# Pleistocene extinctions and recent expansions in an anguid lizard of the genus Pseudopus 

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#### Abstract

Climatic changes during the Pleistocene played an instrumental role in the shaping recent distribution and diversity of the Western Palearctic biota. Range oscillations often lead to allopatric differentiation followed by the establishment of secondary contact zones. As a result, many species are composed of complex networks of phylogenetic lineages with different histories. Pseudopus apodus is the only surviving member of an ancient genus of Western Palearctic anguid lizards (Anguidae) distributed from the Balkans through Anatolia and Caucasus to central Asia. Here, we used mitochondrial and nuclear DNA sequences to reconstruct the evolutionary history of the species in phylogeographic and demographic frameworks. Our analyses revealed three main phylogenetic lineages that diverged during or shortly before the Pleistocene. Two of them more or less correspond to the known subspecies, and their low genetic variability suggests relatively recent dispersal and colonization of vast parts of the range. The third, southern, lineage is more geographically restricted and diversified than the other two. This pattern shows that the Quaternary climatic oscillations presumably caused repeated large-scale population extinctions of the species, depleting most of its diversity. Only a few refugia located in Anatolia, Levant, and Transcaucasia served as sources for subsequent recolonization to the areas of the recent distribution. This is in contrast to many other Western Palearctic reptiles that survived unfavorable climatic conditions in numerous local refugia and sanctuaries, which resulted in more complex phylogenetic structure.


## KEYWORDS

anguidae, phylogeography

## 1 | INTRODUCTION

The origin of most Western Palearctic reptile species dates back to the Miocene (Oosterbroek \& Arntzen, 1992),
although present diversity has been largely affected by unstable climates during the Quaternary (Araújo et al., 2008; Hewitt, 2000). Recurring climatic fluctuations caused successive extinctions and recolonizations, which are reflected
in range contractions and expansions. These climatic pressures fragmented populations of the species and pushed them back to restricted areas during ice ages. Some species survived through glacial cycles in larger areas, sanctuaries of biological diversity, and preserved some of their ancestral diversity (Recuero \& García-París, 2011). These species and species complexes are usually characterized by old, deep divergences among lineages. Other taxa, however, underwent series of general extinctions, lost most of their intraspecific diversity, and their recent ranges were colonized from small areas that preserved only portions of ancestral diversity resulting in shallower diversification of lineages (Recuero \& García-París, 2011).

Legless anguid lizards of the genus Pseudopus Merrem, 1820 have a long history in the Western Palearctic region. Thanks to the robust morphology and integument supported by osteoderms, remnants of at least five species have been well preserved in the fossil record originating from various places and stratigraphic layers of western, central, and eastern Europe, as well as Anatolia and Caucasus (e.g., Blain, 2009, 2016; Čerňanský et al., 2017; Klembara, 1981, 2015; Venczel \& Sen, 1994). They have been present in this region since at least the Early Miocene (Klembara, 2015). However, only one species, Pseudopus apodus (Pallas, 1775), has survived until the present time in a longitudinally oriented range that spreads from the coastal Balkans in the west through Anatolia, western Levant, the Caucasus Isthmus, the Black Sea, and the southern Caspian region to central Asia in the east (Figure 1; Obst, 1981; Sindaco \& Jeremcenko, 2008).

Due to their similar morphologies, P. apodus was once considered a close relative of North American members of
the genus Ophisaurus (e.g., Obst, 1981), which historically also comprised East Asian anguines now belonging to the genus Dopasia, and North African species Hyalosaurus koellikeri (de Pous et al., 2011; Nguyen et al., 2011). However, molecular-phylogenetic analyses suggested that the genus was paraphyletic and P. apodus rather forms a monophyletic clade with partly sympatric Western Palearctic genus Anguis (Macey et al., 1999; Pyron, Burbrink, \& Wiens, 2013). In a region where biota has been shaped by complex geoclimatic history, only two subspecies have been described to date: nominotypic form from the eastern part of the range and Pseudopus apodus thracius (Obst, 1978) from the western regions (Obst, 1978, 1981). Recognition of a separate form was based on morphological differentiation, but doubts were published about exact distribution of the subspecies and sufficiency of the diagnostic characters for an unambiguous identification (Rifai et al., 2005; Szczerbak \& Tertyshnikov, 1989; Thieme, 1979). Recently, Keskin, Tok, Hayretda, Çiçek, and Ayaz (2013) studied genetic structure of northern Anatolian P. apodus and found that there might exist hidden diversity that is not properly reflected in recent taxonomy. This somehow resembles the past situation in the closest recent relative of P. apodus, genus Anguis, in which simplified morphology obscured real diversity before methods of molecular-phylogenetic analysis helped reveal deep divergences among several phylogenetic lineages (Gvoždík, Jandzik, Lymberakis, Jablonski, \& Moravec, 2010; Gvoždík et al., 2013; Jablonski et al., 2016; Thanou, Giokas, \& Kornilios, 2014). Similar to Anguis, P. apodus occurs in the region that harbored refugial populations during the glacial periods and rich fossil material shows presence of the genus


FIGURE 1 Distribution range (based on Sindaco \& Jeremcenko, 2008) and localities of Pseudopus apodus sampled for this study. Colors of the locality circles correspond to the main phylogenetic lineages [Colour figure can be viewed at wileyonlinelibrary.com]
throughout a long period of time. Morphology and ecology of this species also suggest that its distribution and genetic structure might be affected by topography, at least in parts of the range. All of this raises a question whether the evolutionary history of $P$. apodus is similarly complex as that of the genus Anguis or if it rather shows unique patterns and characteristics.

