



Morphological and genetic differentiation in the anguid lizard *Pseudopus apodus* supports the existence of an endemic subspecies in the Levant

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We dedicate this article to Professor Fritz Jürgen Obst (1939–2018) acknowledging thus his precocious contribution to the taxonomy of *Pseudopus (Ophisaurus) apodus* published in *Zoologische Abhandlungen* (1978), today *Vertebrate Zoology* journal, 40 years before the information appeared about the possible existence of the subspecies described here.

Abstract

The Levant represents one of the most important reptile diversity hotspots and centers of endemism in the Western Palearctic. The region harbored numerous taxa in glacial refugia during the Pleistocene climatic oscillations. Due to the hostile arid conditions in the warmer periods they were not always able to spread or come into contact with populations from more distant regions. One large and conspicuous member of the Levantine herpetofauna is the legless anguid lizard *Pseudopus apodus*. This species is distributed from the Balkans to Central Asia with a portion of its range running along the eastern Mediterranean coast. Mitochondrial and nuclear DNA sequences, microsatellite genotypes, and morphology show that populations in this region differ from the two named subspecies and presumably had a long independent evolutionary history during the Quaternary. Here we describe the Levantine population as a new subspecies and present biogeographic scenarios for its origin and diversification. The new subspecies is genetically highly diverse, and it forms a sister lineage to *Pseudopus* from the remaining parts of the range according to mtDNA. It is the largest-bodied of the three subspecies, but occupies the smallest range.

Abstract in Hebrew

הלבנט מהווה את אחד האזורים העשירים ביותר במינים בכלל, במינים אנדמיים ובמגוון פילוגנטי של זוחלים בממלכה הפליארקטית. מינים רבים התקיימו באזור במהלך תקופות הקרח, כשהוא שימש מפלט מהאקלים הקר בצפון. אחד ממיני הזוחלים הגדולים והבולטים בלבנט הוא קמטן החורש, *Pseudopus apodus*. מין זה נפוץ מהבלקן בצפון אל מרכז אסיה במזרח, ומגיע בחוף הים התיכון המזרחי לגבול תפוצתו הדרומי. לפי רצפי DNA מיטוכונדריאלי, DNA גרעיני ומיקרוסטליטים, כמו גם נתוני צורה, גודל ודגמי צבע, נראה כי אוכלוסיות הלבנט של מין זה שונות משמעותית משני תתי המינים המתוארים המצויים מצפון ללבנט. הנתונים מצביעים על כך שאוכלוסיות הלבנט נפרדו מתתי המינים האחרים ומראות היסטוריה פילוגנטית עצמאית לאורך הרביעון. אנו מתארים את אוכלוסיות הלבנט כתת מין חדש, ומציגים תרחישים אפשריים להסבר מוצאו והפרדותו מתתי המינים האחרים. תת המין החדש הוא קבוצה אחת לשני תתי המינים המתוארים על פי הניתוח הגנטי. הוא הגדול בין תתי המינים, אך מאכלס את תחום התפוצה הקטן מבין כל השלושה.

Abstract in Arabic

منطقة الشرق الأوسط تعتبر إحدى أغنى المناطق بانواع الحيوانات المختلفة بشكل عام، وبالعديد من الحيوانات المتوطنة ومجموعة متنوعة من الزواحف من مملكة Palearctic. كانت المنطقة تآوي العديد من الأنواع في الفترة الجليدية، إذ إن المنطقة كانت بمثابة ملجأ لها من برد المناطق الأكثر شمالاً. من إحدى أنواع الزواحف الكبيرة والبارزة في منطقة الشرق الأوسط هي السحلية *Pseudopus apodus*. هذا النوع من الزواحف ينتشر في الشمال من منطقة البلقان إلى مركز آسيا في الشرق ويصل انتشاره حتى شواطئ البحر الأبيض المتوسط الشمالية، والتي تعد أقصى منطقة جنوبية ينتشر بها. استناداً لتسلسل الحمض النووي الميتوكوندريالي والحمض الخلوئي الصبغي، بالإضافة إلى معلومات أخرى منها الشكل والحجم ونماذج من الألوان، نرى أن هذا النوع من السحلية التي تعيش في منطقة الشرق الأوسط يختلف كثيراً عن أنواع أخرى المنتشرة في المناطق الأكثر شمالاً. تشير المعلومات إلى أن مجموعات السحلية عديمة الأرجل القاطنة بمنطقة الشرق الأوسط انفصلت من التوزيعات (أنواع فرعية) الأخرى وتظهر تاريخ تطوري مستقل خلال العصر الرباعي. نصف هنا أن هذا النوع من السحالي يُصنّف كنوع مختلف وجديد ونعرض أحداث ممكنة لشرح أصلها وانفصالها من التوزيعات الأخرى. يبدو أن النوع الفرعي الجديد له علاقة قريبة (أخت) مع النوعين الآخرين، ورغم أنه الأكبر حجماً لكنه يحتل أصغر نطاق انتشار من بينها.

Keywords

Middle East, mitochondrial DNA, microsatellites, *Ophisaurus*, phenotype, Reptiles, sheltopusik, Squamata

Introduction

The Levant forms a biogeographical crossroad between European, African, and Asian biotas. It is currently formed by territories of Cyprus, Israel, Jordan, the West Bank, Lebanon, western Syria, and southern Turkey. The Levant is known for high endemism of amphibians and reptiles at the genus (*Latonina nigriventer*, *Phoenicolacerta*; Biton et al. 2013; Tamar et al. 2015), species (e.g. *Ommatotriton vittatus*, *Blanus alexandri*, *Ophiomorus latastii*, *Mediodactylus amictopholis*, *Daboia palaestinae*, *Eirenis levantinus*, *Hemidactylus dawudazraqi*, *Rhynchocalamus* spp., or *Xerotyphlops syriacus*; Schmidtler 1993; Sindaco and Jeremcenko 2008; Moravec et al. 2011; Sindaco et al. 2013; Tamar et al. 2016; Van Riemsdijk et al. 2017; Jablonski and Sadek 2019; Kornilios et al. 2020) as well as subspecies (*Pelobates syriacus syriacus* or *Zamenis hohenackeri tauricus*; Dufresnes et al. 2019c; Jandzik et al. 2013) levels. Genetically estimated splits of these taxa from their sister lineages often extend back to the Oligocene or Miocene (Biton et al. 2013; Kornilios 2017; Kotsakiozi et al. 2018) and recent studies confirm their high within-taxon diversity (e.g. Jandzik et al. 2013, 2018; Ahmadzadeh et al. 2013; Tamar et al. 2015, 2016; Skourtanioti et al. 2016; Dufresnes et al. 2019a–c; Gumbs et al. 2020). In general, a very similar distribution pattern is observed in multiple taxa and phylogenetic clades occurring approximately between the Nur Mountain Range of south-central Turkey in the north, to the northern border of the Negev Desert in Israel in the south (e.g. Tamar et al. 2016; Van Riemsdijk et al. 2017).

A very similar pattern of distribution is also typical for the Levantine populations of *Pseudopus apodus* (Pallas, 1775) from the southernmost part of the species range (Sindaco and Jeremcenko 2008). This anguid species is the only living member of the genus *Pseudopus* (previously considered congeneric with *Ophisaurus*; e.g. Obst 1981), a large and robust legless lizard that is among the relatively common inhabitants of the Mediterranean maquis leaf litter. Some of us (Jandzik et al. 2018) recently studied its genetic structure using the analyses of the mitochondrial and nuclear DNA sequences, and showed clear genetic divergence partly corresponding to the traditionally recognized, morphologically defined, subspecies: *P. a. apodus* distributed in Crimea, Caucasus, south Caspian Region, and Central Asia, and *P. a. thracicus* (Obst, 1978) from northern and western Anatolia and the Balkans (Obst 1978, 1980). Beside these two subspecific lineages, the phylogeny revealed a lineage, occurring exclusively in the Levant (Jandzik et al. 2018), with unclear morphological affiliations (Rifai et al. 2005). This Southern lineage represents an early divergence within recent populations of *P. apodus*. Despite very limited sampling from the Levant ($n = 3$), Jandzik et al. (2018) proposed that the biogeographic history of the Southern lineage differed from histories of *P. a. apodus* and *P. a. thracicus*. These two subspecies have large ranges, are genetically less diverse, and their present distribution pattern, and genetic structure, indicate they only recently expanded to

inhabit most of their ranges. The Southern lineage, on the other hand, has a relatively small range and appears to be genetically more diverse than the other two. This may reflect historical fragmentation and disruptions in gene flow between populations inhabiting a relatively small range surrounded by hostile arid environment unsuitable for this agnoid species (Jandzik et al. 2018).

Here we studied genetic diversity and morphology of *P. apodus* from across its range taking an advantage of an improved geographic sampling, especially in the Levant, which had previously not been analyzed in depth. This allows us to better understand the patterns of genetic differentiation and obtain deeper insights into the biogeographic history of the species. As a result, we propose to adjust the intraspecific taxonomy by describing a third *P. apodus* subspecies.

Material and methods

We used 156 tissue samples of *P. apodus* for genetic analyses and we analyzed the morphology of 479 specimens covering the entire distribution range of the species (Table S1, S2 and Figure 1).

Genetics

Tissue sampling and laboratory procedures

As a source of DNA, we used buccal swabs, blood (taken from the caudal vein) or scale clips from living individuals, and liver or muscle biopsies from ethanol-preserved museum specimens and road-killed animals. We studied variation in two mitochondrial DNA (mtDNA) markers and eight microsatellite loci. In total, we newly obtained DNA data from 52 individuals for mtDNA and 155 individuals for microsatellites and combined them with previously published data (Table S1).

Total genomic DNA was extracted from the tissue samples using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. For molecular-genetic analyses, we used fragments of two mtDNA markers (NADH dehydrogenase subunit 2, *ND2*, and cytochrome b, *Cyt b*) and eight microsatellite loci. The PCR strategy for mtDNA is described in detail in Jandzik et al. (2018) and was followed without modifications. The complete *ND2* dataset of *P. apodus* contained 33 sequences (21 newly obtained, 12 published) and the *Cyt b* dataset contained 85 sequences (51 newly obtained, 34 published; Table S1).

Eight microsatellite loci (Geiser et al. 2013; Mikulíček et al. 2018) were amplified in two multiplex PCRs (multiplex 1: AF38-VIC, AF44-FAM, AF47-PET, AnFr21-NED; multiplex 2: AF19-FAM, AF37-NED, AnFr12-

VIC, AnFr35-NED) using the Type-it Microsatellite PCR Kit (Qiagen, Hilden, Germany). PCRs were performed in a total volume of 10 μ L containing 1 \times Type-it Multiplex PCR Master Mix, 0.2 μ M of each primer pair (forward primers were fluorescently labeled with FAM, VIC, NED, and PET), 2 μ L of DNA, and RNase-free water. PCR amplification involved an initial cycle of denaturation at 95°C for 5 min and 30 subsequent cycles of 95°C for 30 s, 58°C (multiplex 1) or 55°C (multiplex 2) for 90 s, and 72°C for 30 s, followed by a final extension step at 60°C for 30 min. PCR products were run on an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA) with a LIZ-500 size standard. Microsatellite fragments were visualized using the software GeneMapper 3.7 (Applied Biosystems) and were scored manually by a single observer.

Analyses of mitochondrial DNA

After manual aligning and trimming the low-quality sequence ends the resulting alignments contained sequences of 727-bp fragment of *ND2* and 805-bp fragment of *Cyt b*. These were used in the tree analysis, along with a concatenated *ND2+Cyt b* dataset (1532-bp) which was constructed by combining the two former alignments. For the network analysis of all available sequences, we used the same length fragment of *ND2*, although only 423-bp fragment of *Cyt b*. Only the 575-bp long fragment of *Cyt b* was used for network analysis from the Southern lineage. In both cases this was due to variable lengths of the available sequences. No stop codons were detected when checked in the program DNASP 6.00 (Rozas et al. 2017).

