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Short Communication

Multilocus species-delimitation in the *Xerotyphlops vermicularis* (Reptilia: Typhlopidae) species complex

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ABSTRACT

Scolecophidia (worm snakes) are a vertebrate group with high ecomorphological conservatism due to their burrowing lifestyle. The Eurasian or Greek blindsnake *Xerotyphlops vermicularis* is their only European representative, a species-complex with an old diversification history. However, its systematics and taxonomy has remained untouched. Here, we extend previous work that relied heavily on mitochondrial markers, following a multi-locus approach and applying several species-delimitation methods, including a Bayesian coalescence-based approach (STACEY). Four “species” delimitation analyses based on the mtDNA (ABGD, bGMYC, mPTP, parsimony networks) returned 14, 11, 9 and 10 clusters, respectively. By mitotyping twice as many specimens as before, we have a complete picture of each cluster’s distribution. With the exception of the highly-divergent Levantine lineage, the three independent nuclear markers did not help with phylogenetic resolution, as demonstrated in haplotype networks, concatenated and species-trees, a result of incomplete lineage sorting. The prevailing model from the coalescence-based species-delimitation identified two species: the lineage from the Levant and all others. We formally recognize them as distinct species and resurrect *Xerotyphlops syriacus* (Jan, 1864) to include the Levantine blindsnakes. Finally, *X. vermicularis* and *X. syriacus* may represent species-complexes themselves, since they include high levels of cryptic diversity.

1. Introduction

Species are the fundamental units of biodiversity used in many biological disciplines such as biogeography, ecology and evolution, and applied fields such as conservation biology and management. Historically, morphological traits and characters have been used for the identification of species, but in recent years it has been the analysis of molecular markers that gained great attention for the discovery of species, especially cryptic ones. Additionally, the increased utilization of molecular and genetic data has been accompanied by advances in analytical methods and tools, referred to as “DNA-based species delimitation” (Carstens et al., 2013).

The field of molecular systematics can prove particularly helpful when studying species-limits for organisms that exhibit high morphological (and ecomorphological) conservatism. The Scolecophidia (worm snakes) are a largely understudied group of vertebrates with more than 400 species. They present extremely conserved morphology mainly due to their exclusively burrowing lifestyle, compared to all other snakes, the Alethinophidia (true snakes) which are ecologically and morphologically much more diverse (Miralles et al., 2018). Worm snakes are predominantly distributed in tropical and subtropical regions, with the Eurasian or Greek blindsnake *Xerotyphlops vermicularis* (Merrem, 1820) the only native European representative of the group. The Eurasian blindsnake, although currently recognized as one species, occupies a relatively large part of the geographic distribution of its family, the Typhlopidae (Supplementary Fig. S1), and it includes very high levels of genetic diversity (Kornilios et al., 2012). It represents a species-complex that has diverged from its sister-taxon *X. socotranus* (Boulenger, 1889) roughly during the Eocene-Oligocene transition (Kornilios et al., 2013), while the diversification within the complex has occurred during the early Miocene (Kornilios et al., 2012; Kornilios, 2017). The biogeographic history of the complex has been investigated in several studies with the use of molecular data (Kornilios et al., 2011; 2012; 2013; Kornilios, 2017) but it is still a work-in-progress, since

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several phylogenetic relationships have not been resolved or are unstable, while most conclusions have been drawn from the analysis of mitochondrial (mtDNA) markers.