With the aim to better understand the evolutionary history of $P$. apodus, we analyzed mitochondrial DNA (mtDNA) and nuclear DNA ( nDNA ) nucleotide sequences of individuals originating from the entire range, reconstructed the phylogeography and demography, and present a possible scenario of biogeographic history and intraspecific differentiation of the species.

## 2 | MATERIALS AND METHODS

As a source of DNA, we used oral swabs, blood from the caudal vein or scale clips from living individuals, and liver or muscle biopsies from ethanol-preserved or road-killed animals. In total, we analyzed DNA from 46 specimens of P. apodus collected at 38 localities from across the species range (Figure 1, Table 1).

Total genomic DNA was extracted from tissue using standard phenol-chloroform extraction followed by ethanol precipitation. For molecular-genetic analyses, we used fragments of two mitochondrial and two nuclear protein-coding genes. Gene coding NADH dehydrogenase subunit 2 (ND2) was amplified using primers L4437n and H5934 (Gvoždík et al., 2010; Macey et al., 1999), a fragment of cytochrome $b$ (Cytb) with primers L14910 and H16064 (Burbrink, Lawson, \& Slowinski, 2000), the protein-coding gene for prolactin receptor $(P R L R)$ with primers PRLR_f1 and PRLR_r3 (Townsend, Alegre, Kelley, Wiens, \& Reeder, 2008), and re-combination-activating gene 1 (RAG1) with primers R13 and R18 (Groth \& Barrowclough, 1999). We used standard PCR protocols with varying annealing temperatures and elongation times for each gene: $49.5^{\circ} \mathrm{C}$ annealing temperature and 1:15 min elongation time for $N D 2,46^{\circ} \mathrm{C}$ and $1: 30 \mathrm{~min}$ for Cytb, and $48.5^{\circ} \mathrm{C}$ and $1: 40 \mathrm{~min}$ for $P R L R$ and $R A G 1$. The purified PCR products were sent for sequencing to Macrogen Inc. (Seoul, South Korea, http://www.macrogen.com). The PCR amplicons were sequenced with internal primers: ND2 with AND2inR2 (Gvoždík et al., 2010), Cytb with AcbHin (5'-GGTGAAATCGGATTTTG-3'), $P R L R$ with PRLRiF1 ( $5^{\prime}$-AGGAAACAGACAGTGACTCA- $3^{\prime}$ ), and $R A G 1$ with RAG1iF1 ( $5^{\prime}$-TTCAAAGGAAAKTCAGCAAT- $3^{\prime}$ ). All newly obtained sequences were deposited in GenBank under accession numbers MF547716-MF547739 (Table 1). For molecular-phylogenetic analyses, we supplemented our datasets with Pseudopus or other anguid sequences from the following studies: Macey et al. (1999), Slowinski and Lawson
(2002), Castoe, Jiang, Gu, Wang, and Pollock (2008), Gvoždík et al. (2010), and Keskin et al. (2013) (see details in Table 1).

After manual aligning and trimming, low-quality sequence ends the resulting alignments contained sequences of $727-\mathrm{bp}$ fragment of ND2, 591-bp fragment of Cytb, 463-bp fragment of PRLR, and 580-bp fragment of RAG1. No stop codons were detected when checked in the program DnaSP 5.00 (Librado \& Rozas, 2009). We constructed Bayesian (BI) and maximum likelihood (ML) phylogenetic trees, separately for ND2 and Cytb datasets, using all obtained sequences. The best-fit model of sequence evolution was selected by the Bayesian information criterion as implemented in MrModeltest 2.3 (Nylander, 2004) and jModelTest 2.1.3 (Darriba, Taboada, Doallo, \& Posada, 2012), respectively. Bayesian trees were constructed using MrBayes 3.2 (Ronquist et al., 2012). The analysis was set with partitions for each codon position within ND2 (1st/2nd/3rd codon position: HKY + I/HKY/ HKY) and Cytb (K80 + I/HKY + I/GTR + I) with parameters optimized during the run. Two independent BI analyses were run each with four coupled chains that were run for six million generations. Parameter and tree samples were saved every 100th generation and the sampled trees resulted into a $50 \%$ majority-rule consensus tree after discarding the first $1 / 10$ of trees as the burn-in. The posterior probabilities were calculated as the frequency of samples recovering any particular clade (Ronquist et al., 2012). PhyML 3.1 (Guindon et al., 2010) was used for ML analysis with the best approach combining the nearest neighbor interchanges with the subtree pruning and regrafting algorithm and using the $\mathrm{TrN}+\mathrm{I}$ and HKY + G models of sequence evolution for ND2 and Cytb datasets, respectively. Branch support was quantified as bootstrap values based on 100 resampled datasets (Guindon et al., 2010).

