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# The crested newt *Triturus cristatus* recolonized temperate Eurasia from an extra-Mediterranean glacial refugium

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We assess the role of the Carpathians as an extra-Mediterranean glacial refugium for the crested newt *Triturus cristatus*. We combine a multilocus phylogeography (one mitochondrial protein-coding gene, three nuclear introns, and one major histocompatibility complex gene) with species distribution modelling (projected on current and Last Glacial Maximum climate layers). All genetic markers consistently show extensive genetic variation within and genetic depletion outside the Carpathians. The species distribution model suggests that most of the current range was unsuitable at the Last Glacial Maximum, but a small suitable area remained in the Carpathians. *Triturus cristatus* dramatically expanded its postglacial range, colonizing much of temperate Eurasia from a glacial refugium in the Carpathians. Within the Carpathians, *T. cristatus* persisted in multiple geographically discrete regions, providing further support for a Carpathian ‘refugia within refugia’ scenario. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **114**, 574–587.

**ADDITIONAL KEYWORDS:** Carpathians – genetic depletion – Last Glacial Maximum – major histocompatibility complex – mitochondrial DNA – nuclear DNA – phylogeography – species distribution modelling.

## INTRODUCTION

Phylogeography and species distribution modelling are useful tools for the inference of past range dynamics (Avice, 2000; Kozak, Graham & Wiens, 2008). Populations persisting in glacial refugia have a long and stable demographic history relative to those established postglacially and as a consequence are characterized by relatively high levels of genetic diversity (Provan & Bennett, 2008). Species distribution models projected on past and present environmental reconstructions can be used to identify areas able to sustain populations and can also be used to determine whether suitability increased or decreased through time (Svenning *et al.*, 2011). Phylogeography and species distribution modelling are especially informative when used together to infer distribution shifts (Alvarado-Serrano & Knowles, 2014).

The glacial–interglacial cycles of the Quaternary greatly influenced species distributions. The overarching pattern for temperate terrestrial species is one of glacial range contraction and interglacial range expansion (Hewitt, 2000). In Europe, this entailed southward regression during glacial periods and northward expansion during interglacials. The southern European peninsulas (Iberian, Italian, and Balkan) have traditionally been regarded as glacial refugia, where temperate terrestrial species persisted during glacial periods and from which they recolonized Europe during interglacials (Provan & Bennett, 2008). A recent realization is that, for some temperate terrestrial species, glacial refugia were also situated to the north of these traditionally recognized regions (Provan & Bennett, 2008; Stewart *et al.*, 2010; Schmitt & Varga, 2012; Tzedakis, Emerson & Hewitt, 2013).

The Carpathians are increasingly thought to have acted as an extra-Mediterranean glacial refugium;

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the region experienced relatively mild and stable climatic conditions during glacial cycles and maintained forest cover (Ronikier, 2011; Tzedakis *et al.*, 2013). The Carpathians are an area of endemism, with species thought to have been present long-term and having survived glaciations *in situ* (e.g. the newt *Lissotriton montandoni*, Zieliński *et al.*, 2013). Pollen and fossil data have been insightful in revealing the region's role as a glacial refugium (Willis & van Andel, 2004; Sommer & Nadachowski, 2006). Phylogeographic studies have contributed to this realization in particular. Some species contain distinct genetic clades restricted to the Carpathians, again suggesting long-term survival *in situ* (e.g. the newt *Lissotriton vulgaris*, Babik *et al.*, 2005; and the toad *Bombina variegata*, Fijarczyk *et al.*, 2011). Such geographical subpopulations also contributed to the postglacial recolonization of Europe, in addition to populations from traditional southern glacial refugia (e.g. the tree *Malus sylvestris*, Cornille *et al.*, 2013; and the vole *Clethrionomys glareolus*, Kotlík *et al.*, 2006). Hence, evidence accumulated from diverse taxa indicates that climatic conditions in the Carpathians were sufficiently favourable for survival of diverse temperate communities throughout multiple glacial cycles.

For some species, the Carpathians could have acted as the sole source for colonization of temperate Europe. A likely candidate for such a scenario is the crested newt *Triturus cristatus* (Amphibia: Salamandridae). The range of *T. cristatus* stretches across most of temperate Europe, north of the European peninsulas, and encompasses the Carpathians (Fig. 1). Note that a small part of the range of *T. cristatus* is situated south of the Danube River, in and around the Serbian Carpathians. Although politically this region covers the Balkan Peninsula, geologically and climatologically it is part of the Carpathians.

