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Running head: **Carpathian refugia-within-refugia**

Title: **The Carpathians hosted extra-Mediterranean refugia-within-refugia during the Pleistocene Ice Age: genomic evidence from two newt genera**

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Abstract

Part of Europe's temperate species survived the Pleistocene glacial cycles in refugia north of the Mediterranean peninsulas. For one such extra-Mediterranean refugia, the Carpathians, an intricate 'refugia-within-refugia' scenario has been suggested, involving species surviving in multiple discrete glacial refugia. We test the Carpathian refugia-within-refugia hypothesis, employing genome-wide multilocus datasets for two newt species (Triturus cristatus and Lissotriton montandoni). We first use Bayesian clustering to delineate intraspecific evolutionary lineages. The number of intraspecific lineages identified, and the allocation of localities to these lineages, were used to construct testable hypotheses on the spatial arrangement of glacial refugia in both newt species. Next we employ approximate Bayesian computation to date whether these lineages are of Holocene (< 12 Ka) or Pleistocene (> 12 Ka) origin. We identify three intraspecific evolutionary lineages for T. cristatus and two for L. montandoni. For both newt species, intraspecific divergence is rooted in the Pleistocene, in line with species survival in distinct range fragments during the last glacial period. Hence, our findings firmly support the Carpathian refugia-within-refugia hypothesis. Furthermore, we show that mitochondrial DNA overestimates the age of intraspecific evolutionary lineages and we urge caution in basing refugia-within-refugia scenarios on mitochondrial DNA alone.

Keywords: Approximate Bayesian computation; Bayesian clustering; Historical biogeography; Lissotriton montandoni; Next-generation sequencing; Triturus cristatus; Quaternary

Introduction

The climate oscillations of the Pleistocene Ice Age moulded intraspecific genetic structuring by repeatedly reducing temperate species' ranges during glacial cycles (Hewitt, 2000). The refugia-within-refugia concept addresses the evolution of intraspecific geographical genetic structuring, as species survive glacial conditions in fragmented pockets of suitable habitat within a single, wider refugial area (Abellán & Svenning, 2014; Gómez & Lunt 2007). Refugia-within-refugia have been reported from Europe's canonical glacial refugia: the Iberian (Gómez & Lunt 2007), Italian (Canestrelli et al., 2014) and Balkan (Poulakakis et al., 2015) Peninsulas. As regions situated north of Europe's southern peninsula are increasingly appreciated as sources of postglacial recolonization of temperate Europe (Schmitt & Varga, 2012; Stewart et al., 2010), this raises the question whether such areas also facilitated intraspecific Pleistocene differentiation. The Carpathians are arguably the most significant extra-Mediterranean refugium and accumulating phylogeographic studies suggest a refugia-within-refugia scenario applies (Mráz & Ronikier, 2016). We test this hypothesis here, using two newt species from different genera as a system.

The Northern crested newt Triturus cristatus (Laurenti, 1768) is a species of lowland and hills, distributed over much of temperate Europe and adjacent Asia, while the Carpathian newt Lissotriton montandoni (Boulenger, 1880) is a montane species, endemic to the Carpathians (Fig. 1a). Despite their ecological differences (Speybroeck et al., 2016), both species survived the Pleistocene glaciations in the Carpathians (Wielstra, Babik & Arntzen, 2015; Wielstra et al., 2013; Zieliński et al., 2014a; Zieliński et al., 2013). As genetic data show geographical sub-structuring and species distribution models suggest glacial range fragmentation within the Carpathians, these species are particularly suitable to test the Carpathian refugia-within-refugia hypothesis. We sequence several dozen nuclear DNA markers and use Bayesian clustering to delineate intraspecific geographical structure within each species. Subsequently, we test in an approximate Bayesian computation framework whether the observed intraspecific structure indeed arose during the Pleistocene, which would indicate species survival in multiple discrete glacial refugia and thus support the Carpathian refugia-within-refugia hypothesis.

Material and methods

Sampling

For T. cristatus we included 28 Carpathian breeding ponds (Fig. 1b) and an additional seven positioned in postglacially colonized area and sampled up to three (2.9 on average) individuals per pond (see Table S1 in Appendix S1). For L. montandoni we included 31 Carpathian breeding ponds (Fig. 1c) and sampled up to three (1.3 on average) individuals per pond (Table S2 in Appendix S1). Individual ponds were treated as localities.

