

## The genus *Blanus* (Reptilia: Blanidae) in Iraq: distribution, species identification and genetic affiliation

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The genus *Blanus* Wagler, 1830 is known to be a part of fauna within the territory of modern Iraq since 1966, when A. Alexander published a record from “Salahussin-shaklauva, near Arbil,” with the voucher number HM (Zoologisches Museum, Hamburg) 1216 (Alexander 1966). Another record from “Ashawa village, 5 km SW of Sarsang” in the Dohuok Governorate (voucher number of the Baghdad Museum of Natural History, University of Baghdad, IMN 426) was published by Al-Jumaily (1971). Al-Jumaily considered this record to be the first for Iraq due to missing information from Alexander (1966). These initial records of the genus in Iraqi territory represent data from two governorates of Iraqi Kurdistan, Dohuk, and Erbil, with a geographic distance between them of approximately 100 km. This suggests that the genus *Blanus* can be found, although rarely, in a wide area of northern Iraqi Kurdistan, where its range probably follows the Anatolian distribution of the genus.

Until 2014, all populations from Anatolia and surrounding regions were attributed to *B. strauchi* (Bedriaga, 1884) with a well-developed subspecific taxonomy. However, after the description of *B. alexandri* Sindaco, Kornilios, Sacchi, Lymberakis, 2014, three species of the genus *Blanus* were recognized in the region. This brings an interesting and new perspective on the evolution and biogeography of these morphologically uniform reptiles and raises questions about the genetic diversity in unstudied areas or at the edges of their ranges. By combining morphology and genetics, the distribution range of *B. alexandri* was identified in the region from south-central to south-eastern Turkey, with the easternmost genetically investigated population found in Karalar, Mardin province (Sindaco *et al.* 2014). Subsequently, Jablonski and Sadek (2019) genetically and morphologically identified the species in Lebanon, thus significantly extending its distribution to the south to the Levant region.

Both studies demonstrated that the species contains at least seven distinct mitochondrial lineages (in the analysed NADH dehydrogenase subunit 4 [ND4] marker) with significant genetic distances of up to 11% (uncorrected p-distances; Jablonski and Sadek 2019). Such mitochondrial divergence in reptiles of the region is very high (e.g., Kornilios *et al.* 2020) and may imply past speciation events that might require further taxonomic changes. On the other hand, *B. alexandri* shows a very low level of nuclear divergence (the nuclear protein-coding gene of the prolactin receptor [PRLR]; Sindaco *et al.* 2014). However, to date, a study focusing on the genetic affiliation of populations from the western foothills of the Zagros Mountains, which likely represent the easternmost limit for the distribution of the genus and the family Blanidae worldwide, has not been conducted.

The genetically investigated adult individual of the genus *Blanus* was found by the first author on 24 March 2019 near the Banoka village (36.6356°N, 44.3217°E, 863 m a. s. l.) under a stone. The site featured soft, damp soil (following rainfall at around 1 pm) accompanied by rocky slopes. The vegetation consisted of small oaks or bushes. Notably, the soil beneath the rock where the individual was discovered was dry. Morphometric (mm) and meristic measurements (according to Albert and Fernández 2009) of the specimen are: snout-to-vent length (SVL): 160.5; head length (HL): 7.68; head width (HW): 5.34; pre-frontal length (PFL): 2.97; pre-frontal width (PFW): 2.98; number of dorsal segments (DS): 17; number of ventral segments (VS): 18; number of body annuli (BA): 125; pores: 3+3.