Haplotype networks for all genetic markers were constructed using the statistical parsimony algorithm implemented in TCS 1.21 (Clement, Posada, \& Crandall, 2000) under the $95 \%$ limit of parsimony.

Uncorrected $p$-distances among the sequences of both analyzed mtDNA genes were calculated using PAUP* 4.0b10 (Swofford, 2003) and then manually averaged between the taxa.

Historical demography of the two main clades representing two subspecies of $P$. apodus was analyzed using several approaches and the more numerous Cytb dataset. First, we tested the hypothesis of rapid expansion with the Fu's $F_{\mathrm{S}}$ (Fu, 1997) and $R_{2}$ (Ramos-Onsins \& Rozas, 2002) neutrality tests implemented in the program DnaSP 5.10.01 (Librado \& Rozas, 2009). The significance of the Fu's $F_{\mathrm{S}}$ and $R_{2}$ tests was obtained by examining null distribution of 10.000 coalescent simulations of these statistics. We also used DnaSP to graphically present the mismatch distributions, which are distributions of observed vs. expected pairwise nucleotide
TABLE 1 List of sampling localities and ND2, cytochrome $b$ (Cytb), PRLR, and RAG1 genotypes of Pseudopus apodus and outgroup taxa

| Locality no. | Taxon | Country | Locality | $N$ | Lat. | Long. | Genotypes ND2/ Cytb/PRLR/RAG1 | GenBank | Reference |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| 1 | Pseudopus apodus thracius | Croatia | Krk | 1 | 45.07 | 14.57 | Pat1/--/- | FJ666589/--/- |  |
| 2 |  |  | Starigrad | 1 | 44.31 | 15.47 | Pat1/c6/P1/R1 | FJ666589/MF547730/MF547734/MF547737 |  |
| 3 |  |  | Gradac | 1 | 43.11 | 17.35 | Pat1/c6/-/- | FJ666589/MF547730/-/- |  |
| 4 |  |  | Bacina | 1 | 43.08 | 17.39 | Pat1/c8/-/- | FJ666589/MF547731/-/- |  |
| 5 |  |  | Dubrovnik | 1 | 42.65 | 18.13 | Pat1/-/-/- | FJ666589/--/- |  |
| 6 |  | Albania | Zogaj | 1 | 42.07 | 19.39 | Pat1/c6/P1/R1 | FJ666589/MF547730/MF547734/MF547737 |  |
| 7 |  |  | Diviakë | 1 | 40.95 | 19.47 | Pat1/c6/-/- | FJ666589/MF547730/-/- | ND2 from Gvoždík et al. (2010) |
| 8 |  | Greece | Corfu | 1 | 39.49 | 19.86 | Pat1/c6/-/- | FJ666589/ MF547730/-/- |  |
| 9 |  |  | Lefkada | 1 | 38.59 | 20.56 | Pat1/c6/P1/R1 | FJ666589/MF547730/MF547734/MF547737 |  |
| 10 |  |  | Menikio | 1 | 41.10 | 23.91 | Pat1/c6/-/R1 | FJ666589/MF547730/-/MF547737 |  |
| 11 |  |  | Ládi | 1 | 41.48 | 26.22 | Pat1/-/-/- | FJ666589/--/- |  |
| 12 |  |  | Mavroklissi | 2 | 41.36 | 26.31 | Pat1/c6/P1/R1 | FJ666589/MF547730/MF547734/MF547737 |  |
| 13 |  | Bulgaria | Dimchevo | 1 | 42.40 | 27.43 | Pat1/c6/P1/R1 | FJ666589/MF547730/MF547734/MF547737 |  |
| 14 |  |  | Primorsko | 1 | 42.30 | 27.77 | Pat2/c12/P1/R1 | MF547717/MF547732/MF547734/MF547737 |  |
| 15 |  | Turkey | Üsküp | 1 | 41.63 | 26.68 | Pat1/c6/-/- | FJ666589//MF547730/-/- |  |
| 16 |  |  | Bergama | 2 | 39.13 | 27.18 | Pat1/c6/-/- | FJ666589/ MF547730/-/- |  |
| 17 |  |  | *Kuzulimanı |  | 40.23 | 25.95 | -/c9/--/- | -/JX987428/-/- | Keskin et al. (2013) |
| 18 |  |  | *Gökçeada 1 |  | 40.23 | 25.90 | -/c6/--/ | -/JX987429/-/- | Keskin et al. (2013) |
| 19 |  |  | *Gökçeada 2 |  | 40.18 | 25.87 | -/c6/--/ | -/JX987438, JX987439/-/- | Keskin et al. (2013) |
| 20 |  |  | *Behramlı |  | 40.13 | 26.28 | -/c6/--/ | -/JX987424, JX987425/-/- | Keskin et al. (2013) |
| 21 |  |  | *Kampüs |  | 40.11 | 26.42 | -/c6/--/ | -/JX987422/-/- | Keskin et al. (2013) |
| 22 |  |  | *Assos |  | 39.49 | 26.35 | -/c6/--/ | -/JX987427/-/- | Keskin et al. (2013) |
| 23 |  |  | *Kavak |  | 40.61 | 26.89 | -/c6, -c11/-/- | -/JX987421, JX987423/-/- | Keskin et al. (2013) |
| 24 |  |  | *Uzunköprü |  | 41.27 | 26.70 | -/c6/--- | -/JX987434/-/- | Keskin et al. (2013) |
| 25 |  |  | *Sarköy |  | 40.60 | 27.11 | -/c6/--/- | -/JX987433/-/- | Keskin et al. (2013) |
| 26 |  |  | *Uçmakdere |  | 40.80 | 27.37 | -/c6/--/- | -/JX987426/-/- | Keskin et al. (2013) |
| 27 |  |  | *Bigadiç |  | 39.40 | 28.13 | -/c10/-/- | -/JX987431/-/- | Keskin et al. (2013) |
| 28 |  |  | *Bursa |  | 40.17 | 29.12 | -/c7/-- | -/JX987435/-/- | Keskin et al. (2013) |
| 29 |  |  | *Sarıkum |  | 42.02 | 35.17 | -/c13/-/- | -/JX987430/-/- | Keskin et al. (2013) |
| 30 |  |  | *Bafra |  | 41.66 | 36.06 | -/c13, c14/-/- | -/JX987436, JX987437/-/- | Keskin et al. (2013) |

