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A molecular phylogeny of the eastern group of ocellated lizard genus *Timon* (Sauria: Lacertidae) based on mitochondrial and nuclear DNA sequences

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Abstract. *Timon*, a small genus of lacertid lizards, includes four species distributed in two separate ranges in the western and eastern part of the Mediterranean Basin. Phylogenetic relationships between the two groups have not been resolved, and the taxonomic situation of the two subspecies of the eastern representative of the genus, *Timon princeps*, is not clear. To address these questions, partial DNA sequences of two nuclear (*β-fibrinogen* intron 7 and *C-mos*) and three mitochondrial (cytochrome *b*, 12S rRNA and 16S rRNA) genes were analyzed. Based on the high genetic distance between the two subspecies of *T. princeps* we promote their taxonomic status to full species, *Timon princeps* and *Timon kurdistanicus*. Divergence time estimates based on other lacertid species suggest that the separation of the green (*Lacerta*) and ocellated (*Timon*) lizards took place around 12 My ago, and that the Eastern group underwent speciation around 4-5 my ago, perhaps associated with the uplifting of the Zagros mountains. As expected given this ancient divergence and complex paleogeography, considerable levels of genetic diversity are recovered within both taxa, with geographically close individuals showing very divergent haplotypes.

Keywords: lacertid lizards, mtDNA, nDNA, phylogeny, *Timon kurdistanicus*, *Timon princeps*.

Introduction

The evolutionary history of lacertid lizards has been the subject of various investigations (Lutz and Mayer, 1984; Arnold, 1989; Mayer and Bischoff, 1996; Arribas, 1997; Fu et al., 1997; Harris et al., 1998; Fu, 2000; Carranza et al., 2004; Godinho et al., 2005; Arnold et al., 2007; Pavlicev and Mayer, 2009). However, most of these attempts to reconstruct the phylogeny of lacertids resulted in unresolved bush-like topologies (Pavlicev and Mayer, 2009). In one of the most recent efforts, based on morphological and molecular characterization, the then polyphyletic genus *Lacerta* s. l. was split into several small but generally monophyletic units (Arnold et al., 2007). However, the monophyly of some units (such as *Algyroides*) was not tested, while others (such as *Timon*) were not supported.

Timon as currently accepted is a small genus comprising four species of large ocellated lizards, *Timon princeps* (Blanford, 1874), *Timon lepidus* (Daudin, 1802), *Timon tangitanus* (Boulenger, 1889) and *Timon pater* (Lataste, 1880) distributed across two main geographic regions: the eastern and the western Mediterranean. The *Timon lepidus* group, consisting of *T. lepidus* (with three recognized subspecies, but see Miraldo et al., 2011), *T. pater* and *T. tangitanus*, occupies the western part of the Mediterranean basin including the Iberian Peninsula, southern France and northwestern Italy in Europe and Morocco, Algeria and Tunisia in North Africa (Arnold et al., 2007; Paulo et al., 2008; Perera and Harris, 2010; Miraldo, 2011). On the other hand, *Timon princeps* is distributed in eastern Turkey and in Iran from Kurdistan through western Zagros to Fars Province (An-

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derson, 1999; Ilgaz and Kumlutas, 2008; Sindaco and Jeremcenko, 2008), and includes two currently recognized subspecies (Eiselt, 1968, 1969, 1970; Anderson, 1999).