However, the systematics and taxonomy of the group has remained relatively untouched. In Kornilios et al. (2012), several highly-divergent mtDNA clades were identified by analysing 130 *X. vermicularis* specimens. These corresponded to 10 evolutionary significant units (ESUs), using statistical-parsimony networks and following Fraser and Bernatchez (2001). However, the resulting polytomies and sole use of mtDNA may have overestimated the number of ESUs or "species" within the complex. Later, in Kornilios (2017), some of the polytomies of the mtDNA phylogeny were resolved with the addition of more mtDNA markers but with the analysis of fewer samples. Although that study aimed at a better phylogeographic inference for the Eurasian blindsnake, three levels of divergence could be identified leading to three respective hypotheses for the number of potential "species" within the complex. The most conservative hypothesis includes two species: the first, named clade A in all aforementioned studies, is distributed in south Syria and Jordan (possibly also Israel, Lebanon and Egypt), while the second includes all remaining *X. vermicularis* populations, presenting a much larger distribution and a strong internal genetic structure. The second hypothesis recognizes a total of four species within the complex: clade A and three more species distributed in south-west Turkey (clade B), Cyprus (clade D) and the remaining populations. Finally, according to the third and least conservative hypothesis, seven potential species occur within the Eurasian blindsnake complex: clades A, B and D, with the addition of four species distributed west (clades G + K), east (clades I + H + M + E), south (clade F) and south-east (clade C) of the Amanos or Nur Mountains in south-central Turkey and north-west Syria (Fig. 1), with the east lineage having spread to the westernmost and easternmost parts of the blindsnake's range (Fig. 1). However, these working hypotheses that are based solely on observed levels of mtDNA divergence have not been tested with any other type of data or with species-delimitation tools. Finally, the morphological investigation of the samples we have at hand reveals that they do not differ with regard to all available meristic characters (scale counts) and body measurements, while the preservation of our samples in different mediums does not allow a safe comparison of scale measurements (e.g. length or width of certain head scales; see Kornilios et al., 2018 for a comparison of formalin-preserved to ethanol-preserved specimens).

The scope of this study is to extend previous work that relied heavily on mitochondrial markers, take advantage of molecular species-delimitation techniques and provide an updated taxonomy for the *X. vermicularis* species complex. For this purpose, we have analyzed twice as many specimens compared to our previous works, with a better targeted and more thorough geographical representation that fills several gaps in our sampling. Additionally, we followed a multi-locus approach by also including several independent nuclear markers (nDNA), and applied different species-delimitation methods and tools of modern molecular systematics, including coalescent based approaches. Many recent studies of east Mediterranean reptiles that used current molecular-taxonomy methods on multi-locus data, have uncovered new cryptic species, elevated subspecies to the species level or resurrected old ones (e.g. Sindaco et al., 2014; Kornilios et al., 2018; Jablonski et al., 2019). These studies, including the present one, can provide a better assessment of biodiversity levels and a more stable taxonomic framework for the implementation of conservation actions.

2. Material and methods

2.1. Taxon and DNA sampling

A total of 243 *X. vermicularis* samples (113 new) were sequenced for the mtDNA markers, while, after preliminary species-delimitation analyses, nDNA markers were sequenced for a subset of 65 samples representing all mtDNA clusters. Sampling localities are shown in Fig. 1 and specimen data (working codes, localities and GenBank Accession Numbers) are given in Supplementary Table S2.

The five target-loci were PCR-amplified and sequenced using conditions and primers described in Kornilios et al. (2012) and Kornilios et al. (2013); these were the mtDNA markers 12S rRNA (*12S*) and NADH dehydrogenase subunit 2 (*ND2*), and the nuclear protein-coding genes of the brain derived neurotrophic factor (*BDNF*), neurotrophin 3 (*NT3*) and prolactin receptor (*PRLR*). Heterozygous positions for the nuclear markers were phased using the Bayesian algorithm of Phase 2.1 (Stephens and Scheet, 2005) implemented in DnaSP 5.10 (Librado and Rozas, 2009), with 1,000 iterations after a burn-in of 100. All estimated