TABLE 1 (Continued)

| Locality no. | Taxon | Country | Locality | $N$ | Lat. | Long. | Genotypes ND2/ Cytb/PRLR/RAG1 | GenBank | Reference |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| 31 | Pseudopus apodus apodus | Ukraine | Foros | 1 | 44.40 | 33.78 | Paa1/--/- | AF085623/--/- |  |
| 32 |  |  | Mt. Kyz-Kermen | 2 | 44.71 | 33.92 | Paa4/c1/P2/R2 | MF547721/MF547725/MF547735/MF547738 |  |
| 33 |  |  | Masandra | 1 | 44.52 | 34.21 | Paa1/c1/P2/R2 | AF085623/MF547725/MF547735/MF547738 |  |
| 34 |  |  | Cape Kazantip | 1 | 45.47 | 35.85 | Paa1/c1/P2/R2 | AF085623/MF547725/MF547735/MF547738 |  |
| 35 |  |  | Kerch | 2 | 45.38 | 36.06 | Paal/c1/-/- | AF085623/MF547725/-/- |  |
| 36 |  | Russia | Kabardinka | 1 | 44.63 | 37.93 | Paal/cl/-- | AF085623/MF547725/-/- |  |
| 37 |  |  | Sochi | 1 | 43.57 | 39.75 | Paa1/c1/P2/R3 | AF085623/MF547725/MF547735/MF547739 |  |
| 38 |  |  | Voskresenskaya | 1 | 43.35 | 46.10 | Paa1/--/- | AF085623/--/- | Macey et al. (1999) |
| 39 |  | Turkey | *Digor/Kars |  | 40.24 | 43.61 | -/c1/-/- | -/JX987432/-/- | Keskin et al. (2013) |
| 40 |  | Georgia | Pitsunda | 1 | 43.02 | 40.35 | Paa1/c1/P2/R3 | AF085623/MF547725/MF547735/MF547739 |  |
| 41 |  |  | Ldzaa | 1 | 43.17 | 40.37 | Paal/c1/-/- | AF085623/ MF547725/-/- |  |
| 42 |  |  | Sagarejo | 1 | 41.74 | 45.33 | Paa1/c3/P1P2/ R2R3 | AF085623/MF547727/MF547734, MF547735/MF547738, MF547739 |  |
| 43 |  |  | Dedop'lis Tskaro | 1 | 41.43 | 46.10 | Paa1/c1/-- | FJ666588/MF547725/-I- | ND2 from Gvoždík et al. (2010) |
| 44 |  | Azerbaijan | Shemakha | 3 | 40.65 | 48.62 | $\mathrm{Pa} 2 / \mathrm{c} 4 /-$ - Pa 2 2/c5/-- | MF547719/MF547728/-/-,MF547719/MF547729/-- |  |
| 45 |  |  | Divichi | 3 | 41.29 | 49.05 | Paal/c4/-/- | AF085623/MF547728/-/- |  |
| 46 |  | Iran | Khorkhora | 1 | 37.85 | 45.80 | Paa3/c1/P1/R2 | MF547720/MF547725/MF547734/MF547738 |  |
| 47 |  |  | Torbat-e-Jam | 1 | 35.23 | 60.62 | Paal/c1/--- | AF085623/MF547725/-/- |  |
| 48 |  | Uzbekistan | Samarkand | 1 | 39.45 | 66.97 | Paa1/c2/P2/R2 | AF085623/MF547726/MF547735/MF547738 |  |
| 49 |  |  | Jalair | 1 | 39.88 | 68.58 | Paa1/c1/P1P2/R2 | AF085623/MF547725/MF547724, MF547725/MF547738 |  |
| 50 | Pseudopus apodus Southern lineage | Turkey | Antakya 1 | 1 | 36.20 | 36.17 | PaS1/c15/P1P3/R2 | MF547722/MF547733/MF547724, MF547725/MF547728 |  |
| 51 |  |  | Antakya 2 | 1 | 36.20 | 36.18 | PaS2/-/P1P2/- | MF547723/-/MF547734, MF547735/- |  |
| 52 |  | Israel | Wadi Keziv | 1 | 33,04 | 35,22 | PaS3/-/P2/R2 | MF547724/-/ MF547735/ MF547738 |  |
|  | Anguis fragilis | Czech <br> Republic | Stráž nad Ohří | 1 | 48.15 | 17.07 | f1/--/- | FJ66654/--/- | Gvoždík et al. (2010) |
|  |  |  | Unknown |  | - | - | -/unnamed/-/- | -/AY099996.1/-/- | Slowinski \& Lawson (2002) |
|  | Hyalosaurus koellikeri | Morocco | Kenitra | 1 | 34.27 | $-6.60$ | unnamed/--/- | AF085621/--/- | Macey et al. (1999) |
|  | Ophisaurus attenuatus | United States of America | Unknown |  | - | - | -/unnamed/-/- | -/EU747729.1/-/- | Castoe et al. (2008) |