One of Europe's first phylogeographic studies used the genus *Triturus* as a model, employing mitochondrial DNA (mtDNA) restriction fragment length polymorphism to assess patterns of regional diversity (Wallis & Arntzen, 1989). Little or no genetic variation was detected in *T. cristatus*, except in the southernmost part of the range in Serbia and Romania. This pattern was substantiated based on a study employing mtDNA sequence data (Wielstra *et al.*, 2013b). A study on variation in class II of the major histocompatibility complex (MHC) showed high polymorphism in four Romanian populations in comparison with genetic depletion in five populations from temperate Europe, a pattern mirrored by microsatellites and allozymes (Babik *et al.*, 2009).

These previous studies did not focus specifically on *T. cristatus* and/or included limited sampling in terms

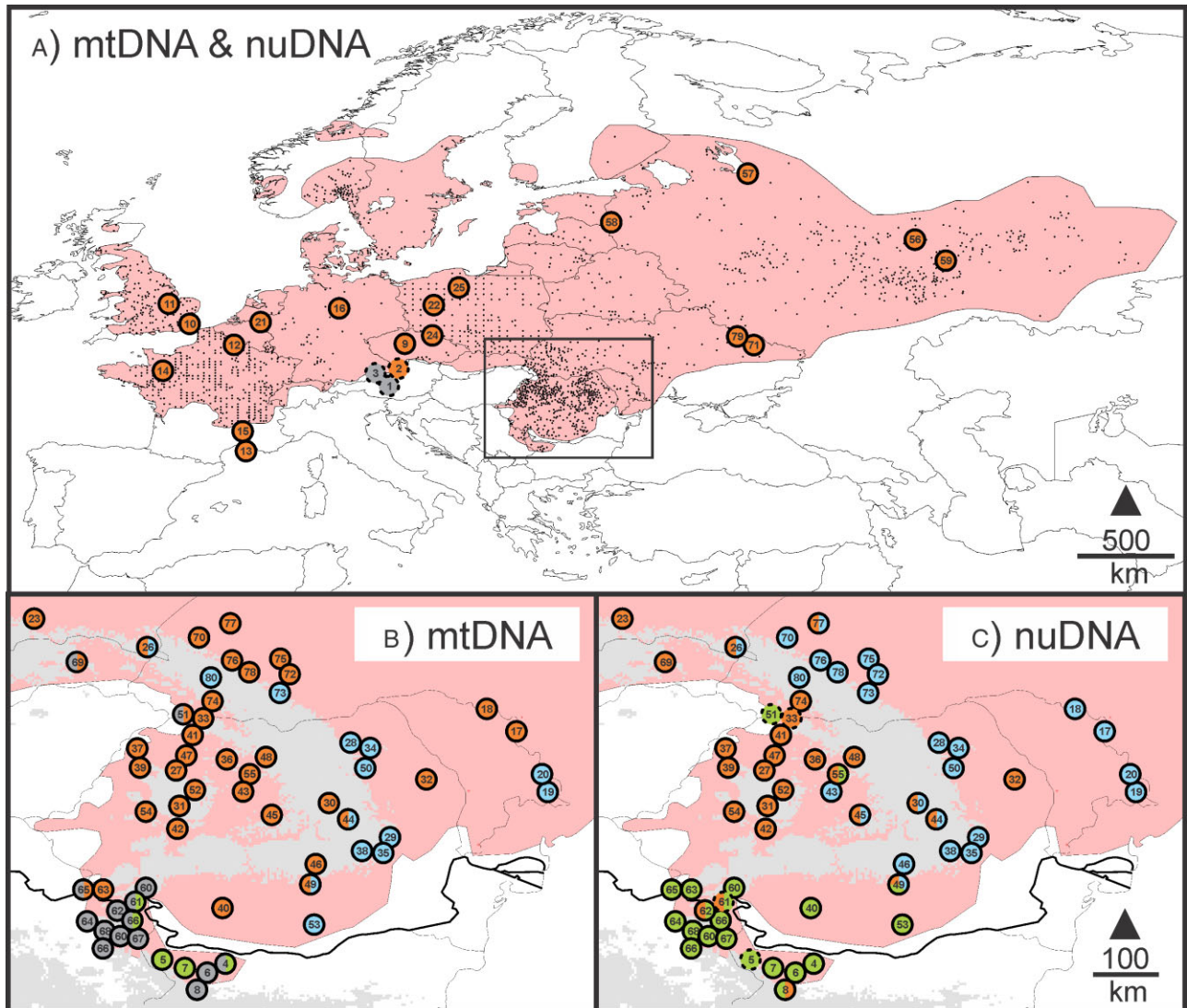
of populations and/or markers. However, they strongly suggested that *T. cristatus* had a glacial refugium in the Carpathians. Here, we aim to trace the glacial contraction and postglacial expansion of *T. cristatus* by conducting a range-wide phylogeographic analysis of five unlinked genetic markers (one mitochondrial protein-coding gene, three nuclear introns, and one major histocompatibility complex gene) and by constructing species distribution models of *T. cristatus* at the Last Glacial Maximum.