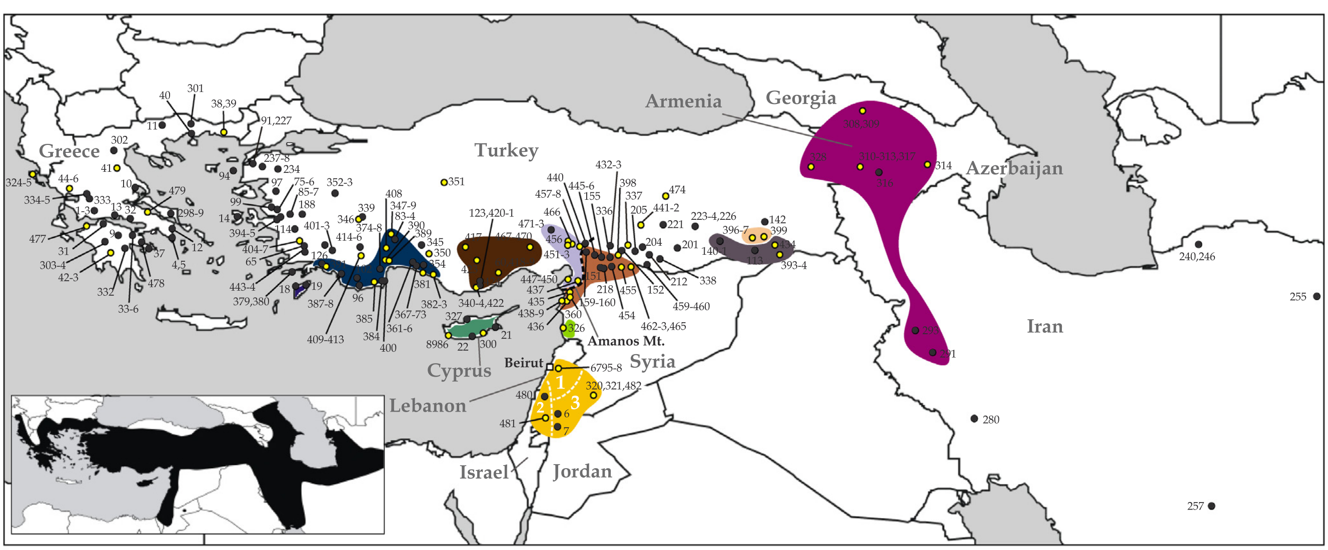

Fig. 1. Map showing the sampling localities of the current study: black circles for samples with mtDNA data and yellow circles for samples with mtDNA and nDNA data. The distribution of main phylogenetic clades is displayed with different colours that correspond to the clades of Fig. 1. All sampling localities that do not have a specific distribution-colour belong to the widespread clade I. Numbers refer to specimen codes given in the Supplementary Table S2. The geographic distribution of the *Xerotyphlops vermicularis* species complex is presented in the lower left inset (Sindaco and Jeremčenko, 2008). Finally, the names of important geographic areas and countries mentioned in the text are also presented. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

haplotypes had probability values of 1.0. The genetic divergences between and within major mtDNA-groups were estimated using MEGA 6 (Tamura et al., 2013) as uncorrected *p*-distances.

2.2 Mitochondrial gene tree, single-locus species delimitation and haplotype networks

A Maximum Likelihood (ML) gene-tree was estimated for the mtDNA using IQ-TREE 1.4.3 (Nguyen et al., 2015). A free rate of heterogeneity was selected and nodal support was tested via SH-aLRT tests with 10,000 replicates (Guindon et al., 2010), 10,000 ultrafast bootstrap alignments (Minh et al., 2013) and 100 standard bootstraps (Felsenstein, 1985). During the analysis, the best partitioning scheme (one partition) and best substitution model (HKY + G), were also evaluated (Chernomor et al., 2016; Kalyaanamoorthy et al., 2017).

