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Zoology in the Middle East is a journal which publishes original papers and review papers on ecology, zoogeography, animal biology, biodiversity, faunistics, systematics and morphology of the Middle East. It seeks to further the understanding of the Middle East as a zoogeographic unit and aims at improving the interchange of knowledge and ideas between specialists on different subjects and taxa.

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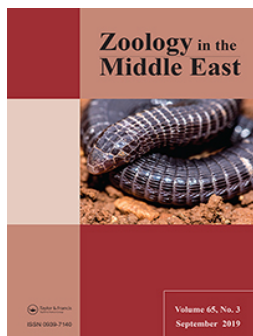
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Cover picture of the current issue: *Blanus alexandri* from Maad, Lebanon (Daniel Jablonski and Riyad A. Sadek).



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The species identity and biogeography of *Blanus* (Amphisbaenia: Blanidae) in Lebanon

Daniel Jablonski & Riyad A. Sadek

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The species identity and biogeography of *Blanus* (Amphisbaenia: Blanidae) in Lebanon

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The genus *Blanus* Wagler, 1830 represents limbless, burrowing reptiles of the family Blanidae with disjunct circum-Mediterranean distribution. The recently described species *Blanus alexandri* Sindaco, Kornilios, Sacchi & Lymberakis, 2014 is known from south-eastern Turkey with a presumed occurrence in the Levant and Iraq. We provide here records from Lebanon and confirm the affiliation of Lebanese populations to this species by mitochondrial and nuclear DNA. *Blanus alexandri* comprises at least seven deeply evolved phylogenetic clades with up to 11% of uncorrected *p*-distances in their mitochondrial DNA. This suggests a probably older than the Miocene origin of some of these clades. Populations from Lebanon form a different clade with a genetic diversity that is close to populations from southern Turkey.

Keywords: Levant; eastern Mediterranean; cryptic diversity; phylogeography; morphology; taxonomy

Introduction

The genus *Blanus* Wagler, 1830 (Amphisbaenia: Blanidae) comprises seven limbless and burrowing species with a disjunct Mediterranean distribution (Albert & Fernández, 2009; Sindaco, Kornilios, Sacchi, & Lymberakis, 2014; Ceriaco & Bauer, 2018). Three of them are found in the eastern Mediterranean and had until recently been regarded as *B. strauchi*: *B. strauchi* (Bedriaga, 1884) s. str. with two subspecies distributed in western Anatolia and some East Aegean islands, *B. aporus* Werner, 1898 in south-central Anatolia and *B. alexandri* Sindaco, Kornilios, Sacchi & Lymberakis, 2014 in south-east Anatolia (Sindaco et al. 2014). Sindaco et al. (2014) expect that the distribution of *B. alexandri* includes Lebanon, Syria and northern Iraq, but molecular data to support this assumption were missing. In Lebanon *Blanus* sp. is known from six localities (Alexander, 1966; In Den Bosch et al., 1998; Hraoui-Bloquet et al., 2002; Supplementary Table S1 and Figure S1) and these had previously been assigned to *B. strauchi aporus* Werner, 1898 (e.g. Hraoui-Bloquet et al., 2002). These populations form probably the southernmost distribution of the genus in the eastern Mediterranean (Göçmen, Disi, & Yıldız, 2008) as the occurrence in Israel is still questionable (Alexander, 1966; Werner, 2016). We present here new material of *Blanus* sp. from Lebanon and examined the identity of these populations and analysed their genetic diversity in a biogeographic context.

Material and Methods

One adult individual of *Blanus* was collected by the first author on 3 April 2018 at Maad (34.19°N, 35.68°E, 509 m) and two at Ghabline (34.18°N, 35.69°E, 362 m) under stones (Fig-

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ure 2). Habitat at the sampled localities consisted of reddish soft soil with rocks and bushy vegetation on the edge with a cultivated area (olive trees; Supplementary Table S1 and Figure S1).

Blood collected from live specimens was used as a DNA source. Tissue samples were either preserved in 96% ethanol or frozen and stored at -25 or -80°C. The specimens are deposited in the collection of Department of Zoology, Comenius University in Bratislava under voucher numbers DJ6808 (Maad), DJ6827-6828 (Ghabline), with GenBank accession numbers MK107995 and MK107996-97, respectively. For DNA extraction, we used a commercial DNA extraction kit (Qiagen DNeasy® Blood and Tissue Kit) according to the manufacturer's protocol. The complete mitochondrial DNA, the NADH dehydrogenase subunit 4 (ND4) and the nuclear protein-coding gene of the prolactin receptor (PRLR) were PCR-amplified with primers ND4 and LEU (Arévalo et al., 1994) and PRLR_f1 and PRLR_r3 (Townsend, Alegre, Kelley, Wiens, & Reeder, 2008), respectively. The laboratory procedure followed Albert, Zardoya, & García-Paris (2007), Albert & Fernández (2009) and Sindaco et al. (2014). The sequencing was performed by Macrogen Inc. (Amsterdam, The Netherlands; <http://www.macrogen.com>). In our analyses, we combined our newly obtained sequences with those stored in GenBank that were used for the phylogeny presented by Sindaco et al. (2014; see Tab. 1, p. 313).

