analysis, those groups were differentiated from each other. Strong sub-population division is often common in low vagility organisms like reptiles. They are more susceptible to climatic oscillation than other organisms (GONÇALVES et al. 2009). Most of the delimitation test (GMYC, ABGD, RESL and network analysis) that are used in this study showed obvious genetic subdivision among groups in *S. stellio*. The future genetic structure of those groups is to be shaped up according to their interactions and geographic obstacles among them.

The nomenclature of *Trapelus ruderatus* is also debated among researchers. RASTEGAR-POUYANI (2000) re-evaluated the systematic status of *Trapelus* from Iran. He has proposed *T. lessonae* as the oldest available name used for all the populations of (conventional) *T. r. ruderatus*, and the spe-

cies name “*ruderatus*” is assigned to all the populations of (conventional) *T. persicus*. In contrast, ANANJEVA et al. (2013) designated the holotype of *Agama lessonae* as the neotype of *A. ruderata* (now *Trapelus ruderatus*) aiming to stabilise the nomenclature. My sequences were designated as *T. ruderatus* in the BOLD system. According the tree-based identification in BOLD, the sequences of this study are placed close to other sequences of *T. ruderatus* from Konya. A high pairwise genetic distance was observed between Konya and Iran (type locality of *T. ruderatus*) sequence data retrieved from BOLD (Table 3). SINDACO & JEREMCENKO (2008) have suggested that *T. ruderatus* is not distributed in Turkey but have identified *T. lessonae* in their studies. It is obvious that members of this genus from Iran, Azerbaijan and Turkey have been differentiated from each other. *Trapelus ruderatus*, *T. lessonae* and *T. persicus* cannot be considered as synonymous according to their genetic interactions. Members of *Trapelus* in Anatolia must be considered under a name differing from names of their congeneric species from neighbouring areas. More specimens from different parts of Anatolia are needed for understanding the exact geographical range of the detected genetic lineages and for improving their taxonomy. Detailed taxonomic revision is inevitable for the Middle East population of the genus *Trapelus*.

*Phrynocephalus horvathi* is also a member of the Anatolian Agamidae with a controversial taxonomic status. Some researchers have placed this taxon as a subspecies of *P. persicus* (SOLOVYEVA et al. 2011, MILTO & BARABANOV 2012), whereas others considered it as a distinct species (MELNIVKOV et al. 2013, TUNIYEV et al. 2014). Low pairwise genetic distance was detected among sequences of Iğdır specimens and BOLD submissions (submitted as a *P. persicus horvathi* from Armenia; Table 4). Pairwise genetic distance showed that specimens from Armenia and Anatolia are genetically similar to each other, while Iranian and Azerbaijan specimens of *Phrynocephalus* were genetically distinct from them. There are so many suture zones between Azerbaijan-Armenia, Armenia-Iran and Anatolia-Iran (ADAMIA et al. 2017). Apart from the other *P. persicus* members from Iran or Azerbaijan, I suggest to affiliate Armenian and Anatolian members to the same species. SOLOVYEVA et al. (2018) stated that future studies can address further taxonomic reassignments within the *P. persicus* complex.

The PCR amplification of the barcode region in the species under study was more successful with the universal Folmer's barcode primers (92.31% for *S. stellio*, 80% for *P. caucasia*, 80% for *P. horvathi* and 100% for *T. ruderatus*) as compared to the reptilian barcode primer sets given in the literature (VENCES et al. 2005a, b, NAGY et al. 2012, CASTAÑEDA & QUEIROZ 2011). Other DNA barcoding studies on reptiles have also indicated that Folmer's barcode primers (LCO and HCO) worked better as compared to RepCOI-F and RepCOI-R (NAGY et al. 2012, HAWLITSCHKEK et al. 2013)

In the phylogenetic tree, we observed lower support values in basal branches than intermediate and terminal ones (ORTIZ & FRANCKIE 2016). In addition, the tree approved the previous data (SOLOVYEVA et al. 2014) and indicated that *Trapelus* is phylogenetically close to *Phrynocephalus*. Tree topology also confirmed the distance-based delimitation test and indicated paraphyly of *S. stellio*.

Species delimitation based upon GMYC and PTP tests is very similar to the currently accepted taxonomy of Agamidae. The main advantages of GMYC and PTP methods are the objective assessment of phylogenetic entities and OTUs based upon branch length dynamics rather than sequence similarities (MONAGHAN et al. 2009, PONS et al. 2006).

In conclusion, the species delimitation test was successfully applied to the Anatolian agamids for the first time and confirmed the confusion in the taxonomy of this family. Many species had become extinct before they were discovered. More OTUs than ever

were assigned to *S. stellio* in this study. Maybe they will deserve a species status in the future. The results of the present study indicate that the Agamidae from Anatolia, especially *S. stellio*, should be earmarked for future re-examination. Moreover, the subpopulation division and structuring of *S. stellio* need further detailed survey. Also, ascertaining the status of *T. ruderatus* in Anatolia is urgent. In future, studies on population genetics may cast light on the species complexes identified in this study.

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