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Identification and characterization of a small sized morph of ocellated lizard (*Timon lepidus* DAUDIN, 1802) from central Spain

RON PEEK, January 2017

Abstract

This report describes the discovery and detailed description of a new small sized morph of ocellated lizard from the northern slopes of the Sierra de Gredos (Castilla y Leon, Spain). Morphological and molecular analysis showed that this new morph is different from the common species of ocellated lizard (*Timon lepidus*) on the Iberian peninsula in body size, sexual size dimorphism and cytochrome b haplotype. DNA sequencing revealed a unique and highly divergent cytochrome b haplotype indicating a long-term separation from other species of ocellated lizards. Evolutionary mechanisms that may have contributed to the formation of the small sized morph are discussed. The results presented in this paper suggest that this new morph is likely to be recognized as a full species in the near future.

Keywords: *Timon lepidus*, small sized morph, Sierra de Gredos.

Introduction

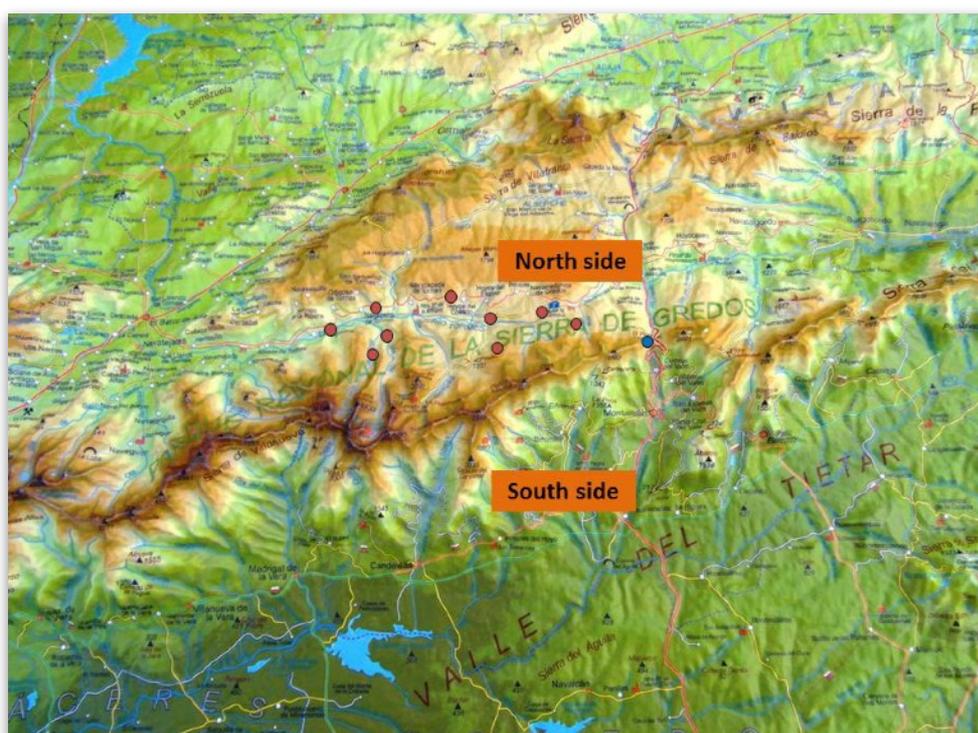
The genus *Timon* currently comprises six recognized species on three different continents. The Asian species *Timon princeps* and *Timon kurdistanicus* inhabit regions in Turkey, Iran and Iraq, while in Africa *Timon tangitanus* and *Timon pater* are found in Morocco, Tunisia and Algeria. The distribution of *Timon nevadensis* and *Timon lepidus* is restricted to Europe. *Timon nevadensis* is present in the south-eastern part of Spain, while *Timon lepidus* is found over much of the Iberian Peninsula and parts of France and Italy. Recent phylogeographic studies have indicated that the Iberian Peninsula has been an important glacial refugium for survival of ocellated lizards during adverse climate conditions during the Quaternary (MIRALDO et al. 2011). These refuges have led to the formation of evolutionary lineages of ocellated lizards by processes of population fragmentation, contraction and expansion. On the Iberian Peninsula the distribution of the different (sub)species of *Timon* is relatively complex. Currently, three subspecies of *T. lepidus* are widely recognized; *T. lepidus ibericus* (LÓPEZ-SEOANE, 1884) in the northwestern part of Portugal and Spain, *T. lepidus oteroi* (CASTROVIEJO & MATEO, 1998) from the island of Salvora and *T. lepidus lepidus* which inhabits the remainder of the distribution area. These subspecies were initially described based on morphological differences only, but analysis of part of the mitochondrial cytochrome b (cytb) gene from

a large number of sampling localities on the Iberian Peninsula has substantiated the status of subspecies for *T. lepidus ibericus*, and has provided compelling evidence that *T. nevadensis* should be considered as a separate species (MIRALDO et al. 2011, 2013).