DNA sequences were manually checked, aligned, and inspected using BioEdit 7.0.9.0 (Hall, 1999). No stop codons were detected when the sequences were translated using the vertebrate mitochondrial genetic code in the program DnaSP 5.10 (Librado & Rozas, 2009). The same program was used to calculate uncorrected p -distances among the main clades as percentages values as in Albert et al. (2007) and to estimate the number of haplotypes (h), haplotype diversity (h_d), number of segregating sites (S), average number of nucleotide differences (k), nucleotide diversity (π), and Watterson's theta per site (θ_w). The best-fit codon-partitioning schemes and the best-fit substitution models were selected using PartitionFinder v1.1.1 (Search algorithm: all, branch-lengths = linked; Lanfear, Calcott, Ho, & Guindon, 2012), according to Bayesian information criterion (BIC). Phylogenetic trees were inferred using the Bayesian approach (BA) and maximum likelihood (ML) by MrBayes 3.2 (Ronquist et al., 2012) and RAxML 8.0. (Stamatakis, 2014), respectively. The best-fit substitution model with each codon position was as follows: HKY+G (1st), HKY+I (2nd), GTR+G (3rd). For the ML analysis, GTR+G model in each codon position was used. The ML clade support was assessed by 1,000 bootstrap pseudoreplicates. MrBayes analysis was set as follows: two separate runs with four chains for each run, 10 million generations with samples saved every 100th generation. The convergence of the two runs was confirmed by the convergence diagnostics (average standard deviation of split frequencies, potential scale reduction factor). First 20% of trees were discarded as the burn-in after inspection for stationarity of log-likelihood scores of sampled trees in Tracer 1.6 (Rambaut, Suchard, Xie, & Drummond, 2013) (all parameters had effective sample size >200). A sequence of *B. tingitanus* Busack, 1988 was included as an outgroup in tree analyses (KJ584062; Sindaco et al., 2014).

We built the phylogenetic network using the NeighborNet algorithm (Bryant & Moulton, 2004) implemented in the software SplitsTree 4.10 (Huson & Bryant, 2006) for all in-group clades. We also constructed mitochondrial haplotype networks for phylogenetically closest clades to those from Lebanon using the 95% limit of parsimony (TCS algorithm; Clement, Posada, Crandall, 2000) as implemented and visualized in the software PopArt (<http://popart.otago.ac.nz>). The same procedure was used for PRLR loci (Sindaco et al. 2014).

Colouration in life was estimated by examination in the field and using colour digital photographs. Following Albert and Fernández (2009), eight morphological variables were taken: head-length (HL), snout-to-vent length (SVL), head width (HW), pre-frontal width (PFW), pre-frontal length (PFL), number of dorsal segments (DS), number of ventral segments (VS), number of body annuli (BA) (Supplementary Table 2 and Figure S2).

Results

We analysed a data matrix of 31 *Blanus* sequences (three new presented by this study together with 28 published by Sindaco et al. 2014) of 657 bp long fragment of ND4. We have observed essentially identical topology of both ML and BA trees (ND4), thus only the BA tree is depicted with BA posterior probabilities and ML bootstrap values for

Table 1. Uncorrected p -distances calculated among the ND4 clades of *Blanus alexandri* (in %). The highest and lowest value of p -distance is highlighted in bold.

	I	II	III	IV	V	VI	VII
I	0						
II	7.6	0					
III	9.9	6.7	0				
IV	7.1	6.7	4.5	0			
V	11.1	6.9	7.2	4.5	0		
VI	8.5	7.3	5.2	5.0	3.8	0	
VII	6.3	7.3	4.7	5.5	3.4	4.0	0

Table 2. Summary of genetic polymorphism of *Blanus alexandri* ND4 sequences: sample size (n), number of haplotypes (h), number of polymorphic sites (S), average number of nucleotide differences (k), nucleotide diversity (π), haplotype diversity (hd), Watterson's theta per site (θ_w). SD = standard deviation.

