

Phylogeography of the genus *Blanus* (worm lizards) in Iberia and Morocco based on mitochondrial and nuclear markers — preliminary analysis

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Abstract. The evolutionary relationships of amphisbaenians of the genus *Blanus* have long been controversial. Presently two species are recognized for Morocco — *Blanus tingitanus* and *B. mettetali* and one for Iberia — *B. cinereus*. Using mitochondrial and nuclear DNA sequences, 25 samples from Portugal, Spain and Morocco were analysed. Both markers indicate the existence of three well-separated clades: one for the Northern Moroccan samples, corresponding to *B. tingitanus* and two to the *B. cinereus* samples. One sample from Taza, Morocco, was identical to specimens from one of the Iberian forms. These results point to the necessity of a systematic revision. These new groups reinforce the need for geographically broader sampling accompanied by morphological studies to fully determine the phylogenetic history of this species complex.

Introduction

Amphisbaenians or worm lizards, are limbless fossorial reptiles belonging to an ancient group predating the geological split of Pangea 200 million years (My) ago (Macey et al., 2004). The evolutionary relationships of amphisbaenians have long been controversial for a number of reasons, especially the difficulty in obtaining tissues for molecular analysis because of their secretive habits in their natural habitat and the highly derived morphological conditions that can confound comparative studies (Kearney & Stuart, 2004). Until very recently the genus *Blanus* Wagler 1830 was considered to be composed of only two species, *Blanus strauchi* Bedriaga 1884 with a range restricted to part of Asia Minor and *Blanus cinereus* (Vandelli 1797) with a western distribution in almost all of the Iberian Peninsula and Mediterranean Morocco. Within this last taxa two subspecies were recognized for Morocco — *B. c. cinereus* for the Northern part and *B. c. mettetali* (Bons 1963) for the rest of Morocco south of the

Zebu river (Bons & Geniez, 1996). Later a new species was established, restricted to North Morocco, *B. tingitanus* Busack 1988, based on allozyme electrophoretic data (Busack, 1986) and morphological studies on pholidosis and allometry (Busack, 1988). The same author elevated *B. c. mettetali* to the species level supported by a higher number of pre-anal pores than the northern individuals. *Blanus cinereus* was presumed to have evolved separately from the Moroccan populations since the reopening of the Gibraltar Strait during the late Miocene.

Blanus mettetali is found in the plains South of Rabat and West of the Atlas massif in habitats with temperate to warm winters and sub-humid and semi-arid climate. The other endemic Moroccan species, *B. tingitanus*, is located all over the Tinges peninsula north of a line Rabat-Meknés-Djbel Tazzeke in the areas with cool to warm winter in the humid and sub humid zones (Bons & Geniez, 1996). *Blanus cinereus* is distributed in the drier and humid areas with Mediterranean climate of the Iberian Peninsula at low altitudes (Gasc et al., 1997; Barbadillo et al., 1999; Almeida et al., 2001). This species is cited in the annexe III of the Bern Convention and although is considered as “Not Threaten”, this is because of the almost nonexistent ecological information concerning it (Almeida et al., 2001).

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In another amphisbaenian species (Mulvaney et al., 2005) and in other Iberian reptiles such as the *Podarcis hispanica* species complex (Harris et al., 2002; Pinho et al., 2006) new lineages and more complex phylogeographic histories than expected have been recovered. Further, the opening of the strait of Gibraltar is not always directly related to genetic diversity in nonvolant terrestrial animals in this area. Some species crossed relatively recently (e.g. *Macroprotodon* — Carranza et al., 2004) while others, such as *Pleurodeles*, have southern Iberian forms related to Moroccan population (Batista et al., 2004; Carranza & Arnold, 2004). Therefore, the aim of this study was to test the present systematic groups by sampling in the Iberian Peninsula and Morocco, discussing taxonomic and conservation implications of distinct evolutionary lineages identified within the genus *Blanus* and highlighting key matters that need to be addressed in future studies.