The latter two (sub)species were found to display unique mitochondrial phylogroups. Three additional phylogroups have been identified in the eastern and central part of the Iberian Peninsula. These groups have not been described as subspecies of *T. lepidus* and are therefore likely to be morphologically identical or very similar to *T. lepidus lepidus* (MIRALDO et al. 2011).

In this paper I describe the identification and characterization of a population of remarkably small bodied ocellated lizards from the northern slopes of the Sierra de Gredos (Spain) at altitudes between 1400 and 2000 meters. I will address this new morph of *Timon lepidus* in this paper as small sized morph (*SSM*). Besides small stature, these lizards are characterized by reduced sexual dimorphism compared to the neighboring populations of *T. lepidus*, including those from the southern side of the Sierra de Gredos. Sequence analysis of part of the cytb gene of the *SSM* revealed a private and highly divergent haplotype that indicates long-term separation from other species of ocellated lizards.

Fig 1.
Overview of the Sierra de Gredos.
The locations where the *SSM* were observed are indicated by red dots.



Material and methods

Study area

The regional park Sierra de Gredos is situated about 100 kilometers west of Madrid and constitutes the highest mountain range of central Spain (2592 meters). The south side has a Mediterranean climate and starts at approximately 300 meters above sea level. The north side starts at 1300 meters and consequently has a completely different climate, vegetation and fauna compared to the south side. This large difference in altitude has also a strong effect on the distribution of lizards in the Sierra de Gredos, with cold resistant species being present on the north side only (*Lacerta schreiberi*, *Iberolacerta cyreni*) and other species found exclusively (*Acanthodactylus erythrurus*, *Tarentola mauritanica*), or predominantly (*Psammodromus algirus*) on the south side (PEEK 2011). The ocellated lizard is present on both the south and the north sides of the mountain range but is much more abundant on the north side, despite the seemingly less favorable climate conditions in this region. For details on sampling- and observation locations see figure 1.

Morphological characteristics

Lizards were captured by noosing. Measurements were done within three minutes and the animals were released back into the wild in the place of capture. Snout-vent length (SVL) and tail length (vent-tail tip) of lizards with original non-regenerated tails were recorded. Pholidosis for 9 adult *SSM* was performed by counting the number of supralabialia, collaria, ventralia, dorsalia, supracilliarialia and femoral pores. For adult and subadult lizards head width and length were taken to the nearest millimeter (figure 2). A single adult male *T. lepidus lepidus* was captured on the south flank of the Sierra de Gredos and measured.

DNA extraction, amplification and sequencing

Tail tissue was recovered from two *SSM* that were found as fresh traffic casualties on the road AV-941 that runs parallel to the northern slopes of the Sierra de Gredos. This road has been described as a death trap for lizards (PEEK 2011). In addition, a 1 cm tail tip was clipped from a captive bred *Timon lepidus lepidus* as a positive control.

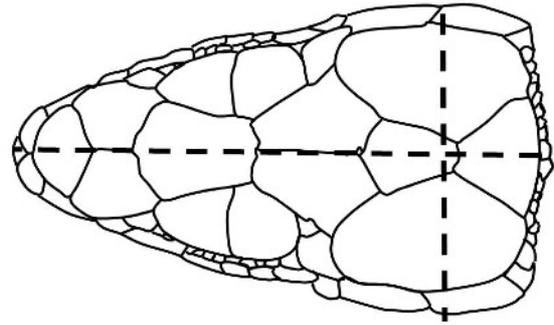


Fig 2. Schematic representation of the head of an adult male of the *SSM*. Head width and length were determined by measuring along the dotted lines.

Tissue was ethanol preserved and transported to the laboratory. For DNA extraction a small piece of the tissue was homogenized and digested for 16 hours at 58 °C in 450 μ l TSE/SDS/proteinase K buffer (10 mM Tris-Cl, 400 mM NaCl, 2 mM EDTA, pH 7.4, 0.5% SDS) and 1.6 mg/ml proteinase K. The next day, 180 μ l 5M NaCl was added and the solution was centrifuged for 15 minutes at 14,000 g to remove debris. The DNA was precipitated from the supernatant using 2 vol EtOH. The DNA was pelleted and dissolved in 500 μ l TE buffer (10mM Tris, 1 mM EDTA, pH 8.0). Fifty nanogram of DNA was subjected to PCR to amplify a 627 bp fragment of the mitochondrial DNA cytochrome b gene, with forward and reverse primers, as described by MIRALDO et al. 2011. As a negative control a PCR reaction was performed containing no input DNA. PCR products were sequenced from both sides using standard methods.

Alignment and statistical analysis

To investigate the relationships within the genus *Timon*, three separate cytb haplotypes were used for each of the six recognized species of *Timon*. *Podarcis muralis* was included as a significant outgroup. For the alignment of cytb DNA and the construction of the phylogenetic tree the Clustal Omega multiple alignment web tool was used (available online at <http://www.ebi.ac.uk/Tools/msa/clustalo>). The GraphPad software was used for graphing and for the statistical analysis of the morphological measurements (available online at www.graphpad.com).