We constructed Bayesian Inference (BI; MRBAYES 3.2.6; Ronquist et al. 2012) and Maximum Likelihood (ML; RAXML 8.0.0; Stamatakis 2014) phylogenetic trees, for all three datasets, using all available sequences. The best-fit model of sequence evolution was selected using PARTITIONFINDER 2 (Lanfear et al. 2017) with the following parameters (single locus trees): Bayesian approach (BA) – linked branch length; all models; BIC model selection; greedy schemes search; data blocks by codons for each used marker. The best partitioning scheme and models of nucleotide substitutions were as follows: the first position K80+G, second position HKY+I, third position HKY for *Cyt b* and HKY+I, HKY, and HKY+G for *ND2*. The ML analysis followed the same approach; the best model in this case was GTR+G with two subsets in *Cyt b* and three subsets in *ND2*. The concatenated dataset was divided to six subsets based on four user schemes (unpart, gene-part, 3rd-pos-extra, codon-part) with following final models for BI analysis: HKY+I (subset 1), HKY (2, 3, 5, 6), and JC+I (4), and GTR+G in all subsets for ML analysis. The BI analysis was set as follows: two separate runs, with four chains for each run, 10 million generations with trees sampled every 100th generation. The 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 et al. 2013; all parameters had effective sample sizes [ESSs] of > 200). A majority-rule

consensus tree was drawn from the post-burn-in samples and posterior probabilities were calculated as the frequency of samples recovering any particular clade. Nodes with posterior probability values 0.95 were considered as strongly supported. The ML clade support was assessed by 1,000 bootstrap pseudoreplicates.

Genealogical relationships between mtDNA haplotypes were separately assessed with haplotype networks (32 sequences in *ND2*, 85 in *Cyt b*) based on alignments of the network analyses as mentioned above, and from the concatenated dataset. Haplotype networks of both analyzed markers were constructed and drawn using POPART (<http://popart.otago.ac.nz>) and the implemented parsimony network algorithm of TSC (Clement et al. 2000), with 95% connection limit. Well defined networks are considered distinct evolutionarily significant units, following Fraser and Bernatchez (2001), thus this analysis was also used to infer genetic structure within the studied taxa.

DNASP 6.00 (Rozas et al. 2017) was used to estimate the number of haplotypes (*h*), haplotype diversity (*Hd*), and nucleotide diversity (π) for particular detected lineages of the species based on single locus datasets. The same program was used for uncorrected *p* distance calculations.

Principal Component Analyses (PCAs) of mtDNA markers were carried out using 33 sequences of both the concatenated and *ND2* datasets, and 85 sequences of *Cyt b* dataset in the R package ADEGENET (Jombart 2008) implemented in the R statistical environment (R Core Team 2020).

Analyses of microsatellites

We used two approaches to assess genetic structure among the samples: a Bayesian-based method implemented in the STRUCTURE 2.3.4 software (Pritchard 2000) and a Principal Component Analysis (PCA) implemented in the R package ADEGENET (Jombart 2008). Using STRUCTURE, direct posterior probabilities for the number of clusters *K* [$\text{LnP}(K)$] as well as the ad hoc statistic ΔK (Evanno et al. 2005) were estimated assuming uniform prior values of *K* between one and ten. ΔK statistic was calculated using online program STRUCTURE HARVESTER 0.6.94 (Earl and vonHoldt 2012). In STRUCTURE, both admixture and non-admixture models, always in combination with uncorrelated allele model, were applied. The analyses were based on runs of 10^6 iterations, following a burn-in period of 10^4 iterations. A series of five independent runs for each *K* was made with the same parameters to test the accuracy of the results. PCA were run using the same data set as STRUCTURE.

To assess genetic diversity and differentiation between clusters, the samples were pooled into three geographical regions based on the outcomes of the mtDNA phylogenetic, Bayesian and PCA analyses (see below). A number of different (N_A) and private (N_P) alleles, allele frequencies, percentage of polymorphic loci (*P*), and observed (H_O) and unbiased expected (H_E) heterozygosity were calculated using GENALEX 6.5 (Peakall and Smouse 2006). Genetic differentiation among the three clusters (subspecies)

was estimated using both unbiased Nei's genetic distance D_S (Nei 1972) and F_{ST} statistics calculated via an Analysis of Molecular Variance (AMOVA) (Meirmans 2006). The significance of genetic differentiation (F_{ST}) among clusters was based on 9999 permutations. To understand how genetic variation is partitioned within and among clusters, we applied AMOVA based on F_{ST} statistics. Analysis of genetic diversity, AMOVA, and the calculation of genetic distances were carried out using GENALEX 6.5.

Morphology

Studied material and morphological data

We examined the external morphology of 479 *P. apodus* specimens (Fig. 1C): 358 specimens of *P. a. apodus* (189 males, 72 females, 35 juveniles, and 62 adult specimens of unknown sex originated in Armenia, Azerbaijan, Crimea, Georgia, Iran, Kazakhstan, Russia, Tajikistan, eastern Turkey, Turkmenistan, and Uzbekistan), 17 specimens of *P. a. thracicus* (11 males and 6 females; from Bulgaria and western Turkey), and 104 specimens from the Southern lineage (39 males, 19 females, and 46 juveniles; all from Israel). The subspecific status of the examined specimens was determined based on distribution data, morphological characters, and molecular analyses. Differences between the total number of examined specimens and the number of analyzed specimens for each character are due to poor condition of some specimens (e.g. damaged parts of the head or body), which only allowed some characters to be recorded. The complete list of analyzed specimens and their geographic origin (if georeferenced) are available in Table S3 and Fig. 1C. The material was obtained either directly in the field or from the collections of the following institutions: The Steinhardt Museum of Natural History, Tel Aviv University, Tel Aviv, Israel (TAU); Institute of Zoology of the Republic of Kazakhstan, Almaty, Kazakhstan (IZRK); Zoological Museum in Kiev, Ukraine (ZMK); Zoological Museum of the Zoological Institute of the Russian Academy of Sciences (ZISP); Museum of Nature of V. N. Karazin Kharkiv National University, Kharkiv, Ukraine (SMNK); Museum of Zoology of the Senckenberg Natural History Collection Dresden, Dresden, Germany (MTD). Manipulation of live individuals was limited to minimize animal discomfort. No specimens from Israel were collected specifically for this study. All originated from the TAU collections.

Metric characters were taken with a measuring tape (± 0.1 cm; body and tail lengths) and with a digital caliper (± 0.1 mm; other measurements); meristic characters, and other morphological characters, were observed using a stereomicroscope when needed (e.g., for small juveniles). Metric characters are (all in mm; abbreviation in parentheses, for details see Table S4): snout-vent length (SVL); length of the intact tail (TL); total length (TotL); head length 1 (HL1); head length 2 (HL2); pileus length (PL); head width 1 (HW1); head width 2 (HW2); head width 3 (HW3); eye-nostril distance (ENL); frontal shield length (FL); frontal shield width (FW); Frontal shield-

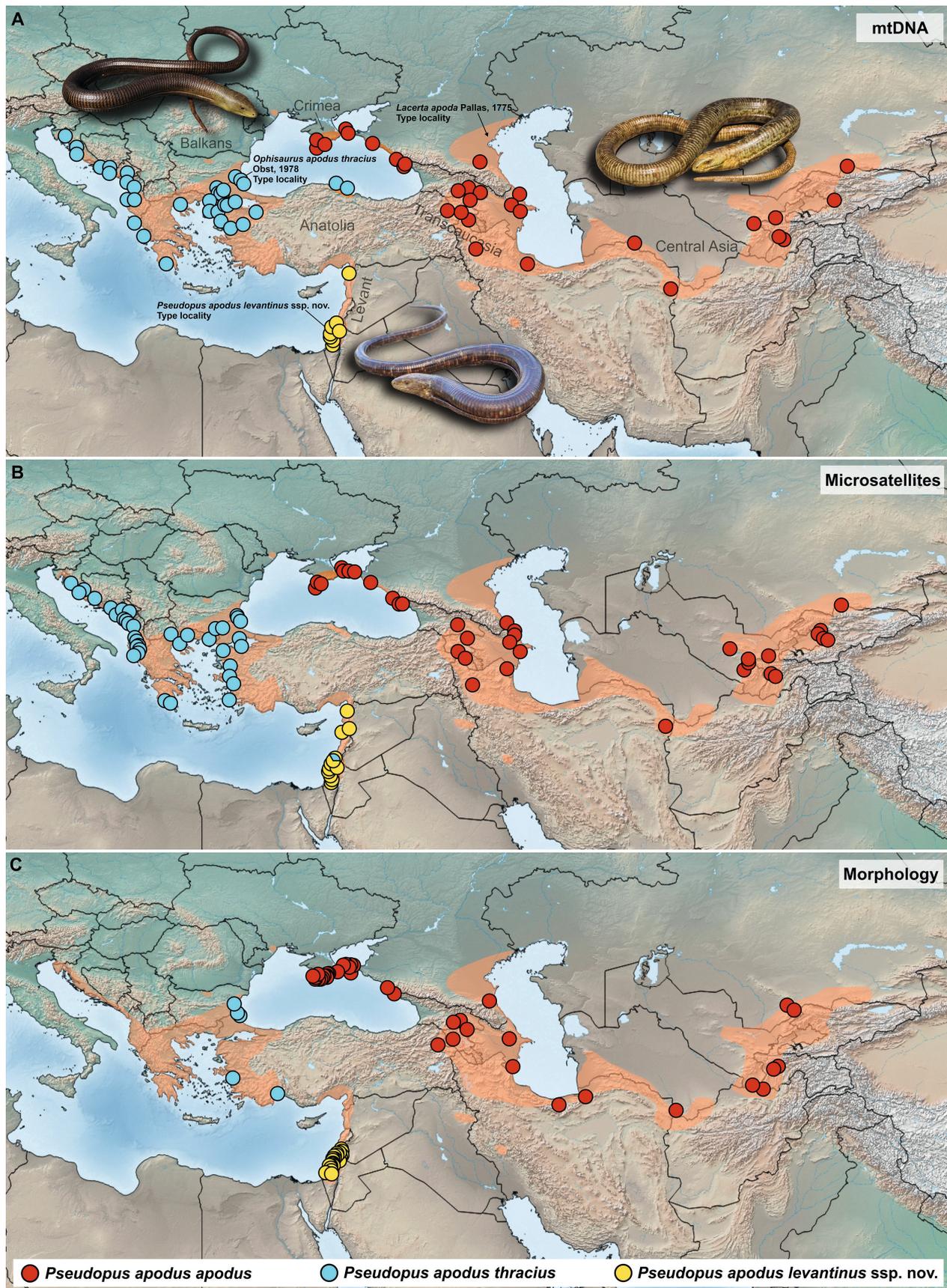


Figure 1. Geographic distribution of the samples and specimens used in the molecular-phylogenetic (A, B) and morphological analyses (C). Some specimens used in morphological analyses were from unknown localities, so those are not indicated in the map. Color scheme corresponds with the one used in Fig. 2 and follows Jandzik et al. (2018). For more details on samples, specimens, and localities, refer to Tables S1–3. The distribution range follows Sindaco and Jeremcenco (2008) and Jandzik et al. (2018). The pictured individuals represent currently recognized subspecies originating from (left to right) Lefkimi, Greece (*P. a. thracicus*), Hirbat Tzura, Israel (*P. a. levantinus ssp. nov.*), and Tyolyok, Kyrgyzstan (*P. a. apodus*). Photos by Daniel Jablonski and David David.

snout length (FSL); snout width (SW); intermaxillar shield height (IMH); intermaxillar shield width (IMW); rudiments length (RL).

We used the following meristic characters (abbreviations in parentheses, for details see Table S4): dorsal scales longitudinal (DSL); ventral scales longitudinal (VSL); dorsal scales transversal (DST); ventral scales transversal (VST); subcaudal scale rows (SCR); preanal scales (PAN); and supralabial scales (left and right sides; SPL/l/r).

Coloration of the body was recorded via direct observation in the field or from photographs of live specimens (when available). Coloration in preservative is based on observation of the specimens the abovementioned collections.

Analysis of external morphology

For the initial analysis of the differences in metric and meristic characters among the three groups (*P. a. apodus*, *P. a. thracius* and the Southern lineage, below described as a new subspecies) we used analysis of co-variance (ANCOVA) with subspecies and sex as main effects and SVL as a covariate. Analyses were conducted in R statistical environment (R CoreTeam 2020). Next, we performed three principal component analyses (PCAs): 1) with all adult specimens (SVL \geq 310 mm, sensu Kukushkin and Dovgal 2018) and all characters, 2) with only adult males, and 3) with only adult females. Sexual dimorphism in the Southern lineage was analyzed using MANOVA for the meristic characters, t-test for SVL and Ltot, and MANCOVA, with SVL as a co-variate, for the remaining measures. These analyses were carried out in SPSS STATISTICS 17.0.0. (SPSS Inc., Chicago, IL, USA).