## MATERIAL AND METHODS

### DNA SEQUENCING

Samples were collected throughout the range of *T. cristatus*, with a particular focus on the Carpathians. Breeding ponds were interpreted as populations. We included 194 newts from 80 ponds: 59 ponds and 153 newts from the Carpathians (as defined in Fig. 1; details in Supporting Information, Appendix S1); and 21 ponds and 41 newts from elsewhere. We sequenced one mitochondrial protein-coding gene (ND4, 658 bp), three nuclear introns ( $\beta$ fibint7, 499 bp; *CalintC*, 419 bp; and *Pdgfra*, 653 bp), and the second exon of class II of the MHC (200 bp). For details on primers and laboratory methods for the mtDNA and nuclear intron data, see Wielstra *et al.* (2013b) and Espregueira Themudo, Wielstra & Arntzen (2009). mtDNA data for 188 newts and nuclear intron data for 22 newts were taken from these two studies; other data were new. Sequences were manually aligned and identical ones were merged into haplotypes using MacClade 4.08 (Maddison & Maddison, 2005). We excluded indels (present in the nuclear introns) from the analyses, following the recommendations of Lemmon *et al.* (2009).

We sequenced a 200 bp fragment of the second exon of the MHC class II for all newts, using primers that amplify both pseudogenes and functional alleles (see Babik *et al.*, 2009). Primers contained additional 6-bp tags (ten variants in the forward primer and ten in the reverse, giving 100 combinations) to allow identification of reads derived from a particular individual. Amplicons were pooled equimolar into two pools. Each pool was barcoded with a unique Ion Xpress™ Barcode Adapter (Life Technologies) and sequenced using an Ion-318-chip (Ion PGM 400 Sequencing Kit) on the Ion Torrent platform (Rothberg *et al.*, 2011). Sequences were processed with jMHC (Stuglik, Radwan & Babik, 2011), which assigned reads to individuals on the basis of barcode sequences and provided counts of sequence variants per individual. As there are several co-amplifying



**Figure 1.** Distribution of and sampling scheme for *Triturus cristatus*. A, sampling outside the Carpathians. The cut-out (B, C) shows sampling in the Carpathian region in more detail, with areas of > 500 m altitude shaded grey and the Danube River shown as a thick black line. Localities sampled for genetics are shown as open circles, and small black dots represent additional localities used for the species distribution modelling. A, B, populations belonging to mtDNA clades 1, 2, and 3 are coloured blue, green, and orange, respectively (as in Fig. 2), and populations containing introgressed mtDNA derived from other *Triturus* species are coloured grey. A, C, populations belonging to BAPS groups 1, 2, and 3 are coloured blue, green, and orange, respectively [the two grey populations in (A) also belong to BAPS group 3] and those populations for which (part of the) individuals are genetically admixed as a result of hybridization with other *Triturus* species have an interrupted outline. Sampling details can be found in the Supporting Information, Appendix S1. nuDNA, nuclear DNA.

pseudogene loci in *T. cristatus*, and assignment of pseudogene variants to loci was not possible, we only genotyped the functional DAB locus. True alleles were distinguished from artefacts at the level of the entire dataset following Radwan *et al.* (2012), and genotyping was performed manually on the basis of read counts.

#### GENETIC ANALYSES

Introgressed mtDNA haplotypes derived from other *Triturus* species (identified based on Wielstra *et al.*, 2013b) were excluded from further analyses. Because introgression from other *Triturus* species could inflate genetic diversity (Arntzen, Wielstra & Wallis, 2014),



we excluded individuals showing signs of genetic admixture based on the nuclear DNA introns, by conducting pairwise comparisons with each of the four species that have a hybrid zone with *T. cristatus*: *Triturus carnifex*, *Triturus dobrogicus*, *Triturus ivanbureschi*, and *Triturus macedonicus* (sampling details in Supporting Information, Appendix S1; data from Wielstra, Baird & Arntzen, 2013a) with NewHybrids 1.1 (Anderson & Thompson, 2002), using a burn-in and formal run of 10 000 iterations each. NewHybrids infers for every individual the probability with which it belongs to a purebred (either of the two parental species under consideration) or a hybrid (F1, F2, or a backcross with one of the two parental species) class. In each of the four runs we set the foreign species as the second parent species using the z option; all tested individuals belonging to *T. cristatus* with a *P*-value of < 0.95 were considered admixed. For the MHC it was less straightforward to identify alleles potentially derived from other *Triturus* species. Populations showing signs of introgression for the other genetic markers were treated as potentially introgressed.

