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Extensive cytonuclear discordance in a crested newt from the Balkan Peninsula glacial refugium

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Integration of multilocus data and species distribution modelling into phylogeography allows mitochondrial DNA (mtDNA)-based scenarios to be fine-tuned. We address the question of whether extensive mtDNA substructuring in the crested newt *Triturus macedonicus* from the Balkan Peninsula is matched in the nuclear genome. We determine the intraspecific population structure based on 52 nuclear DNA markers and project a species distribution model on climate layers for the Last Glacial Maximum. We show that *T. macedonicus* accumulated nuclear DNA population structure in an area predicted to have been climatically stable during the Pleistocene, with four nuclear DNA groups in the western part of the species range. The distribution of these nuclear DNA groups shows little agreement with that of mtDNA structuring, which shows three highly distinct species-specific clades and a fourth one introgressed from another crested newt species. This cytonuclear discordance conveys that historical biogeographical scenarios based on mtDNA exclusively should be interpreted with caution. Our findings further highlight the important role the Balkan Peninsula has played in the evolution and preservation of European biodiversity.

ADDITIONAL KEYWORDS: glacial refugium – historical biogeography – Ion Torrent – nuclear DNA – postglacial expansion – *Triturus macedonicus*.

INTRODUCTION

The aim of phylogeography is to reconstruct the biogeographical history of species from geographical patterns in genetic structure (Avise, 2000). Historically, phylogeographical surveys have relied mainly on mitochondrial DNA (mtDNA; Beheregaray, 2008), which is more susceptible to geographical structuring than the rest of the genome (Petit & Excoffier, 2009). This raises the question: how representative is the mtDNA geographical structure of the overall history of the taxon under study? With 'next-generation phylogeography' (Puritz *et al*., 2012), in which many unlinked nuclear markers are consulted, this question can now be addressed (Ekblom & Galindo, 2011; McCormack *et al*., 2013; Garrick *et al*., 2015).

The field of phylogeography has been particularly prolific in unravelling the influence of the climate cycles of the Pleistocene Ice Age on biodiversity (Hewitt, 2004). During glacial cycles, many temperate species had their ranges reduced to glacial refugia, whereas during interglacials, they could expand their ranges (Bennett & Provan, 2008). This cyclical contraction-and-expansion pattern has left its mark on genetic variation within species, with populations that persisted in glacial refugia showing relatively high genetic diversity and populations established during recent colonization being genetically depleted (Wallis & Arntzen, 1989; Taberlet *et al*., 1998; Hewitt, 2000). Furthermore, interglacial range expansion allowed secondary contact among populations and species, facilitating genetic admixture and introgression (Hewitt, 2011*a*; Garrick *et al*., 2019). The Last Glacial Maximum (LGM) is considered reasonably representative of previous glaciations (Hewitt, 2011b). Hence, species distribution models for the LGM can provide coarse estimates of those areas that were able to sustain populations during the glacial periods of the Pleistocene and those that became suitable during interglacials (Svenning *et al*., 2011; Alvarado-Serrano & Knowles, 2014).

In Europe, the Mediterranean peninsulas (the Iberian, Italian and Balkan Peninsulas) are

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considered the dominant refugial areas for European temperate species (Taberlet *et al*., 1998; Provan & Bennett, 2008; Hewitt, 2011b). Phylogeographical studies have also uncovered genetically distinct, geographically coherent populations within the Mediterranean peninsulas, suggesting intraspecific fragmentation and differentiation throughout the Pleistocene (Hewitt, 2011b). The presence of multiple, discrete glacial refugia existing in an area formerly recognized as a single continuous glacial refugium has been coined 'refugia-within-refugia' (Gómez & Lunt, 2007; Abellán & Svenning, 2014). Many species in the Balkan Peninsula show extensive mtDNA structuring (Poulakakis *et al*., 2015), which raises the question: to what extent is the geographical structure observed in mtDNA paralleled in the nuclear genome?

We applied next-generation phylogeography to the crested newt *Triturus macedonicus* (Karaman, 1922), a Balkan endemic (Fig. 1). This species shows a degree of intraspecific mtDNA differentiation unparalleled in the genus *Triturus*, in line with a long-term presence in the Balkan Peninsula and a complex history of genetic differentiation *in situ* (Wielstra *et al*., 2013; Fig. 1). We here compare and evaluate the spatial correspondence of the geographical substructuring in the nuclear and mitochondrial genomes of *T. macedonicus*.