Methods

Blanus specimens were collected in the field from Portugal, Spain and Morocco and released after a small clip of material was taken and stored in ethanol. Genomic DNA was extracted following a standard high-salt protocol. Part of the ND4 and *C-mos* genes were amplified by Polymerase Chain Reaction using the primers ND4 and LEU (Arévalo et al., 1994)/Mos-F and Mos-R (Godinho et al., 2005) and conditions described by Harris (2001). The amplified products were sequenced on an automated sequencer (ABI 310 by Amersham Biosciences) and then checked by eye and aligned against the others using Bio Edit (Hall, 1999). The location and taxa identification of the 25 samples sequenced in this study are presented in table 1 and fig. 1. New sequences were deposited on GenBank, accession numbers DQ324863 to 66 and DQ329241 to 65.

Analytical methods

For the mitochondrial DNA (mtDNA), in total 25 ND4 sequences of 800 base pairs (bp) length — four from Portugal, 12 from Spain and nine from Morocco — were studied. This corresponds to 654 bp of ND4 and 146 bp of adjacent tRNAs. One specimen of *Blanus strauchi* was also sequenced as an outgroup. The same fragment of *Bipes biporus* (Macey et al., 2004) was included as an additional outgroup.

Mitochondrial DNA sequences were imported into PAUP* 4.0b10 (Swofford, 2002) for phylogenetic analysis.

For the phylogenetic analysis maximum likelihood (ML) and maximum parsimony (MP) were used. The approach outlined by Huelsenbeck and Crandall (1997) was used to test 56 alternative models of evolution, employing PAUP* 4.0b10 and Modeltest (Posada & Crandall, 1998). Once a model of evolution was chosen, it was used to estimate a tree employing ML with random sequence addition (10 replicate heuristic search). The MP analysis was also performed with random sequence addition (100 replicate heuristic searches). In MP and ML support for nodes was estimated using the nonparametric bootstrap technique (Felsenstein, 1985) with 1000 and 100 replicates respectively.

In order to confirm the groups resulting from the analysis using the mitochondrial marker four of the referred samples (B2, B3, B4 and B5), belonging to different mitochondrial lineages, were analysed for part of the nuclear gene, *C-mos*, of 314 base pairs length. These were joined in a median-joining network (Bandelt et al., 1999) together with three *Blanus* sequences downloaded from GenBank, from Harris (2003) and Kearney & Stuart (2004).

Results

The most appropriate model for the combined data was the GTR model with a discrete approximation of the gamma distribution (0.402). The ML heuristic search using this model found one tree of $-\ln 3375$. For MP 192 characters were informative, and the MP search found 4 trees of 555 steps (fig. 2).

In both estimates of relationships based on mtDNA sequences three major lineages were detected, as shown in fig. 2. The first clade (A), well supported as a monophyletic group (100% bootstrap), included all the *B. tingitanus* Moroccan samples, except B3. The divergence between the Northern samples from the Rif Mountains and those from Taza is quite deep (4.2% sequence divergence) and both groups are well supported (100% bootstrap). The second clade (B) includes Iberian Peninsula *B. cinereus* samples and one sample from Taza Caves (B3) that presented a paler appearance than other specimens collected in Taza (D.J. Harris, pers. obs.). There is almost no genetic diversity in this group. The third clade (C) corresponds to samples collected from the Iberian Peninsula but that present a 10 to 12% sequence divergence from the other Iberian clade (B).

Table 1. Location of the samples sequenced for this study with respective codes. Samples marked with * were also sequenced for the nuclear marker *C-mos*.