We applied several mtDNA-based species-delimitation analyses, inspired by the phylogenetic species concept and the DNA barcoding approach, that utilize different algorithms and tools, incorporating different theoretical backgrounds. Specifically, the Automatic Barcode Gap Discovery (ABGD; Puillandre et al., 2012) is a distance-based method, that can identify β-diversity relying on sequence similarity-thresholds but ignoring the relationships of the studied taxa. The Generalized Mixed Yule-Coalescent analysis (GMYC; Pons et al., 2006) and Poisson Tree Processes model (PTP; Zhang et al., 2013) are not distance-based and account for the phylogenetic relationships of the sequences. GMYC requires an ultrametric tree as input and uses absolute or relative ages to separate the branches into two processes (speciation and coalescence) and to differentiate within and between species. PTP directly uses substitutions to determine the transition from a between- to a within-species process by assuming that a two parameter model (one for speciation and one for coalescence) best fits the data. On the other hand, the multi-rate PTP (mPTP; Kapli et al., 2017), also accounts for different levels of intraspecific genetic diversity deriving from differences in the evolutionary history or sampling of each species, consistently yielding more accurate delimitations with respect to the actual taxonomy (Kapli et al., 2017).

We used the online version of ABGD (http://wwwabi.snv.jussieu.fr/public/abgd/), calculated *p*-distances and used the default priors for the relative gap width (1.5), *Pmin* (0.001) and *Pmax* (0.1). All analyses involved 50 steps and 20 bins of distance distribution.

We used the Bayesian version of GMYC (Reid and Carstens, 2012), bGMYC v.1.0.2 in R Studio, implementing 50,000 MCMC steps with 40,000 steps as burn-in and a thinning of 100 steps. As input, we used 1,000 posterior (ultrametric) trees of mtDNA haplotypes constructed with BEAST v1.10.4 (Drummond et al., 2012). Two independent runs were conducted with a chain length of $3*10^7$ iterations, under the uncorrelated longnormal relaxed clock approach with a Yule tree prior. TRACER v1.6 (Rambaut and Drummond, 2007) was used to check for convergence and adequate effective sample size (ESSs). Independent runs were combined using Logcombiner, discarding the first 25% of each run as burn-in, and the tree was summarized with TreeAnnotator. For the bGMYC analysis we used a conservative posterior-probability threshold of 0.5 to identify putative species, compared to higher values that could overestimate the species' number.

The ML mtDNA tree was used as input in mPTP: two independent analyses ran with 10^8 generations, a thinning of 10^4 and a burn-in of 10%. Again, we used a conservative posterior-probability threshold of 0.95 to identify speciation events.

Finally, we constructed independent haplotype networks with statistical parsimony, using TCS v.1.21 (Clement et al., 2000) under the 95% connection limit of parsimony. Independent mtDNA networks were considered distinct evolutionarily significant units (ESUs), following Fraser and Bernatchez (2001). Similarly, we constructed haplotype networks for each of the nuclear markers.

2.3. Multilocus coalescent-based species tree and species delimitation

Multilocus coalescent-based species delimitation can test alternative hypotheses of lineage divergence allowing for gene tree discordance under genetic drift (Fujita et al., 2012). To estimate a species tree and to test species' boundaries in this framework, we performed the Species Tree and Classification Estimation, Yarely – STACEY v.1.2.5 (Jones, 2017), in BEAST2 v.2.6.0 (Bouckaert et al., 2019). All 65 samples and all loci were included in the analysis (the two mtDNA genes were considered a single locus), and for species' assignments (minimal clusters) we used the maximum number of mtDNA clusters, as estimated from the combination of our single-locus species delimitation analyses (13 species). Two runs of 10^9 generations were conducted, with an uncorrelated longnormal relaxed clock and a Yule tree prior [CollapseHeight = 0.001; CollapseWeight = beta (1.1) around (0.1); bdcGrowthRate = lognormal (M = 4.6, S = 1.5); pop-PriorScale = lognormal (M = − 7, S = 2); relativeDeathRate = beta (1.1)].