For mtDNA and the nuclear DNA introns, median joining networks were created using Network 4.6.11 (<http://www.fluxus-engineering.com>). Phylogenies were constructed with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003), employing two, four-chain, twenty-million-generation runs, with a sampling frequency of 0.001 and a heating parameter of 0.1. The most appropriate models of sequence evolution (GTR+I for ND4, GTR+G for  $\beta$ fibint7, F81+I for *CalintC*, and HKY+I for *Pdgrfr*) were identified with MrModeltest 2.2 (<http://www.abc.se/~nylander/>), based on the Akaike Information Criterion. The first quarter of the sampled trees was discarded as burn-in after analysis of the output in Tracer 1.5 (<http://tree.bio.ed.ac.uk/software/tracer/>) and the inference was drawn from the remaining 'forest'.

Pairwise distances (*p*) among the main mtDNA clades identified were determined using MEGA 5 (Tamura *et al.*, 2011). To estimate divergence times for mtDNA clades we used BEAST 1.8 (Drummond *et al.*, 2012) and followed the calibration scheme of Wielstra *et al.* (2010). A fossil dated at 24 Mya was interpreted as a minimum estimate for the most recent common ancestor of the genus *Triturus* and was appointed a lognormally distributed prior with a mean of 24, an offset of 10, and a default standard deviation (1.0). The origin of the Adriatic Sea at 5.33 Mya, at the end of the Messinian Salinity Crisis, was interpreted as the vicariant event causing the split between *T. carnifex* and *T. macedonicus* and was appointed a normally distributed prior with a small standard deviation (0.001). We applied the uncorrelated lognormal relaxed clock model and a Yule speciation model and used the

GTR+I model of sequence evolution. We conducted two independent hundred-million-generation runs, with a sampling frequency of 0.0001. The first half of the sampled trees was discarded as burn-in after analysis in Tracer 1.5; runs converged and effective sample sizes were at least 200.

We conducted a Bayesian analysis of population structure for the three nuclear DNA introns combined with the program BAPS v.6 (Cheng *et al.*, 2013). BAPS assigns individuals to distinct gene pools probabilistically, based upon multilocus genetic data, in which each individual allele is coded as a haplotype (two alleles per marker, which may or may not be identical). BAPS does not make a-priori assumptions about the number of gene pools (*k*) but a fixed number can be set. We used BAPS in two ways. First, we let BAPS determine the most probable number of distinct gene pools, evaluating *k* over a  $1 \leq k \leq 80$  range. Second, we enforced BAPS to partition the individuals in three groups (*k* = 3), as the mtDNA data revealed the presence of three distinct clades (see the Results). For both searches we used ten replicates.

For mtDNA and the nuclear DNA introns we used DnaSP 5 to determine the number of haplotypes and nucleotide diversity. We tested for the signal of a bottleneck using Fu's *F<sub>s</sub>* statistic, for which significance was assessed with 1000 coalescent simulations. Newts form discrete demes, corresponding to breeding ponds, which undergo extinctions and recolonizations, and hence the regional population can be regarded as a metapopulation (Smith & Green, 2005). If one gene copy per locus is sampled per deme, the ancestral process converges to the unstructured coalescent (Wakeley & Aliacar, 2001). Therefore, we used a randomly selected haplotype to represent each population as this greatly simplifies interpretation of coalescent-based tests. We conducted these analyses for all populations and for those positioned inside or outside the Carpathians. Because sampling is skewed towards the Carpathians, the hypothesis that genetic diversity outside the Carpathians is lower than inside was tested using randomization tests with 1000 replicates. In each replicate, sequences were randomly assigned to the two groups, whilst keeping sample sizes equal to the observed, thus accounting for the differences in sampling density inside and outside the Carpathians (Fig. 1). The difference in nucleotide diversity was calculated for each permutation and the fraction of permutations that produced a difference higher than or equal to that observed was taken as the one sided *P*-value.

For MHC the allelic richness (AR) was computed in FSTAT (Goudet, 1995). Differences in AR between the three groups of populations, namely (1) those showing signs of introgression based on the other genetic markers, (2) those genetically pure inside the

Carpathian region, and (3) those genetically pure outside the Carpathian region, were assessed using randomization tests in PopTools (<http://www.poptools.org>) as well as by using the standard *t*-test allowing unequal variance in the two groups.

#### SPECIES DISTRIBUTION MODELLING

We constructed a dataset of 2136 *T. cristatus* localities as in Wielstra *et al.* (2014; Fig. 1). For climate layers we used bioclimatic variables at 2.5 arcminute resolution (approximately  $5 \times 5$  km), available from the WorldClim database 1.4 (Hijmans *et al.*, 2005; <http://www.worldclim.org>). Following Guisan & Thuiller (2005) and Peterson (2011), we selected a subset of bioclimatic variables considered to reflect physiological limitations of the study species (reflecting seasonality) that showed little multicollinearity (a Pearson's correlation of  $r < 0.7$ ): 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). Bioclimatic variables are also available for the Last Glacial Maximum (~21 kya), derived from the Paleoclimate Modelling Intercomparison Project phase 2 (Braconnot *et al.*, 2007; <http://pmip2.lsce.ipsl.fr/>). We used bioclimatic values for the Last Glacial Maximum based on two climate simulations: the Model for Interdisciplinary Research on Climate version 3.2 (MIROC) ([http://ccsr.aori.u-tokyo.ac.jp/~hasumi/miroc\\_description.pdf](http://ccsr.aori.u-tokyo.ac.jp/~hasumi/miroc_description.pdf)); and the Community Climate System Model version 3 (CCSM) (Collins *et al.*, 2006).