MATERIAL AND METHODS

Sampling and sequence data

We included three individuals from each of 39 populations, i.e. 117 individuals in total (Fig. 1; Supporting Information, Table S1). We re-analysed data previously used in studies that focused on interspecific hybridization, rather than intraspecific phylogeography. Sequence data for an mtDNA marker (*ND4*, 658 bp), including representatives of the eight other *Triturus* species and the related newt genus *Calotriton*, were taken from Wielstra *et al*. (2013). Sequence data for 52 nuclear DNA markers were taken from Wielstra *et al*. (2017). To obtain these nuclear markers, we used the Ion Torrent next-generation sequencing protocol described by Wielstra *et al*. (2014a). In brief, we amplified markers of ~140 bp in length (excluding primers), positioned in 3′ untranslated regions, in five multiplex polymerase chain reactions. We pooled the multiplexes for each individual and ligated unique tags to be able to recognize the product belonging to each individual. We sequenced the amplicons on the Ion Torrent next-generation sequencing platform and processed the output with a bioinformatics pipeline that filters out poor-quality reads, identifies alleles and converts data

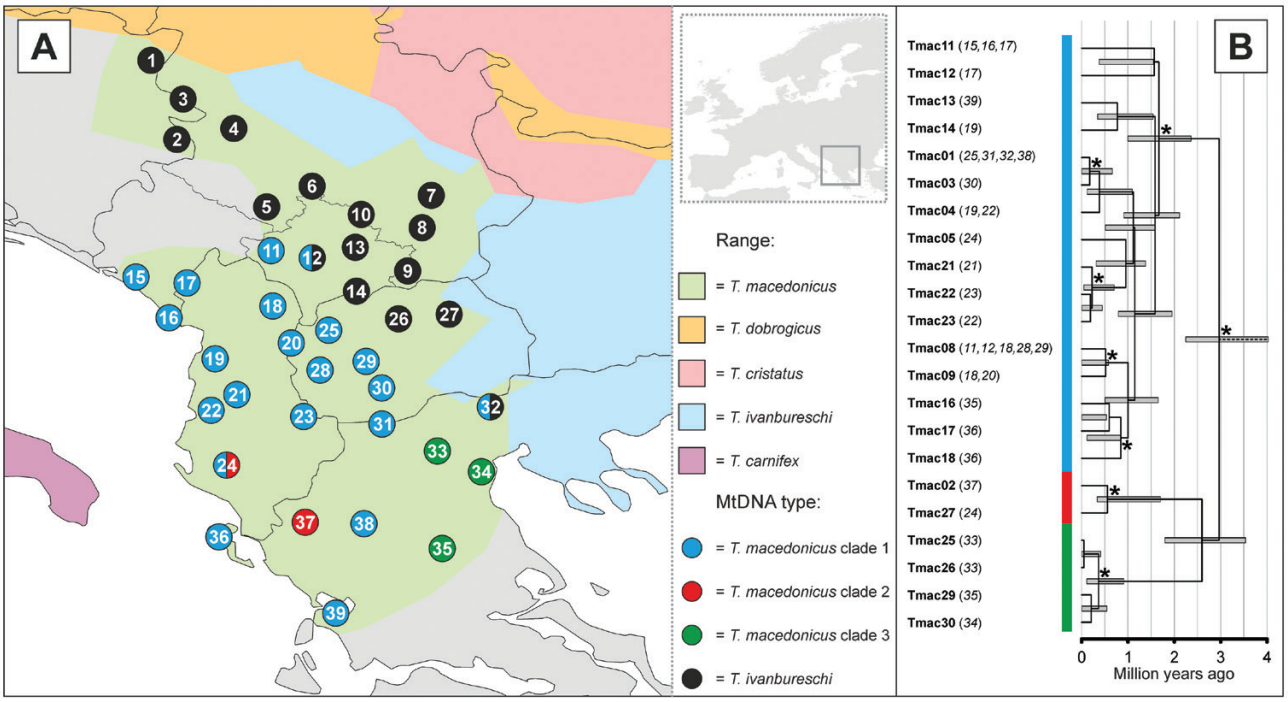

Figure 1. Distribution and sampling of *Triturus macedonicus*. In A, background colours reflect the range of *T. macedonicus* and neighbouring crested newt species. Populations are coloured according to the mitochondrial DNA (mtDNA) clade; those containing introgressed *Triturus ivanbureschi* mtDNA are shown in black. B, the calibrated mtDNA phylogeography. Asterisks denote nodes with a posterior probability ≥ 0.95. Population numbers and mtDNA haplotype codes correspond to those in the Supporting Information (Tables S1, S2).

to a genotypic format that can easily be converted to other formats with the program CREATE (Coombs *et al.*, 2008). The total number of aligned Ion Torrent reads after data filtering was 6 293 299. The mean coverage was 791 reads (range 0–24 444) per marker–individual combination, and 98.9% of marker–individual combinations were considered successful (meaning that they had ≥ 20 reads available).