Sample	Taxa	Locality	Code
B1	<i>B. tingitanus</i>	Morocco, Taza	MT1
B2*	<i>B. tingitanus</i>	Morocco, Taza	MT2
B3*	<i>Blanus sp</i>	Morocco, Taza Caves	MTC1
B4*	<i>B. cinereus</i>	Portugal, Alentejo, S. Mamede	PSM
B5*	<i>B. cinereus</i>	Spain, Badajoz, St. ^a Marta	SSM1
B6	<i>B. cinereus</i>	Spain, Badajoz, St. ^a Marta	SSM2
B7	<i>B. cinereus</i>	Spain, Badajoz, Zafra	SZ
B8	<i>B. cinereus</i>	Spain, Huelva, Castaño del Robledo	SCR
B9	<i>B. cinereus</i>	Spain, Badajoz, Castrejón de Capote	SCC
B10	<i>B. cinereus</i>	Portugal, Alentejo, Carvalhal	PC1
B11	<i>B. cinereus</i>	Portugal, Alentejo, Carvalhal	PC2
B12	<i>B. cinereus</i>	Portugal, Alentejo, Marvão	PM
B13	<i>B. cinereus</i>	Spain, Córdoba, Ruins near Castañar, Hermita road	SHr
B14	<i>B. cinereus</i>	Spain, Córdoba, Mirador road	SMr
B15	<i>B. cinereus</i>	Spain, Málaga, Puerto Sabar	SPS1
B16	<i>B. cinereus</i>	Spain, Málaga, Puerto Sabar	SPS2
B17	<i>B. cinereus</i>	Spain, Cádiz, Laguna de Medina	SLM
B18	<i>B. cinereus</i>	Spain, Granada, El Turro	SeT
B19	<i>B. cinereus</i>	Spain, Ávila, Navalanguilla	SN
B20	<i>B. tingitanus</i>	Morocco, M'idq	MM
B21	<i>B. tingitanus</i>	Morocco, Tétouan	MTe1
B22	<i>B. tingitanus</i>	Morocco, Tétouan	MTe2
B41	<i>B. tingitanus</i>	Morocco, Taza Caves	MTC2
B42	<i>B. tingitanus</i>	Morocco, Taza Caves	MTC3
B43	<i>B. tingitanus</i>	Morocco, Taza Caves	MTC4

A total of four haplotypes were established using the nuclear marker, *C-mos*, that reinforced the groups found with the mtDNA marker. From this analysis one haplotype appeared, shared by the GenBank sequences classified as *Blanus cinereus* — Bc₁, from an unknown locality in Portugal and Bc₂ from Benalup de Sidonia, Southern Spain — B3 and B5; one haplotype shown by the B2 sample from Morocco, corresponding to the *B. tingitanus* clade, that presented two mutational differences from this Iberian haplotype and another by B4, with one difference, representing the divergent Iberian clade (C). The *Blanus strauchi* individual — Bs from Turkey, Hurma Köyü — presented a distant haplotype with six mutational steps from this cluster.

Discussion

Although there is little information regarding expected levels of intraspecific variation

within amphisbaenians, the high level of divergence detected between the three mitochondrial *Blanus* lineages indicates that these may correspond to separated species. These were also diagnosable with the nuclear marker — *C-mos*. Even with a low number of differences, the *C-mos* data reinforced the suggestion that specimens from Iberia may belong to two distinct species and that *B. cinereus* is a species complex in the Iberian Peninsula, since many reptiles species are separated by only one or two mutations with this marker (Harris et al., 2004). Moreover the high degree of divergence (10 to 12%) shown by the mtDNA sequences is unusual within a species and alone could indicate that *B. cinereus* is a species complex in the Iberian Peninsula. Indeed in a recent analysis of spiny-tailed lizards (*Ctenosaura*) 2-4% divergence for the same ND4 gene region was considered an indicator of species status (Hasbun et al., in press). However, the divergence between