We created a species distribution model for *T. cristatus* using Maxent 3.3.3k (Phillips, Anderson & Schapire, 2006). We restricted the feature type to hinge features as this produces a smoother model fit that emphasizes trends rather than idiosyncrasies of the data (Elith, Kearney & Phillips, 2010). The environmental range covered by pseudo-absence data, used by Maxent to discriminate presence data from the environmental background, should neither be too narrow nor too broad (VanDerWal *et al.*, 2009; Elith *et al.*, 2011; Stokland, Halvorsen & Støa, 2011). Following VanDerWal *et al.* (2009), we drew a 200-km-radius buffer around known *Triturus* localities. We projected the species distribution model on the current and past climate layers. To determine whether the model performs better than expected by random chance, its area under the curve of the receiver operating plot (AUC) value was tested for statistical significance against a null model of AUC values from 99 species distribution models based on random localities (*sensu* Raes & ter Steege, 2007). Random point data were created using ENMTools 1.3 (Warren, Glor & Turelli, 2010).

#### DATA ACCESSIBILITY

GenBank accession numbers and locality data are given in the Supporting Information (Appendix S1). Raw Ion Torrent reads in FASTQ format, together with a decoding table and an alignment of the MHC class II DAB haplotypes, input files for MrBayes, BEAST, Newhybrids, BAPS, and bioclimatic layers used for species distribution modelling are available via Dryad Digital Repository entry doi:10.5061/dryad.s9v06..

## RESULTS

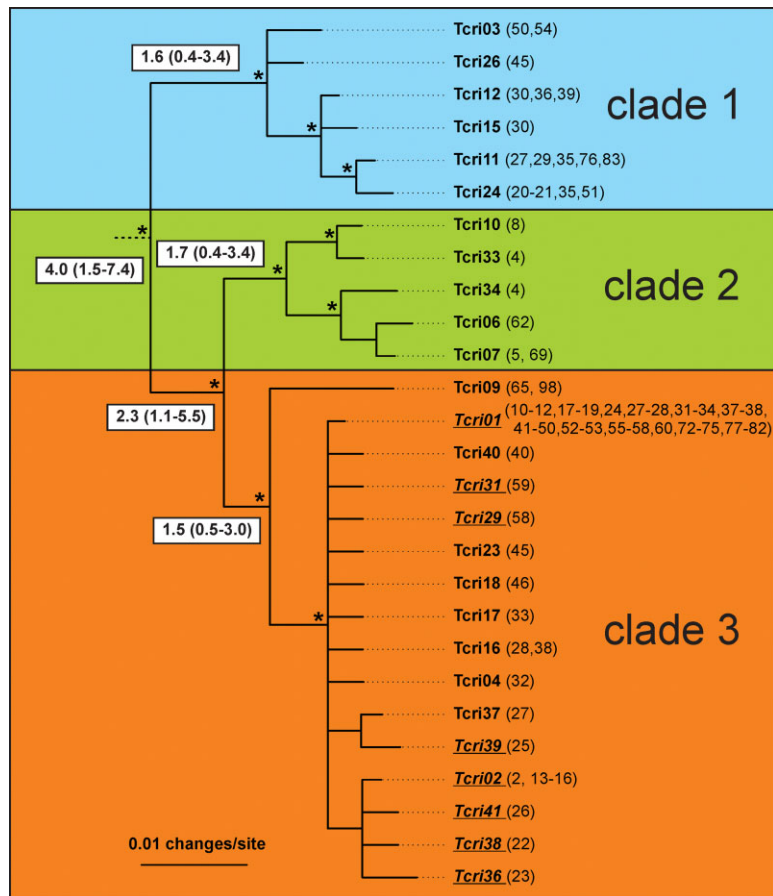
#### MITOCHONDRIAL DNA

Introgressed mtDNA, derived from *T. carnifex* and *T. dobrogicus*, is found in *T. cristatus* near the contact zones with these species, and mtDNA derived from *T. ivanbureschi* is found in an extended area south of the Danube River (Fig. 1; Supporting Information, Appendices S1 and S2). For *T. cristatus* we distinguished three monophyletic, significantly supported groups of mtDNA haplotypes (posterior probability  $\geq 0.95$ ), here named mtDNA clades 1–3 (Fig. 2). A sister relationship was found for mtDNA clades 2 and 3. The pairwise distance was 2.3% between mtDNA clades 1 and 2, 2.2% between mtDNA clades 1 and 3, and 1.4% between mtDNA clades 2 and 3. The most recent common ancestor of *T. cristatus* is dated at approximately 4.0 Mya, the most recent common ancestor of mtDNA clades 2 and 3 at 2.3 Mya, and the crowns of mtDNA clades 1, 2, and 3 at 1.6, 1.7, and 1.5 Mya, respectively (Fig. 2).