For the species-delimitation analysis, we applied equal ploidy for the nDNA and mtDNA loci since this is considered a more robust approach that avoids disproportionate influence of mtDNA data (Busschau et al., 2019). We used SpeciesDelimitationAnalyser (Jones, 2015) to process output files and examine the clusters of species assignments.

Finally, we reconstructed a ML tree using the concatenated dataset (mtDNA and nDNA markers) in IQ-TREE, using the same parameters for the analysis and nodal support as for the mtDNA. We used five partitions, one for each marker, with their own best-fit substitution model (TN + G for ND2; K2P + I for 12S and the nuclear loci). For the phylogenetic analysis, heterozygous positions of the nuclear markers were coded according to the IUPAC ambiguity codes.

3. Results and discussion

3.1. MtDNA: gene-tree and clusters

The mitochondrial phylogeny within the *X. vermicularis* species complex has been a long standing issue: it presents a large number of highly divergent mtDNA lineages with many unresolved relationships due to a combination of soft and hard polytomies (Kornilios et al., 2011; 2012), while, even a multi-marker mtDNA dataset of more than 3,700 bp has failed to resolve some of them (Kornilios, 2017). The dense sampling of the present study with almost twice as many analyzed samples and the inclusion of unrepresented areas (and lineages) especially from Anatolia (Fig. 1), which is the diversification center of the complex, has returned a similar phylogenetic result of many mtDNA clades with ambiguous relationships (Fig. 2). However, by mitotyping a plethora of samples we have now an almost complete picture of each clade's geographic distribution (Fig. 1), while we have identified two new clades (K and M) that had been undetected.

The four single-locus cluster delimitation analyses, based on the mtDNA, returned relatively similar results with some differences regarding both the number of clusters and the appointment of samples into the clusters. Specifically, ABGD, bGMYC, mPTP and parsimony networks favored the occurrence of 14, 11, 9 and 10 clusters, respectively (Fig. 2; Supplementary Figure S3). According to all analyses, each of the clades B, C, E, F, G and K constitute different clusters. Three of the four analyses favoured clade D of Cyprus as one cluster, with ABGD, the most prone to splitting, showing it might be two. The monophyletic unit of clades IHM can be considered either as one cluster or as three, corresponding to the individual clades. Finally, the very divergent clade A was regarded either as one cluster in two of the analyses, or two clusters, splitting the Lebanon population (subclade A1) or even three clusters, further splitting the individuals from Israel (A2) from those of Jordan and south Syria (A3).

Conclusively, the most conservative outcome that keeps the species-splitting to a minimum, is the existence of at least nine mtDNA clusters within *X. vermicularis* corresponding to clades A, B, C, D, E, F, G, K and