Results

Genetics

Mitochondrial diversity and phylogeography

Our comprehensive sampling (Fig. 1A and Table S1) split *Pseudopus apodus* into three lineages in all analyses and using all sequence alignments (Fig. 2, S1, S2). These lineages correspond to the subspecies *P. a. apodus*, *P. a. thracius*, and to the Southern lineage (below described as a new subspecies; Fig. 2A,C,E). The lineage corresponding to the nominotypic subspecies is distributed from Crimea, through the north-eastern Black Sea coast and southern Russia, Georgia, Azerbaijan, Armenia, northern Iran, Turkmenistan to Tajikistan, Uzbekistan and Kyrgyzstan. The lineage corresponding to the subspecies *P. a. thracius* inhabits the Mediterranean regions of the Balkan Peninsula, and western and northern Turkey. The Southern lineage inhabits southernmost Turkey, and northern and central Israel as well as western Syria and Lebanon, though we have no mtDNA data from these countries (but see data from Syria in *Microsatellite genotyping*).

The network visualization from the concatenated dataset corresponded to the phylogenetic division into three groups, *P. a. apodus*, *P. a. thracius*, and the Southern lineage. We found seven haplotypes of *P. a. apodus* forming a star-like pattern with shallow genetic substructure, four distinct haplotypes of *P. a. thracius*, and eight haplotypes of the Southern lineage, showing deep intra-lineage diversity. More than 40 mutation steps were recognized between haplotypes of *P. a. apodus* and the Southern lineage, and more than 33 between *P. a. thracius* and the Southern lineage (Fig. 2B).

In the 727 bp long fragment of *ND2* (32 sequences) used to build the haplotype network, we found four haplotypes of *P. a. apodus*, two haplotypes of *P. a. thracius*, and six haplotypes of the Southern lineage. The most distant in-group haplotypes (*P. a. apodus* and the Southern lineage) spanned 31 mutation steps. Between the most distant haplotypes of *P. a. apodus* and *P. a. thracius* there were 19 mutation steps. A star-like pattern was partially found in *P. a. apodus* haplotypes (Fig. 2D). The highest number of mutations and the most divergent haplotypes were found in the Southern lineage – 13 mutations steps between populations from southern Turkey and central Israel (and eight within Israel; see details below; Fig. 2D).

In the 423 bp long fragment of *Cyt b* (85 sequences) used for the haplotype network, we found seven *P. a. apodus* haplotypes, ten haplotypes of *P. a. thracius*, and four haplotypes of the Southern lineage. Between the most distant in-group haplotypes (*P. a. thracius* and the Southern lineage) there were 29 mutation steps, nine mutation steps between *P. a. apodus* and *P. a. thracius*, and 13 mutation steps between *P. a. apodus* and the Southern lineage. *Pseudopus a. thracius* had the highest number of haplotypes, and all lineages showed star-like pattern (Fig. 2F).

The dataset of the 727 bp long *ND2* fragments of the Southern lineage revealed four, well-diverged haplogroups: two in southern Turkey and two in Israel. Three of these haplogroups comprised single haplotypes each. The most common haplogroup (19 sequences), detected in central and northern Israel, contains three haplotypes. All haplogroups are separated by at least six mutation steps. The most distant haplogroup was found on the southern border of the species range (at least eight mutation steps from the adjacent haplotypes; Fig. 3A).

In the 575 bp long *Cyt b* fragments dataset of the Southern lineage we detected lower haplotype separation with two main haplogroups separated by three mutation steps from each other. One haplogroup containing a single haplotype was detected in southern Turkey, another, containing four haplotypes and 21 sequences separated by one mutation step, was found in Israel (Fig. 3B).

We found clear differences in mtDNA polymorphism between the lineages with values of $Hd = 0.800$ and $\pi = 0.18\%$ for *P. a. apodus* (n = 6 sequences), $Hd = 0.667$, $\pi = 0.09\%$ for *P. a. thracius* (n = 3), and $Hd = 0.458$, $\pi = 0.34\%$ for the Southern lineage (n = 23) on the *ND2* dataset and $Hd = 0.722$ and $\pi = 0.28\%$ for *P. a. apodus* (n = 28), $Hd = 0.494$, $\pi = 0.17\%$ for *P. a. thracius* (n = 35), and $Hd = 0.271$, $\pi = 0.11\%$ in the Southern lineage (n = 22) in the *Cyt b* dataset.

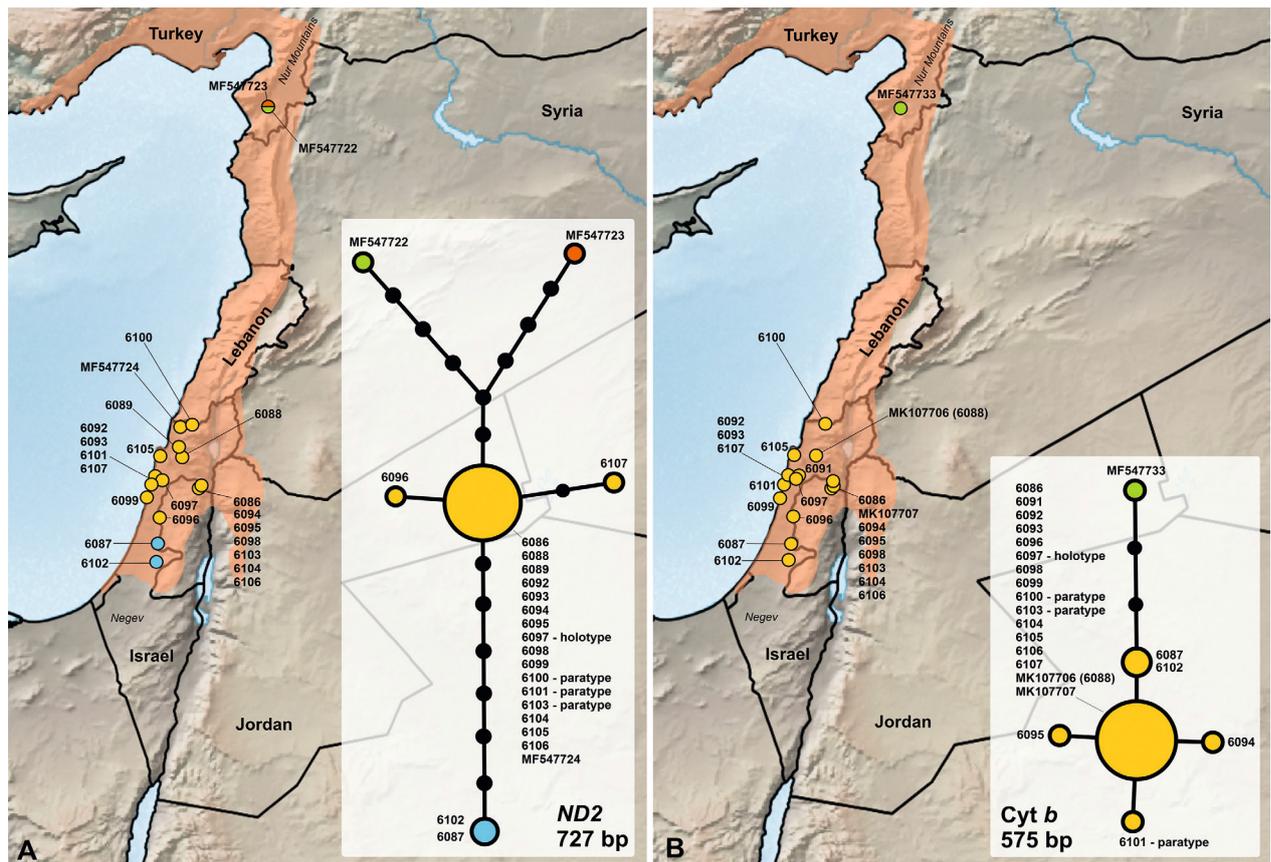


Figure 3. Maps showing the localities and sampling numbers related to the collection with DJab acronym or GenBank number (see Table S1) of the genetically investigated populations of *Pseudopus apodus levantinus* ssp. nov. (*ND2* & *Cyt b*) in the Levant with the haplotype networks for each DNA marker. Circle sizes in the haplotype networks are proportional to the relative frequency of haplotypes and small black circles represent missing haplotypes. The expected distribution range (orange) of the Levantine subspecies (see also Figure S4) follows Sindaco and Jeremcenko (2008) and Jandzik et al. (2018).

The ranges of uncorrected p distances were as follows: *ND2* + *Cyt b* (concatenated dataset, 1532 bp) 2.6% between *P. a. apodus* and *P. a. thracicus*, 3.5% between *apodus* and the Southern lineage, and 3.6% between *thracicus* and the Southern lineage. In *ND2* alone (727 bp): 3.0% between *apodus* and *thracicus*, 3.8% between *apodus* and the Southern lineage, 3.6% between *thracicus* and the Southern lineage. With *Cyt b* (805 bp): 2.6% between *apodus* and *thracicus*, 3.3% between *apodus* and the Southern lineage, and 3.7% between *thracicus* and the Southern lineage.

In accordance with the phylogenetic and haplotype network analyses, the PCAs revealed three highly distinct clusters corresponding to *P. a. apodus*, *P. a. thracicus* and the Southern lineage with non-overlapping 95% confidence intervals (Fig. 4A). Due to the similarity of sequences and haplotypes, the individual values appear only as few dots despite the large numbers of samples. First (PC1) and second (PC2) principal components explain 45.9% and 21.3% of the observed variance in the concatenated dataset, 44.9% and 17.4% in the *ND2* dataset, and 26.3% and 20.7% in the *Cyt b* dataset, respectively.

Microsatellite genotyping

For detection of genetically homogeneous groups of individuals in STRUCTURE, we used two approaches: direct

posterior probabilities [$\text{LnP}(K)$] and ad hoc statistic ΔK . Direct posterior probabilities increased sharply from $K=1$ to $K=3$ and then, with larger K s, increased just slightly and reached a plateau. The statistic ΔK estimated the most likely number of clusters as $K=2$, followed by $K=3$. For higher K s the analysis obtained much lower probabilities.

Assuming $K=2$, all individuals from the Balkans and western Turkey were assigned to cluster 1, corresponding to *P. a. thracicus*. Specimens from the Crimea, through the Caucasus region, Iran up to Central Asia were assigned to cluster 2, corresponding to *P. a. apodus*. Lizards from the Levantine region (Israel, Syria, and an adjacent part of southern Turkey) were either assigned to cluster 1 or showed admixture. Assuming $K=3$, Levantine specimens formed a separate cluster 3, corresponding to the Southern lineage (Figs. 1B and 4, Table S2). The only exceptions were two individuals from Biranit (Israel) and Misayaf (Syria), which showed admixed genomes in the admixture model but were assigned to the Southern lineage in the non-admixture model.

PCA analysis of microsatellite data showed similar results (Fig. 4B) to those obtained using mtDNA and the STRUCTURE clustering ($K=3$; Fig. 4C), presented a clear separation among the three sampling regions, i.e. 1) the Balkans and western Turkey (*P. a. thracicus*), 2) Crimea eastward to Central Asia (*P. a. apodus*), and 3) the Levant