Results

During visits in June of 2010, 2012, 2014 and 2015 a large number (>500) of ocellated lizards was observed on the northern slopes and adjacent areas of the Sierra de Gredos (Castilla y Leon, Spain). Due to the patchy distribution of suitable lizard habitat no reliable estimate can be given on the population density. However, based on my experiences and reports describing population density of ocellated lizards in other locations (DIAZ et al. 2006; MARTIN & LOPEZ 1996), the population density of ocellated lizards in the Sierra de Gredos appeared to be exceptionally high in suitable habitat (figure 3).

Frequently, up to four individual lizards could be observed simultaneously. Remarkably, both males and females (figure 4) were small-bodied and were only slightly larger than the sympatric occurring



Fig 3. Typical hardpan habitat of the small sized ocellated lizard population on the northern slopes of the Sierra de Gredos at 1500 meters above sea level.

Iberian emerald lizard (*Lacerta schreiberi*). The remarkable difference in body size between adult



Fig 4. *SSM* adult males (panels A and B) and adult females (panels C and D).

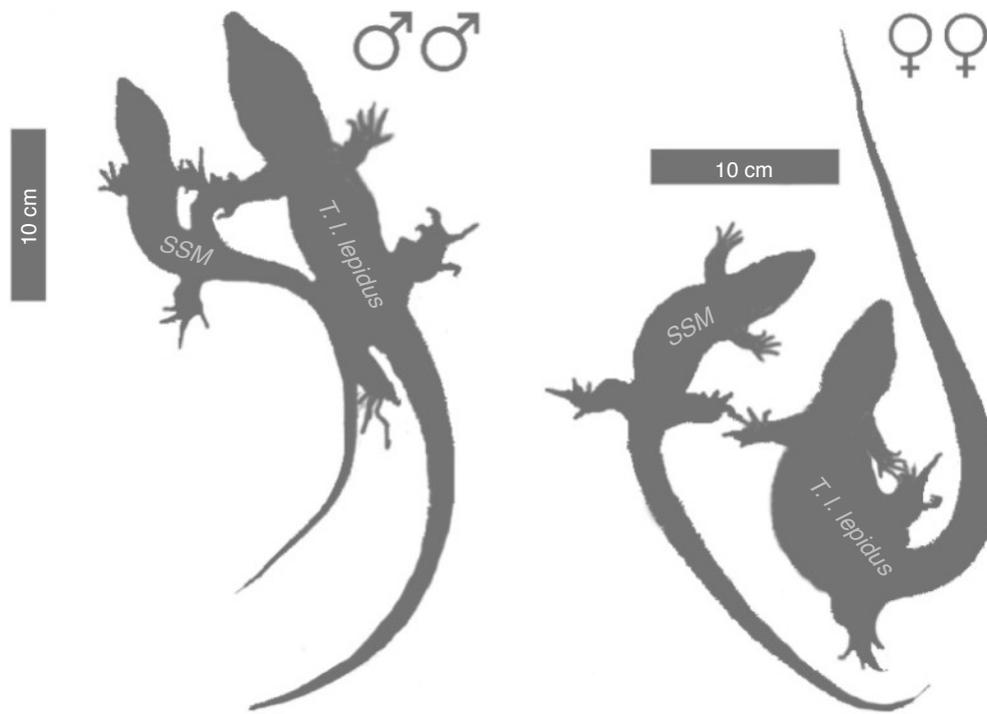


Fig 5. Body size differences between adult males (left panel) and adult females (right panel) of the *SSM* (animals at the left side in both panels) and *Timon lepidus lepidus*.

All animals depicted were over 5 years of age. Bars represent 10 cm.

SSM from the northern slopes of the Sierra de Gredos and adult *T. lepidus lepidus* originating from a population of ocellated lizards from northern Spain is illustrated in figure 5.

Interestingly, I also captured a single adult male from the southern side of the Sierra de Gredos. This lizard with a snout-vent length (SVL) of 20.7 cm was considerably larger than all of the *SSM* adult males observed on the northern slopes (mean SVL 14.0 cm). Also the shape, length and width of the head of this particular male were substantially different from those of the *SSM*. In addition, the teeth of the male from the south slope were very small and hardly visible, while the *SSM* has relatively large, clearly pronounced teeth (figure 6).