These eastern and western species appear to be related on the basis of albumin immunology (Lutz and Mayer, 1984) and some morphological features (Eiselt, 1968, 1969). Harris et al. (1998), based on mitochondrial DNA sequences showed that the two discrete groups are monophyletic. On the other hand, Arnold et al. (2007), despite recognizing *Timon* as a full genus, did not recover the monophyly of the group, with *T. princeps* unrelated to other *Timon* species albeit without strong support. None of the available DNA studies provide a fully resolved hypothesis of relationships between the two main groups, although "western" *Timon* species are usually found to be the sister taxa of *Lacerta* s. str. (Harris et al., 1998; Fu, 2000; Arnold et al., 2007). Regarding the variation within each group, several recent studies investigated the phylogeographic patterns within the *T. lepidus* group (Paulo et al., 2001, 2008; Perera and Harris, 2010; Miraldo et al., 2011). Concerning the eastern group, although the morphology of *T. princeps* has been discussed extensively by Eiselt (1968, 1969), its genetic variation and phylogeographic patterns remain unknown. This author recognizes the existence of two different subspecies: *T. princeps kurdistanicus* and *T. p. princeps*. Both subspecies are separated on the basis of pholidotic and meristic characteristics, but as far as we know, no genetic data regarding the validation of these two morphologically differentiated groups exist.

Therefore, the three main objectives of this study are: 1) what are the phylogenetic relationships between the eastern and western groups? In particular, are "western" *Timon* more closely related to *Lacerta* s. str.? 2) what is the level of genetic variability between the two subspecies of *T. princeps*? and finally, 3) what shaped the current biogeographic pattern of the eastern populations, and when did they start to diverge and how?

Materials and methods

In total, 16 samples of *T. princeps* covering all the distribution range of the species were analysed. Specimen codes used in this study and their respective locations can be found in table 1. Total genomic DNA from each individual was extracted from tail tips using standard saline methods (Sambrook et al., 1989). Polymerase chain reaction (PCR) amplifications were performed for all samples using three different mitochondrial DNA fragments (cytochrome *b*, 16S rRNA and 12S rRNA). Additionally, in order to investigate the phylogenetic relationships between *Timon* and other lacertids, two nuclear DNA fragments (β-fibrinogen and *C-mos*) were amplified for two individuals of each recognized subspecies (*T. p. princeps* and *T. p. kurdistanicus*). The amplification and sequencing of three mtDNA gene fragments was performed using the primers GluDG/Peil (modified from Palumbi et al., 1991 and Engstrom et al., 2007), 16SL/16SH (Palumbi et al., 1991), and 12Sa/12Sb (Kocher et al., 1989). For the amplification of intron 7 of the *β-fibrinogen* and the *C-mos* gene, the primers FIB-BI7U and FIB-BI7L (Prychitko and Moore, 1997), and LCS1 and LCS2 (Godinho et al., 2005) were used, respectively. These five sets of primers amplified regions of approximately 950, 550, 450, 700 and 550 base pairs, respectively.

In all cases, PCR mix was carried out in a 25 μl total volume, containing 2.5 μl reaction Buffer, 1.5 mM of $MgCl_2$, 0.5 mM each dNTP, 0.5 mM each primer, 0.1U Taq DNA polymerase (Invitrogen) and approximately 100 ng of template DNA with the following conditions: an initial cycle of 92° for 2 min, followed by 35 cycles of 92°C for 30 s, 50° for 40 s (54° for nuclear genes) and 72° for 45 s, and a final cycle of 72° for 5 min. All amplified fragments were sequenced by a commercial company (Macrogen, Korea).

Sequences were imported to Bioedit (Hall, 1999) where they were first aligned using ClustalW with default parameters and then adjusted by hand. Sequences are available in GenBank (table 1).

In order to investigate the relationships of the genus *Timon* with other lacertids sequences, a phylogenetic analysis using the five amplified fragments (both mtDNA and nDNA) was performed. Separate and combined analyses were performed using MP. Conflict between partitions was assessed using the qualitative evaluation of Wiens (1998). Since there was no strong conflict between gene regions, concatenated analyses were performed. All genes were concatenated resulting in a final fragment of 2713 bp. From the GenBank database, previously published sequences of the same genes belonging to five species of *Lacerta* (*L. agilis*, *L. pamphylica*, *L. schreiberi*, *L. viridis*, *L. strigata*), as well as three species of the western group of *Timon* (*T. lepidus*, *T. pater* and *T. tanginatus* – Godinho et al., 2005; Paulo et al., 2008) were added to our analysis (table 1). Due to their shorter length, only 2084 bp (350 bp 12s rRNA, 420 bp 16 rRNA, 306 bp cytochrome *b*, 317 bp *C-mos*, 691 bp β-fibrinogen) fragments could be included in the final analysis. *Archaeolacerta bedriagae* was included as the closest available outgroup (Pavlicev and Mayer, 2009; Savli, unpub. data).