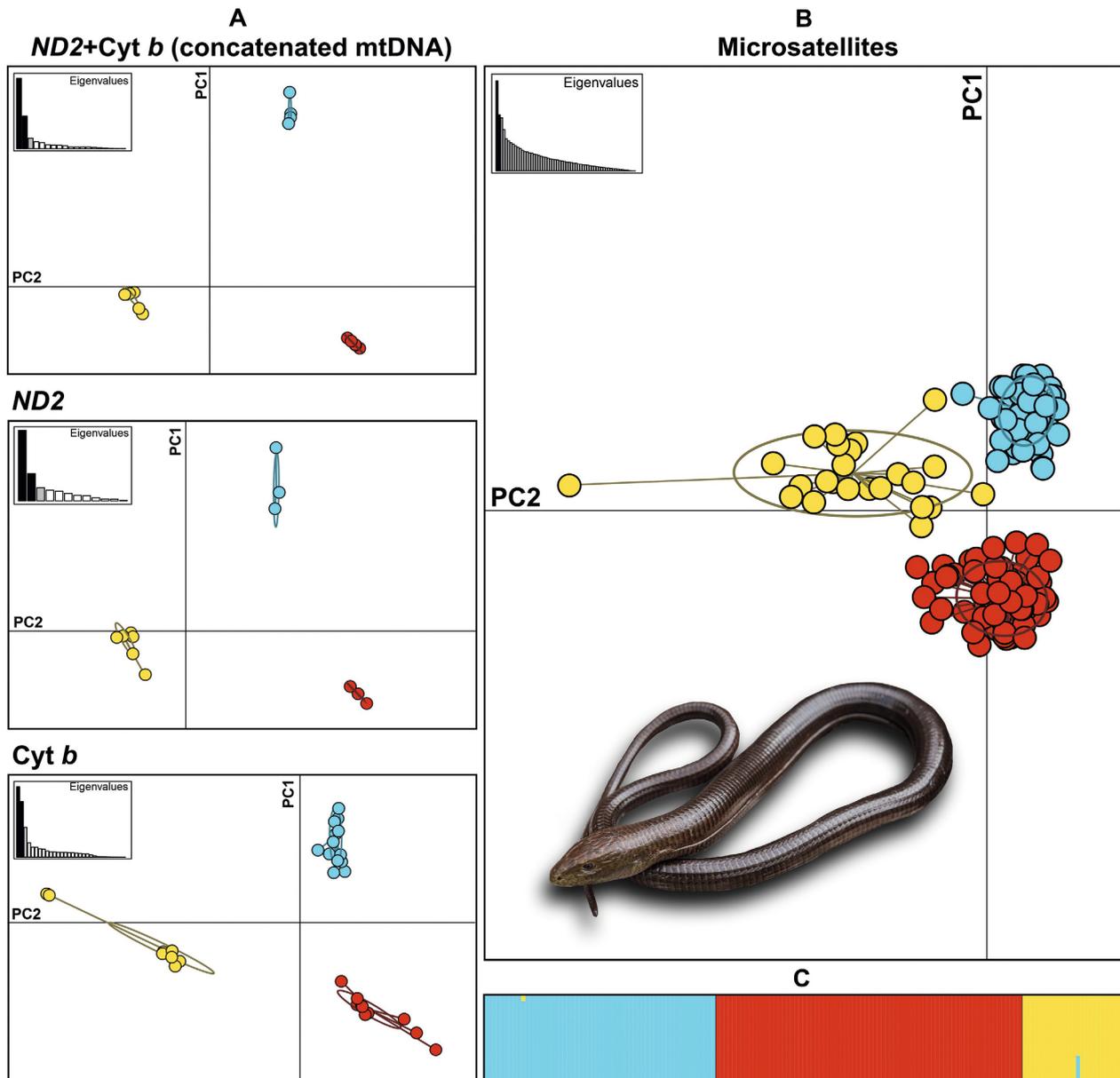


Figure 4. Principal Component Analysis (PCA) of three lineages of *Pseudopus apodus* for mitochondrial DNA (A) and microsatellite data (B) with the STRUCTURE plot of posterior probabilities for $K=3$ (C). The oval outlines in PCAs represent 95% confidence intervals. First (PC1) and second (PC2) principal components explain 45.9% and 21.3% of the observed variance in the concatenated dataset, 44.9% and 17.4% in *ND2* dataset, 26.3% and 20.7% in *Cyt b* dataset, and 10.8% and 6.7% in microsatellites, respectively. The plot of two principal components shows a clear separation between three groups of samples. Color scheme corresponds with the one used in Fig. 2: *P. a. apodus* (red), *P. a. thracius* (blue) and *P. a. levantinus* **ssp. nov.** (yellow).

(Southern lineage). First (PC1) and second (PC2) principal components explain 10.8% and 6.7% of the observed variance (Fig. 4B).

To assess genetic diversity and differentiation, all samples were assigned to three groups corresponding to the STRUCTURE and PCA clusters. The percentage of polymorphic loci (P) was lower in *P. a. apodus* and *P. a. thracius* (87.5% polymorphic loci) than in the Southern lineage, in which all microsatellite loci were polymorphic. Individuals from the Levant showed substantially higher genetic diversity [numbers of different (N_A) and private (N_p) alleles and observed (H_o) and expected (H_e) heterozygosity; Table 1] followed by lizards assigned to *P. a. apodus* and *P. a. thracius*, respectively.

Genetic differentiation, as measured by unbiased Nei's genetic distance (D_S) and F_{ST} statistics, reached similar values between the Southern lineage and both of the subspecies (Table 2). The highest differentiation was found between *P. a. thracius* and *P. a. apodus* ($D_S = 0.796$, $F_{ST} = 0.395$). Average F_{ST} summarized over all samples reached the value of 0.333. Genetic differentiation among all three groups was highly significant (Table 2). As shown by AMOVA, genetic variance within individuals was the highest (51%, $DF = 155$, $SS = 244.00$, $MS = 1.574$), followed by genetic variance among the three geographical regions (33%, $DF = 2$, $SS = 199.92$, $MS = 99.96$) and among individuals (15%, $DF = 152$, $SS = 383.28$, $MS = 2.52$).

Table 1. Parameters of genetic variation in three subspecies of *Pseudopus apodus* based on eight microsatellite loci. N – number of analysed individuals, P – percentage of polymorphic loci, N_A – a number of different alleles, N_p – a number of private alleles, H_o – observed heterozygosity, H_E – unbiased expected heterozygosity, SD – standard deviation.

Subspecies	N	P (%)	N_A (mean±SD)	N_p (mean±SD)	H_o (mean±SD)	H_E (mean±SD)
<i>P. a. apodus</i>	74	87.5	5.875±1.217	1.625±0.625	0.388±0.089	0.473±0.096
<i>P. a. thracius</i>	56	87.5	3.625±0.625	1.125±0.125	0.342±0.080	0.397±0.076
<i>P. a. levantinus ssp. nov.</i> (Southern lineage)	25	100	6.375±1.017	1.750±0.453	0.563±0.086	0.639±0.092

Table 2. Genetic differentiation among three subspecies of *Pseudopus apodus* estimated using F_{ST} statistics (above diagonal) and unbiased Nei's genetic distance D_S (below diagonal) based on microsatellites. Genetic differentiation between the subspecies based on F_{ST} was highly significant (***) $P < 0.001$.

Subspecies	<i>P. a. apodus</i>	<i>P. a. thracius</i>	<i>P. a. levantinus ssp. nov.</i> (Southern lineage)
<i>P. a. apodus</i>	—	0.395***	0.247***
<i>P. a. thracius</i>	0.796	—	0.248***
<i>P. a. levantinus ssp. nov.</i> (Southern lineage)	0.475	0.388	—

Morphology

Quantitative analyses of morphological data

Snout-vent length (SVL) differed significantly between subspecies and sexes. Males are longer than females, specimens of the Southern lineage are longer than *P. a. apodus*, and *P. a. thracius* specimens are the shortest (Tables 3 and 4). All other characters were therefore compared between the three groups while controlling for SVL and sex (no interactions were tested). All metric and meristic characters differed significantly among all three groups, except for the frontal-snout length (FSL; Table 3). Morphological differences among the three groups revealed variable degrees of divergence between the Southern lineage and *P. a. apodus* and *P. a. thracius*. The Southern lineage has the longest pileus (PL) and the highest intermaxillar shield (IMH). It also has wider head (HW1 and HW2) and intermaxillar shield (IMW), and longer rudiment (RL) than *P. a. apodus* but does not differ in these characters from *P. a. thracius*. The head at the level of the suture between the third and fourth supra-orbital scales (HW3) is wider in the Southern lineage than in *P. a. apodus*, and narrower than in *P. a. thracius*. The Southern lineage does not differ from *P. a. apodus* but has significantly longer heads than *P. a. thracius* (HL1 and HL2) and frontal shields (FL), and wider snouts (SW). The frontal shield (FW) is narrower in the Southern lineage than in both *P. a. apodus* and *P. a. thracius*. In meristic characters, the Southern lineage has the highest number of preanal scales (PAN), and the number of these scales increases with SVL. Members of the Southern lineage have more supralabials (SPL) than *P. a. apodus* and fewer than *P. a. thracius* for a given SVL. The Southern lineage has fewer dorsals (DSL), ventrals (VSL), and subcaudals (SCR) than *P. a. apodus*, and more ventrals

(VSL), and subcaudals (SCR) than *P. a. thracius* (Table 3). A summary of the variation of the characters in *P. a. apodus*, *P. a. thracius*, and the Southern lineage is presented in Table 4.

The three PCA analyses with (1) both males and females; 2) only males; 3) only females, presented similar results. The first component explained 98.7, 98.6, and 98.9%, respectively, of total variance of the 17 analyzed characters, with the most important character being SVL (Table S5). Most of the specimens from Israel (the Southern lineage) had much higher PC1 scores than any specimen of the other subspecies, while the majority of *P. a. apodus* and *P. a. thracius* specimens had a combination of high scores on PC2 and low scores on PC1, not seen in the Southern lineage. However, there were areas of morphological overlap among all three lineages, especially between *P. a. apodus*, and *P. a. thracius*, which showed near-complete overlap (Fig. 5, Table S5).

We found no evidence for sexual dimorphism in meristic characters in the Southern lineage. None of the eight measures significantly differ between males and females and three characters (DST, VST, PAN) do not vary at all. The sexes do not differ in their overall size, but males have generally wider heads than females (significant in HW1–3, SW, and IMW; Table S6). In the PCA analysis of the sexual dimorphism of the Southern lineage the first axis explained 99.2% of the total variance of the four analyzed characters, and each analyzed character was presented as the most important in each component (HL1 in the first component; HW1 in the second; PL in the third one; and HW2 in the fourth component; Fig. S3).

Systematic account

Pseudopus apodus comprises three clearly genetically and slightly morphologically differentiated allopatric populations (Obst 1978, Jandzik et al. 2018, this study).

Table 3. Intercepts (in mm) of the analysis of co-variance (ANCOVA) models with subspecies and sexes main effects and SVL as a covariate (for details see Table S4).

Metric data	<i>P. a. levantinus</i> ssp. nov.	<i>P. a. apodus</i>	<i>P. a. thracius</i>	Pairwise comparisons
Snout-vent length (DF = 281)	1.818	2.609 (p = 0.2373)	5.538 (p = 0.0005)	<i>thracius</i> > <i>apodus</i> = <i>levantinus</i>
Head length 2 (DF = 291)	0.808	1.28 (p = 0.4345)	3.233 (p = 0.0132)	<i>thracius</i> > <i>apodus</i> = <i>levantinus</i>
Pileus length (DF = 259)	3.979	5.977 (p = 0.0008)	8.662 (p = 5.03e-07)	<i>levantinus</i> > <i>apodus</i> = <i>thracius</i>
Head width 1 (DF = 329)	-0.717	-1.73 (p = 0.0368)	-0.014 (p = 0.3752)	<i>levantinus</i> = <i>thracius</i> > <i>apodus</i>
Head width 2 (DF = 245)	7.835	3.488 (p = 3.11e-16)	6.700 (p = 0.367)	<i>levantinus</i> = <i>thracius</i> > <i>apodus</i>
Head width 3 (DF = 297)	8.288	5.763 (p = 1.87e-09)	10.054 (p = 0.0067)	<i>thracius</i> > <i>levantinus</i> > <i>apodus</i>
Frontal shield length (DF = 266)	2.558	2.730 (p = 0.5528)	4.196 (p = 0.0004)	<i>thracius</i> > <i>apodus</i> = <i>levantinus</i>
Frontal shield width (DF = 266)	0.360	0.989 (p = 0.001)	1.807 (p = 1.78e-06)	<i>apodus</i> = <i>thracius</i> > <i>levantinus</i>
Frontal shield-snout length (DF = 203)	1.530	1.727 (p = 0.4982)	0.902 (p = 0.1412)	<i>levantinus</i> = <i>apodus</i> = <i>thracius</i>
Snout width (DF = 203)	3.409	3.418 (p = 0.9748)	4.975 (p = 0.0002)	<i>thracius</i> > <i>apodus</i> = <i>levantinus</i>
Intermaxillar shield height (DF = 300)	2.178	1.590 (p = 8.23e-07)	1.770 (p = 0.0312)	<i>levantinus</i> > <i>apodus</i> = <i>thracius</i>
Intermaxillar shield width (DF = 299)	0.569	0.127 (p = 0.0165)	0.808 (p = 0.4199)	<i>thracius</i> = <i>levantinus</i> > <i>apodus</i>
Rudiments length (DF = 239)	-1.675	-1.163 (p = 0.0094)	-2.042 (p = 0.2079)	<i>thracius</i> = <i>levantinus</i> > <i>apodus</i>
Meristic data				
Dorsal scales longitudinal (DF = 249)	98.250	103.779 (p = 1.25e-09)	97.492 (p = 0.5860)	<i>apodus</i> > <i>thracius</i> = <i>levantinus</i>
Ventral scales longitudinal (DF = 268)	120.227	121.571 (p = 0.0145)	116.397 (p = 2.08e-05)	<i>apodus</i> > <i>levantinus</i> > <i>thracius</i>
Subcaudal scale rows (DF = 162)	213.670	240.662 (p = 0.0006)	238.797 (p = 0.0593)	<i>apodus</i> > <i>levantinus</i> = <i>thracius</i>
Preanal scales (DF = 259)	7.640	6.060 (p = 9.59e-10)	4.614 (p = 1.17e-12)	<i>levantinus</i> > <i>apodus</i> = <i>thracius</i>
Supralabial scales left (DF = 190)	10.146	9.626 (p = 0.0108)	9.582 (p = 0.0595)	<i>thracius</i> = <i>levantinus</i> > <i>apodus</i>

Table 4. Summary of the variation of the characters in *Pseudopus apodus apodus*, *P. a. thracius*, and *P. a. levantinus* ssp. nov. Counts and measurements (in mm) are presented as minimum–maximum (mean±standard deviation); n = total number of specimens studied. Only intact and non-regenerated tails were considered in SCR, TL, and TotL (for details see Table S4).