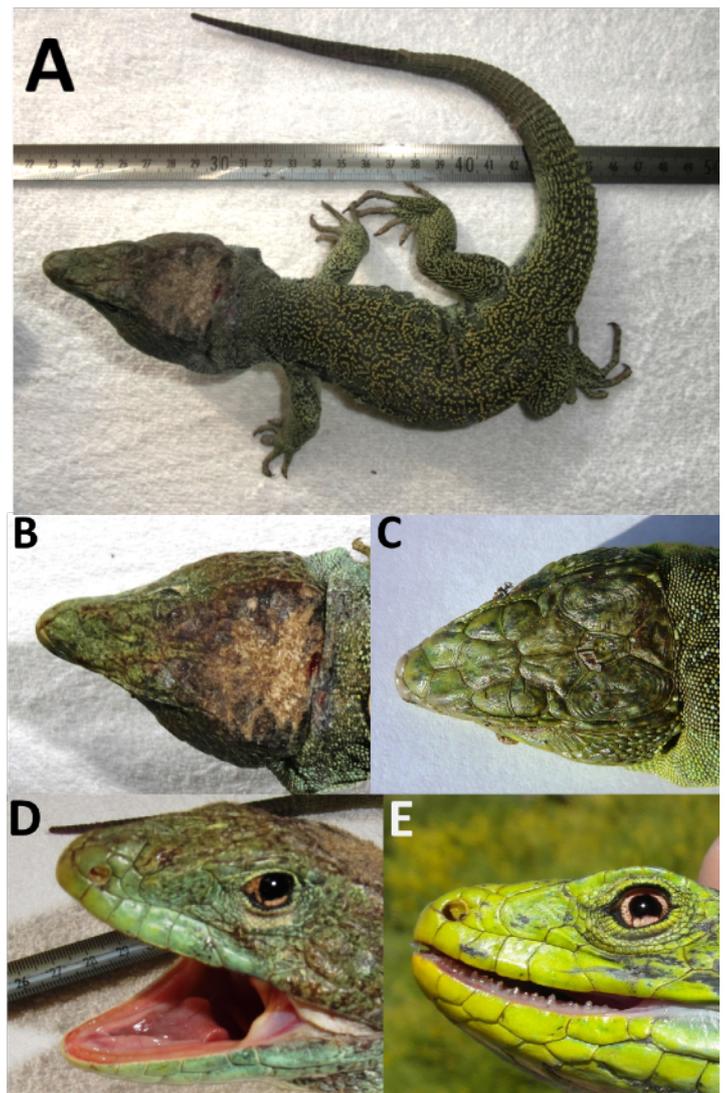


Fig 6. Comparison of an adult male *T. lepidus lepidus* from the southern side of the Sierra de Gredos with an adult male of the *SSM* from the northern slopes. The body proportions of the male *T. l. lepidus* (A and B) were very different from the *SSM* (C), with a very large head and pointy snout. In panels D and E the difference in teeth size is shown. The *SSM* (E) has relatively large irregular sized teeth compared to the hardly visible teeth of the male from the southern side (D).

Exact body measurements were obtained from a number of (sub)adult males and (sub) adult females. In addition, measurements were obtained from two juvenile lizards (figure 7). These measurements demonstrate that adult males have an average snout-vent length (SVL) of 14.0 cm (range 13.0 – 15.0 cm), which was statistically not different ($p=0.84$) from the SVL of the adult females (mean 13.9 cm; range 13.0 – 15.5 cm). Similar results were obtained when comparing the SVL of subadult males and females, with maximum SVL of 11.6 cm and 12.0 cm, respectively.

Length of original non-regenerated tails was very different ($p=0.001$) between adult males (mean 27.0 cm; range 23.5 – 29.0 cm) and females (mean 21.3 cm; range 19.0 – 24.5 cm). This remarkable difference was not observed in subadult males and females (figure 8).

The typical difference in head size between adult male and adult female of *T. lepidus* was not obvious for the *SSM* (see also figures 4 and 5). Exact measurements, however, showed that both head length ($p=0.002$) and head width ($p=0.01$) were slightly but statistically larger in adult males

compared to adult females (figure 9). This difference was not yet observed in subadults. The ratio between head length and width is 2.1 in both adult males ($n=9$) and females ($n=12$) of the *SSM*.

Pholidotic analysis of 3 male and 6 female specimens of the *SSM* showed 8 supralabialia, 9-12 collaria, 8-10 horizontal ventral scale rows, 29-32 vertical ventral scale rows, 63-70 dorsal scale rows around mid-body, 5-6 supraciliaria and 11-14 femoral pores on each side.

From the SVL measurements of 28 individual *SSM* three different size classes could be deduced. The smallest animals were juveniles that most likely were born in the previous year. The lizards with intermediate sizes were subadults (figures 7 and 10), of which the females were apparently not gravid nor displayed the characteristic mating marks at the ventral side that were obvious on all adult females. Furthermore, the morphological characteristics of sexual differentiation like relative tail length and head size were not statistically different between subadult males and females (figure 8). These subadults were likely to be two years of age.