mtDNA clade 1 is restricted to eastern Romania, Moldova, south-western Ukraine, and south-eastern Poland; mtDNA clade 2 is distributed south of the Danube River in the east of Serbia and north-western Bulgaria; and mtDNA clade 3 occurs in the small Serbian section of the *T. cristatus* range north of the Danube River, in most of the Romanian part of the Carpathians, and throughout Europe (Fig. 1). The three mtDNA clades are largely geographically separated from each other, but mtDNA clades 1 and 3 are found in syntopy in three localities (Fig. 1). mtDNA clade 3 is the only mtDNA clade that occurs outside the Carpathians. The eight haplotypes from mtDNA clade 3 that occur outside the Carpathians (coloured orange in Fig. 3) are similar to one another (Table 1). The same or similar haplotypes are found in the Carpathians. Fu's  $F_s$  statistic is significantly negative outside the Carpathians (Table 1) and nucleotide diversity there is significantly lower ( $P < 0.001$ , randomization test).

#### NUCLEAR DNA INTRONS

Ten newts from contact zones with other *Triturus* species were identified as genetically admixed in the



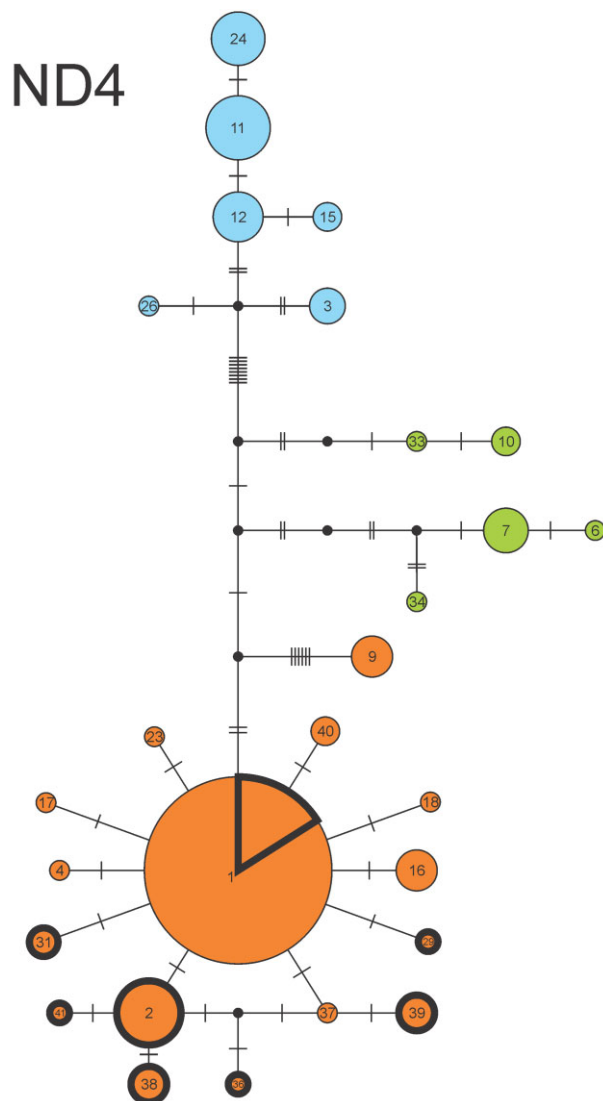
**Figure 2.** A Bayesian mtDNA phylogeny for *Triturus cristatus*. Branches supported with a posterior probability of  $\geq 0.95$  are marked with an asterisk. The outgroup is not shown. Haplotype codes correspond to those given in the Supporting Information, Appendix S1. Numbers in parentheses refer to the populations in which each haplotype is found and correspond to Figure 1 and Supporting Information, Appendix S1. The mtDNA clades 1, 2, and 3, are coloured blue, green, and orange, respectively (see the text for details). The italicized, underlined haplotypes (found in mtDNA clade 3 only) are (also) found outside the Carpathians. Results of molecular dating (mean values and 95% confidence intervals are given in Mya) are provided for splits between and crowns of the three mtDNA clades.