Summary of sequencing

For T. cristatus we newly sequenced 52 nuclear markers. See Wielstra et al. (2014) for a detailed description of the laboratory and bioinformatics protocol. In brief, we amplified markers of c. 140 bp in length (excluding primers), positioned in 3' untranslated regions of protein-coding genes, in five multiplex PCRs. We pooled the multiplexes for each individual and ligated unique tags to be able to recognize the amplicons 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 to a genotypic format directly usable for population genetic analysis. Mean coverage was 777 reads (range 0-13,622) per marker-individual combination. Marker-individual combinations with ≥ 20 reads (1.73%) were considered successful.

For L. montandoni we sequenced 74 nuclear markers. See Zieliński et al. (2014b) for a detailed description of the laboratory and bioinformatics protocol. In brief, we amplified markers of ca. 500 bp in length (excluding primers), positioned in 3' untranslated regions of protein-coding genes, in ten multiplex PCRs. Again, multiplexes for each individual were pooled and given unique tags. We sequenced the amplicons on the Illumina MiSeq next-generation sequencing platform to the average per base coverage of 1017 ± (SD) 1181. Sequence data were further processed using standard, freely available bioinformatic tools, producing phase-resolved variants. Fasta files were obtained from vcf files using custom python script. Marker-individual combinations with < 10 reads were considered failed. These data were previously used in another study (Zieliński et al., 2016).

Bayesian clustering analysis – constructing hypotheses

Triturus and Lissotriton newts hybridize with congeneric species at their contact zones (Arntzen, Wielstra & Wallis, 2014; Zieliński et al., 2016) and while introgression of mitochondrial DNA in T. cristatus is restricted to the contact zone with congeneric species (Wielstra et al., 2015), it has led to the complete replacement of the original mitochondrial DNA of L. montandoni (Babik et al., 2005; Zieliński et al., 2013). Including individuals showing recent genetic admixture with another species (early generation hybrids) could have unpredictable effects in downstream analyses of intraspecific genetic divergence, while limited nuclear DNA introgression (via ancient hybridization) simply constitutes a part of intraspecific genetic diversity and as such does not require separate treatment in our models.

To confirm there were no early generation hybrids present in our dataset we took a two-step approach. We first used STRUCTURE 2.3.4 (Pritchard, Stephens & Donnelly, 2000) to confirm that our set of T. cristatus and L. montandoni individuals did not show significant genetic admixture (STRUCTURE $Q \geq 0.05$) with Triturus or Lissotriton species with abutting ranges. We did so by enforcing the number of gene pools (k) to 2 in pairwise species comparisons (Tables S1-S2 in Appendix S1). Entire haplotypes were treated as alleles at each locus. 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 five replicates, which were summarized with CLUMPAK (Kopelman et al., 2015). As T. cristatus has parapatric range boundaries with four other Triturus species, namely T. carnifex, T. dobrogicus, T. ivanbureschi and T. macedonicus, we took reference data for these species, four localities per species with three individuals per locality, from Wielstra et al. (2014). The only species that L. montandoni has a parapatric range boundary with is L. vulgaris and we took reference data for this species, 45 individuals from 38 localities, from Zieliński et al. (2016).

Next, we used STRUCTURE to determine the number of intraspecific evolutionary lineages in both newt species. We used the same settings as before but tested over multiple values of k. The upper k limit was 35 for T. cristatus and 31 for L. montandoni, as defined by the total number of localities included. We used Evanno's Δ k criterion (Evanno, Regnaut & Goudet, 2005) to select the optimum k

value. The number of intraspecific lineages identified, and the allocation of localities to these lineages, were used to construct testable hypotheses on the spatial arrangement of glacial refugia in both newt species.