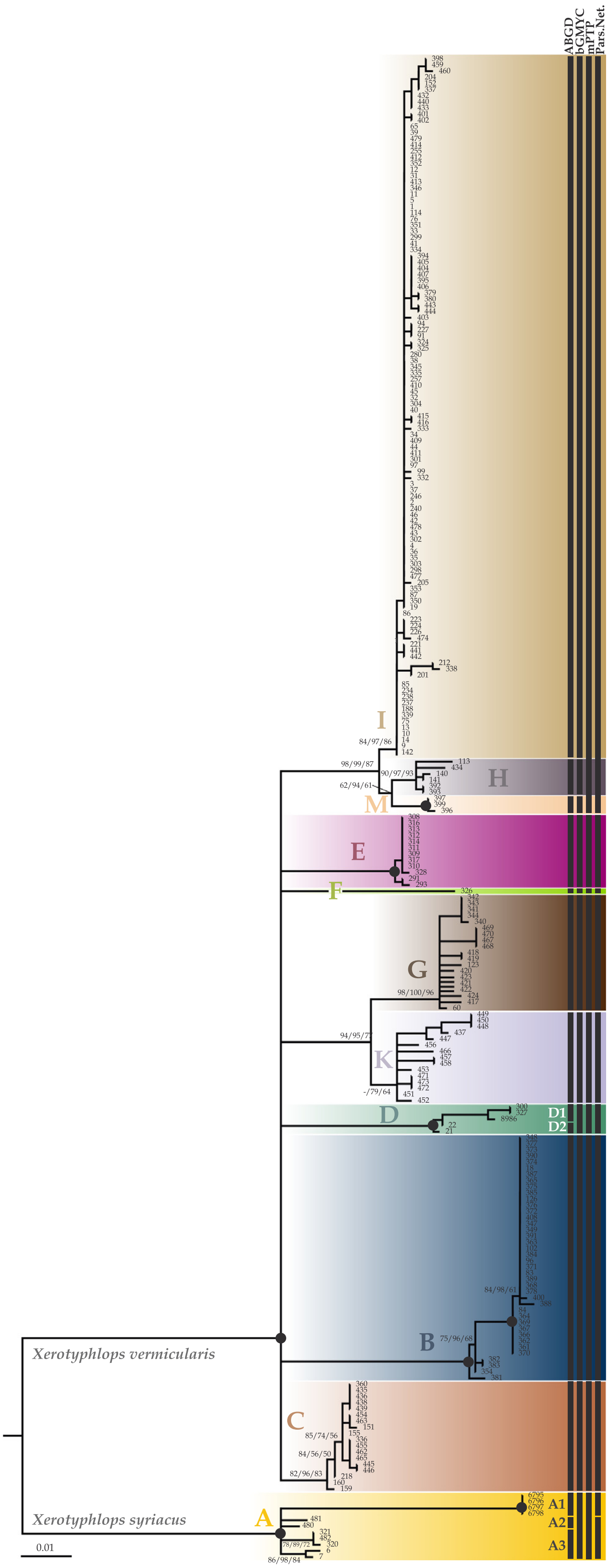

Fig. 2. Maximum Likelihood (ML) tree, reconstructed with IQ-TREE, based on the mtDNA dataset. Numbers in terminal nodes refer to specimen code numbers presented in Supplementary Table S2 and in the map of Fig. 1. Numbers next to nodes are statistical support values: SH-aLRT tests/ultrafast bootstrap alignments/standard bootstraps. Nodes with values ≤ 50 have been collapsed, while nodes with black closed circles indicate full support (100). Black vertical bars on the right of the clades are the results of the single-locus species-delimitation analyses (ABGD, bGMYC, mPTP, parsimony networks), with each segment representing a distinct candidate species according to the respective method.

the unit IHM. The maximum number of clusters, from the combination of all approaches, was 14 and was used in the coalescence-based species delimitation as the potential species, with the exception of D2 (samples 21, 22) due to failure in the PCR amplifications of the nuclear markers.

3.2. Multilocus coalescence-based species delimitation

Besides the clear separation of clade A, the analysis of three independent nuclear markers did not help with the phylogenetic resolution, as demonstrated in the independent networks (Fig. 3A) and the concatenated tree (Fig. 3B). In the species-tree, produced with STACEY (Supplementary Figure S4), all relationships were unsupported with the exceptions of the ancestor of clade A and the ancestor of all remaining clades that had a posterior probability value of 1.0. The observed patterns in the nuclear markers that are in conflict with the mtDNA, can be attributed either to introgression or to incomplete lineage sorting (ILS). We consider the former highly unlikely because if this is the case it would actually reflect a complete panmixia rather than local introgression and it would render the results from the mtDNA almost inexplicable.

STACEY returned a total of 145 different models for the number and composition of species within *X. vermicularis*. The prevailing one that is backed up by 80% of the posterior distribution of samples was the identification of two species, namely clade A and all others. Two other models were found in much smaller fractions of the distribution (6.5% and 5.7%); those were a single-species model and the recognition of a third species corresponding to clade D from Cyprus, respectively. All remaining 142 species-models covered less than 1% of the distribution each. As our nuclear-loci dataset is suffering from ILS, it becomes clear that the phylogeny and species-limits within the *X. vermicularis* complex will need a phylogenomic approach for their resolution, with the analysis of a large number of genomic markers and the added power of coalescence and population-genetics tools (work in progress).