Sequences were analysed using Bayesian inference (BI), maximum-parsimony (MP) and maximum likelihood (ML)

Table 1. List of samples analyzed, sampling localities, origin of the samples and GenBank accession numbers for the samples included in this study.

Species	Code	Locality	Origin	GenBank accession numbers (12S, 16S, *cytb*, *C-mos*, β-fib)				
Timon princeps kurdistanicus	DB6235	Iran, Kurdistan, Sarv Abad	This study	JQ425790	JQ425806	JQ425830	–	–
Timon princeps kurdistanicus	DB6236	Iran, Kurdistan, Sarv Abad	This study	JQ425791	JQ425807	JQ425831	JQ425828	JQ425824
Timon princeps kurdistanicus	DB6237	Iran, Kurdistan, Sarv Abad	This study	JQ425792	JQ425808	JQ425832	–	–
Timon princeps kurdistanicus	DB6245	Iran, Kurdistan, Sarv Abad	This study	JQ425793	JQ425809	JQ425833	–	–
Timon princeps kurdistanicus	DB6252	Turkey, Southeastern Anatolia, Mardin	This study	JQ425794	JQ425810	JQ425834	–	–
Timon princeps kurdistanicus	DB6260	Iran, Kurdistan, Sarv Abad	This study	JQ425795	JQ425811	JQ425835	JQ425829	JQ425825
Timon princeps kurdistanicus	DB6451	Turkey, Southeastern Anatolia, Başaran	This study	JQ425796	JQ425812	JQ425836	–	–
Timon princeps princeps	DB6238	Iran, Lorestan, Khoram Abad	This study	JQ425797	JQ425813	JQ425837	–	–
Timon princeps princeps	DB6239	Iran, Lorestan, Khoram Abad	This study	JQ425798	JQ425814	JQ425838	–	–
Timon princeps princeps	DB6233	Iran, Fars, Dasht-e Arzhan	This study	JQ425799	JQ425815	JQ425839	JQ425826	JQ425822
Timon princeps princeps	DB6241	Iran, Fars, Dasht-e Arzhan	This study	JQ425800	JQ425816	JQ425840	–	–
Timon princeps princeps	DB6255	Iran, Kermanshah, Eslâm-Abâd-e Gharb	This study	JQ425801	JQ425817	JQ425841	–	–
Timon princeps princeps	DB6257	Iran, Kermanshah, Eslâm-Abâd-e Gharb	This study	JQ425802	JQ425818	JQ425842	–	–
Timon princeps princeps	DB6326	Iran, Yasuj	This study	JQ425803	JQ425819	JQ425843	–	–
Timon princeps princeps	DB6327	Iran, Yasuj	This study	JQ425804	JQ425820	JQ425844	JQ425827	JQ425823
Timon princeps princeps	S3504	Iran, Yasuj	This study	JQ425805	JQ425821	JQ425845	–	–
Lacerta agilis agilis	–	Holland	Godinho et al., 2005	DQ097096	DQ097096	AF080299	AF315397	DQ097109
Lacerta pamphylica	–	Turkey	Godinho et al., 2005	AF149954	AF149954	DQ097089	DQ097142	DQ097103
Lacerta strigata	–	Georgia	Godinho et al., 2005	DQ097094	DQ097094	DQ097091	DQ097137	DQ097107
Lacerta virids guntherpetersi	–	Greece	Godinho et al., 2005	AF149959	AF149959	AF233424	DQ097131	DQ097119
Timon lepidus lepidus	–	Portugal, Serra da Estrela	Paulo et al., 2008	AF378942	AF378948	AF378987	EU365407	EU365429
Timon lepidus nevadensis	–	Spain, Sierra de los Filabres	Paulo et al., 2008	AF378941	AF378950	AF379010	EU365408	EU365421
Timon pater	–	Tunisia, Tabarka	Paulo et al., 2008	AF378945	AF378955	AF378961	EU365410	EU365419
Timon tangitanus	–	Morocco, Xeuen	Paulo et al., 2008	AF378945	AF378955	AF378962	EU365410	EU365420
Lacerta schreiberi	–	Portugal	Paulo et al., 2008	AF206591	AF206591	AF372121	EU365406	EU365412