Approximate Bayesian computation – rationale

Using approximate Bayesian computation (ABC) we evaluated the existence of three T. cristatus and two L. montandoni glacial refugia in the Carpathians (as suggested by STRUCTURE) by testing models assuming: 1) a Holocene (< 12 Ka) and 2) a Pleistocene (> 12 Ka) origin of intraspecific evolutionary lineages. Support for the latter model would imply that the evolutionary lineages diverged prior to the end of the last glacial maximum and hence must have survived glaciations in separate refugia. Therefore our ABC modelling can be considered an explicit test of the refugia-within-refugia hypothesis. Within species, all parameter priors (except topology for T. cristatus) were identical for the tested models and no demographic changes and historical gene flow were allowed to keep models as simple as possible.

Approximate Bayesian computation – data preparation

For T. cristatus we focus on localities in the Carpathian area only (1-28). According to the STRUCTURE results, crested newt localities were assigned to three lineages, one within (**TcB**), one east of (**TcE**) and one south of (**TcS**) the Carpathian mountain belt (figure 1b). We considered three topologies: 1) (**TcE**, **TcS**) **TcB** – supported by a drift tree based on allele frequency data, 2) **TcB**, **TcE**, **TcS** – a polytomy, and 3) (**TcB**, **TcS**) **TcE** - supported by the nucleotide distance between evolutionary lineages (see Fig. S1 in Appendix S2). Two localities (2 and 9) showing a high degree of admixture between evolutionary lineages ($0.3 < Q < 0.7$) were not analysed to exclude the effect of ongoing hybridization and early-generation admixture. Seven markers (agl, clasp, gys, samdb, smo, taf8, and usp) in which more than 5% of individuals did not amplify were removed. Furthermore, 11 individuals for which one or more of the retained markers did not amplify were excluded. Next, alignment columns with missing data (i.e. indels) were removed. We assume that newt breeding ponds correspond to discrete demes which may undergo extinction and recolonization. To minimize the confounding effects of current population structure we randomly subsampled one gene copy per locality. It has been shown (Wakeley, 2004;

Wakeley & Aliacar, 2001) that if one gene copy per locus is sampled per deme in a metapopulation composed of a large number of demes, the ancestral process producing such a sample is identical to the unstructured coalescent process. Our final ABC dataset contained one gene copy per locus from 25 localities, distributed over the three evolutionary lineages as follows: 7 **TcB**, 9 **TcE** and 9 **TcS**.

For L. montandoni STRUCTURE suggested two lineages: one south (**LmS**) and one north (**LmN**) of approximately the centre of the Eastern Carpathians, roughly the Ukrainian/Romanian border (figure 1c). As there are only two evolutionary lineages in L. montandoni we only had to consider a single topology: **LmN**, **LmS** (Fig. S2 in Appendix S2). We excluded two individuals from localities with considerable admixture between evolutionary lineages ($0.3 < Q < 0.7$). For the ABC analysis, we excluded eight markers that were fully coding or amplified inconsistently so that the final data set included 66 markers. Furthermore, five individuals for which one or more of the retained markers did not amplify were excluded. Next, alignment columns with missing data were removed. As explained above for T. cristatus, one gene copy per marker was sampled per locality. Our final ABC dataset contained one gene copy per locus from 24 localities, distributed over the two evolutionary lineages as follows: 9 **LmN** and 15 **LmS**.

Approximate Bayesian computation – summary statistics

We focused on a set of basic summary statistics, likely to be informative about the time of the split between intraspecific evolutionary lineages, and other demographic parameters. For each evolutionary lineage we calculated average and variance of: number of fixed polymorphisms (SF), number of shared polymorphisms (SS), number of private polymorphisms (SP), nucleotide F_{ST} (FST_nuc) calculated between evolutionary lineages and between a particular evolutionary lineage and the remaining ones pooled (in three-lineage models), Tajima's D (D), nucleotide diversity (Pi), number of haplotypes (nHap), haplotype diversity (HapW), d_{xy} calculated between lineages (PiA) and the number of haplotypes shared between all lineages and lineage pairs (n_shared_hap). Additionally we calculated average and variance of nHap, HapW, D, Pi and the overall number of segregating sites (S) for the whole dataset. Summary statistics for both observed and simulated data sets were calculated on polymorphic biallelic sites only. Positions with more than two segregating variants were excluded as