site differences under the expansion model in all individuals within the studied group, that is, within each of the two subspecies.

Additionally, we also inferred the past population dynamics in both main recovered mtDNA clades using the Bayesian coalescent-based approach of the Bayesian skyline plots (BSP; Drummond, Rambaut, Shapiro, \& Pybus, 2005) implemented in BEAST 2.1 (Bouckaert et al., 2014). Preliminary analyses were run using both strict molecular clock and uncorrelated lognormal relaxed molecular clock. The parameter of the standard deviation of the uncorrelated lognormal relaxed clock was close to zero, and thus, the final analyses were run enforcing the strict molecular clock model but without setting the clock rate (i.e., clock rate $=1.0$ ). Using PartitionFinder v1.1.1. (Lanfear, Calcott, Ho, \& Guindon, 2012), all codon positions treated together as one partition and the HKY substitution model were selected as the best-fit partitioning scheme and the best-fit model, respectively, for each clade. The final BSP analyses were run in duplicates to check for consistency between runs, each for at least 10 million generations (or more according to each dataset until the effective sample size $[\mathrm{ESS}]>200$ was achieved) and sampled every 1,000 generations. Convergence, ESS, stationarity, and appropriate number of generations to be discarded as burn-in (10\%) were assessed using Tracer 1.6 (Rambaut, Suchard, Xie, \& Drummond, 2013), where also the resulting BSPs were summarized.

## 3 | RESULTS

## 3.1 | Variation in mtDNA and phylogenetic relationships

Both analyzed mtDNA genes, ND2 and Cytb, are variable in $P$. apodus. The 727-bp fragment of $N D 2$ forms seven unique haplotypes and the 591-bp fragment of Cytb revealed nine haplotypes, which were supplemented by an additional six haplotypes obtained from 429-bp fragment of the same gene from the study of Keskin et al. (2013). Phylogenetic reconstructions, performed separately for both fragments due to the different representation of samples in both datasets, inferred three lineages within the species with high supports in both BI and ML analyses (Figure 2). Two of the clades correspond to the recognized and widespread subspecies, Pseudopus apodus apodus and P. a. thracius, while the third clade comprising samples from Southern Turkey and Israel represents a so far undescribed form (see Figure 1). We call this lineage with unresolved nomenclature the "Southern lineage" hereafter. The relationships of these three groups remain somewhat ambiguous, with no statistical support for either relationship in ML trees, high support for sister relationship of P. a. apodus and P. a. thracius
lineages in ND2 BI tree, but only low support in Cytb BI tree (Figure 2).

The genetic distances based on uncorrected p-distances are relatively low in both mtDNA fragments and while they do not reach 3\% between P. a. apodus and P. a. thracius, they are between $3 \%$ and $4 \%$ when these two taxa are compared to the Southern lineage (Table 2).

The subspecies $P$. a. apodus has four unique haplotypes in ND2 and five in Cytb, while P. a. thracius has only two ND2 haplotypes but nine Cytb haplotypes. However, the P. a. thracius ND2 sequences originated from only 16 localities, while the Cytb dataset contained P. a. thracius sequences from additional localities in Turkey (obtained from Keskin et al., 2013), which added more variation to this dataset. Compared to $P$. a. apodus and $P$. a. thracius, a surprisingly high variation was found in the Southern lineage, where three $N D 2$ sequences belong each to a unique haplotype (Table 1, Figure 2).

The structure within the two widespread lineages corresponding to $P$. a apodus and P. a. thracius is very similar as is illustrated in haplotype networks of both mtDNA markers: samples collected from the vast majority of localities belong to one haplotype and the remaining haplotypes are derived more or less directly from the most common haplotype as suggested by the star-like pattern of the networks (Figure 2). This simple picture seems to be a bit more complicated in Cytb of P. a. thracius in which some samples from localities in Turkey (obtained from Keskin et al., 2013) form a complex network of relationships. Haplotypes of the Southern lineage are relatively distant from those of the two widespread lineages, and haplotype network in ND2 marker indicates numerous missing haplotypes (Figure 2). It is worth noting that samples from two very close localities (Antakya, Turkey, Localities 50 and 51 in Figure 1 and Table 1) belong to two separate, relatively divergent, haplotypes.