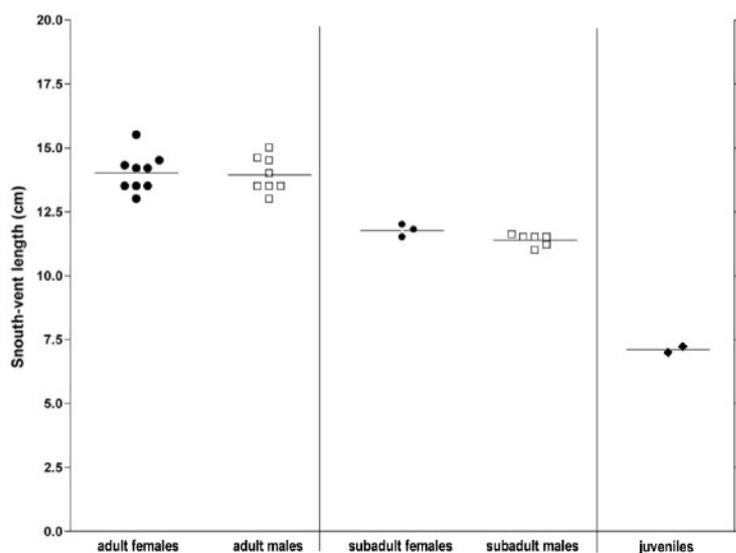


Fig 7. Snout-vent lengths (SVL) in adult females ($n=9$), adult males ($n=8$), subadult females ($n=3$), subadult males ($n=6$) and juveniles ($n=2$) of the *SSM*. The mean value for each group is indicated by a horizontal line.

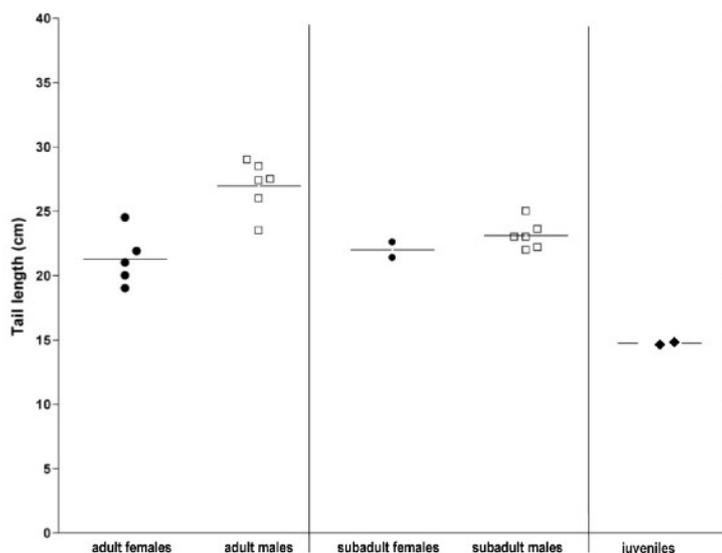


Fig 8. Original non-regenerated tail lengths in adult females ($n=5$), adult males ($n=6$), subadult females ($n=2$), subadult males ($n=6$) and juveniles ($n=2$) of the *SSM*. The mean value for each group is indicated by a horizontal line.

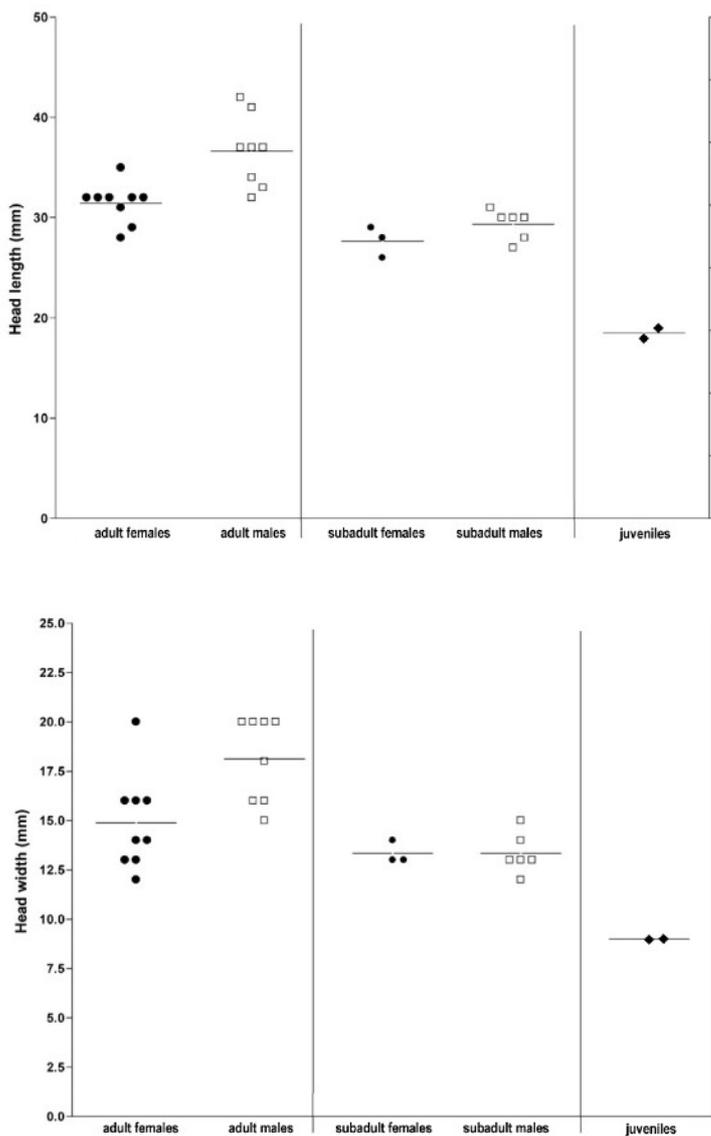


Fig 9. Head length (top panel) and head width (bottom panel) in adult, subadult and juveniles *SSM*. For details see legend to figures 7 and 8.