We collected a blood sample that was used as a DNA source stored in 96% ethanol and frozen at -25°C. The specimen

is deposited in the collection of Department of Zoology, Comenius University in Bratislava under voucher number CUHC 8606. For DNA extraction, we used a commercial DNA extraction kit (Qiagen DNeasy® Blood and Tissue Kit) and followed the manufacturer's protocol. A fragment of the mitochondrial ND4 gene and the nuclear PRLR gene were PCR-amplified with primers ND4 and LEU (Arévalo *et al.* 1994) and PRLR\_f1 and PRLR\_r3 (Townsend *et al.* 2008), respectively. The laboratory procedure followed Albert *et al.* (2007), Albert & Fernández (2009) and Sindaco *et al.* (2014). The sequencing was performed by Macrogen Europe Inc. (Amsterdam, The Netherlands). Both final fragments (868 bp for ND4 and 549 bp for PRLR) were aligned using Clustal W algorithm (Thompson *et al.* 1994) as implemented in BioEdit (Hall 1999). The GenBank accession numbers for the newly generated sequences of ND4 and PRLR are OR487416 and OR664391, respectively.

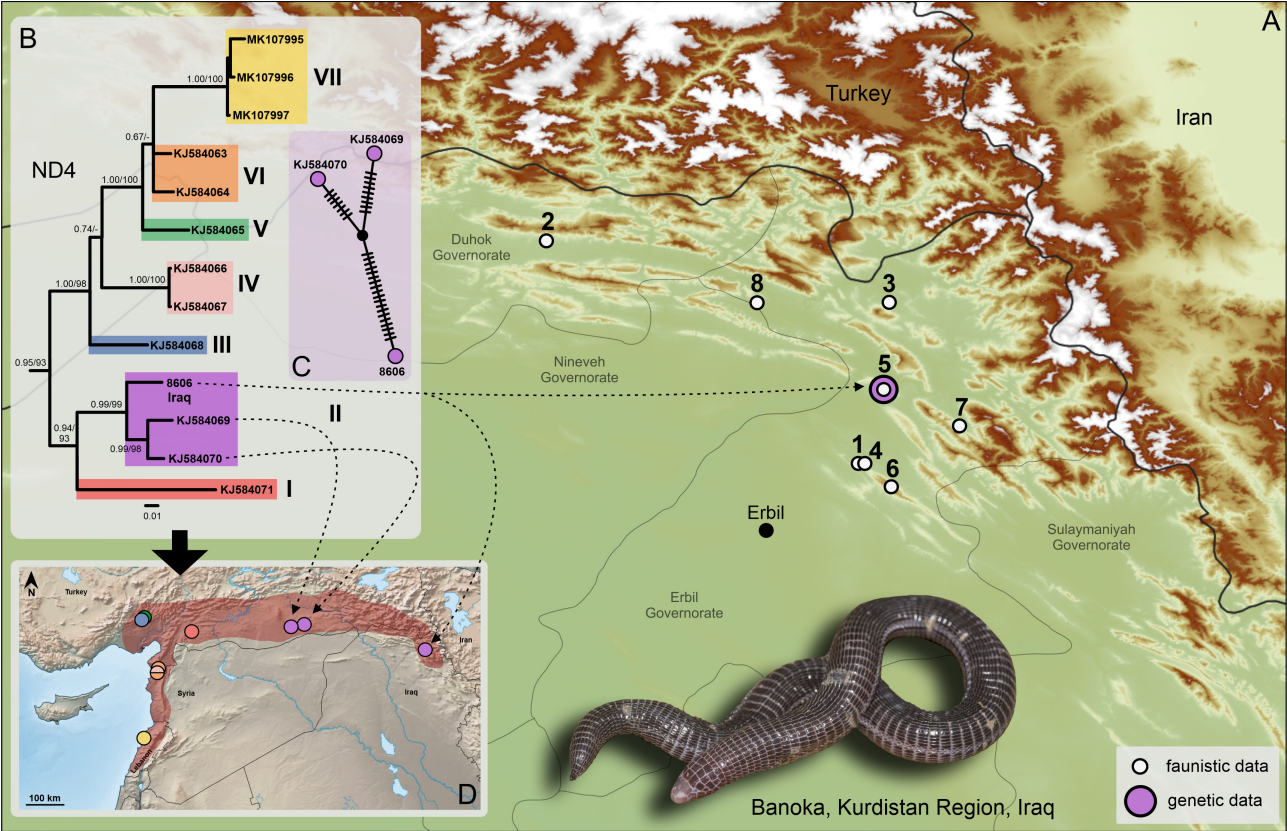
Tree analyses followed the methodology described by Jablonski and Sadek (2019). For basic species affiliation we firstly checked the new sequences via BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and downloaded available sequences of the genus *Blanus* from the published studies of Sindaco *et al.* (2014) and Jablonski and Sadek (2019). Then we used PartitionFinder 2 (Lanfear *et al.* 2017), Bayesian Inference (BI; MrBayes 3.2.6; Ronquist *et al.* 2012) and Maximum Likelihood (ML; RAxML 8.0.0; Stamatakis 2014) phylogenetic approaches to find the best-fit model of sequence evolution and reconstruct tree hypotheses. Final nodes of the tree with posterior probability values 0.95 were considered as strongly supported. The ML tree supports (significant when values reached 70 or higher) was assessed by 1,000 bootstrap pseudoreplicates. Using the 95% limit of parsimony (TCS algorithm; Clement *et al.