Mitochondrial DNA re-analysis

We obtained a dated phylogeny with BEAST v.2.1 (Bouckaert *et al.*, 2014). A fossil dated at 24 Mya was used as a minimum estimate for the most recent common ancestor of the genus *Triturus* (Steinfartz *et al.*, 2007) and given a lognormally distributed prior with an offset of 24 and a mean and standard deviation of 1.0. The origin of the Adriatic Sea at 5.33 Mya, at the end of the Messinian Salinity Crisis, was assumed to be the vicariant event causing the split between *T. macedonicus* and its sister species *Triturus carnifex* (Arntzen *et al.*, 2007; Wielstra *et al.*, 2019) and given a normally distributed prior with a small standard deviation (0.001). The most appropriate model of sequence evolution (GTR+G) was identified with jModelTest v.2.1.7 (Darriba *et al.*, 2012), based on the Akaike information criterion. We applied the relaxed lognormal clock model and a Yule speciation model. We conducted two independent runs, each of 100 million generations, with a sampling frequency of 0.0001. The first half of the sampled trees was discarded as burn-in after analysis in TRACER v.1.6 (http://tree.bio.ed.ac.uk/software/tracer/); runs converged, and effective sample sizes were ≥ 200.

Bayesian clustering analysis

Interspecific

Triturus macedonicus is parapatric with three additional *Triturus* species (Fig. 1). Considering that *Triturus* species hybridize at their contact zones (Arntzen *et al.*, 2014), we took the following approach to counter potential adverse effects of interspecific gene flow on the recovery of population structure within *T. macedonicus*. We conducted Bayesian species assignment using STRUCTURE v.2.3.4 (Pritchard *et al.*, 2000) and compared the *T. macedonicus* dataset with the three crested newt species with which it shares a contact zone: *Triturus cristatus*, *Triturus dobrogicus* and *Triturus ivanbureschi* (reference data; three individuals for four populations per species, taken from the study by Wielstra *et al.*, 2014a). We fixed the number of distinct gene pools (k) at four, because we were dealing with four parental species. We used the admixture model in combination with the correlated allele frequency model with 100 000 iterations, after 50 000 iterations of burn-in, and ran ten replicates, which were summarized with CLUMPAK (Kopelman *et al.*, 2015). Individuals allocated to *T. macedonicus* with a support < 0.95 were excluded.

Intraspecific

To explore population structure within *T. macedonicus*, we conducted non-spatial Bayesian clustering analysis with STRUCTURE v.2.3.4 and spatially explicit Bayesian clustering analysis using BAPS v.6 (Cheng *et al.*, 2013). The upper limit of the range over which the number of gene pools (k) was tested was defined by the total number of populations included in these datasets (35, because four populations were excluded based on interspecific gene flow). To select the optimal value of k for STRUCTURE, we used the Δk criterion (Evanno *et al.*, 2005) as implemented in CLUMPAK (Kopelman *et al.*, 2015). BAPS determines the optimal value of k internally. For each program, we performed five independent runs. In STRUCTURE, we used the admixture model in combination with the correlated allele frequency model with 100 000 iterations, after 50 000 iterations of burn-in. CLUMPAK was used to average the outputs of multiple replicates. In BAPS, we conducted a spatial genetic mixture analysis. Given that individuals within populations share the same coordinates, we allowed BAPS to make input coordinates unique by introducing minor random variation.