## 3.2 | Variation in nuclear genes

Three distinct haplotypes were found in each of the two studied nuclear markers (Figure 3), with two polymorphic sites in the 463-bp PRLR fragment as well as in the 580-bp RAG1 fragment. Surprisingly, all mutations were non-synonymous with the exception of one synonymous substitution in RAG1. Among the analyzed individuals, were four heterozygotes. All of them were heterozygous in one nucleotide position in PRLR, but only one of them was also heterozygous in one position in RAG1 (Table 1). All P. a. thracius belong to one PRLR and RAGl haplotype, while both P. a apodus and the Southern lineage specimens are more variable with two and three haplotypes in PRLR and one and two haplotypes in $R A G 1$, respectively. The most common $P R L R$ haplotype ( P 1 ) is shared among all three lineages, two of the less common PRLR and RAG1 haplotypes (P2 and R2) are shared among P. a. apodus and the Southern lineage, while


FIGURE 2 Phylogenetic relationships of Pseudopus apodus ND2 and cytochrome $b$ sequences presented as Bayesian trees and haplotype networks. The numbers at the tree nodes represent Bayesian Posterior Probabilities/Bootstraps showing branch support. Different colors highlight three main monophyletic groups and the color code corresponds to the code in Figure 1. The names of the terminal tree branches show the locality numberlocality, haplotype name of the analyzed samples (see also Figure 1 and Table 1). Sizes of the circles in haplotype networks are proportional to the relative frequency of haplotypes they represent and, small black circles represent missing haplotypes [Colour figure can be viewed at wileyonlinelibrary.com]
the least common haplotypes (P3, R1, and R3) are exclusive for one clade each (Table 1, Figure 3). This pattern indicates incomplete sorting among all three lineages. Higher variability in nuclear sequences corresponds with the observed number of heterozygotes-none in $P$. a. thracius, but two in both $P$. a. apodus and the Southern lineage.

The haplotype networks are simple in both analyzed markers, with one most common central haplotype that is also present in the most lineages and two derived from this one, each by one mutation step (Figure 3).

## 3.3 | Demography

Values of the $R_{2}$ neutrality tests calculated for $P$. a. apo$d u s$ and $P$. a. thracius were positive suggesting population growth, although only in $P$. a. thracius showed a high statistical significance ( $R_{2}=0.14, p=0.0004$ ). The Fu's $F_{\mathrm{S}}$ neutrality tests indicated population growth only in $P$. a. thracius where the $F_{\mathrm{S}}$ value was negative. However, the test was not statistically significant, presumably due to small sample size as the Fu's $F_{\mathrm{S}}$ is more powerful in larger datasets (Fu, 1997; Ramos-Onsins \& Rozas, 2002).

The mismatch distributions indicated population growth in both tested groups. The diagrams show unimodal distribution suggesting single population under expansion in each group (Figure 4a,b).

The BSPs inferred population growth in both analyzed subspecies based on the median values for the population size (Figure 4c,d). In P. a. thracius, the population growth seems to be starting approximately three times earlier than in P. a. apodus reaching a plateau in the recent while continuing till present in the latter (Figure 4c,d).

## 4 DISCUSSION

## 4.1 | Genetic structure and phylogenetic relationships

Our molecular-phylogenetic analysis of $P$.apodus samples from throughout the entire range revealed three main clades existing within this species. Two of them support the previously proposed concept of two widely distributed forms: (i) nominotypic subspecies covering the eastern part of the range and (ii) P. a. thracius occurring in the west (Obst, 1978, 1981). The

|  | Ophisaurus <br> attenuatus | Anguis <br> fragilis | P.a. <br> apodus | P. a. <br> thracius | P.apodus <br> Southern <br> lineage |
| :--- | :--- | :--- | :--- | :--- | :--- |
| Hyalosaurus <br> koellikeri | 17.8 | 16.7 | 16.2 | 16.4 |  |
| Anguis fragilis | 17.6 |  | 13.8 | 13.6 | 13.4 |
| P. a. apodus | 16.2 | 12.7 | $0.3 / 0.3$ | $\mathbf{2 . 4}$ | $\mathbf{4 . 0}$ |
| P. a. thracius | 17.1 | 13.7 | $\mathbf{2 . 7}$ | $0.1 / 0.5$ | $\mathbf{3 . 1}$ |
| P. apodu Southern <br> lineage | 16.9 | 13.0 | $\mathbf{3 . 1}$ | $\mathbf{3 . 8}$ | $0.9 /-$ |

Distances in ND2 are above diagonal, in Cytb below diagonal, within-lineage distances on diagonal in italics, and within-genus distances in bold.


FIGURE 3 Haplotype networks of $P R L R$ and $R A G 1$ genes in Pseudopus apodus reconstructed using the statistical parsimony algorithm [Colour figure can be viewed at wileyonlinelibrary.com]
third (Southern) lineage has more limited distribution in the Levant along the eastern Mediterranean coast, an area forming the extreme southern part of the species range (Figure 1).