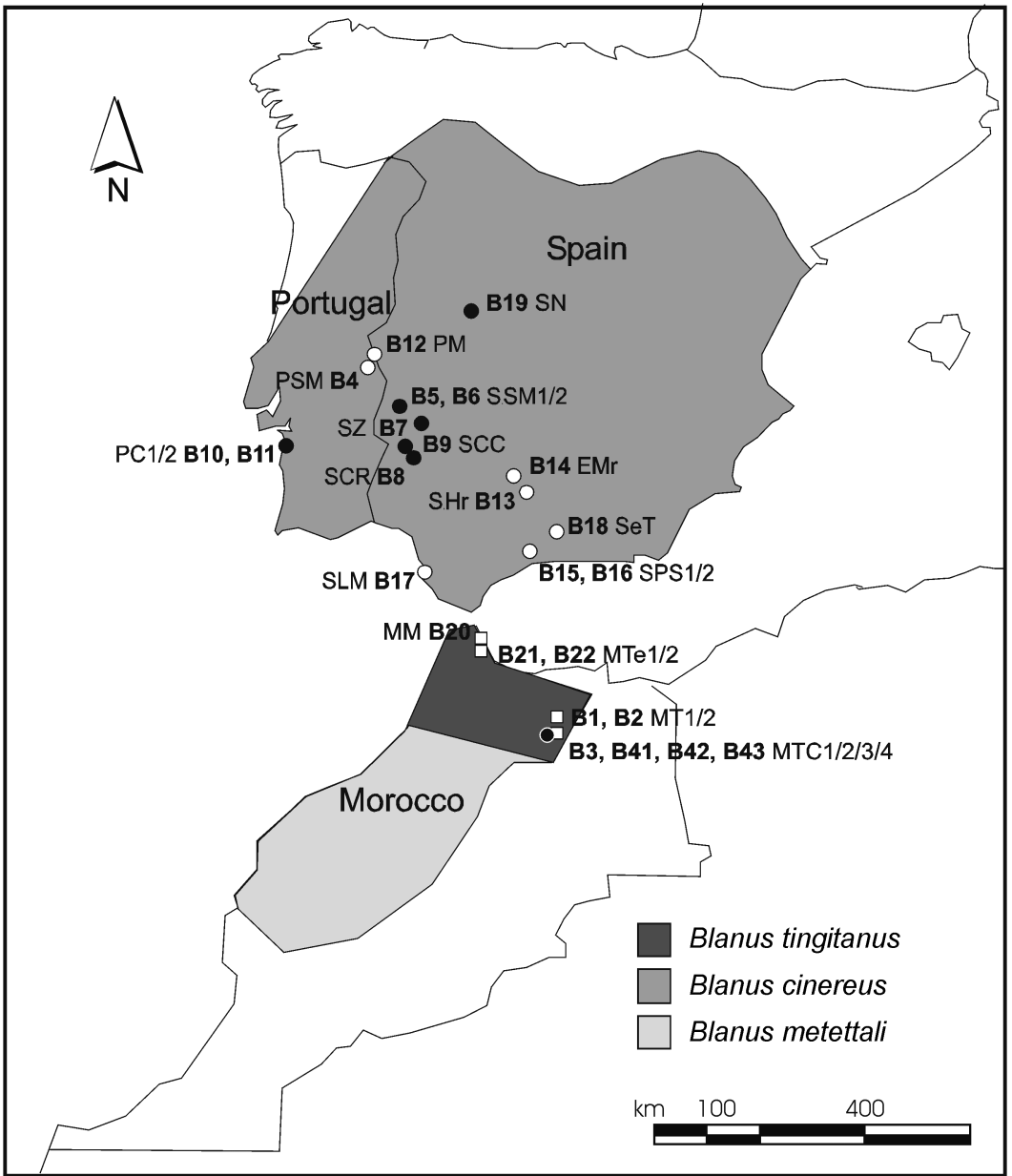


Figure 1. Map showing sampling localities of the *Blanus cinereus* species complex sequenced for this study. Closed circles refer to clade B samples and open circles to the ones included in clade C. Open squares refer to *B. tingitanus* samples.

B. strauchi and the remaining *Blanus* for ND4 is even higher (17-20%).

The presence of a Moroccan sample, B3, within Iberian samples of group B and with the same *C-mos* haplotype as Bc₁, Bc₂ and B5 can be explained in three different ways. The first one implies the existence of a third un-

known lineage in Morocco, in sympatry with *B. tingitanus*, at least in Taza, just sampled once that recently, either naturally or anthropologically, colonised and spread rapidly in the Iberian Peninsula. A similar pattern was already reported in some reptile species with similar distributions, such as *Macroprotodon cucullatus*