Species distribution modelling

We used MaxEnt v.3.3.3k (Phillips *et al.*, 2006) to create a species distribution model, because this program was developed for presence-only data, outperforms other algorithms and can be used reliably to hindcast to past environments (Elith *et al.*, 2006; Hijmans & Graham, 2006). The feature type was restricted to hinge features because this produces a smoother model fit, emphasizing trends rather than data idiosyncrasies (Elith *et al.*, 2010). We used a database of 131 *T. macedonicus* localities presented by Wielstra *et al.* (2014b). For climate layers, we used bioclimatic variables available from the WorldClim database v.1.4 (Hijmans *et al.*, 2005; http://www.worldclim.org) at 2.5 arc-min resolution (~5 km × 5 km). Following Peterson (2011) and Guisan & Thuiller (2005), we selected a subset that showed low multicollinearity (a Pearson's correlation of $r < 0.7$) and was considered to reflect the physiological limitations of the study species (in this case, seasonality: bio10 = mean temperature of warmest quarter; bio11 = mean temperature of coldest quarter; bio15 = precipitation seasonality; bio16 = precipitation of wettest quarter; and bio17 = precipitation of driest quarter). To prevent the environmental range covered by pseudo-absence

data, used by MAXENT to discriminate presence data from the environmental background, being either too narrow or too broad (Elith *et al.*, 2011), we drew a buffer of radius 200 km around known *Triturus* localities (following VanDerWal *et al.*, 2009). We tested that our species distribution model performed better than random expectation by testing its area under the curve of the receiver operating characteristic value against a null model (Raes & ter Steege, 2007) based on 99 models for random point data created in ENMTools v.1.3 (Warren *et al.*, 2010). For hindcasting to the LGM (~21 000 years ago), we used bioclimatic variables, based on downscaled climate data from simulations of two global climate models: CCSM4 (Brady *et al.*, 2013) and MIROC-ESM (Sueyoshi *et al.*, 2013), available from WorldClim.

RESULTS

MITOCHONDRIAL DNA RE-ANALYSIS

Triturus macedonicus possesses three main mtDNA clades that split > 2.5 Mya (Fig. 1). Along the entire north-eastern fringe of its range, *T. macedonicus* has captured mtDNA derived from another crested newt species, *T. ivanbureschi*.

BAYESIAN CLUSTERING ANALYSIS

Interspecific

The comparison in STRUCTURE with the three parapatric *Triturus* species revealed signs of nuclear genetic admixture with *T. ivanbureschi* only, in 15 individuals from seven *T. macedonicus* populations from near the contact zone (Fig. 2; Supporting Information, Table S1). In four populations, all individuals showed introgression, whereas in each of the other three only a single individual was affected.

Intraspecific

Results of the non-spatial and spatially explicit Bayesian population assignment analyses at the level of the population are presented in Figure 2 (details on the assignment of individuals are in Supporting Information, Table S1). The STRUCTURE analysis suggested $k = 2$ as the most likely number of nuclear DNA gene pools based on Evanno's Δk criterion, with a second smaller peak found for $k = 4$. A spatially explicit Bayesian clustering analysis in BAPS also considered $k = 4$ as the most likely number of gene pools. Given the tendency of STRUCTURE to converge on $k = 2$ under the Δk criterion (Janes *et al.*, 2017), we also explored population subdivision under $k = 4$. In all analyses, one 'north-eastern group' was found in the north-eastern part of the range, and three additional groups found in BAPS and STRUCTURE under $k = 4$ fitted in a 'south-western group' recovered by STRUCTURE under $k = 2$. Results for BAPS were similar to those of STRUCTURE under $k = 4$, although less mingling between gene pools was suggested. There was little apparent agreement between the distribution of these nuclear DNA groups and the mtDNA clades (Figs 1, 2).

SPECIES DISTRIBUTION MODELLING

The species distribution model predicted that the western part of the current range of *T. macedonicus* was also suitable during the LGM (Fig. 3). Two