The largest group of measurements were done on the adult lizards that displayed very little variation in SVL and were at least three years old (figure 7). Females from this latter group were all gravid during the study period judged by the their size and the outline of eggs visible through the abdomen (see figure 4).

To determine the phylogenetic relationship of the *SSM* with other known species of *Timon*, the sequence of part of the *cytb* gene of two individual adult *SSM* was determined. As a control the *cytb* sequence of a captive bred *T. lepidus lepidus* originating from the northeastern coast of Spain was determined in parallel. All *cytb* sequences obtained

had open reading frames with no premature stop suggesting that they represented functional mitochondrial DNA copies. The sequence obtained from the control animal was identical to *T. lepidus lepidus* haplotype 1 (GenBank accession number AF378968.1) that has been reported to occur in the northeastern part of Spain (MIRALDO et al. 2011).

The two sequences obtained from the adult *SSM* were found to be identical and deposited in GenBank under accession number KC517380. The *SSM* DNA sequence was used in a similarity search and compared to sequences entries in the Genbank database (BUSACK & LAWSON 2008 ; PAULO et al. 2008 ; AHMADZADEH et al. 2012). From the resulting alignments it was found that the *SSM* *cytb* DNA sequence was different from all known *Timon* *cytb* gene entries available for *T. lepidus*, *T. nevadensis*, *T. pater*, *T. tangitanus*, *T. princeps* and *T. kurdistanicus* with a maximum percentage of 86% identity with *cytb* haplotypes of *T. tangitanus* and *T. pater*. Sequence similarity with *cytb* haplotypes of *T. lepidus* or *T. nevadensis* was 85% or less.

A phylogenetic tree based on the *cytb* DNA sequence was constructed to visualize the evolutionary relationships of the *SSM* with the other taxonomic groups within the genus *Timon*. Published sequences of *cytb* haplotypes of *T. lepidus*, *T. nevadensis*, *T. pater*, *T. tangitanus*, *T. princeps* and *T. kurdistanicus* were retrieved from the GenBank database, and listed in table 1.

In addition, the sequence obtained from two specimens of the *SSM* was used. The results are shown in figure 11 and clearly illustrate the usefulness of phylogenetic analysis using *cytb* haplotypes in determining speciation. The separation between western (*T. lepidus*, *T. nevadensis*, *T. tangitanus* and *T. pater*) and eastern (*T. princeps* and *T. kurdistanicus*) group of ocellated lizards was confirmed. Also the more recently acquired full species status of *Timon nevadensis* (MIRALDO et al. 2013), *Timon princeps* and *Timon kurdistanicus* (AHMADZADEH et al. 2012) was evident. The sequence of the *SSM* branches after the separation of the eastern and western ocellated lizards, but before the separation of the African (*T. tangitanus* and *T. pater*) and the European (*T. lepidus* and *T. nevadensis*) species. This suggests a separate and relatively ancient evolutionary lineage for the *SSM*.

A



B



C



Fig 10. *SSM* juvenile (panel A), subadult male (panel B) and subadult female (panel C).

Discussion

In this paper I describe a population of ocellated lizards from the northern slopes of the Sierra de Gredos that is different from *T. lepidus*, the common species of ocellated lizard in central Spain, by several morphological features including body size, sexual size dimorphism and teeth size. *T. lepidus* may reach SVL of up to 24 cm (DIAZ et al. 2006 ; BUSACK 1987) which is considerably larger than the maximum of 15,5 cm SVL for the *SSM* from the northern slopes of the Sierra de Gredos. Pholidosis counts for the *SSM*, including the supralabialia, collaria, ventralia, dorsalia, supracilliarlia and femoral pores, did not show significant differences with the counts reported for *T. lepidus* (BISCHOFF et al. 1984 ; BUSACK 1987 ; PETERS 1961).

All specimens of the *SSM* were observed at altitudes between 1300 and 2000 meters. Generally, animal species fit to Bergmann's rule; the tendency for animals to exhibit larger body size in the cooler climates at elevated heights (BLACKBURN et al. 1999).

In contrast to most species, lizards were found to have larger body size in warmer climates, with over 70 percent of squamates exhibiting reversed Bergmann's clines (ASHTON & FELDMAN 2003).