For the time being and based on the current strong evidence, we are able to update and improve the systematic and taxonomic situation of the Eurasian blindsnake, by formally recognizing at least two valid species and identifying the populations of clade A as a distinct species from *X. vermicularis*.

3.3. The resurrection of *Xerotyphlops syriacus* and future work

The Italian zoologist, botanist and herpetologist Giorgio Jan (1791–1866) had described a blindsnake species from the area where we now find clade A (Jan, 1864). He named that species *Typhlops syriacus*, later synonymized with *T. vermicularis*, by describing a specimen found in Beirut, which is currently the capital of Lebanon but at that time was part of Syria. The sole specimen was unfortunately destroyed together with the entire collection of the Natural History Museum of Milan during the bombings of World War II (Scali, 2010). The samples from Lebanon included in our study come from a locality very close to Beirut, the type locality of *T. syriacus* (approximately 25 km west; Fig. 1). In this context, we resurrect the species *Xerotyphlops syriacus* (Jan, 1864) to formally include the populations belonging to our clade A, i.e. the Levantine blindsnake (Israel, Jordan, Lebanon, south Syria and probably Egypt). Although the original specimen is lost, a holotype still exists since Jan (1864) provided illustrations of the type specimen that should be designated as the holotype (Supplementary Figure S5). According to his brief morphological description of the single specimen from Beirut and a *X. vermicularis* representative from Cyprus, the main diagnostic character between them was the nasal furrow that did not surpass the nostril in the former while it did in the latter. The inspection