179 departing from the infinite sites model. For each statistic, mean and variance across all loci were
180 calculated using MSSTATSPOP v.0.998980-beta (Ramos-Onsins et al., unpublished) and custom Python
181 scripts.
182
183 Approximate Bayesian computation – simulations and analysis
184 Coalescent simulations were performed using FASTSIMCOAL2.01 (Excoffier et al., 2013). We simulated
185 data using the finite site mutation model (as our data did not fit the infinite site model) and a single,
186 fixed mutation rate of = 5.7 x 10^{-9}per site, per generation, as previously estimated for smooth and
187 Carpathian newts using fossil-based dating of divergence within genus Lissotriton (Pabijan et al., 2015;
188 Zieliński et al., 2016). Considering that Triturus and Lissotriton are relatively closely related (Zhang et
189 al., 2008) and we use highly similar genetic markers (Wielstra et al., 2014; Zieliński et al., 2014b), we
190 considered it appropriate to use the same mutation rate for both systems. These markers are known to
191 be unlinked in both newt systems (Wielstra et al., 2017; Zieliński et al., 2016). Loci were simulated as
192 independent chromosomes. The ABC analysis was performed within the ABCTOOLBOX (Wegmann et
193 al., 2010). We used a generation time of 4 years based on the synthesis of the literature (Nadachowska
194 & Babik, 2009) and assumed it appropriate to use this value for Triturus as well (Duellman & Trueb,
195 1994). Our recombination rate priors were based on a previous estimate for smooth and Carpathian
196 newts (Zieliński et al., 2016) (Tables S3-S4 in Appendix S1). Parameter values were sampled from
197 uniform prior distributions, priors for population sizes were uniform on a log10 scale (Tables S3-S4 in
198 Appendix S1) and were set to cover biologically plausible values. Analyses were based on 10^6 datasets
199 simulated under each demographic model. We retained the 0.1% (10^3) best simulations for each model
200 and computed the marginal likelihood of the observed and retained datasets under a Generalized Linear
201 Model (Leuenberger & Wegmann, 2010). For each species we compared all models in a single model
202 selection procedure and selected the best fitting ones based on posterior probabilities. We inspected
203 posterior probability curves and the fraction of retained simulations with the marginal likelihood smaller
204 or equal to that of the observed data (observed P-value) to determine if models could faithfully
205 reproduce the observed data. The best fitting model was selected based on Bayes factors (ratios of model
206 marginal densities). To estimate the power to distinguish between models we generated 1,000 pseudo-

observed datasets for each model and checked how often the ABC model choice procedure correctly predicted the true model (the one that produced the dataset). Each pseudo-observed dataset was treated as the observed data and used to calculate marginal densities of all compared models. Bayes factors were then used to select the best model. As we were interested in both the overall power to identify the true model as well as the power in the observed summary statistics space, the pseudo-observed datasets for each model were chosen from both random and retained simulations. To check whether the marginal posterior distributions estimated from the best models were biased, we generated 1,000 pseudo-observed data sets for each best model and tested uniformity of the posterior quantile distributions (the position of the true values within the posterior distribution) for each parameter with a Kolmogorov–Smirnoff test. If the parameter values for these pseudo-observed data were randomly chosen from the prior distribution, we expect the posterior quantiles to be uniformly distributed. Because for T. cristatus (while a Holocene divergence was confidently rejected) the posterior validation suggested potential overestimation of divergence time in the preferred model, we further explored this matter by rerunning the preferred model 1) without a fixed lower prior boundary for split time and 2) without a fixed lower prior boundary for split time and with an upper prior boundary for split time fixed to 0.5 Ma.

Results

Bayesian clustering analysis – intraspecific evolutionary lineages

STRUCTURE confirmed our set of T. cristatus and L. montandoni individuals showed no significant recent genetic admixture with congenerics. STRUCTURE suggested k = 3 as the most likely number of gene pools for T. cristatus and k = 2 for L. montandoni (Tables S1-S2 in Appendix S1). The three T. cristatus lineages roughly correspond to within (**TcB**), east of (**TcE**) and south of (**TcS**) the Carpathian mountain belt (figure 1b). Lineage **TcB** is also the one that postglacially colonized temperate Eurasia. The two L. montandoni lineages show a different geographical configuration, with an evolutionary lineage south (**LmS**) and north (**LmN**) of approximately the centre of the Eastern Carpathians, roughly the Ukrainian/Romanian border (figure 1c).