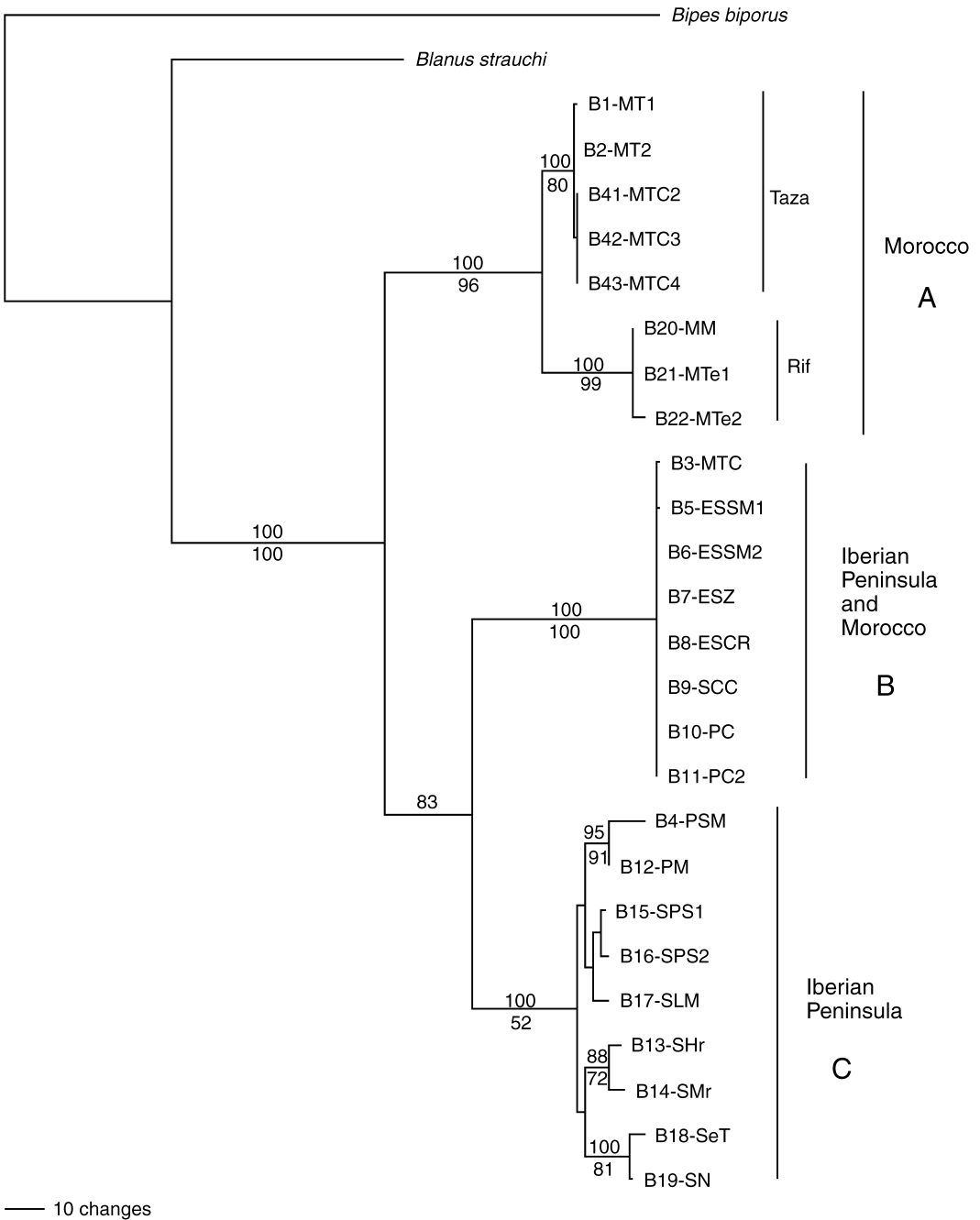


Figure 2. One of four MP trees inferred from ND4 mitochondrial DNA sequences. Bootstrap support for MP and ML analyses are indicated above and below nodes respectively. Codes for sequences are given in table 1. The tree was rooted using *Bipes biporus*.