NewHybrids analyses (Fig. 1; Supporting Information, Appendix S1) and were excluded from further analyses. The individual haplotype networks (Fig. 4) and gene trees (Supporting Information, Appendix S2) show little resolution. With BAPS allowed to determine the optimal value of  $k$ , 22 genetic clusters are resolved. Almost all fit within the three groups delimited by BAPS when  $k = 3$  is fixed (guided by the mtDNA results), but four of the 22 clusters are shared between groups 1 and 3 and one is shared between groups 2 and 3, suggesting ambiguous placement of ten individuals in the three groups under  $k = 3$  (Supporting Information, Appendix S1). The spatial distribution of the three BAPS groups under  $k = 3$  generally coincides with mtDNA, but the geographical distribution is less clear cut and groups 1 and 2 are more widely distributed than mtDNA clades 1 and 2, and group 3 is also found, albeit in low frequency,

south of the Danube River (Fig. 1). Fu's  $F_s$  statistic is positive, although not always significantly so, for the populations outside the Carpathians (Table 1), and nucleotide diversity there is significantly lower for all three introns ( $P < 0.001$ , randomization tests).

#### MHC DATA

The MHC class II DAB locus could be genotyped for 191 of 194 newts. Of these, five individuals have three alleles (which may reflect copy-number variation or laboratory artefacts) and were not analysed further (Supporting Information, Appendix S1). All 37 MHC alleles are present in the Carpathians, whereas only a single allele (*Trcr-DAB\*01*) is present outside (excluding population 3, which is heavily affected by interspecific gene flow based on the other markers).



**Figure 3.** Haplotype network for mtDNA. Haplotype frequency is expressed by the diameter of the circles, and substitutions are represented by bars. Pies are coloured according to the mtDNA clade to which each individual newt belongs: blue, green, or orange for mtDNA clades 1, 2, or 3, respectively. Pie slices representing haplotypes sampled outside the Carpathians are highlighted with bold lines (details in the Supporting Information, Appendix S1).

Allelic richness, calculated only for populations with MHC genotypes available for three individuals that showed no signs of introgression based on the other markers, is significantly higher in populations positioned inside the Carpathians than in those located outside (randomization test,  $P = 0.003$ ; Welch's  $t$ -test,  $P = 7.8 \times 10^{-9}$ ). However, differences in AR between populations with introgressed DNA (AR = 3.1) and populations from the Carpathians without introgressed DNA (AR = 3.2) are small and

not significant (randomization test,  $P = 0.896$ ;  $t$ -test,  $P = 0.85$ ). Within the Carpathians, differences in allelic richness between more northern localities (Ukraine and south-eastern Poland; AR = 2.75) and the remaining localities (Bulgaria, Serbia, Romania, and Moldova; AR = 3.38) are not significant (randomization test,  $P = 0.29$ ;  $t$ -test,  $P = 0.15$ ). We found no evidence for an increased number of private alleles in the group of 18 populations with signals of introgression from other *Triturus* species (one-sided randomization test,  $P = 0.57$ ). This test includes all sampled populations with at least one individual genotyped for MHC.

#### SPECIES DISTRIBUTION MODEL

The species distribution model for *T. cristatus* performs significantly better than random expectation (Fig. 5A). The predicted suitable area for the present day largely overlaps with the actual range of *T. cristatus* (Fig. 5B). However, an area south of the range of *T. cristatus*, occupied by other *Triturus* species (Supporting Information, Appendix S2), is additionally predicted as suitable. The two different climate simulations used for the Last Glacial Maximum (CCSM and MIROC) both suggest that at the Last Glacial Maximum the area predicted as suitable for *T. cristatus* was much reduced, but differ in the extent of this area, with a wider extent for the MIROC climate simulation (Fig. 5C, D). Both simulations agree that a suitable area was present at the Last Glacial Maximum in continental western Europe and to the south of the current range of *T. cristatus*. In the Carpathians, both climate simulations show a suitable area in the south, but the extent of this area is limited, especially based on the CCSM climate simulation (Fig. 5E, F).