Approximate Bayesian computation – polymorphism and observed summary statistics

The T. cristatus ABC dataset included 45 markers of the average length 139 bp (6,248 bp). There were 106 haplotypes out of which 51 (48%) were shared between evolutionary lineages. We observed 67 polymorphic sites out of which four (6%) were private to **TcB**, 19 (28%) to **TcE** and 23 (34%) to **TcS**. The L. montandoni ABC data set comprised of 66 markers of the average length of 484 bp (31,929 bp). There were 391 haplotypes out of which 105 (27%) were shared between evolutionary lineages. We observed 652 polymorphic sites out of which 156 (24%) were private to **LmN**, 283 (43%) to **LmS**. In both species the percent of sites segregating in all lineages was similar, 31% in T. cristatus and 33% in L. montandoni. We found no fixed differences between lineages in either species (Tables S5-S6 in Appendix S1).

Approximate Bayesian computation – model choice for Triturus cristatus

The P-values calculated under the Generalized Linear Model were used to check whether tested models were able to reproduce the observed data (Table S5 in Appendix S1). For all T. cristatus models assuming a Pleistocene split (M2, M4, M6), the observed data fell well within the distribution of retained simulated data (Table S7 in Appendix S1). Models assuming a Pleistocene divergence were always favoured and the polytomy model (M4) had the highest posterior probability (PP=0.95) (Table S7 in Appendix S1). The mean power to identify the true model was 0.59 and in the case of the preferred M4 model it was 0.74 (Table S9 in Appendix S1). Although within the observed summary statistics space the M4 model power decreased to 0.37, there was no case in which simulations produced under other models would choose M4 as the true model more often than the model of origin. Importantly, only simulations under other models of a Pleistocene divergence selected M4 more often than expected by chance (Table S9 in Appendix S1). The selected M4 model indicates a Middle Pleistocene 172 Ka (77-281 Ka) divergence between lineages (Fig. S3 in Appendix S2, Table S3 in Appendix S1).

According to the posterior validation (Fig. S5 in Appendix S2, Table S3 in Appendix S1), divergence time might be overestimated for model M4, so the estimates should be treated with caution. It needs to be stressed here, however, that hypothesis testing was based on model selection, not on parameter estimates. Therefore, the bias in the divergence time estimates does not affect the main results

of our test, which firmly supports the Pleistocene divergence and rejects a Holocene divergence. Still, to interpret whether this bias affected the actual divergence time estimate within the preferred Pleistocene divergence model, we reran the preferred model without a fixed lower prior boundary for split time (M7) and without a fixed lower prior boundary for split time and with an upper prior boundary for split time fixed to 0.5 Ma (M8). While M7 showed a similar bias as M4, bias was considerably reduced in M8 (details on Dryad). Yet, the inferred divergence time was almost identical (details on Dryad). Hence we conclude that the divergence time estimated in M4 is reliable.

Approximate Bayesian computation – model choice for Lissotriton montandoni

A model assuming Pleistocene divergence (M2) performed better than one assuming post-Pleistocene divergence (M1; Table S8 in Appendix S1). The power to correctly predict the true model was high for both models, regardless of statistics space (Table S10 in Appendix S1). According to the Kolmogorov–Smirnoff test results (Table S4 in Appendix S1) all parameter distributions were biased. However, visual inspection of the distributions of divergence time posterior quantiles (Fig. S6 in Appendix S2) suggests that the true values were more often found in the centre of the distribution, which is a consequence of overly wide priors. Importantly, this kind of bias may only slightly decrease precision of the estimates. Hence, while our simple models were not able to faithfully reproduce the observed data (Table S6, S8 in Appendix S1), we nevertheless consider it safe to interpret the estimated divergence time from the best performing model. The selected M2 model again indicates a Middle Pleistocene 202 Ka (54-347 Ka) divergence between lineages (Fig. S4 in Appendix S2, Table S4 in Appendix S1).