methods. The appropriate models of evolution were determined through jModeltest (Posada, 2008). Bayesian analysis was implemented using Mr. Bayes v.3.1 (Huelsenbeck and Bollback, 2001) with parameters estimated as part of the analysis. The analysis was run for 1×10^6 generations, saving one tree in each 100 generations. The log likelihood values of the sample points were plotted against the generation time, and all the trees prior to reaching stationarity (10%) were discarded, ensuring that burn-in samples were not retained. Remaining trees were combined in a 50% majority consensus tree. MP and ML analyses were carried out in PAUP 4.0b10 (Swofford, 2002). MP analysis was carried out using heuristic searches involving tree bisection and reconnection (TBR) branch swapping with 100 replicates. Bootstrap support (Felsenstein, 1985) was estimated for MP and ML with 1000 replicates.

To further assess variation within *T. princeps* across its distribution range we constructed a Median Joining Network using the software Network 4.5.1.0 (©Fluxus Technology; Bandelt et al., 1999). For this analysis, all mitochondrial fragments (12S rRNA, 16S rRNA and *cytb*) were concatenated for each subspecies and poorly aligned positions were detected and eliminated using the Gblocks software (online version 0.91b; Castresana, 2000) considering stringent parameters. A final dataset of sequences of 1605 bp (for *T. p. princeps*) and 1606 bp (for *T. p. kurdistanicus*) were used in the networks construction.

Uncorrected p-distances for the cytochrome *b* gene fragment were calculated using the software MEGA version 5 (Tamura et al., 2011).

Divergence times were estimated using a relaxed molecular clock approach with the program BEAST 1.5.3 (Drummond and Rambaut, 2007). A mutation rate of 2% for cytochrome *b* was used, given the lack of calibration points and that this rate has been applied to other lacertids (e.g. Paulo et al., 2008). The analysis was run for 10 million generations, sampling every 1000 generations. The first 10% of samples were discarded as burnin.

Results

A total of 2084 bp were included in the analysis. From them, 1076 bp of mitochondrial fragments including 340 variable sites, corresponding to 87 sites (24%) for 12S rRNA, 138 sites (32%) for 16S rRNA and 115 sites (37%) for cytochrome *b* were used. From the 1008 bp of nuclear genes analyzed, 150 positions were variable including 130 sites (18%) for the β-fibrinogen and 20 sites (6%) for *C-mos*. For the combined dataset the model $GTR+G$ was identified by jModelTest as the most appropriate nucleotide substitution model of molecular evolution.

We inferred separate gene trees for each of the five studied fragments (not shown) and a final tree based on combined dataset. All methodologies (ML, MP and BI) supported the same tree topology with high bootstrap support values for the ML and MP and posterior probabilities values for BI (fig. 1). The tree shows two distinct and well-supported evolutionary lineages (fig. 1), one grouping all species of genus *Lacerta*, and another one comprising all samples of the genus *Timon*. Within the *Timon* clade, two subclades, corresponding to the eastern (*T. princeps*) and western (*T. lepidus*, *T. tangitanus* and *T. pater*) groups, were found. The differentiation between the two *T. princeps* subspecies, *T. p. princeps* and *T. p. kurdistanicus* is strongly supported (fig. 1). The uncorrected p-distance for cytochrome *b* between the two lineages ranged from 14-16%, while divergences within them were from 0-3.9% (table 2). The nuclear genes also support this separation, with two fixed differences between *T. p. princeps* and *T. p. kurdistanicus* within the *C-mos* sequences (both transitions) and four fixed differences with the β-fibrinogen.