	n	h	S	K	$\pi \pm SD$ (%)	$Hd \pm SD$	$\theta_w \pm SD$ (%)
<i>Blanus alexandri</i>	12	11	151	52.0	7.9 \pm 0.8	0.99 \pm 0.04	7.6 \pm 3.0
I	1	1	-	-	-	-	-
II	2	2	16	16.0	2.4 \pm 1.2	1.0 \pm 0.5	2.4 \pm 1.7
III	1	1	-	-	-	-	-
IV	2	1	-	-	-	-	-
V	1	1	-	-	-	-	-
VI	2	2	17	17.0	2.6 \pm 1.3	1.0 \pm 0.5	2.6 \pm 1.8
VII	3	3	8	5.3	0.8 \pm 0.3	1.0 \pm 0.3	0.8 \pm 0.5

each main branch (Figure 1A). PRLR dataset (549 bp) formed the same haplotype with KJ584093, published by Sindaco et al. 2014 (code B12, from Gaziantep, Turkey; network not showed in this study and our PRLR sequences not provided for GenBank). Both used markers showed an affiliation to species *Blanus alexandri* with respect to the current phylogenetical and taxonomical view.

In the in-group of *B. alexandri*, sequences clustered in seven clades, not well supported in all branches. Some clades are represented by a single sample. A similar pattern was recovered by phylogenetic network (Figure 1C). Although we have limited samples, most of clades exhibit distinct geographical pattern (Figure 1B), and some of them also have a conspicuous internal structure as can be seen on the phylogenetic tree, haplotype as well as phylogenetic networks (Figure 1A, C, D). On the other hand, some of the phylogenetically distant clades are geographically very close to each other (e.g. III and V or IV and VI). Moreover, clade IV (KJ584066-067) and one sequence of the clade VI (KJ584064) were recorded in sympatry at one locality in southern Turkey (1.3 km E Sungur, Hatay; see Sindaco et al. 2014). Specimens from Lebanon formed a very well supported independent clade (VII; Figure 1A).

Preliminary analysis of complete ND4 *B. alexandri* dataset under the 95% parsimony limit in PopArt did not resolve relationships between main clades due to high genetic

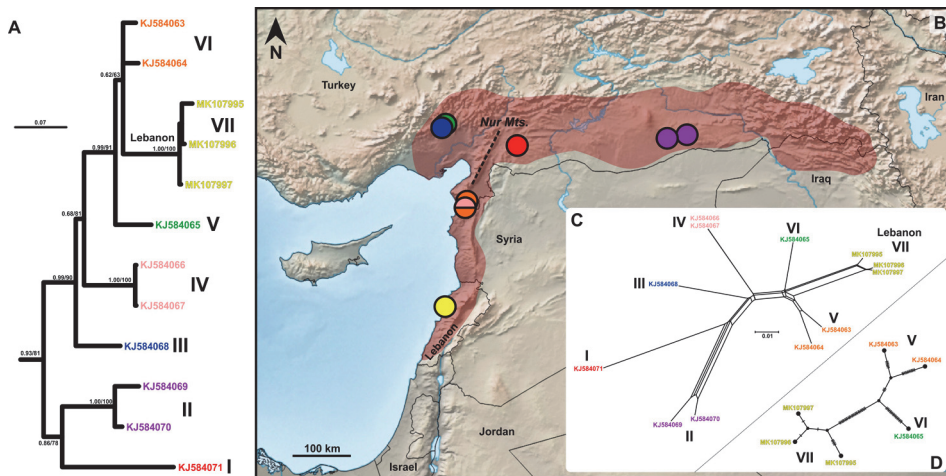


Figure 1. (A) Bayesian tree reconstructed from the dataset of mtDNA ND4 gene sequences of *Blanus alexandri* (657 bp; see Materials and Methods). Outgroup not shown. Numbers above the branches show posterior probabilities/maximum likelihood bootstrap support values. Each terminal branch represents GenBank accession number. (B) Geographic distribution of sequences with colours corresponding to the main recovered phylogenetic clades. The approximate distribution range of the species is highlighted in brown. (C) SplitsTree phylogenetic network reconstructed using the same dataset of mtDNA as for tree analysis. (D) 95% parsimony haplotype network of Lebanese sequences (clade VII) and two closest clades of the species.

distances between them. Therefore, we run this analysis only with closest clades (V and VI) to the clade VII from Lebanon. As shown in the final haplotype network (Figure 1D), clade VII is distant more than 30 mutation steps from two other clades and the distance between Maad and Ghabline is seven mutation steps. All three samples from Lebanon represent unique mitochondrial haplotypes.