Metric data	<i>P. a. apodus</i>	<i>P. a. thracius</i>	<i>P. a. levantinus</i> ssp. nov.
Snout-vent length (SVL)	310–487 (387.61±37.60) (n=304)	320–410 (360.06±31.47) (n=17)	317–610 (490.22±76.15) (n=55)
Length of the tail (TL)	320–765 (567.38±75.61) (n=232)	463–645 (552.71±55.56) (n=17)	416–757 (600.70±107.04) (n=27)
Total length (TotL)	635–1230 (948.40±99.98) (n=232)	796–1025 (912.76±73.52) (n=17)	733–1367 (1072.30±194.60) (n=27)
Head length 1 (HL1)	28.63–58.00 (38.61±4.90) (n=219)	31.10–49.50 (39.55±5.15) (n=17)	29.30–63.75 (47.60±7.99) (n=53)
Head length 2 (HL2)	23.60–47.20 (35.77±4.67) (n=226)	27.20–43.40 (35.40±4.76) (n=17)	27.59–59.58 (44.12±7.65) (n=53)
Pileus length (PL)	26.40–46.30 (35.82±4.02) (n=197)	28.80–44.10 (36.93±4.56) (n=17)	26.85–54.79 (41.98±6.78) (n=53)

Table 4 continued.

Metric data	<i>P. a. apodus</i>	<i>P. a. thracius</i>	<i>P. a. levantinus</i> ssp. nov.
Head width 1 (HW1)	17.96–36.50 (24.47±3.62) (n=267)	20.10–29.50 (24.35±2.95) (n=17)	16.62–44.90 (32.04±6.34) (n=53)
Head width 2 (HW2)	15.20–28.76 (19.44±2.37) (n=183)	17.30–26.30 (22.33±2.48) (n=17)	16.06–39.33 (28.21±4.95) (n=53)
Head width 3 (HW3)	13.00–26.87 (17.13±2.26) (n=235)	16.40–25.00 (20.74±2.34) (n=17)	14.21–28.59 (22.62±3.31) (n=53)
Eye-nostril distance (ENL)	6.99–13.90 (10.29±1.28) (n=176)	8.10–11.20 (10.02±0.91) (n=17)	6.58–15.23 (10.13±1.94) (n=54)
Frontal shield length (FL)	8.00–18.50 (11.59±1.60) (n=202)	9.50–15.30 (12.50±1.76) (n=17)	8.63–19.74 (13.75±2.49) (n=52)
Frontal shield width (FW)	5.10–11.75 (8.72±1.09) (n=202)	6.50–10.60 (9.05±1.10) (n=17)	5.69–15.78 (10.23±2.01) (n=52)
Frontal shield-snout length (FSL)	11.00–18.00 (13.90±1.64) (n=137)	10.30–15.98 (12.58±15.98) (n=17)	10.28–23.39 (17.27±2.96) (n=54)
Snout width (SW)	5.40–12.81 (7.76±1.31) (n=137)	6.30–12.77 (9.17±1.74) (n=17)	5.40–12.41 (8.95±1.57) (n=54)
Intermaxillar shield height (IMH)	3.00–9.00 (4.32±0.63) (n=235)	3.30–5.20 (4.32±0.48) (n=17)	3.22–7.78 (5.61±0.86) (n=53)
Intermaxillar shield width (IMW)	4.00–12.60 (6.49±1.09) (n=234)	5.30–8.20 (6.73±0.90) (n=17)	4.22–11.62 (8.53±1.69) (n=53)
Rudiments length (RL)	1.00–6.55 (3.50±1.01) (n=169)	2.00–4.80 (3.31±0.77) (n=17)	2.68–8.56 (5.55±1.53) (n=55)
Meristic characters			
Dorsal scale transversal (DST)	11–14 (12.11±0.40) (n=103)	12 (n=17)	12 (n=55)
Ventral scale transversal (VST)	10–11 (10.01±0.10) (n=83)	10 (n=17)	10 (n=55)
Dorsal scales longitudinal (DSL)	88–119 (105.71±3.88) (n=182)	92–110 (99.30±4.09) (n=17)	88–119 (100.71±5.43) (n=55)
Ventral scales longitudinal (VSL)	111–129 (123.30±2.53) (n=201)	108–124 (118.00±3.61) (n=17)	115–137 (122.40±3.19) (n=55)
Subcaudal scale rows (SCR)	175–249 (228.68±15.43) (n=78)	173–237 (214.22±24.72) (n=9)	199–222 (208.67±7.54) (n=28)
Preanal scales (PAN)	5–11 (7.94±1.44) (n=197)	5–8 (6.37±0.72) (n=17)	10 (n=55)
Supralabial scales left (SPLI)	9–13 (11.17±0.98) (n=124)	10–12 (11.06±0.43) (n=17)	11–14 (12.13±0.85) (n=54)

Two of them are taxonomically recognized as subspecies: *P. a. apodus* (Pallas, 1775) and *P. a. thracius* (Obst, 1978). The type locality of *P. a. apodus* (Pallas, 1775), described under the name *Lacerta apoda* Pallas, 1775, was extensively but not precisely reported by Pallas (1775: 448–449 pp.) as a wide area between “... *sabulosi* Naryn inter Rhymnum et Volgam ... *Rarius etiam ad Sarpam rivum occurrit; sed frequentior in eodem, ex quo Sar-*

pa fluit, deserto Kumano versus ipsum Kumam fluvium ... circaque Terekum lecta fuit.” In 1776 (703 p.), Pallas published an abbreviated version of the report on the occurrence of the species: “*Habitat in conuallibus herbidis deserti Naryn et ad Sarpam, Kumam, Terekum fluuios*”. Additionally, for unclear reasons, Pallas subsequently restricted the range of the species to the Caucasus (with the Ciscaucasia, i.e. around Terek region) and

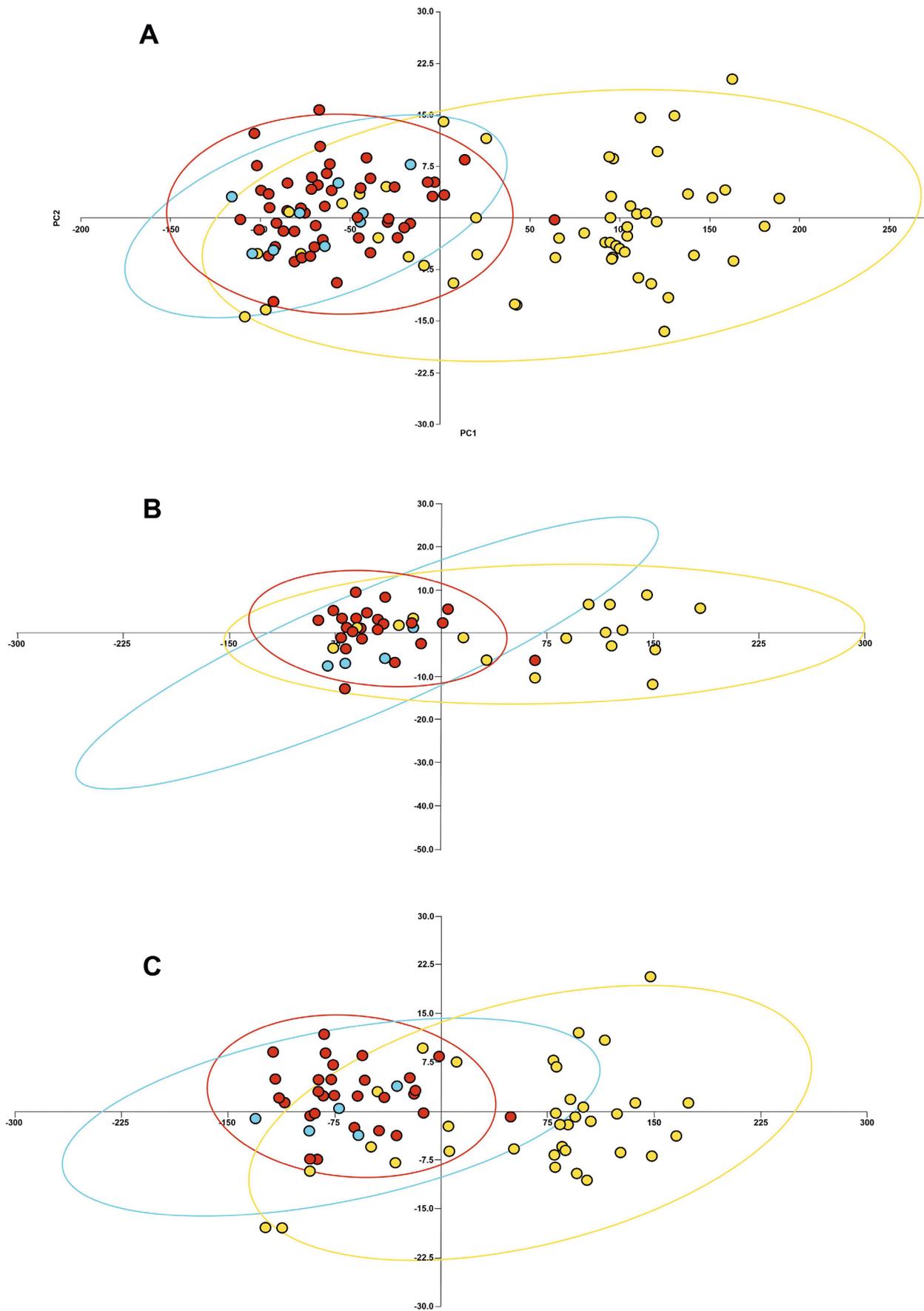


Figure 5. Principal Component Analysis (PCA) of the examined phenotypic characters among the adult specimens of *Pseudopus apodus apodus* (red dots), *P. a. thracicus* (blue dots), and *P. a. levantinus* ssp. nov. (yellow dots): A - adult females and males together, B – females only, C – males only. The oval outlines correspond to 95% confidence intervals. For A, PC1 explains 98.7% of variance, PC2 0.59%; for B, PC1 explains 98.9% of variance, PC2 0.45%; for C, PC1 explains 98.6%, PC2 0.59% (see details in Table S5), respectively.