The Median Joining Network using all three mitochondrial fragments shows high haplotype diversity. For *T. p. kurdistanicus*, four different haplotypes were found in seven individuals from three different localities. Interestingly, despite being separated by only 300 km, the individual further north from Turkey (6252) is separated by 34 mutational steps from the closest one (6451), also from Turkey (fig. 2). The remaining samples, all from the Kurdistan area in Iran, are separated by 16-35 mutational steps from the Turkish samples, and have two different haplotypes, separated by just one mutational step (fig. 2). Regarding *T. p. princeps*: 5 haplotypes from a total of 7 individuals from 5 different locations were retrieved. The distribution of the haplotypes is concordant with a geographic pattern. One haplotype includes the samples from Central Iran (Kermanshah and Yasuj areas), separated from the other individual analysed in this region (S3504) by 12 mutational steps. The samples from the southern part of the distribution range form a haplogroup with

Table 2. Pairwise uncorrected genetic divergence (p-distance) within *Timon princeps* populations, derived from the mitochondrial gene cytochrome *b* (codes correspond to samples as in table 1).

	DB6235	DB6236	DB6237	DB6245	DB6252	DB6260	DB6451	DB6238	DB6239	DB6233	DB6241	DB6255	DB6257	DB6326	DB6327	S3504
DB6235																
DB6236	0.00															
DB6237	0.00	0.00														
DB6245	0.00	0.00	0.00													
DB6252	0.04	0.04	0.04	0.04												
DB6260	0.00	0.00	0.00	0.00	0.04											
DB6451	0.02	0.02	0.02	0.02	0.04	0.02										
DB6238	0.15	0.15	0.15	0.15	0.14	0.15	0.15									
DB6239	0.15	0.15	0.15	0.15	0.14	0.15	0.15	0.00								
DB6233	0.15	0.16	0.16	0.15	0.15	0.15	0.15	0.01	0.01							
DB6241	0.15	0.16	0.16	0.15	0.15	0.15	0.15	0.01	0.01	0.00						
DB6255	0.15	0.15	0.15	0.15	0.14	0.15	0.15	0.00	0.00	0.01	0.01					
DB6257	0.15	0.15	0.15	0.15	0.14	0.15	0.15	0.00	0.00	0.01	0.01	0.00				
DB6326	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.01	0.01	0.00	0.00	0.01	0.01			
DB6327	0.15	0.16	0.16	0.15	0.15	0.15	0.15	0.01	0.01	0.00	0.00	0.01	0.01	0.01		
S3504	0.16	0.16	0.16	0.16	0.15	0.16	0.16	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	