In their mtDNA study, Keskin et al. (2013) mentioned the possibility of the existence of another species based on a sample from eastern Anatolia. However, this individual (locality 39, Digor in Figures 1 and 2) bears the most common Cytb haplotype of P. a. apodus (c1) widespread in the Caucasus and northeastern Turkey, while all other sequences from their analysis cluster with our P. a. thracius samples.

The relationships between the two large clades are not fully resolved, although the sister relationship of P. a. apodus and P. a. thracius with basal-branching position of the Southern lineage is partly supported (Figure 2). Also, genetic distances between this Southern lineage and any of the other two clades are larger than the distance between the two clades corresponding to the recognized subspecies. This shows that current intraspecific taxonomy does not fully reflect phylogenetic structure of P. apodus and that Southern lineage deserves the status of a third subspecies.

## 4.2 | Distribution and taxonomy

The most recent comprehensive map of $P$. apodus distribution (Sindaco \& Jeremcenko, 2008; Figure 1) indicates that Southern lineage presumably comes into parapatry with P.a. thracius in southern Turkey, although ranges of both
lineages might be in fact isolated from each other. This would add a geographic argument for legitimate description of the subspecies; however, the gap in our sampling and thus uncertainty about molecular-genetic identity of populations from southwestern Anatolia leaves us only to speculate at this time.

Pseudopus apodus thracius was described based on morphological differentiation from the nominotypic form, but presently there are no solid data morphologically differentiating specimens of the southern populations from those from other parts of the range. Individuals from Jordan that most probably belong to the Southern phylogenetic lineage (these were not investigated molecularly, the hypothesis is based on the distribution pattern) resemble more those of $P$. a. thracius, while in some characters (head size, scalation, coloration), they are more similar to the nominotypic subspecies (Rifai et al., 2005). Thus, before describing a new subspecies, our molecular-phylogenetic results ought to be confirmed by a thorough morphological study that would supplement it with solid evidence of differentiation observed in independent characters.

Besides revealing previously unrecognized form of P. apodus, our results show that morphological diagnosis of P. a. thracius did not allow for unambiguous identification of the populations belonging to the corresponding phylogenetic lineage. According to Obst (1978, 1981), populations from the region of western Caucasus and possibly Crimea (specimens from Crimea were not analyzed by Obst) belong to $P$. a thracius, but also show some intergradation between both subspecies (cf. also Thieme, 1979). However, in our mtDNA phylogeny they clearly cluster with the eastern subspecies and similarly the sequences of nuclear markers relate them to other $P$. a. apodus populations. Specimens from Crimea also seem to show clear morphological characters of nominotypic subspecies (O. V. Kukushkin, unpublished data). So the extreme eastern and western range limits of each subspecies lie on the shores of the Black Sea-the nominotypic form occurs along the northern coast, while P. a. thracius is distributed along the southern coast.


FIGURE 4 Demography of Pseudopus apodus. (a-b) Mismatch distributions of observed frequencies (dashed line) in two subspecies of P. apodus compared to the expected frequencies (solid line) under the expansion model of population size. (c-d) Demographic history of both subspecies of $P$. apodus estimated with the Bayesian skyline plots. The central line shows the median values of the population size $\left(N_{\mathrm{e}} \times \tau \times \mu ; N_{\mathrm{e}}=\operatorname{effective}\right.$ population size, $\tau=$ generation length in units of time [substitution/site], $\mu=$ mutation rate) on the logarithmic scale, and the gray area represents the $95 \%$ highest posterior density. The maximum time is given as the median of the root height parameter [Colour figure can be viewed at wileyonlinelibrary.com]

## 4.3 | Biogeographic history

Remnants of the genus Pseudopus are commonly found in fossil record dated from the Early Miocene to Holocene, mainly from Europe, but also from Anatolia and Caucasus (Blain, 2016; Klembara, 1981, 2015; Venczel \& Sen, 1994). During the Pleistocene a large, widespread, and ecologically plastic species Pseudopus pannonicus was gradually replaced with P. apodus, which has remained the only survivor of its genus until the present time despite its lower ecological plasticity (Klembara, Böhme, \& Rummel, 2010). Lower plasticity might be one possible explanation of smaller European range of extant $P$. apodus in comparison with both its extinct relatives as well as the extant relative genus Anguis. Also in contrast to the individual species of Anguis, whose origin dates back to the Late Miocene (Gvoždík et al., 2010, 2013), paleontological evidence suggests younger, the middle Pliocene origin of P. apodus (Klembara, 1986; Klembara et al., 2010). On the other hand, the genus Pseudopus dates back to at least 20 Mya (Early Miocene; Klembara et al., 2010; Klembara, 2015) and genetic data suggest split of the two genera in the Late Miocene, about 9 Mya (Macey et al., 1999). Genetic distances between the main clades $(2.4 \%-4.0 \%$; see Table 2) place the basal split within $P$. apodus to about 2-3 Mya (based on the molecular evolution rate of $0.65 \%-0.69 \%$ change per million years in one lineage and $0.0228 \pm 0.00806$ substitutions/site/million years between two divergent lineages, for ND2 and Cytb, respectively; Macey et al., 1999; Carranza \& Arnold, 2012). Direct comparison of genetic data obtained
from a single locus (mtDNA) with incomplete and potentially hard-to-interpret fossil record (Gauthier, 1982; Macey et al., 1999) is not without complications; however, our data are at least not in conflict with the species age hypothesized using the fossils.