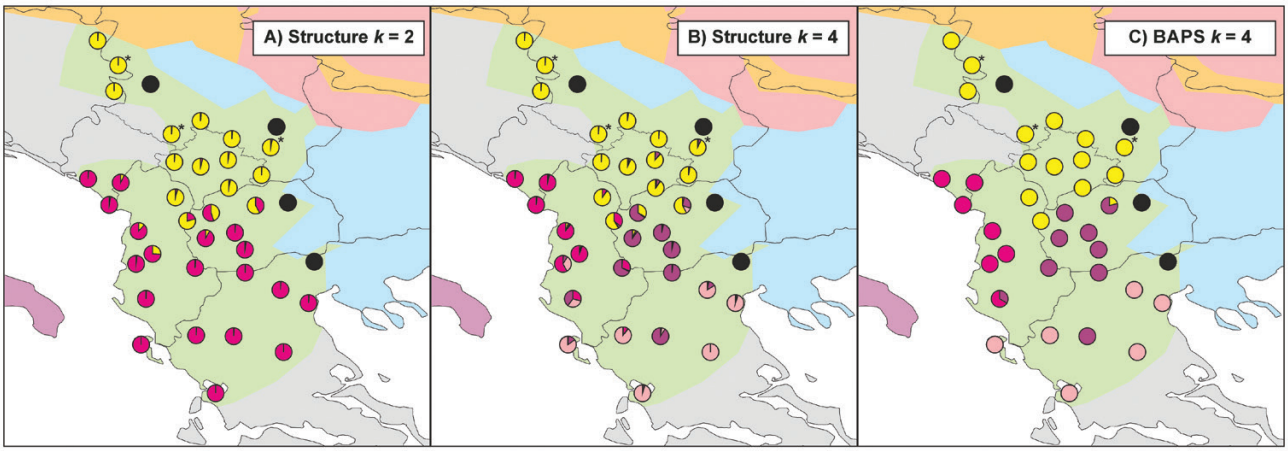

Figure 2. Bayesian structuring results for *Triturus macedonicus*. Pies reflect admixture proportions to genetic clusters at the level of the population for STRUCTURE under k=2 (A) and k=4 (B) and BAPS under k=4 (C). Populations affected by interspecific gene flow are marked with an asterisk if one individual was affected (and only the remaining two individuals were included) and coloured black if all individuals were affected (and the entire population was excluded).

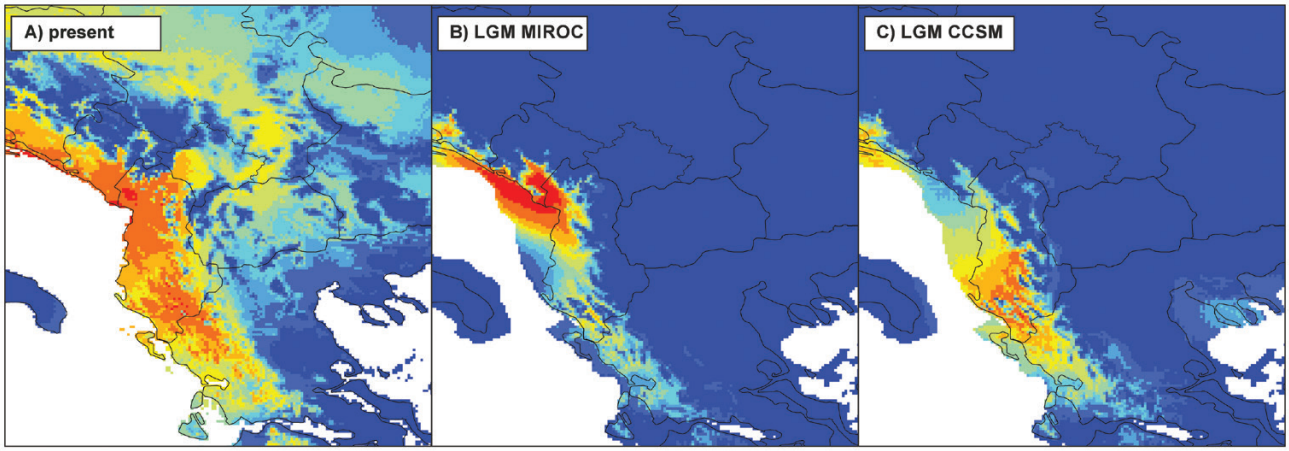

Figure 3. Species distribution model for *Triturus macedonicus*. The species distribution model is projected on climate layers for the present (A) and the Last Glacial Maximum based on the MIROC (B) and CCSM (C) climate models. Warmer colours indicate higher predicted suitability.

clades of species-specific mtDNA and all four nuclear DNA groups occur in this area today. There was no suggestion that the distribution range was fragmented at the LGM. The eastern part of the current range became suitable only after the LGM. This includes the area where mtDNA clade 3 occurs, where introgressed mtDNA is found and where the 'north-eastern nuclear DNA group' has its main distribution.

DISCUSSION

Although mtDNA has driven the field of phylogeography for more than two decades (Avise, 2000; Beheregaray, 2008), there is currently a shift taking place towards the use of large panels of unlinked nuclear markers (Garrick *et al*., 2015). Next-generation phylogeography allows us to test, refine and adjust the biogeographical hypotheses derived from mtDNA (Spinks *et al*., 2014; Dufresnes *et al*., 2020). Here, we have explored intraspecific genetic structuring of mitochondrial and nuclear genomes within the Balkan Peninsula glacial refugium, using the crested newt *T. macedonicus* as a model (Wallis & Arntzen, 1989).