* 2000) and the PopArt software (Leigh and Bryant 2015) we also created a haplotype network for the ND4 gene, focusing on the length of available sequences (657 bp) within the lineage to which the specimen from Iraq belongs. Except for the records presented by Alexander (1966) and Al-Jumaily (1971), the additional data presented here represents an extension of the species' distribution in the Iraqi part of Kurdistan (Fig. 1A). By combining our own field efforts and citizen science data from [www.inaturalist.org](http://www.inaturalist.org), we have gathered a total of only eight localities of the species in Iraq since the first evidence (Table 1), with most of them located in the Erbil governorate. Despite numerous existing research gaps in the herpetologically understudied region of Iraqi Kurdistan (Al-Barazengy *et al.* 2015; Jablonski *et al.* 2020), our data suggest that this area likely represents the edge of the distribution for the genus *Blanus* (Sindaco and Jeremčenko 2008; Sindaco *et al.* 2014) and possibly indicates its rarity in the region. This distribution pattern resembles that of several other species such as *Ommatotriton vittatus* (Gray in Jenyns, 1835), *Salamandra infraimmaculata* (Martens, 1885), *Pseudopus apodus* (Pallas, 1775), or *Dolichophis jugularis* (Linnaeus, 1758), whose distribution is influenced by the Zagros Mountains barrier in this part of their distribution ranges (Sindaco and Jeremčenko 2008; Sindaco *et al.* 2013; Dufresnes 2019). This could be affected by the specific bioclimatic and environmental conditions in the region (Şahin *et al.* 2021; Vaissi 2021) such as annual precipitation or elevation (*Blanus* individuals were recorded between ~500 and 1900 m a. s. l.). Unlike some other species as for example *S. infraimmaculata*, we speculate that the range of *B. alexandri* does not extend into the Iranian territory, and its eastern distribution is strictly confined to the steppe and forest-steppe regions of northern and north-eastern Iraq. However, it remains uncertain where the south-eastern border in Iraqi Kurdistan lies, as similar habitats can be found along the foothills of the Zagros Mountains, extending up to the Sulaymaniyah and Halabja governorates, where the species has never been recorded so far. The presence in the Nineveh Governorate is also highly probable (Fig. 1A).