In contrast to Anguis (cf. Gvoždík et al., 2010, 2013; Jablonski et al., 2016), but similar to another close anguid relative H. koellikeri from North Africa (de Pous et al., 2011), genetic structure within both widely distributed P. apodus subspecies is shallow, with only a few haplotypes recovered in ND2 and most of the variability in Cytb coming from relatively limited part of the range in Turkey (Figure 2; Keskin et al., 2013). Vast areas of the P. a. thracius and P. a. apodus ranges are inhabited by the same or very closely related haplotypes (e.g., from Istria to central Anatolia in P. a. thracius or from Crimea to Central Asia in P. a. apodus). This strongly suggests that both lineages have colonized their ranges relatively recently and there has not been enough time to develop a more pronounced genetic structure. Most of the observed genetic diversity within these two lineages is found in Anatolia and the eastern Transcaucasia. This region is characterized with relatively stable climate affected less severely by Pleistocene climatic oscillations than more northerly and easterly situated parts of the range (Kahle et al., 2011; Kornilios et al., 2011). As such it might have served as refugial areas for P. apodus populations and a source for later expansions to the Balkans (in P. a. thracius) and Transcaspia (in P.a.apodus), respectively. This scenario is supported by demographic analyses indicating that each
lineage represents one growing population (Figure 4). In most Western Palearctic temperate herpetofauna, including the closest relative of Pseudopus, genus Anguis, Quaternary climatic oscillations caused heavy range fragmentations and isolation of populations surviving in numerous local glacial refugia or sanctuaries (Gvoždík et al., 2013; Jablonski et al., 2016; Joger et al., 2007; Recuero \& García-París, 2011). The areas isolating the refugia during glaciations as well as the northernmost parts of the ranges were repopulated from these source populations later during interglacials, which often resulted in complex genetic structure of recent populations. In Pseudopus however, the very shallow genetic structure outside the presumed refugia in Anatolia, Levant, and Transcaucasia suggests that populations from most of the recent range went extinct during glacials and these areas have been recolonized relatively recently. The BSPs indicate that the population growth started earlier in P. a. thracius, that is, within the Mediterranean biome, while the Caucasus and central Asian regions, which were supposedly more affected by glaciations, were recolonized by P. a. apodus later.

Biogeographic history of the Southern lineage seemingly differs from that of P. a. apodus and P. a. thracius, just as recent distribution does. Only three samples that were available in our study recovered three distinct haplotypes, and moreover, the haplotype network shows several missing haplotypes that would presumably be identified if the sampling from this region was more extensive. This lineage occurs in the extreme southern part of the range where the surrounding deserts prevent the species from dispersal in other directions. A long-term survival of P. apodus in partially isolated populations was presumably accompanied by some level of restricted gene flow. These temporary isolations continued to occur even during the Late Pleistocene, when the region was exposed to periodic eras of higher aridity (Bartov, Goldstein, Stein, \& Enzel, 2003).

Bilgin (2011) in his review described two basic phylogeographic patterns observed in Anatolia. Pseudopus apodus clearly follows the pattern, in which the split between lineages lies within Anatolia rather than between Anatolia and the Balkans. This can be attributed to the mountain ranges of central Anatolia forming the so-called Anatolian Diagonal that have strong isolating effects observable in a great taxonomic variety of organisms spanning from plants to vertebrates (Davis, 1971; Nilson, Andrén, \& Flärdh, 1990). In a more detailed view, the split to three or more lineages, with western, eastern, and southern distributions, and ranges abutting in south-central Anatolia, is often observed in other reptile taxa, although usually, the divergences between lineages are older than in P. apodus. Beside species in which these lineages represent subspecies or are not recognized taxonomically, such as the snakes Zamenis hohenackeri (Jandzik, Avci, \& Gvoždík, 2013) and Natrix tessellata (Guicking, Joger, \& Wink, 2009), a similar pattern is known in complexes of
related species with much deeper splits such as Mauremys caspica-rivulata terrapins (Fritz et al., 2008), the Ablepharus kitaibelii complex (Skourtanioti et al., 2016), the Lacerta trilineata complex (Ahmadzadeh et al., 2013), Ophisops elegans (Kyriazi et al., 2008), and Xerotyphlops vermicularis (Kornilios, in press; Kornilios et al., 2011, 2012). This indicates that the Anatolian geology, geography, and climate had profound and long-lasting effects on shaping the herpetofauna of this region. This was not only restricted to Quaternary climatic oscillations, but also affected much older histories of the species in this region. Phylogeography and biogeographic history of $P$. apodus nicely illustrate on the intraspecific level the importance of Anatolia and Caucasus/Transcaucasia as refugia and diversity centers for colonization of more distant regions such as the Balkans and central Asia (Bilgin, 2011; Joger et al., 2007; Sindaco \& Jeremcenko, 2008).

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