the mountainous coast of the Crimean peninsula: “*Ad omnem Caucasum, in hortis ad Terekum et in montosae orae Tauricae Chersonesi ...*” (Pallas, 1814: 33 p.). This Pallas’ auto-correction provided ground for the assumption that the information about the occurrence of *Lacerta apoda* in the Volga-Ural interfluvium (Naryn steppe) was erroneous. Obst (1978) probably overlooked the above mentioned original range description provided by Pallas and because he knew that *P. apodus* did not occur in the Naryn Steppe (northern coast of the Caspian Sea; today’s Kazakhstan lying outside of the species range according to the current knowledge), restricted the type locality to “Terek-Gebiet” (Obst 1978: 136 p.). The valid type locality of *P. a. apodus* however, encompasses all the localities given by Pallas (1775, 1776) and mentioned above, i.e. from the Terek region to the Naryn Steppe the Naryn Steppe, included. Pallas (1776) clearly mentioned *P. apodus* from the more humid parts of Naryn Steppe though we wish to stress here that the current occurrence in this region has not been confirmed (Bakiev et al. 2020). Therefore, we used a sequence from a geographically closely related specimen from the locality of Voskresenkoe (approx. 25 km NNW of Gudermes, Tersko-Kumskaya, Terek River region, lowland Russia, 43.55°N, 46.38°E) of the *ND2* marker (GenBank accession number AF085623, Macey et al. 1999; the specimen from the herpetological collection of the California Academy of Sciences [CAS]: 182911) and microsatellite data from the locality Bilbil’-Kazmalyar, Samur Forest, Russia-Dagestan, (41.90°N, 48.48°E, distance 230 km from the Terek River; see Fig. 1, Table S1) as proxies of the genetic lineage of the nominotypic subspecies from the type locality of the species. The type locality of *P. a. thracicus*, described as *Ophisaurus apodus thracicus* Obst, 1978 (holotype MTD 5464, J. Fritzsche leg. May 1972; see Fritz 2002 for the complete details on the type series), is on the Black Sea coast of Bulgaria, Primorsko, District Burgas (~42.27°N, 27.74°E). It is related to the sequences from Primorsko i.e. MF547732 (*ND2*) and MF547717 (*Cyt b*) analyzed by Jandzik et al. (2018) and by us, and to microsatellite data from the Bulgarian Black Sea coast (Fig. 1, Table S1). Our data also confirm that *P. a. thracicus* has a wide distribution (Fig. 1). This range also includes the type locality of the name *Pseudopus Durvillii* Cuvier, 1829, which is “L’Archipel”, Greece. Therefore, we designate the name *Ophisaurus apodus thracicus* Obst, 1978 as *nomen protectum* and *P. Durvillii* as *nomen oblitum* according to the Article 23.9.1.2 of the ICZN (1999; see Appendix to this article). The animals from the Levant (analyzed here from the territory of southern Turkey, Syria, and Israel), represent a separate, allopatric lineage (Jandzik et al. 2018; Lavin and Girman 2019), genetically and morphologically differentiated from the two described subspecies (see details below). The Southern lineage of *P. apodus*, according to our results and under the current practices in reptile taxonomy and nomenclature (see Kindler and Fritz 2018; Fritz and Schmidler 2020 based on Moritz 1994), can be taxonomically defined and we described it here as a new subspecies:

***Pseudopus apodus levantinus* ssp. nov.**
Jablonski, Ribeiro-Júnior, Meiri, Maza,
Mikulíček, Jandzik

<http://zoobank.org/D1621AC7-53A4-4CD5-A958-659510199169>

The proposed common name in English, Hebrew, and Arabic: Levantine Glass Lizard, קמטן חורש לבנשתי, الشرقي, respectively.

Holotype. TAU-R 16895 (Fig. 6, Table S7), an adult male, collected on the 16th of May, 2014 by Erez Maza, at Giv’at Ada (גבעת עדה), Israel (32.52°N, 35.01°E, Fig. 7). The molecular-genetic data of the holotype are available for *ND2* (MW400924), *Cyt b* (MW400903), and microsatellites (Fig. 2, Table S1, S2).

Paratypes. TAU-R 17076 (MW400927, MW400906), an adult male, collected on 19th May 2014 by Talia Oron, at Biranit (בירנית), Israel (33.05°N, 35.34°E); TAU-R 17235 (MW400928, MW400907), an adult male, collected on 6th May 2015 by Ron Elazari, at Hadera (חדרה), Israel (32.47°N, 34.89°E); TAU-R 17895 (MW400930, MW400909), an adult male, collected on 18th April 2016 by Ofer Shimoni, on Mt. Gilboa’ (הר הגלבוע), Israel (32.45°N, 35.43°E); TAU-R 19403, an adult male, collected on 16th May 2019 by Amir Arnon, at Ramat HaNadiv (רמת הנדיב), Israel (32.55°N, 34.95°E) (Figs. 2, 8, Table S1, S2, S7).

Diagnosis. A large *Pseudopus* (up to 610 mm snout-vent length, 1,367 mm total length and a mass of 1,100 g) that can be distinguished from the other two subspecies by a combination of the following characters (means followed by standard deviations; Table 4): (1) preanal scales (PAN) 10; (2) long body (SVL; 490.22 mm ± 76.15); (3) long tail (TL; 600.70 mm ± 107.04); (4) long head (HL1; from the ear aperture to the tip of the snout, 47.6 mm ± 7.99); pileus length (PL; mean 41.98 mm ± 6.78); and distance from the frontal shield to the tip of the snout (FSL; 17.27 mm ± 2.96); (5) wide head (HW1; maximum width, 32.04 mm ± 6.34); and width at the level of the posterior edge of the eyes orbits (HW2; 28.21 mm ± 4.95); (6) relatively long limb rudiments (RL; 5.55 mm ± 1.53) (see and Tables 3 and 4 for comparisons to *P. a. apodus* and *P. a. thracicus*).

Comparisons. *Pseudopus apodus levantinus* ssp. nov. can be distinguished from *P. a. apodus* and *P. a. thracicus* by having 10 preanal scales (PAN) in all morphologically examined specimens (vs. 5–11, with the mean of 8 in *P. a. apodus*, and 5–8 and mean of 6 in *P. a. thracicus*). Other differences can be used in combination to support differences between *P. a. levantinus* ssp. nov., *P. a. apodus*, and *P. a. thracicus*: longer body (SVL; 610 mm maximum length and mean of 490.22 mm in *P. a. levantinus* ssp. nov., vs. 487 mm maximum and mean of 387.61 mm in *P. a. apodus*, and 410 mm maximum and mean of 360.06 mm in *P. a. thracicus*); longer distance between the ear aperture and the tip of the snout (HL1; 63.75 mm maximum length and mean of 47.60 in *P. a. levantinus* ssp. nov., vs. 58 mm maximum and mean of 38.61 mm in *P. a. apodus*, and 49.50 maximum and mean of 39.55 mm in *P. a. thracicus*); longer pileus (PL; 54.79 mm maximum length and mean of 41.98 in *P. a. levantinus* ssp. nov., vs. 46.30 mm maximum and mean of 35.82 mm in *P. a. apodus*, and 28.80 mm maximum and mean of 36.93 mm in *P. a. thracicus*); longer distance between the anterior

TAU 16895 - holotype

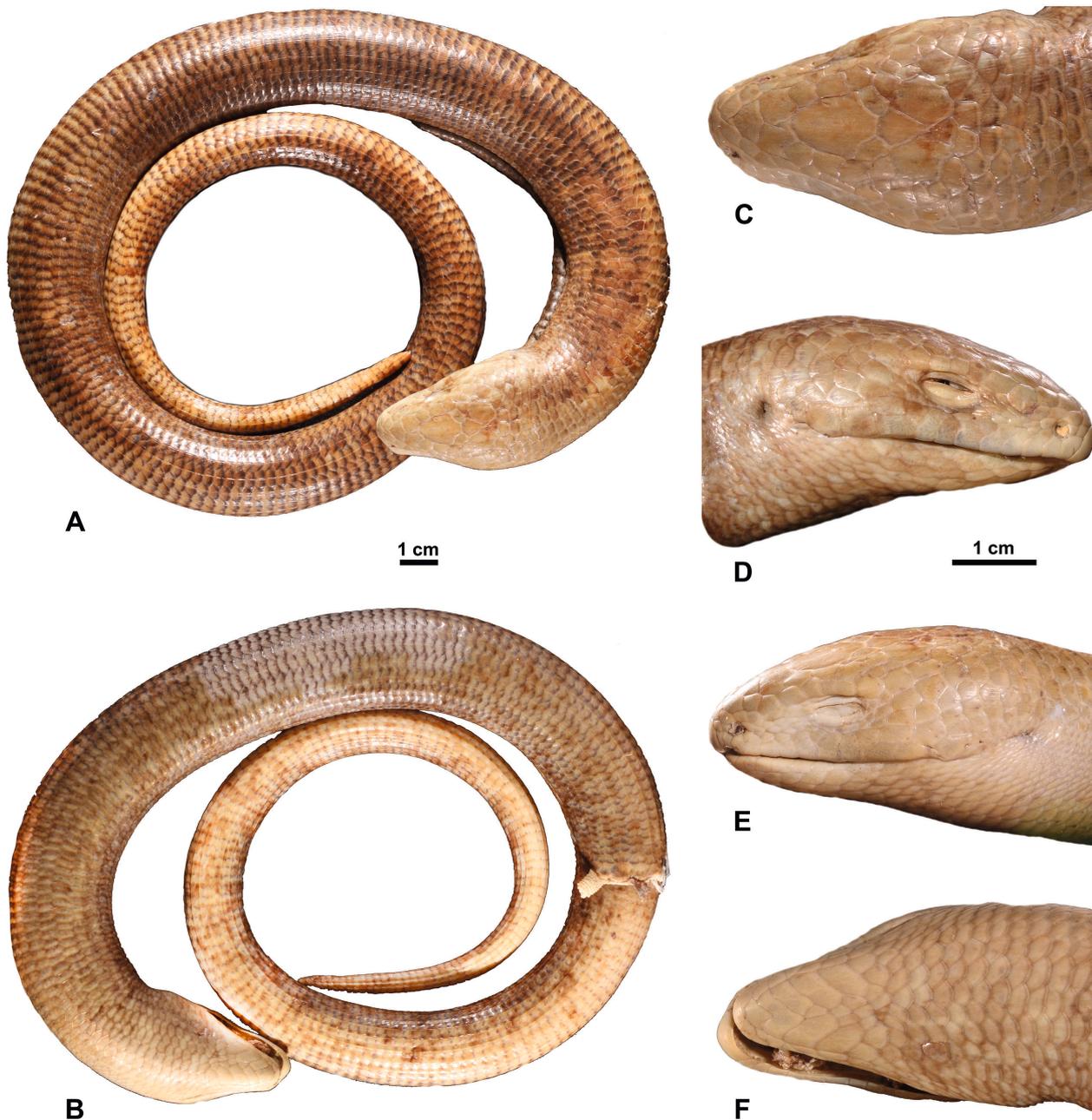


Figure 6. Holotype (TAU 16895) of *Pseudopus apodus levantinus* ssp. nov. from Giv'at Ada (גבעת עדה), Israel. A – dorsal view, B – ventral view, C – dorsal view of the head, D, E – lateral views, F – ventral view. For details and measurements see Tables S1, S2 and S7.

frontal scale to the tip of snout (FSL; 23.39 mm maximum length and mean of 17.27 mm in *P. a. levantinus* ssp. nov., vs. 18 mm maximum and mean of 13.90 mm in *P. a. apodus*, and 15.98 mm maximum and mean of 12.58 mm in *P. a. thracicus*); wider head (HW1; 44.90 mm maximum width and mean of 32.04 mm in *P. a. levantinus* ssp. nov., vs. 36.5 mm maximum and mean of 24.47 mm in *P. a. apodus*, and 29.50 mm maximum and mean of 24.35 mm in *P. a. thracicus*); wider distance between the posterior edge of the orbits (HW2; 39.33 mm maximum width and mean of 28.21 mm in *P. a. levantinus* ssp. nov., vs. 28.76 mm maximum and mean of 19.44 mm in *P. a. apo-*

dus, and 26.30 mm maximum and mean of 22.33 mm in *P. a. thracicus*); and longer limb rudiments (RL; 8.56 mm maximum length and mean of 5.55 mm in *P. a. levantinus* ssp. nov., vs. 6.55 mm maximum and mean of 3.5 mm in *P. a. apodus*, and 4.8 mm maximum and mean of 3.31 mm in *P. a. thracicus*). The remaining differences are presented in Tables 3–4.

Description of the holotype. An adult male (TAU 16895; Fig. 6, Table S7), specimen in a good state of preservation in 70% ethanol, midbody oval, and robust. All measurements of the holotype were tak-



Figure 7. Habitat of *Pseudopus apodus levantinus* **ssp. nov.** at the type locality Giv'at Ada (גבעת עדה) in Israel (photo by Ilana Rosenstein).

en post-preservation. Snout-vent length 532 mm, tail length 602 mm, weight 900 g. The number of transversal dorsal scale rows at midbody 12 (DST), the number of transversal ventral scale rows 10 (VST). Head large, length (HL1) 59.01 mm, clearly distinct from the neck. The pileus length is 50.52 mm, maximum head width 42.25 mm. The right part of the mouth is slightly open. The number of supralabials on both parts of the head is 11. Head and body scales smooth. Slightly keeled scales are visible on the ventral part of the tail. Rostral curved toward the top of the head. Frontal scale well visible and big with the length 17.73 mm and width 13.15 mm. Eyes oval, both closed. The ear and nose openings are visible. The hind limb rudiments are well visible with a length 7.42 mm. The complete tail is clearly differentiated from the body by the cloacal opening and 10 preanal scales on the ventral side. There are 154 subcaudal scale rows (SCR) overall, which is very similar to the specimens with regenerated tails. It has 106 longitudinal dorsal scale rows (DSL), and 121 ventral longitudinal scale rows (VSL).