Relative genetic distances among most clades of *B. alexandri* vary around 3–5% as expressed by uncorrected p -distances, with the maximum of 11.1% between clade I and V. The lowest average distance was recorded between clade V and VII with value 3.4% (Table 1). The values of DNA polymorphism for species and particular clades are presented in Table 2.

Morphological data of three available Lebanese specimens do not exceed characters of the species from other populations except here recorded a higher number of body annuli (Supplementary Table 2). Colouration of the species goes from grey to dark violet with depigmented areas of the body (Supplementary Figure 1).

Discussion

While our results show that populations of Lebanese *Blanus* clearly correspond with *B. alexandri*, our overall results suggest a complex evolutionary history of the species. The results are consistent with the phylogeographic patterns observed in other Anatolian and the Levant species (Tamar et al., 2015; Baier et al., 2017; Kornilios, 2017; Jandzik et al., 2018; Kotsakiozi et al., 2018). We recovered here seven divergent clades that have more or less distinct geographic distribution pattern (Figure 1). Populations from Lebanon are phylogenetically and geographically different but close to clade VI from the Hatay



Figure 2. *Blanus alexandri* from Maad, Lebanon.

Province in southern Turkey. As is suggested by the network analysis (see Figure 1C, D), haplotypes are clearly distinct even in geographically close localities (the airline distance between the two analysed Lebanese localities is about 1.5 km). A similar pattern was found and discussed by Albert et al. (2007) from the Iberian Peninsula as recent dispersal events.

Our results of the mean *p*-distances among the clades in ND4 vary in the range of 3.4% to 11.1% (Table 1). The highest distance was recorded between Turkish clades I and V separated by Nur Mountains. It suggests important divergence seen in the congeneric species and closest relatives, *B. cinereus* and *B. vandellii* (10.5–12.4%; Albert et al., 2007; Albert & Fernández, 2009; Ceriaco & Bauer 2018) or even between *B. alexandri* and *B. aporus* (10.0%; Sindaco et al., 2014). The distances between Lebanese clade (VII) and others are between 3.4% and 7.3%. On the other hand and in the respect to mtDNA, divergence in nuclear PRLR loci is generally very shallow in *B. alexandri* and populations from Lebanon correspond with a known haplotype from Turkey (see Sindaco et al., 2014). Although genetic distances among ND4 sequences suggest very old split between some of clades (estimated rates of evolution for this locus is 0.4–0.6% per lineage per Mya, Caccone, Amato, Gratry, Behler, & Powell, 1999; i.e. older than the time of the Messinian Salinity Crisis that is probably the main factor leading to the Miocene/Pliocene reptile speciation in the Mediterranean, e.g. Kornilios, 2017), only these two markers cannot clearly say if *B. alexandri* is an old deeply evolved species or may represent a separate species complex. A fossil record of *B. cf. strauchi* is, however, known already from the Miocene of Anatolia (Georgalis, Halaçlar, Mayda, Kaya, & Ayaz, 2018). The genus *Blanus* comprises species whose dispersal ability is limited by fossorial lifestyle and high site tenacity (Werner, 2016). Such a combination of life history, past geological events, climatic shifts in the region (see Tchernov, 1992) and habitat characteristics (altitudinal differences, steep exposed slopes, heterogeneous

landscapes), provides suitable predispositions for isolation and subsequent genetic divergence due to numerous effective barriers preventing dispersal of small legless reptiles (Jablonski et al., 2016).

Supplementary Material

Supplementary Table S1 is available via the “Supplementary” tab on the article’s online page (<http://dx.doi.org/10.1080/09397140.2019.1604471>).

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Supplementary Material to:

**The species identity and biogeography of *Blanus* (Amphisbaenia:
Blanidae) in Lebanon**

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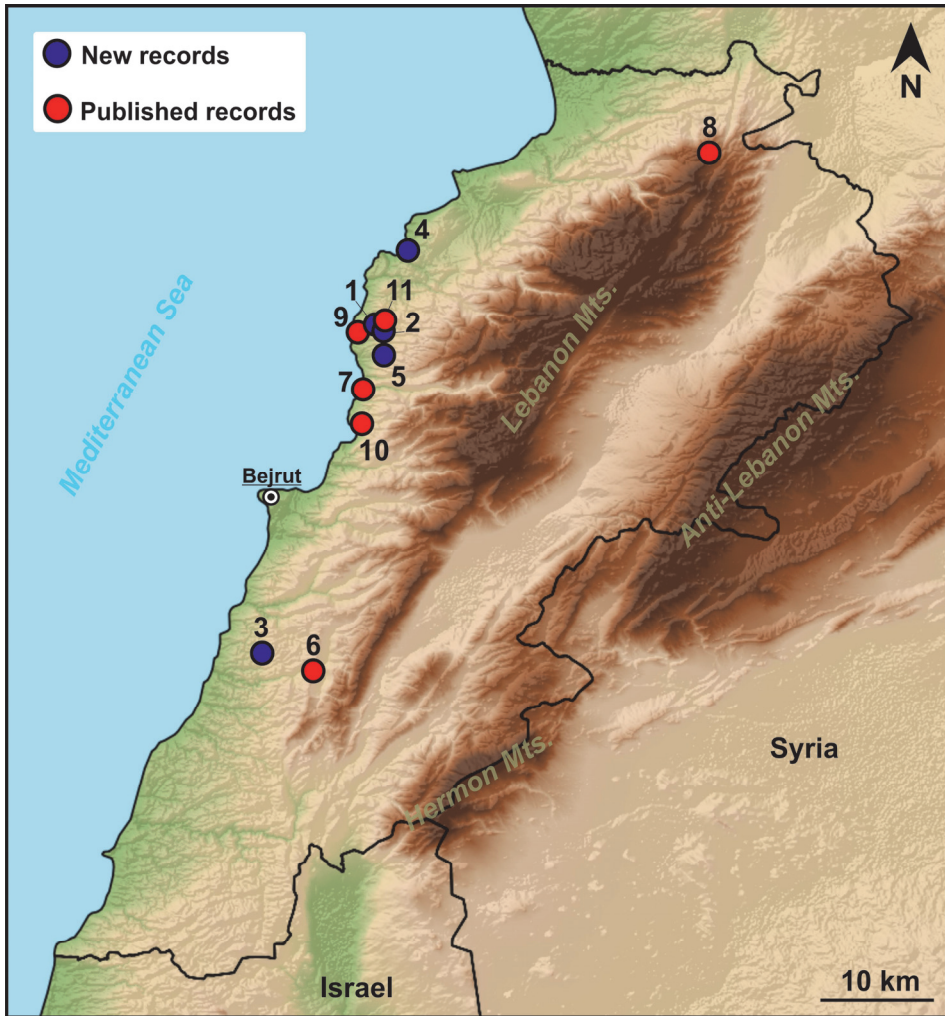
<http://dx.doi.org/10.1080/09397140.2019.1604471>

Supplementary Table S1. Published and new records of *Blanus alexandri* in Lebanon

No.	Locality	N	E	Elevation	Observer	Sources
1	Maad	34.19	35.68	509	D. Jablonski	This study
2	Ghabline	34.18	35.69	362	D. Jablonski	This study
3	Chehim	33.62	35.48	483	R. Sadek	This study
4	Chekka	34.32	35.74	?	A. Cluchier	This study
5	Bentael Nature Reserve	34.13	35.70	650	J. Fenianos	This study
6	Jounieh	33.59	35.57	?	?	Hraoui-Bloquet et al., 2002
7	Halat	34.08	35.66	?	?	Hraoui-Bloquet et al., 2002
8	10 km S Qoubaiyat	34.49	36.27	1350	H. In Den Bosch	In Den Bosch et al., 1998
9	Monsef	34.18	35.65	?	?	Alexander, 1966
10	Ghazir	34.02	35.66	?	?	Alexander, 1966
11	Sghar	34.20	35.70	?	?	Alexander, 1966

Supplementary Table 2. Measurements (in mm) and meristic characters of examined *Blanus alexandri* from Lebanon. SVL = snout to vent length, HL = head width, PFL = pre-frontal length, PFW = pre-frontal width, BA = body annuli, DS = dorsal scales, VS = ventral scales.

<i>Voucher number</i>	<i>SVL</i>	<i>HL</i>	<i>HW</i>	<i>PFL</i>	<i>PWW</i>	<i>BA</i>	<i>DS</i>	<i>VS</i>	<i>pores</i>
6808	169	10.0	7.0	3.0	3.5	114	19	20	3+3
6827	167	10.5	6.5	4.0	4.0	114	18	22	3+3
6828	182	9.5	6.5	3.0	3.0	114	18	21	3+3



Supplementary Figure S1. Compilation of published and own records of *Blanus alexandri* from Lebanon. For details see Table S1.



Supplementary Figure S2. Individuals of *Blanus alexandri* from Lebanon and their habitat. Overall and head view of an unsexed individual from Maad (A, B). Dorsal view on head part of individual from Ghabline (C). Detailed view on the microhabitat shortly after finding of an individual, Ghabline (D). Localities of capture in Maad (E) and Ghabline (F) and their macrohabitats. All photos: D. Jablonski.