This is explained by the behavioral thermoregulation of lizards that greatly influences both their daily and seasonal period of activity. At higher altitudes, such as at the northern slopes of the Sierra de Gredos, temperatures are relatively low during a large part of the year. Energy intake by the *SSM* might therefore

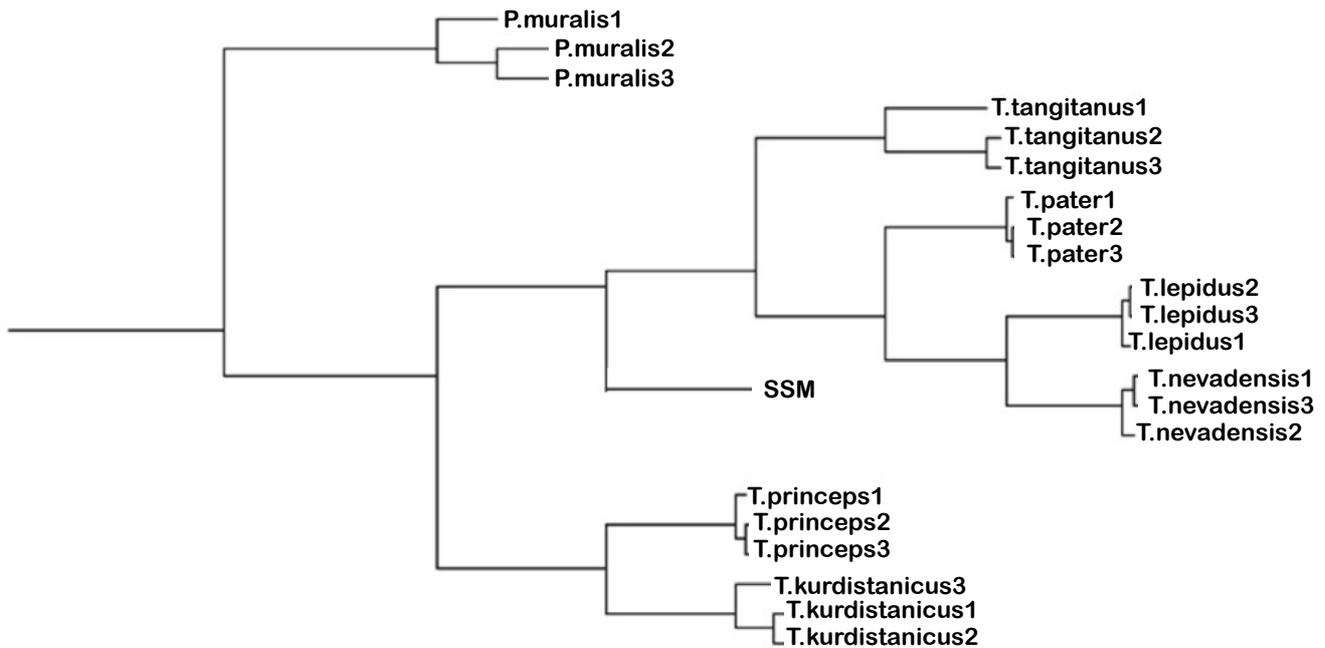


Fig 11. Phylogenetic tree of cytochrome b DNA sequences of the *SSM*, *T. lepidus*, *T. nevadensis*, *T. pater*, *T. tangitanus*, *T. princeps* and *T. kurdistanicus*. Cytb DNA sequences of *Podarcis muralis* were used as a relevant outgroup. The number of sequence character changes is represented by the horizontal branch lengths. The numbers 1-3 after each species indicate cytb haplotypes from different lizards. GenBank accession numbers are listed in table 1.

be restricted compared to lizard populations in regions with more temperate climates. This subsequently would lead to an increase in the number of years required to reach adulthood. The *SSM* may have compensated for this by a reduction in body size of the reproductive (adult) stage in both males and females. Subsequently, the relatively large teeth I observed in the *SSM* could be an adaptation for this small body size. *T. lepidus* eats predominantly beetles which are crushed before ingestion (ARETS 2015). The small heads of the *SSM* clearly lack the pronounced jaw musculature present in *T. lepidus* and might have developed more pronounced teeth to compensate for loss of biting force.

From our SVL measurements it became apparent that in spring the *SSM* population displays three different size classes. Although I could only measure a limited number of subadults and juveniles, it can be concluded that the *SSM*, identical to individuals of most populations of *T. lepidus*, become reproductive in their fourth year, albeit at much smaller body size.

Another factor that may have contributed to the reduced body size of the *SSM* is the limited number

of suitable retreat sites in their hardpan environment. In the majority of my observations these hiding places consisted of the narrow and relatively shallow crevices in large boulders that would be unsuitable for larger bodied lizards. *Timon lepidus* is exposed to a high predation pressure and the availability of stone refuges has been shown to be an important factor for the maintenance of viable populations (CORBETT 1989 ; DIAZ et al. 2006 ; MARTIN & LOPEZ 1996). Although no exact information could be obtained on population density, I observed large numbers of the *SSM* in suitable habitat. Possibly the small size of these lizards allows for higher population density than normally observed in larger bodied species of ocellated lizards.