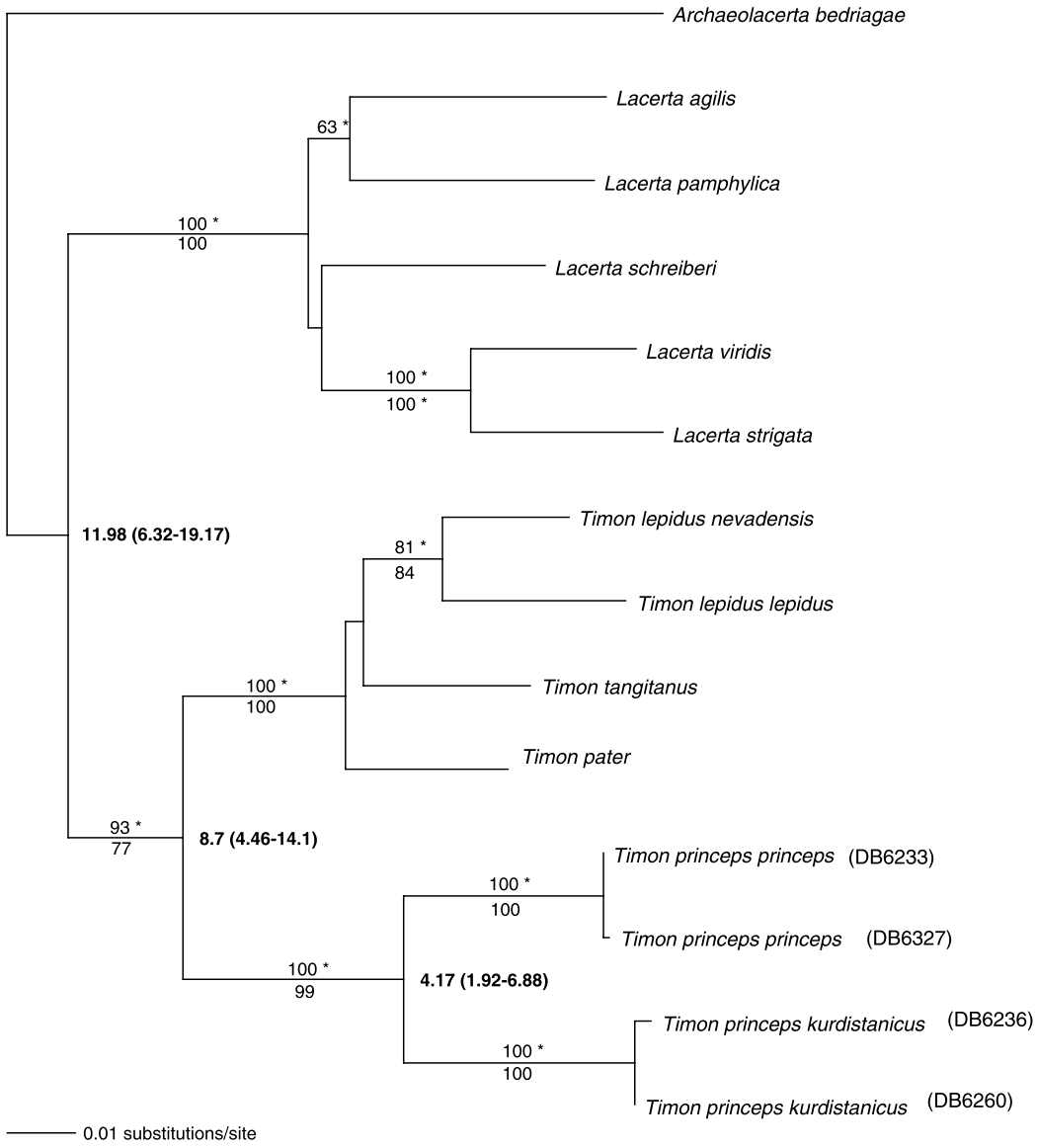

Figure 1. Phylogram derived from the combined data set sequences. As ML, MP and Bayesian resulted in similar tree topologies, only the ML tree is presented. Numbers on branches are bootstrap support values for ML (above) and MP (below) analyses, and (*) correspond to Bayesian posterior probabilities above 95%. The numbers in bold near the branches represents divergence dates.

three different haplotypes separated between 2 and 6 mutational steps (fig. 2).

Discussion

Based on a considerable dataset of morphological characters, Arnold (1973) suggested that green lizards (*Lacerta* s. str.) and *Timon lepidus* group (then including *T. lepidus*, *T. pater* and *T. princeps*) formed a clade, although this was not found with a chemosystematic approach (reviewed in Böhme and Corti, 1993). Subsequently, evidences from two studies using mtDNA sequences suggested that the *T. lepidus* group is the sister clade to the green