(Carranza et al., 2004; Vasconcelos & Harris, 2006). Another possibility is that individual B3 is the result of colonization from Iberia into Morocco. This assumption is less probable because

of the low amount of variation in the Iberian samples that in this scenario are assumed to be belonging to the original population. The probability of an anthropogenic introduction is also

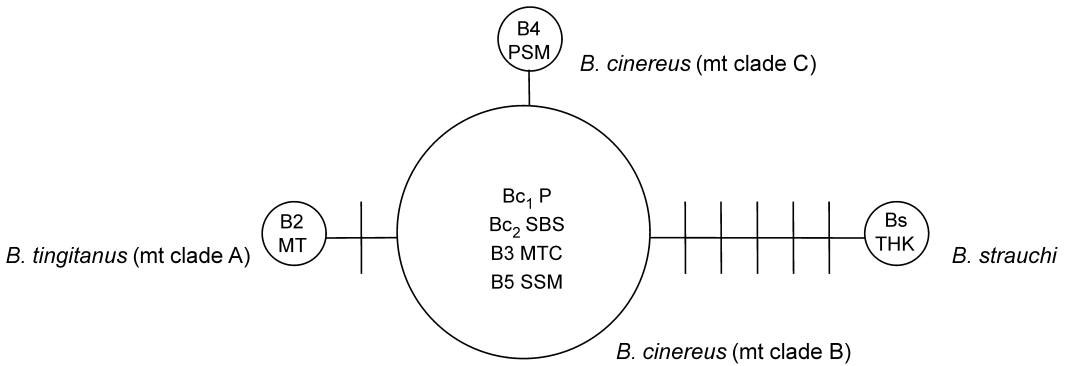


Figure 3. Median-joining network of the *C-mos* nDNA sequences for *Blanus*. The GenBank sequences are referred as Bs for *Blanus strauchi* (No AY444024); Bc₁ (No AY234227) and Bc₂ (No AY444019) for *Blanus cinereus*. Vertical lines indicate presumed missing haplotypes. SBS — Spain, Benalup de Sidonia; P — Portugal, unknown locality; THK — Turkey, Hurma Köyü. Other codes for the sequences are given in table 1.

low because of its isolated sampling location in an undisturbed habitat in Tazzeka Natural Park.

Finally, the high degree of genetic divergence (10 to 12%) between B and C lineages and the typical estimated rates of evolution of the ND4 gene (0.4–0.6% per lineage per My, Caccone et al., 1999), would imply that all the lineages had diverged from each other much earlier than the reopening of the strait of Gibraltar around 5.4 My ago (Duggen et al., 2003). However this calibration may be inappropriate, as shown by a recent study in chameleons that has suggested that ND4 may be evolving faster than this (Rocha et al., 2005) and thus any suggested date for divergence of these lineages should be treated cautiously. It does seem likely though that *Blanus* is another nonvolant species in which the opening of the strait of Gibraltar is not directly related to observed patterns of genetic diversity as already observed in *Podarcis* lizards (Harris et al., 2002; Pinho et al., 2005) and in *Pleurodeles* (Batista et al., 2004; Carranza & Arnold, 2004).

Conclusions

Despite the sampling limitation of this study, it seems that *Blanus cinereus* presents two lineages that should perhaps be described as dif-

ferent species, if additional genetic and morphological data support this data. In this way, this work reiterates the importance of extensive geographic sampling to phylogenetic studies, particularly around the geographically complex region of the strait of Gibraltar (Harris et al., 2002).

It is now important to carry out a detailed morphological analysis of the Iberian individuals in order to better understand the ancient divergence shown in the two *B. cinereus* subgroups. Additional sampling in the *Blanus sp.* distribution range, especially Iberia and Morocco would be an important step to resolve outstanding questions regarding the historical biogeography, population genetic structure and taxonomy of this taxa. In this way, taxonomical recognition and range determination of apparently divergent evolutionary lineages within *B. cinereus* and definition of units for conservation would be possible. Genetic analysis should help prioritise the areas most in need of protection (i.e. Tazzeka National Park).

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