The coloration of the holotype in life was not recorded. The coloration of the holotype in preservative is brownish or slightly orange with some of the scales on the body that have darker coloration creates an impression of tiny dark spots on the body. The head is lighter than the body, which is especially apparent on its dorsal side. The dorsal side of the body is light brown to gray, again with the impression of darker spots on the tail.

Variation. Details on variation among the type specimens of *P. a. levantinus* **ssp. nov.** are presented in Table S7. The overall morphology and coloration of the paratypes (TAU 17076, 17235, 17895) are very similar to that of the holotype; TAU 19403 was recently collected and has an evident dark-brown coloration pattern (TAU 19403, Fig. 8).

Etymology. No name is available for the glass lizards from the Levantine region. We hence suggest a new name, *Pseudopus apodus levantinus*, as a reference to the isolated and allopatric distribution of this subspecies exclusively in the Levant region. This region covers present-day (western) Syria, Lebanon, (north-western) Jordan, Israel, West Bank, Cyprus, and most of Turkey south-east of the middle Euphrates, which is almost identical to the known distribution range of the new subspecies. The term “Levant” is derived from the Italian “Levante”, meaning “rising” and implying the rising of the Sun in the east as an equivalent to the Arabic “al-Mashriq” (المشرق) and the Hebrew “Mizrah” (מִזְרָח) both meaning “east”.

Distribution. According to the genetic data, *P. a. levantinus* **ssp. nov.** occurs in southern Turkey, western Syria, northern and central Israel (Jandzik et al. 2018; this study). Based on the published distribution data, the new subspecies could also be expected in the Mediterranean regions of Lebanon and north-western Jordan (In den Bosch et al. 1998; Disi et al. 2001; Hraoui-Bloquet et al. 2002; Rifai et al. 2005; Sindaco and Jeremcenko 2008),

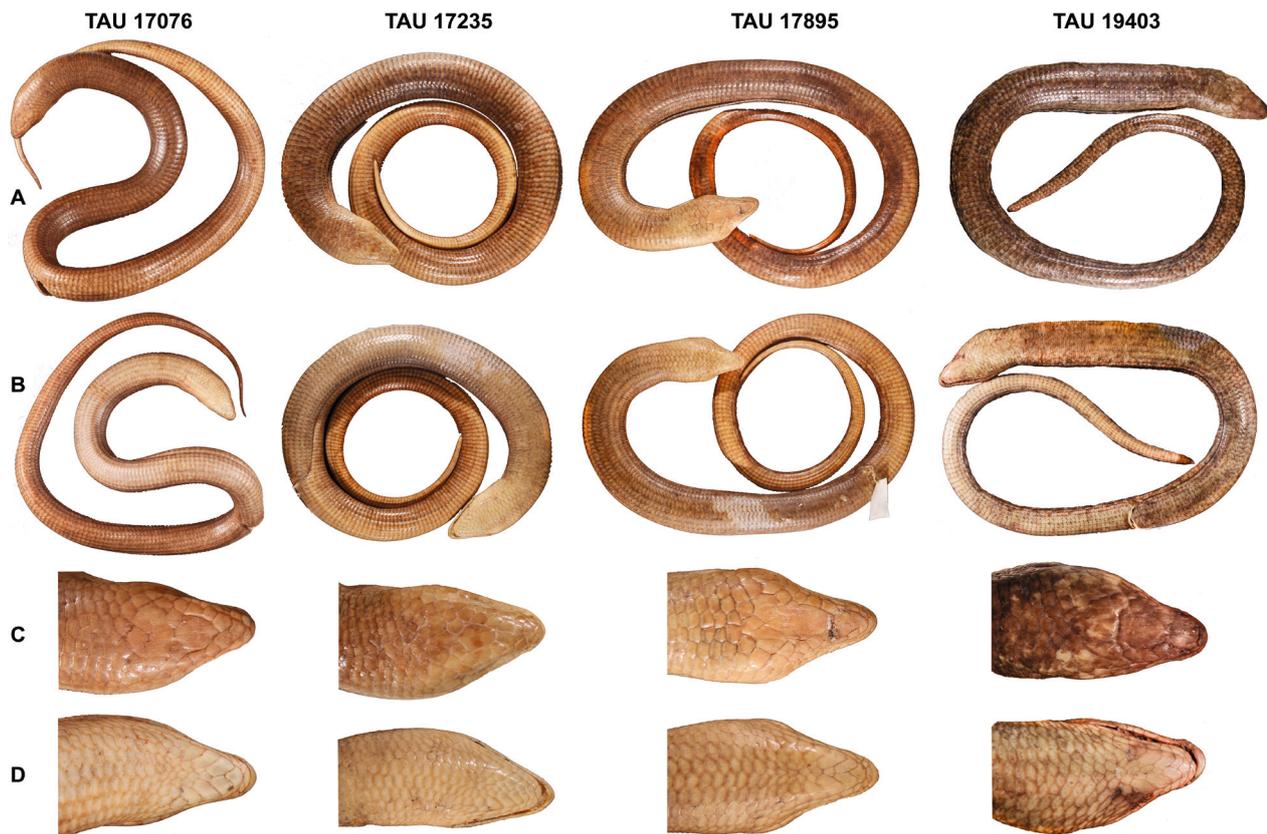


Figure 8. Paratypes of *Pseudopus apodus levantinus* **ssp. nov.** from Israel showing A – dorsal part of the body, B – ventral part of the body, C – dorsal part of the head, D – ventral part of the head. For the locality details and measurements see Tables S1, S2, and S7.

although we did not examine specimens from these regions. We therefore hypothesize that it ranges from southern Turkey (Hatay province) to central Israel (throughout the Mediterranean zone but excluding the deserts to the south and east; Figs. 3 and S4). Its northern distribution edge is presumably south of the Nur Mountains, the biogeographic barrier for the split between Anatolian and Levantine populations of other species (Jandzik et al. 2013; Tamar et al. 2015; Jablonski and Sadek 2019; Šmíd et al. 2021). This, however, needs further investigation as there is a possibility that the new subspecies could be found further north in Mersin or Adana provinces (Figs. 1, S4 and Baran et al. 1988; Sindaco and Jeremcenko 2008; see also Hofmann et al. 2018). The southern limit of the subspecies range is near the border of the Negev desert, around Lahav (31.38°N, 34.87°E), and Kisufim (31.37°N, 34.40°E) in Israel, and Dhibam (31.47°N, 35.80°E) in Jordan (Fig. S4; Disi and Amr 1998; Roll et al. 2017, the Steinhardt Museum records).

Habitat and ecology. The new subspecies is known in Mediterranean habitats of the Levant (see the type locality Fig. 7), preferring relatively well-shaded light woodlands (maquis and garrigue), dry and warm hillsides, stream banks, and agricultural fields. It is found from ~50 m below sea level (in Hefzibah, Israel; 32.52°N, 35.42°E) to the hilly sub-mountain areas in Israel, Lebanon, Syria, and Turkey (elevation maximum ~1000 m; In den Bosch et al. 1998; Disi et al. 2001; Rifai et al. 2005; Werner 2016; the Steinhardt Museum records). *Pseudopus a. levantinus* **ssp.**

nov. is a leaf-litter diurnal carnivore (Bar and Haimovich 2012). It is oviparous and females typically lay a single clutch comprised of 3–12 eggs (data from the records of the Zoological Research Garden, Tel Aviv University). Its habitat characteristics, ecology, and diet were studied in Jordan and presented by Rifai et al. (2005), however data from other countries (see Distribution) are scarce (see Arbel 1984; In den Bosch et al. 1998; Bouskila and Amitai 2001; Bar and Haimovich 2012; Werner 2016).

Diversity. The specimens analyzed in this study showed certain variation in both genetic and morphological markers. Some intra-lineage variation in *P. a. levantinus* **ssp. nov.** can be found in *ND2* and *Cyt b* sequences as well as in microsatellites (see details in the Results). The haplotype pattern of nuclear genes *PRLR* and *RAG1* indicates incomplete lineage sorting among all three subspecies, with one recorded heterozygote of *P. a. levantinus* **ssp. nov.** (Jandzik et al. 2018). The calculated genetic distances among the three main mtDNA lineages (~2.6–4.0%) and the estimated time of divergence in *Pseudopus* mostly based on the nuclear loci (~8 to 3 Mya) support the subspecies status of the Levantine population (Jandzik et al. 2018, Lavin and Girman 2019). This is in accordance with subspecific taxonomy of the closely related species *Anguis colchica* (approx. split between 2.8–2.5 Mya, genetic distances ~3.1–4.7% among defined subspecies/lineages based on *ND2* mtDNA fragment; Gvoždík et al. 2010; Jablonski et al. 2016), while the genetic distance



Figure 9. Color and pattern variation in the *Pseudopus apodus* subspecies: A – adult male of *P. a. apodus* in a typical steppe habitat from Samarkand, Uzbekistan (photo by Daniel Jablonski). B – juvenile individual of *P. a. apodus* from Kyz-Kermen, Bakhchisarayi, Crimea (photo by Oleg V. Kukushkin). C – adult female of *P. a. thracicus* from Dadia, Greece (photo by D. Jablonski). D – juvenile individual of *P. a. thracicus* from National Park Paklenica, Croatia (photo by D. Jablonski). E – adult female of *P. a. levantinus ssp. nov.* from Antakya, Turkey (photo by David Jandzik). F – juvenile individual of *P. a. levantinus ssp. nov.* from Ness Ziyona, Israel (photo by David David).

among the *Anguis* species are significantly higher (7.0% between *A. fragilis* and *A. colchica* and even 9.2% between *A. fragilis* and *A. veronensis*; Gvoždík et al. 2013). We also recorded some variation in the measurements and coloration both among the three subspecies (Figs. 5, 9) and within *P. a. levantinus ssp. nov.* Males of *P. a. levantinus ssp. nov.* differ from females by having relatively wider heads (HW1–3, SW, IWM; Table S6). The coloration of this subspecies is similar to the nominotypic subspecies (Rifai et al. 2005) but more data are needed to allow for drawing any more robust conclusions. A melanistic individual has been recorded in the Levantine population (Jablonski and Avraham 2018).

Conservation. Based on the data presented here, the distribution range of *P. a. levantinus ssp. nov.* covers approximately 30,000 km². Together with human overpopulation and accelerated development in the Mediterranean parts of the Levant, high traffic density (most individuals nowadays brought to the Steinhardt Museum of Natural History in Tel Aviv are road-kills), development of mass tourism, extensive use of pesticides in the agricultural areas, proliferation of human commensals such as domestic cats, cattle egrets, dogs, rats, and golden jackals, and challenging political situation have potential to worsen the conservation status of this endemic subspecies. Pending a formal assessment, we preliminary recommend the

IUCN category of Vulnerable (VU) based on the criteria A2c,e and strongly encourage further surveys benefiting from international collaboration allowing to open a dialogue across the conflict zones (e.g. EcoPeace Middle East, <http://ecopeaceme.org>; Roulin et al. 2017). Interestingly, recent data suggest that populations of *P. apodus* from the Levant were likely a part of modern human diet for millennia (Natufian culture, around 13,050 to 7,550 BC), which provides a rare piece of evidence of a long-term civilization pressure on the local biota (Lev et al. 2020).

Discussion

Pseudopus apodus: a species of a monotypic genus with three subspecies

Pseudopus apodus is the single extant species of the genus *Pseudopus* that has been known as a relatively common member of the fossil assemblages from the Early Miocene or possibly even the Oligocene (Klembara et al. 2010; Klembara 2015; Čerňanský et al. 2016; Villa and Delfino 2019). It has been suggested that the species gradually replaced a larger, ecologically more plastic species *P. pannonicus* during the Pleistocene (Klembara et al. 2010). The possibly lower ecological plasticity of *P. apodus* has been used to explain its more limited distribution in comparison to its closest relatives from the genus *Anguis* (Jandzik et al. 2018).