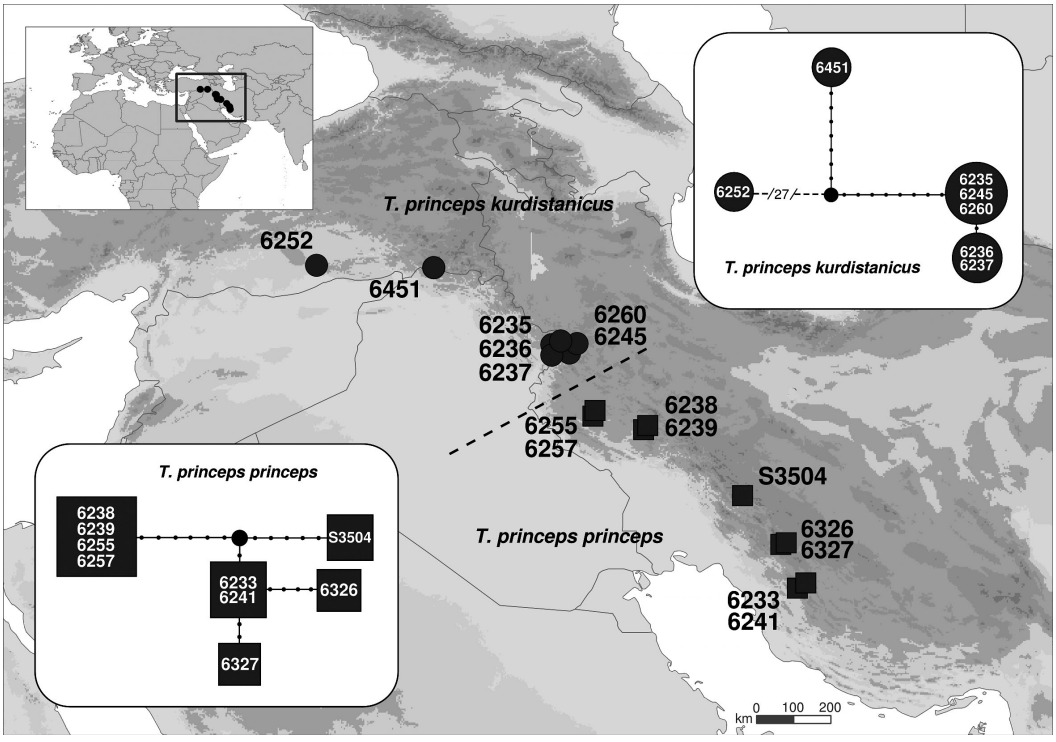

Figure 2. Distribution map of the *T. princeps* samples included in the study and haplotype Networks for the two *T. princeps* subspecies. Networks were constructed for the two species separately based on the mitochondrial fragments (12S rRNA, 16S rRNA and *cytb* concatenated). (a) Median Joining Network for *T. p. kurdistanicus* based on a 1606 bp fragment; (b) Median Joining Network for *T. p. princeps* based on 1605 bp. Grey circles represent *T. p. kurdistanicus*. Squared symbols represent *T. p. princeps*.

lizards (Harris et al., 1998; Fu, 2000). Although Arnold et al. (2007) found *Timon* to be polyphyletic, support levels were very low, and essentially relationships remaining unresolved. Paraphyly of *Timon* seemed unlikely given that the species share a chromosomal arrangement unique among lacertids (36 diploid with two biarmed chromosomes instead of the typical 38 diploid number (Rykena and Nettmann, 1986). However, assessing the monophyly of *Timon* is complicated by the lack of consensus regarding the possible sister taxa (Arnold et al., 2007; Pavlicev and Mayer, 2009; Hipsley et al., 2011). Without additional data from other possible sister taxa, it is therefore not possible to confirm the monophyly of *Timon*.

Our results also confirm the separation between eastern and western ocellated lizards, as previously shown (Harris et al., 1998; Arnold et al., 2007). However, this study shows for the first time complex patterns of genetic variation for the eastern group. *Timon princeps kurdistanicus* was originally described by Suchow (1936) from Bydarvaz, Kurdistan, Iran. Eiselt (1968, 1969) provided an extended description of *T. p. kurdistanicus* and distinguished it from *Timon princeps princeps* by the following characters: 17-19 gular scales (20-22 in *T. p. princeps*); 16-21 femoral pores on each side (13-17 in *T. p. princeps*); outer row of ventrals (marginals) keeled as are all flank scales (smooth in *T. p. princeps*), lower edge of subocular half or greater than half maximum length of shield (in *T. p. princeps* less than half maximum length of shield). Sampling across the distribution area using genetic markers revealed high genetic variability between the two subspecies, circa 15% uncorrected genetic distance for cy-