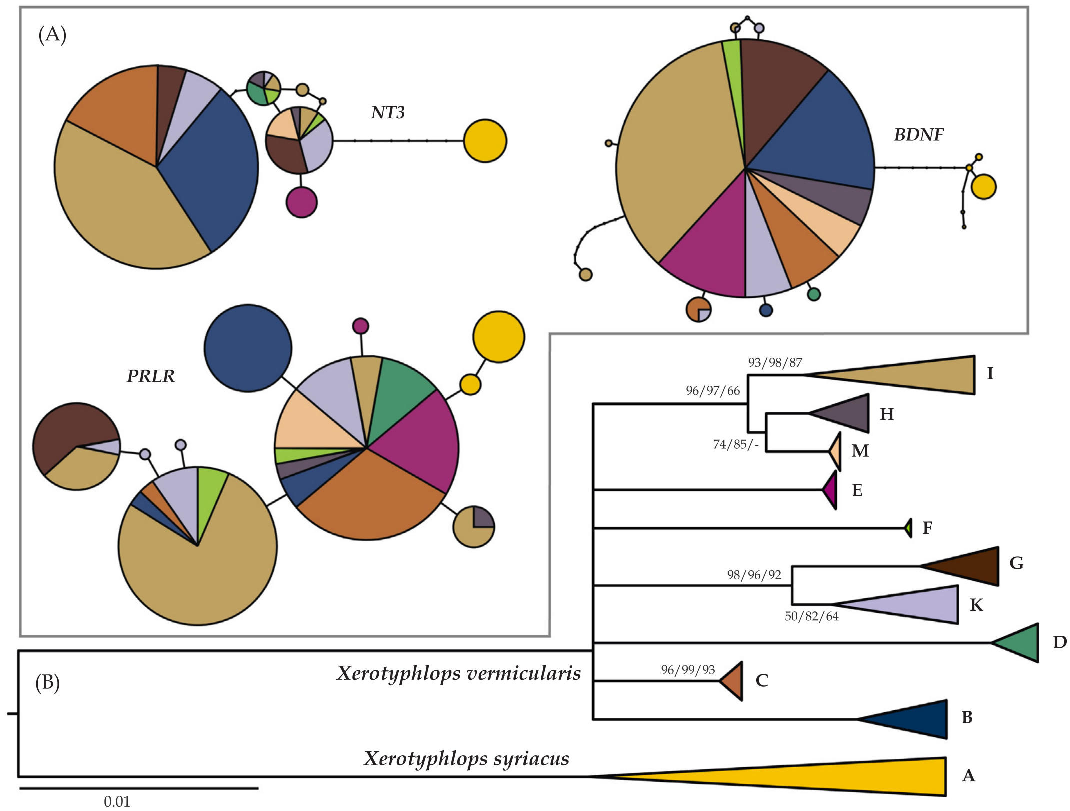

Fig. 3. (A) The statistical-parsimony networks based on the three nuclear markers (*NT3*, *BDNF*, *PRLR*) calculated with TCS with a 95% connection limit. Segment between black dots represents one mutational step, colored circles are haplotypes and colours refer to the clades of the mtDNA tree of Fig. 2. The circle area is proportional to the number of individuals sharing that haplotype. (B) The concatenated (mtDNA and nDNA) Maximum Likelihood (ML) tree, reconstructed with IQ-TREE. Branches of each clade have been collapsed (when not mentioned they have full statistical support, 100). Numbers next to nodes are statistical support values: SH-aLRT tests/ultrafast bootstrap alignments/standard bootstraps. Nodes with values ≤ 50 have been collapsed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of our samples shows that this is not a character that differentiates the two species. A morphological investigation based on skeletal and cranial elements, using x-rays and ct-scans may prove more informative for the diagnosis of these two species, especially in the light of the extreme morphological conservatism that typhlopids and scolecophidians exhibit.

The evidence we have so far mostly from the mtDNA implies that both *X. vermicularis* and *X. syriacus* may represent species-complexes themselves, since they include high levels of cryptic diversity reflecting the existence of more species. The analysis of single-locus nuclear markers has not helped us confirm or reject this hypothesis, which will be tested with the use of genomic approaches and the analysis of introgression/hybridization levels between lineages. The genetic divergence values, as *p*-distances, between *X. vermicularis* and *X. syriacus* are very high, ranging from 10.6 to 13.0% for *ND2* and 4.4–5.7% for *12S* (Supplementary Table S6). However, the respective values among the intraspecific lineages of these two species are also high, comparable to or higher than species-level values from other reptiles (as high as 8.3% for *ND2* and 3.9% for *12S*; Table S3). Additionally, the mtDNA species-delimitation analyses recognize up to three "species" within *X. syriacus* and up to eleven "species" within *X. vermicularis* (Fig. 2). For the latter, the geographic distributions of these potential species coincide with known regions of high endemism and biodiversity (Kornilios et al., 2011; 2012; Jablonski and Sadek, 2019). For the former, according to some of our results, there are indications of genetic differentiation between populations from the north (Lebanon; A1), the west (Israel; A2) and the east (south Syria, Jordan; A3) that agree with known biogeographic barriers that have acted as speciation drivers in other organisms (Dufresnes et al., 2019 and references therein). However, a more thorough sampling is needed in order to evaluate the intraspecific diversity and taxonomic situation within *X. syriacus*.

CRediT authorship contribution statement

P. Kornilios: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **D. Jablonski:** Resources, Data curation, Writing - review & editing, Funding acquisition. **R.A. Sadek:** Resources, Writing - review & editing. **Y. Kumlutaş:** Resources, Data curation, Writing - review & editing, Funding acquisition. **K. Olgun:** Resources, Data curation, Writing - review & editing, Funding acquisition. **A. Avci:** Resources, Data curation, Writing - review & editing, Funding acquisition. **C. Ilgaz:** Resources, Data curation, Writing - review & editing, Funding acquisition.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2020.106922.

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