The simplified morphology and lack of conspicuous markings may be among the reasons why the species had been considered monotypic until F. J. Obst described the subspecies *P. a. thracicus* based on a slightly distinctive morphology (Obst 1978). Recently, an additional support for recognition of *P. a. thracicus* has been provided by a molecular-phylogeographic study (Jandzik et al. 2018). These authors also revealed a genetically distinct lineage in the Levant, which could potentially represent morphologically cryptic endemic subspecies. After adding further evidence from both molecular-genetic markers and from morphology, we described it here as *P. a. levantinus* ssp. nov. The newly described taxon meets the criteria of the subspecific category according to which the subspecies should occupy a distinct geographic range, and should have distinct hereditary characters including mtDNA haplotypes, nuclear alleles and ideally, but not necessarily, morphological traits (Patten 2015, Kindler and Fritz 2018, Hillis 2020). In contrast to species, subspecies are not reproductively isolated and are capable of extensive gene flow. Although we do not have evidence for present or historical gene flow between the three *P. apodus* lineages (see below), we opted for subspecific and not specific rank for it taking into account the expected time of divergence and genetic distances in relation to the divergence of closely related Western Palearctic species within the family Anguidae (see also Diversity).

This new subspecies is currently known from the area south of the Nur Mountains in Turkey, to the Mediterranean regions of Syria and Israel. While we lack direct genetic evidence, it is highly probable that also the populations from the Mediterranean coast of Lebanon and from Jordan belong to *P. a. levantinus* ssp. nov. Not so clear are the taxonomic relationships of the populations from the south-central Turkey around the Gulfs of Iskenderun and Mersin (particularly Mersin and Adana Provinces), the only area where two subspecies likely come into contact and, potentially, hybridize (Fig. 1). While the distribution shows an almost 400 km distribution gap from the closest *P. a. thracicus* populations in the west (see Baran et al. 1988 and Fig. 1), this area also lies across the Nur Mountains which are known to form a biogeographic boundary between lineages of several other reptile taxa (Jandzik et al. 2013; Jablonski and Sadek 2019; Šmíd et al. 2021). Although we expect the new subspecies to occur in these parts of southern Turkey (i.e. similar phylogeographic pattern of *Zamenis hohenackeri*; Hofmann et al. 2018), we cannot exclude that the populations west of this mountain range are distinct (*P. a. thracicus*; Baran et al. 1988) from those in the east and southeast, reflecting a possibly longer separation and range fragmentation. Alternatively, the occurrence of the nominotypic subspecies in the southern-central Turkey, is also possible, although less likely (see the biogeographical pattern of *Bufo verrucosissimus*; Özdemir et al. 2020). The most enigmatic populations of *P. apodus* are, however, in north-eastern Iraq and mid-western Iran (Schmidt 1955; Reed and Marx 1959). They are isolated from the remaining species range (Levant, Turkey, Caspian region) by vast areas of unsuitable arid habitats of the Syrian Desert and Mesopotamia in the west and likely by Zagros Mountains montane habitats in the north (Fig. 1). While the range of the nominotypic subspecies is the closest to this Iraqi and Iranian populations, they could also potentially represent old relict populations or even a distinct evolutionary lineage. It would be thus extremely interesting to learn more about their phylogenetic affiliations. With our present knowledge we cannot exclude the possibility of the historical contact with subsequent extinction but the current species range seems to be fragmented (Fig. 1). This is congruent with our genetic data and, therefore, studying possible contact zones and inter-lineage hybridization in *P. apodus* offers a further challenge.

All three subspecies are genetically differentiated in both mtDNA and nDNA. Strongly supported subspecific lineages in the phylogenetic analysis and relatively long distances among mtDNA haplotypes (Figs. 2, 3) are mirrored in non-overlapping, well-supported and distant clusters of the highly polymorphic microsatellite genotypes (Fig. 4B). All unambiguously correspond to the three subspecies. Microsatellites, as fast evolving neutral markers, show better discrimination among closely related evolutionary lineages than most nuclear genes (e.g. Spilani et al. 2019), including *RAG1* and *PRLR* used in our previous study (Jandzik et al. 2018). In the case of the sequences of these two genes, two and one out of the three identified haplotypes, respectively, were shared be-

tween two or even among all three subspecies (see Fig. 3 in Jandzik et al. 2018), which we interpreted as indicative of incomplete lineage sorting. This is not unusual among closely related evolutionary lineages of reptiles, which show monophyly in mtDNA but paraphyly in nuclear genes (e.g. Gvoždík et al. 2010; Jablonski et al. 2019; Abreu et al. 2020). We discuss alternative biogeographic hypotheses explaining this incongruence below.

In addition to the genetic level differentiation, we provide morphological evidence supporting independent evolutionary history of the three subspecies. The new subspecies differs from *P. a. apodus* and *P. a. thracicus* in several traits, most notably in the overall size. It attains significantly longer average and maximum lengths (Tables 3, 4). We note, however, that the maximum recorded size for the species (1,440 mm; Obst 1981) was reported from a specimen observed in Bulgaria (*P. a. thracicus*). In several other characters *P. a. levantinus* **ssp. nov.** differs from both remaining subspecies, while in some it is more similar to one or to the other (Tables 3, 4). These overlaps are visualized in the PCA plots (Fig. 5), in which *P. a. levantinus* **ssp. nov.** shows greater variation. Its morphospace, as manifested in the first two principle components, encompasses most of the variation existing within both *P. a. apodus* and *P. a. thracicus*, and its range along both axes (especially PC1) is greater than the combined range of the other two subspecies. So far, the only available morphological analysis of the specimens that most likely represent this Levantine subspecies comes from Jordan (not confirmed by molecular-genetic data yet), where Rifai et al. (2005) found that the lizards are characterized by morphology intermediate between *P. a. apodus* and *P. a. thracicus*. The results of our morphological analyses (and partly also of microsatellites) show a similar though not exactly the same picture. We posit that the overlap in morphology could be explained by higher variation within *P. a. levantinus* **ssp. nov.** However, overall size is a very plastic trait, often characterized by high variation even within genetically uniform groups. It can be influenced by a number of environmental variables and their interactions (Horvathova et al. 2013; Mikulíček et al. 2013; Pincheira-Donoso and Meiri 2013; Ortega et al. 2019; Slavenko et al. 2019). Interestingly, Glavaš et al. (2020) recently found striking size differences between two geographically relatively close populations of *P. a. thracicus* in Croatia. The population attaining larger size was an island population from a more extreme environment than the mainland population, generally resembling the situation among *P. a. levantinus* **ssp. nov.** and the other two subspecies. The question whether and to what extent the distinct morphology of *P. a. levantinus* **ssp. nov.** is a result of environmentally driven plasticity or independent evolutionary history of the lineage remains to be answered and would require at least a correlative study between morphological and environmental variation in all three subspecies. Studying environmentally driven phenotypic plasticity of recent *P. apodus* populations could be very informative for re-evaluation of the taxonomic identity of very similar fossil species. These are mainly characterized by different sizes and osteolog-

ical characters associated with size such as ridges, crests, and tubercles of the cranial bones or cranial and vertebral processes (e.g. *P. pannonicus*; Klembara et al. 2010, 2019).

Historical biogeography of *Pseudopus* in the Levant

The Levant is a major biodiversity hotspot (Myers et al. 2000) and has the highest reptile richness (Sindaco and Jeremcenko 2008; Roll et al. 2017) and endemism (Ficetola et al. 2018; Gumbs et al. 2020) in the Western Palearctic. The actual Levantine reptile diversity still appears to be underestimated (Jablonski et al. 2019; Šmíd et al. 2019, 2021; Kornilios et al. 2020). Our description of a new taxon in a very common, large and conspicuous lizard species is a further evidence for this. Endemism in the Levant is often associated with complex paleogeographic events that occurred in the Levant mainly in the middle Miocene and separated Eurasia from Africa-Arabia (e.g. Tamar et al. 2016) with subsequent geological instability, volcanic activity, and environmental changes, mainly aridification (Edgell 2006). All this probably facilitated the emergence of different evolutionary lineages in the Levant. The origin of endemic species in this region can be dated back to the Late Eocene (split of *Latonia* spp. and *Discoglossus* spp., or *Parvilacerta* spp. from *Anatololacerta* spp.; Biton et al. 2013; Garcia-Porta et al. 2019) or the Miocene (split of *Mediodactylus orientalis* and *M. kotschyi*, *Acanthodactylus beershebensis* and *A. maculatus*, *Phoenicolacerta kulzeri* and *P. laevis*, or *Xerotyphlops syriacus* and *X. vermicularis*; Tamar et al. 2015, 2016; Kornilios 2017; Kotsakiozi et al. 2018; Kornilios et al. 2020). Similarly, dating of some of the currently recognized lineages representing endemic subspecies places their splits to the Miocene (*Zamenis h. hohenackeri* and *Z. h. tauricus*; Hofmann et al. 2018), though the Pleistocene origin is more typical (*Pelobates s. syriacus* and *P. s. boettgeri*, *Bufotes* cf. *sitibundus*, and *Platyceps c. collaris* and *P. c. rubriceps*; Dufresnes et al. 2019a,c; Šmíd et al. 2021). Dating of the basal split among the *P. apodus* subspecies remains controversial. Jandzik et al. (2018) placed the basal split to about 2-3 Mya (Plio-Pleistocene) and discussed strong incongruence between the genetic and fossil data in the split of the sister genera *Anguis* and *Pseudopus*, while Lavin and Girman (2019) using more robust genetic data (25 nuclear loci and three mtDNA genes) but limited taxon sampling (missing *P. a. thracicus*) dated the split to the Late Miocene.

Jandzik et al. (2018) presented a biogeographic scenario in which *P. a. apodus* and *P. a. thracicus* recently expanded to their ranges after they survived the Pleistocene climatic oscillations in one or only few refugia. Deeper divergences within *P. a. levantinus* **ssp. nov.** lead us to hypothesize that this lineage did not experience significant glacial retraction and postglacial expansion and that recent diversity is of an older origin than in the other two subspecies. Our new data confirm the highest within-group genetic variation of *P. a. levantinus* **ssp. nov.**,

i.e. the highest number of *ND2* haplotypes and the highest microsatellite polymorphism and diversity (Figs. 3, 4, Tables 1, 2). This distinct structure is also reflected in morphology, with higher variance in the new subspecies than in *P. a. apodus* and *P. a. thracicus* (Fig. 5, Table 4). Several newly revealed, and distantly related, mtDNA haplotypes of *P. a. levantinus ssp. nov.* are separated by numerous missing haplotypes suggesting longer independent evolution (and/or sampling artefacts) in more stable environment, rather than repeated extinctions and expansions.

The molecular-genetic data we obtained from different markers, and by different methods, show some incongruence in detailed relationships among the three subspecies. The genetic distances, network, tree and PCA analyses using mtDNA sequences (Figs. 1–4A) show that *P. a. levantinus ssp. nov.* is more distant from both *P. a. apodus* and *P. a. thracicus* than they are from each other (Figs. 2, 3). However, closer relationships of *P. a. levantinus ssp. nov.* to *P. a. thracicus* than to *P. a. apodus* based on microsatellite analyses (Fig. 4B, Table 1, 2) corroborate findings from the sequences of the nuclear genes (Jandzik et al. 2018), in which *P. a. levantinus ssp. nov.* and *P. a. thracicus* share more nDNA haplotypes than any other combination of the remaining subspecies. This pattern suggests three alternative scenarios: i) it could be an artefact of incomplete lineage sorting, ii) sharing the nuclear haplotypes and higher variability in microsatellites could have resulted from the past hybridizations of *P. a. levantinus ssp. nov.* with the remaining two subspecies over large portions of the range, resulting in massive nuclear introgressions. Alternatively, iii) *P. a. levantinus ssp. nov.* could have split from *P. a. apodus* (since they have the lowest Nei's distance), after the latter diverged from *P. a. thracicus*. Then the mtDNA diversity would be largely influenced by bottlenecks following the range restrictions. Neither of these scenarios is substantially supported by the existing data and a better picture of the biogeographic history of *P. apodus* awaits further study.

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Appendix

Morphological and genetic differentiation in the anguid lizard *Pseudopus apodus* supports the existence of an endemic subspecies in the Levant

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Pseudopus apodus thracius (Obst, 1978) is nomen protectum

To maintain the current usage of *Pseudopus apodus thracius* (Obst, 1978), we qualify the name *Ophisaurus apodus thracius* Obst, 1978 as *nomen protectum* with respect to *nomen oblitum* *Pseudopus Durvillii* Cuvier, 1829 (type locality: “L’Archipel”, Greece). According to the Article 23.9.1.2 of ICZN (1999) the application of the Principle of Priority is moderated, and prevailing usage must be maintained when “the junior synonym or homonym has been used for a particular taxon, as its presumed valid name, in at least 25 works, published by at least 10 authors in the immediately preceding 50 years and encompassing a span of not less than 10 years”. To fulfill the Article 23.9.2 requirements and give evidence that conditions of the Article 23.9.1.2 are met, we present the following list of publications where the name “*thracius*” was used as a valid name:

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