Mitochondrial DNA is a poor predictor for the contemporary population structure of *T. macedonicus* because its geographical structure (Fig. 1) is highly discordant to that of the nuclear DNA groups (Fig. 2). In the west of its range, *T. macedonicus* possesses three highly distinct mtDNA clades that appear to have radiated before the Pleistocene (< 2.58 Mya; green, red and blue in Fig. 1). In fact, *T. macedonicus* shows the deepest divergence in mtDNA of any *Triturus* species (Wielstra *et al*., 2013). Mitochondrial DNA clade 1 (blue in Fig. 1) covers a large area and includes many distinct haplotypes with unclear relationships, indicative of long-term demographic stability (Hewitt, 1999). In contrast, clades 2 (red) and 3 (green) contain few haplotypes and are localized. Clades 1 and 2 occur in areas consistently predicted to have been suitable during the LGM based on species distribution modelling (Fig. 3). Clade 3 occurs slightly further eastwards of this suitable area, suggesting that the LGM distribution by the model is at least somewhat underestimated, because clade 3 must have survived here locally.

The nuclear DNA in *T. macedonicus* is structured into four groups that show extensive genetic admixture in the STRUCTURE analysis (but not in BAPS), all occurring in the west of the *T. macedonicus* range (Fig. 2). The distribution of nuclear DNA groups has little bearing on the distribution of mtDNA clades; one nuclear DNA group contains all three mtDNA clades, and one mtDNA clade is found in three of the nuclear DNA groups (Figs 1, 2). In general, mtDNA appears to be relatively stationary in comparison to the average nuclear marker, resulting in stronger and more permanent geographical structuring (Petit & Excoffier, 2009; Toews & Brelsford, 2012). Hence, this mtDNA structuring could reflect ancient population subdivision, whereas the signal in the nuclear DNA was lost at a later point owing to genetic swamping upon secondary contact, resulting in the maintenance of mtDNA clades that are no longer reflected by nuclear DNA clusters. Several other Balkan newt species are also known to possess such mtDNA 'ghost lineages' (Recuero *et al*., 2014; Pabijan *et al*., 2015).

In most of its postglacially colonized range, as predicted by the species distribution model (Fig. 3), *T. macedonicus* has captured mtDNA derived from another crested newt species, *T. ivanbureschi* (Wielstra *et al*., 2017; Fig. 1).

Although all the populations that carry introgressed mtDNA also belong to the same nuclear DNA group, this nuclear DNA group additionally encompasses individuals from further west that are known not to possess any diagnostic *T. ivanbureschi* nuclear alleles (Wielstra *et al.*, 2017). Hence, rather than introgression of *T. ivanbureschi* nuclear alleles defining this nuclear DNA group, we would argue that strong genetic drift attributable to postglacial expansion from a small source unites these populations in the Bayesian clustering analyses. The source of this expansion would be northern Albania, right at the eastern edge of the area predicted to have harboured suitable conditions during the LGM (Figs 2, 3).

Conclusion

Based on several dozen nuclear DNA markers, we find that *T. macedonicus* comprises multiple geographical genetic groups. Although species distribution modelling suggests a reduced glacial range, it does not support range fragmentation. We find the same number of partitions based on mtDNA and nuclear DNA, but the two datasets show geographical discordance. This 'evolutionary melting pot' pattern (Dufresnes *et al.*, 2016) suggests recurrent interglacial mingling of populations that have been diverging gradually during the glacial periods (Garrick *et al.*, 2019). For *T. macedonicus*, hybridization upon secondary contact even resulted in the capture of mtDNA from a different crested newt species (Wielstra *et al.*, 2017). Our genetic data are in line with a refugia-within-refugia scenario (Abellán & Svenning, 2014). Considering a large number of taxa with deep mtDNA structuring previously identified in the Balkan Peninsula (Poulakakis *et al.*, 2015), the generality of this scenario can be tested in future next-generation phylogeographical studies (Arbogast & Kenagy, 2001).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Sampling details and Bayesian population assignment for *Triturus macedonicus*.
Table S2. GenBank accession numbers for the mitochondrial DNA haplotypes identified in *Triturus macedonicus*.

SHARED DATA

Sequence data and files associated with analyses are available from the Dryad Digital Repository: Wielstra & Arntzen (2020).