Discussion

While the importance of the Carpathians as a glacial refugium has by now become well established, a more intricate pattern of refugia-within-refugia is still emerging (Mráz & Ronikier, 2016). We here tested the Carpathian refugia-within-refugia hypothesis, based on next-generation phylogeography and ABC analysis for two newt species of different genera. For both species, models assuming a Pleistocene divergence were strongly preferred, even though disparate patterns of intraspecific genetic structure

291 highlight that species had idiosyncratic responses to glacial cycles (Fig. 1). The build-up of deep
292 intraspecific differentiation in ecologically distinct species provides strong support for a scenario in
293 which multiple discrete regions within the Carpathians acted as glacial refugia, for a broad range of
294 species. Our findings emphasize the key role that the Carpathians played in Pleistocene survival and
295 radiation of temperate Eurasia's biodiversity.

296 Accuracy of our divergence time estimates, crucial for the interpretation of this test, could be
297 affected by 1) the mutation rate and generation time used to convert ABC estimates into calendar years,
298 and 2) gene flow between evolutionary lineages. Only a several-fold underestimation of mutation time
299 or overestimation of generation time could lead to erroneous support for Pleistocene divergence, but,
300 as the values used are well supported, we consider this unlikely. Furthermore, gene flow would cause
301 under- rather than an overestimation of divergence time, yet post-Pleistocene divergence was still
302 rejected. Hence, we conclude that our ABC analysis strongly supports a pre-Pleistocene divergence of
303 evolutionary lineages and provides robust evidence for Carpathian refugia-within-refugia, illustrating
304 the added value of ABC analysis in Carpathian phylogeography (see also Kolář et al., 2016).

305 Our nuclear DNA results suggest that the intraspecific structuring observed today originated
306 during the penultimate glacial period (130-200 Ka). This is an order of magnitude younger than the
307 divergence of the three mitochondrial DNA lineages present in T. cristatus (with even the most
308 conservative interpretation based on confidence intervals suggesting divergence well over a million
309 years ago), which have a similar distribution as the evolutionary lineages identified in the present study
310 (Wielstra et al., 2015). It should be noted that no comparable mitochondrial DNA data is available for
311 L. montandoni, as its native mitochondrial DNA relatively recently got replaced with that of a congener,
312 via mitochondrial DNA capture (Zieliński et al., 2013). Nuclear gene flow upon secondary contact
313 during Pleistocene interglacials would cause fusion of intraspecific lineages, a realistic scenario given
314 the historical instability of phylogeographic patterns (Hofreiter et al., 2004), and in fact genetic
315 admixture is observed where evolutionary lineages meet today. Phylogeographic structure is often
316 retained longer in mitochondrial DNA than in the nuclear genome (Petit & Excoffier, 2009). While the
317 long-term persistence of geographically structured mitochondrial DNA clades could be interpreted as
318 evidence that the same areas acted as refugia during multiple glacial periods (Hewitt, 2011), our findings

underline that the stability and historical isolation of refugia-within-refugia delineated based on mitochondrial DNA alone could well be overestimated. Considering the strong bias in phylogeographic surveys towards mitochondrial DNA (Riddle, 2016), we suggest that proposed refugia-within-refugia scenarios require re-evaluation with nuclear data.

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Supporting information

Appendix S1 Supplementary Tables S1-S10.

Appendix S2 Supplementary Figures S1-6.

Data accessibility

Sequence data and files associated with analyses are available from the Dryad Digital Repository entries doi:10.5061/dryad.83k00 and [xxxToBeAddedxxx].

Figure legends

Figure 1. Distribution of and Bayesian clustering results for Triturus cristatus and Lissotriton montandoni. In (a) rough outlines of the ranges of both species are shown, with the range of L. montandoni (in blue) superimposed on that of T. cristatus (in red). In (b) the preferred model for each

451 species in the approximate Bayesian computation analysis is shown. Codes for evolutionary lineages
452 are explained in Results and colours correspond to the gene pools identified in the Structure analysis.
453 In (c) pie diagrams represent the allocation by Structure of localities to different gene pools (k) for T.
454 cristatus (k = 3; red tones) and L. montandoni (k = 2; blue tones) and pie diameter reflects sample size
455 of localities (n = 1 or n = 3). Grey shading denotes elevation.