tochrome *b*. This can be compared to two green lizard species, *Lacerta viridis* and *Lacerta bilineata* where genetic differentiation ranges from 4.1% to 5.8% for the cytochrome *b* gene between the two species (Godinho et al., 2005). This uncorrected genetic distance (15%) between two subspecies of *Timon princeps* can also be compared to the average value for reptiles, of 13.6% average uncorrected divergence between species in the same genera and 11% for other lacertids (Harris, 2002). Given the considerable morphological and genetic differences (both mtDNA and nuclear), we recommend considering the former subspecies as two full species, as *Timon princeps* and, following the gender concordance, *Timon kurdistanicus*.

Fossil record and amber-preserved material evidences showed that Lacertidae probably evolved in Central Europe and West Asia after the collision of Africa and Eurasia (Estes, 1983; Böhme and Weitschat, 1998, 2002; Müller et al., 2011). Furthermore, genetic analyses (Harris et al., 1998; Fu, 2000; Arnold et al., 2007) suggest a fast diversification and radiation of the lacertid lizards. Estimations based on both mtDNA sequences (Carranza et al., 2004; Arnold et al., 2007) and albumin immunology and protein electrophoresis (Lutz et al., 1986) indicates this diversification happened about 13-9 My ago in the mid-late Miocene. Hipsley et al. (2009) report much older dates for the main radiation within the lacertini, but use *Timon lepidus* as one of their calibration points, based on Pliocene fossil remains from France. This clearly only gives a minimum age for this species, younger than the age estimated here. Arnold et al. (2007) used a molecular clock based on 12S rRNA and cytochrome *b* sequences and suggested that the separation of the green (*Lacerta*) and ocellated (*Timon*) lizards perhaps took place around 12.5 My ago. We estimate a similar age of 11.98 My. According to these authors, *L. agilis* and *L. pamphylica* split around 9.1 My, while *T. lepidus* split from the ancestor of *T. pater* and *T. tangitanus* around 8 My ago. Given the estimate of phylogeny produced here, it seems that the split between *T. princeps* and *T. kurdistanicus* should be nearly as old as this later date. Although the other ages estimated in this study (fig. 2) are less than those proposed by Arnold et al. (2007), they have large confidence intervals that overlap. Considering that *Timon princeps* and *Timon kurdistanicus* are allopatric, the most plausible speciation scenario would involve vicariance events. The mountain system of the Iranian plateau was formed on pre-Eocene plate boundaries by the Indian and Arabian collisions. The early uplifting of the Zagros Mountains was caused by the Arabian plate indentation into Iran during the late Miocene to early Pliocene (5-10 My), and led to the fragmentation of the western and eastern Iranian plateau (Abdrakhmatove et al., 1996; Macey et al., 1998). In the beginning of the Pliocene (5 My), the Arabian Plate accelerated its separation from Africa (Girdler, 1984) and mountain building on the northern (Lesser Caucasus Mountains, Kopet Dagh) and southern (Zagros) margins of the Iranian Plateau occurred (Macey et al., 1998). The speciation pattern of *Timon princeps* coincides with these events. It seems plausible that the uplifting and formation of the Zagros Mountains acted as a barrier to separate the Eastern *Timon* group, while the ancestor of the Eastern units fragmented due to the continuing Zagros Mountain uplift activity, and speciated following a vicariance event. Such geographic events also explain the patterns of divergence between *T. princeps* and *T. kurdistanicus* both at a morphological (Eiselt, 1968; Anderson, 1999; Ilgaz and Kumlutas, 2008) and molecular level (this study) and the high haplotype diversity found within them, with geographically close individuals showing very divergent haplotypes. This pattern is consistent with phylogeographic results from other genetically divergent groups of populations in this region (Macey et al., 1998; Rastegar-Pouyani et al., 2010).

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