T. lepidus is a species that is sexually dimorphic with respect to features like SVL and head size being considerably larger in males (BUSACK 1987). In the *SSM* no statistical difference in SVL between adult males and females was observed. In fact, the largest lizard recorded was a female with a SVL of 15.5 cm. Sex-related difference in head size was present although less marked than in *T. lepidus*. The ratio between head length and width in *T. lepidus* was 1.37 for males and 1.44 for females (BISCHOFF et al. 1984). In contrast to *T. lepidus*, the ratio between

head length and width in the *SSM* was found to be 2.1 in both adult males (n=9) and females (n=12). This showed that male and female of the *SSM* have the same head shape and, compared to *T. lepidus*, have narrow heads. A reduction in sexual size dimorphism at higher altitudes has been observed in several other species of Lacertids such as *Lacerta agilis boemica* (ROITBERG & SMIRINA 2006) and *Lacerta strigata* (MELKUMYAN 1983).

STAMPS et al. (1997) have hypothesized that this might be the result of differences in intra-sexual selection between lowland populations (high population density and high sexual size dimorphism) and highland populations (low population density and reduced sexual size dimorphism). In case of the *SSM* the reduced sexual size dimorphism is unlikely to be the result of low population density as I observed many of these lizards in patches of suitable habitat.

Another remarkable but puzzling feature of sexual differentiation in the *SSM* was the original non-regenerated tail length in adult lizards. Males were found to have tails that were on average 5.7 cm longer than those of females. Unfortunately I could not retrieve information on sexual dimorphism with respect to tail length in other published studies, precluding comparison between the *SSM* and other species of *Timon*.

Sequence analysis of the mitochondrial *cytb* gene has been extensively and successfully used to analyze the distribution of mitochondrial phylogroups for *T. lepidus* on the Iberian Peninsula and has provided at least 145 unique haplotypes for the species. The validity of phylogrouping *T. lepidus* based on *cytb* haplotyping for determining speciation was confirmed by the close association between the geographic distribution of the recognized subspecies of *T. lepidus* and the distribution of the mitochondrial DNA phylogroups (MIRALDO et al. 2011, 2013). I have therefore determined the sequence of the *cytb* haplotype in DNA isolated from two individuals of the *SSM*. These sequences were found to be identical and unique. Comparison to all known sequences deposited in the EMBL database revealed that similarity of the *SSM* to other *cytb* DNA sequences of *Timon* species was $\leq 86\%$. Sequence similarity between different *cytb* haplotypes of the subspecies

T. lepidus lepidus is $\geq 97\%$, while *cytb* sequence similarity between the two species *T. lepidus* and *T. nevadensis* is $\geq 88\%$ (data not shown). This indicates that the *SSM* *cytb* sequence represents a unique and highly divergent haplotype. Despite the fact that I have obtained only two identical *cytb* haplotype for the *SSM*, the high level of sequence divergence on both DNA and deduced protein divergence (data not shown) strongly suggest that the population of the *SSM* from the northern slopes of the Sierra de Gredos might represent a distinct species of *Timon* that has been separated from surrounding populations of ocellated lizards for a long period of time. Phylogenetic analysis based on *cytb* haplotypes of all known species of *Timon* showed that the *SSM* appeared after the separation of the western and eastern group of species, but before the formation of the European and the African species. Based on a similar analysis of the genus *Timon* by AHMADZADEH et al. (2012), it can be concluded that the *SSM* underwent speciation around 8-9 million years ago. Further research focusing on the identification of additional *cytb* haplotypes for the *SSM* and other informative nuclear gene polymorphisms (AVISE 2004 ; MIRALDO et al. 2013), might further substantiate this notion.

The northern slopes of the Sierra de Gredos are known for their potential to induce speciation. Various species of plants and animals are endemic to this region, including two subspecies of amphibians (*Bufo bufo gredisicola* and *Salamandra salamandra almanzoris*; MÜLLER & HELLMICH 1935) and one subspecies of lizard (*Iberolacerta cyreni casteliana*; CARRANZA et al. 2004). The relative high level of endemism found in the Sierra de Gredos suggests that this part of the Sistema Central has been a refugial area during adverse climate conditions and as such has had a large impact on the evolutionary history of isolated populations that have persisted in this region. The result presented in the current paper indicate that the population of ocellated lizards that inhabits the northern slopes of the Sierra de Gredos displays morphological and genetic features that are indicative of speciation. The *SSM* might therefore represent a new species of ocellated lizard.

Based on the results, I recommend to elevate the status of this new morph of ocellated lizard to a new species of *Timon* in the near future.

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Species	GenBank accession number
<i>T. lepidus</i>	AF198549
	AF198551
	AF198557
<i>T. nevadensis</i>	JX626269
	JX626270
	JX626271
<i>T. tangitanus</i>	AF378960
	AF378961
	AF378962
<i>T. pater</i>	AF378963
	AF378964
	AF378965
<i>T. princeps</i>	AF198549
	AF198551
	AF198557
<i>T. kurdistanicus</i>	AF198542
	AF198543
	AF198548
<i>P. muralis</i>	HQ652924
	HQ652925
	HQ652926

Table 1.
GenBank accession numbers used in the phylogenetic tree of figure 11.