The genetically investigated specimen from Banoka village corresponds with populations of *B. alexandri* from south-eastern Turkey (Sindaco *et al.* 2014), particularly with the population from 4–5 km NE of Derik, Mardin province (KJ584069), the type locality of the species, and from the population from E of Karalar, Mardin province (KJ584070) (Fig. 1B, D). All of them form a well-supported so-called lineage II *sensu* Jablonski and Sadek (2019) within which the sequence from Iraq forms an unique sublineage. According to the haplotype network, the population is separated by 29 and 27 mutation steps from the sequence KJ584069 and KJ584070, respectively (Fig. 1C). This genetic confirmation newly shifts the distribution of the lineage II approximately 250 km to the east (Fig. 1D). The lineage II is sister to lineage I (Fig. 1B), both with the distribution east of Nur Mountains that are considered as a geographic division between different but closely related evolutionary lineages of reptiles in the Middle East (Jablonski and Sadek 2019; Kornilios *et al.* 2020; Šmíd *et al.* 2021). The nuclear PRLR sequence fully matches the sequence KJ584093, which is found within the mitochondrial lineage I (KJ584071) originating from south-central populations in Turkey. There are no heterozygotes present in this sequence.

**TABLE 1.** Records of *Blanus alexandri* in Iraq. The numbers correspond with the map presented in Figure 1.

No.	Locality	N	E	elevation (m)	date	observer	Voucher number	source
1	Salahussin-shaklauva, Erbil Governorate	36.4180	44.2690	~900	?	C. Kosswig	HM 1216	Alexander (1966)
2	Ashaw village, 5 km SW of Sarsang, Dohuok Governorate	37.0400	43.3430	~1000	13 August 1971	?	IMN 426	Al-Jumaily (1971)
3	Banok village, Peran, Erbil Governorate	36.9021	44.3728	866	28 March 2017	S. A. Sleman	KMN 302a, b	This study
4	Shaqlaw, Erbil Governorate	36.3920	44.3340	~1000	?	H. Ibrahim	KMN 280a, b	This study
5	Banoka village, Erbil Governorate	36.6356	44.3217	863	24 March 2019	D. Jablonski	CUHC 8606	This study
6	Shaqlaw, Erbil Governorate	36.3564	44.3577	1937	15 May 2022	C. Langer	-	<a href="https://www.inaturalist.org/observations/126929875">https://www.inaturalist.org/observations/126929875</a>
7	Alana Valley, Erbil Governorate	36.54561	44.45967	989	18 May 2022	C. Langer	-	<a href="https://www.inaturalist.org/observations/131854826">https://www.inaturalist.org/observations/131854826</a>
8	Hamdla, Dohuok Governorate	36.9452	43.9870	534	22 May 2022	N. Barzani	CUHC 12669, 12670	This study

Abbreviations: CUHC = Comenius University Herpetological Collection; HM = Zoologisches Museum, Hamburg; IMN = Baghdad Museum of Natural History University of Baghdad; KMH = Kurdistan Museum of Natural History, Erbil.



**FIGURE 1.** The distribution (A) and genetic affiliation (B, D) of *Blanus alexandri* from Iraq (Kurdistan). The Bayesian tree (B) represents the mitochondrial relationships of the species with numbers above branches showing posterior probabilities/maximum likelihood bootstrap support values. Each terminal branch represents GenBank accession number. (C) 95% parsimony haplotype network of the lineage II. Numbers of distribution points correspond with Table 1. Geographic distribution of sequences with colours corresponding to the lineages *sensu* Jablonski and Sadek (2019). The approximate distribution range (modified after Sindaco and Jeremčenko 2008; Sindaco *et al.* 2014; Jablonski and Sadek 2019) of the species is highlighted in brown (D). The photography of *B. alexandri* from Iraq was taken by D. Jablonski.



Comparing genetic diversity of *B. alexandri* (Fig. 1B), populations of the eastern part are significantly less variable than the Levant and the Anatolian ones. Such result supports the hypothesis about the fast spreading of Anatolian lineage and its populations to the east due to suitable environmental corridors without significant geographic barriers limiting spreading or increasing the evolutionary divergence (Fritz *et al.* 2007; Jablonski *et al.* 2021). However, these conclusions based on mitochondrial lineages should be considered preliminary. It is essential to conduct wide genomic studies to examine the overall genetic diversity in *B. alexandri*. Furthermore, investigating other species in this region would help identify similarities that could lead to broader conclusions about the comparative biogeography of the biota in the Middle East.

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