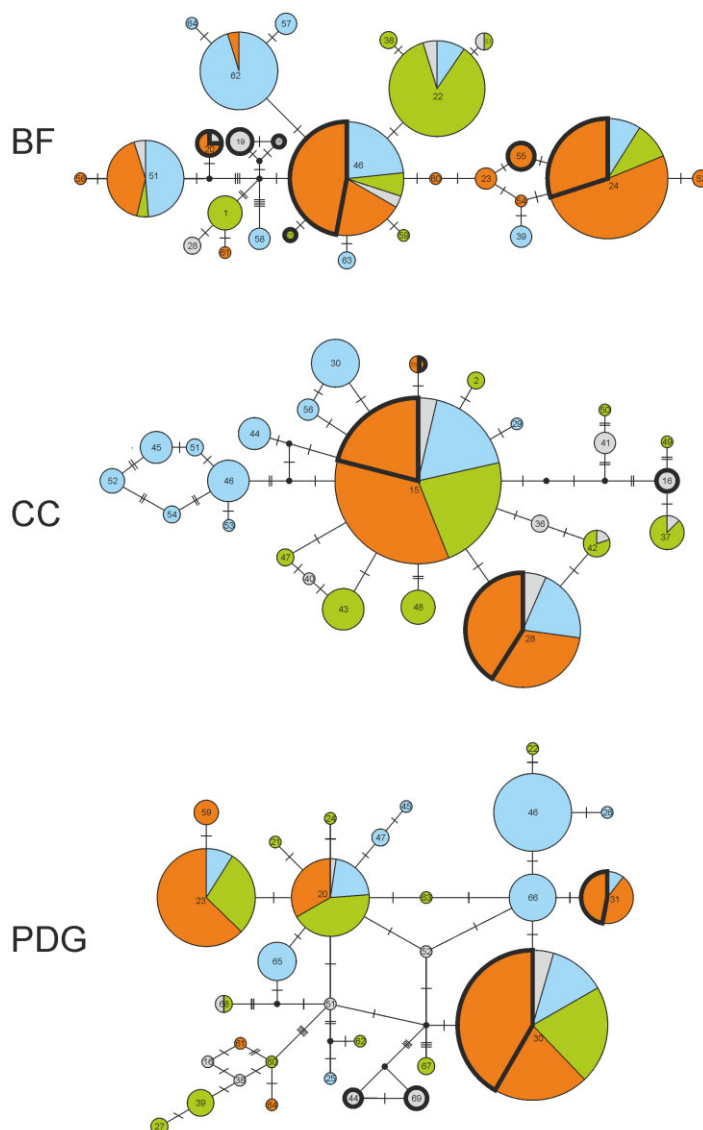
#### DISCUSSION

We have clearly demonstrated that the Carpathians acted as a glacial refugium for *T. cristatus* during the Pleistocene glaciations, harbouring multiple evolutionary lineages with almost non-overlapping distributions, and conclude that *T. cristatus* colonized the remainder of its range – which encompasses a considerably larger area – after the Last Glacial Maximum.

#### MITOCHONDRIAL DNA

All three mtDNA clades of *T. cristatus* are found inside the Carpathians, and one of these, mtDNA clade 3, protrudes into the remainder of the range of *T. cristatus*, covering most of temperate Europe (Fig. 1). In contrast to the genetic richness within the Carpathians, only a few, similar mtDNA clade 3





**Figure 4.** Haplotype networks for each of the three nuclear DNA introns:  $\beta$ fibint7 (BF), *CalintC* (CC) and *Pdgfra* (PDG). Haplotype frequency is expressed by the diameter of the circles, and substitutions are represented by bars. Pies are coloured according to the BAPS group under  $k = 3$  to which individual newts belong: blue, green, and orange for BAPS groups 1, 2, and 3, respectively. Individuals identified as genetically admixed in the NewHybrids analyses are coloured grey (details in the Supporting Information, Appendix S1). Pie slices representing haplotypes sampled outside the Carpathians are highlighted with bold lines. The same haplotype networks, but coloured according to mtDNA clade, are shown in the Supporting Information, Appendix S2.

haplotypes occur outside it, over a much wider area. The situation is best illustrated by the *Tcri01* haplotype, which is found not only throughout the Carpathians, but also at the western and eastern extremes of the *T. cristatus* range (locality 11 in England and locality 59 in Russia, separated by a distance of approximately 3200 km; see Fig. 1). The genetic homogeneity observed outside the Carpathians is in line with leading-edge expansion from a single source population (Excoffier, Foll &

Petit, 2009). Similarly, the presence of low-frequency haplotypes, similar to *Tcri01* and not found in the Carpathians, reflects a starburst pattern and is supportive of a population explosion expected under range expansion (Excoffier *et al.*, 2009).

The estimations for the ages of the crowns of the three mtDNA clades, including their confidence intervals, predate the Last Glacial Maximum (Fig. 2). These findings are in line with a long-term presence in the Carpathians *in situ* for each of the

**Table 1.** Haplotype number and diversity and a test for the signal of a genetic bottleneck for mtDNA and the nuclear DNA introns

		h	$\pi$	Fu's $F_S$	P
ND4	Total	27	0.0088	-2.00	0.304
	Inside	20 (19)	0.0100	1.15	0.700
	Outside	8 (7)	0.0020	-2.88	<b>0.008*</b>
$\beta$ fibint7	Total	26	0.0070	-1.15	0.824
	Inside	19 (13)	0.0074	-0.55	0.865
	Outside	13 (7)	0.0040	2.64	<b>0.992*</b>
CalintC	Total	25	0.0037	-7.42	<b>0.013*</b>
	Inside	24 (18)	0.0042	-6.93	0.036
	Outside	7 (1)	0.0013	1.41	0.935
Pdgr $\alpha$	Total	30	0.0036	-1.81	0.625
	Inside	29 (22)	0.0038	-2.00	0.582
	Outside	8 (1)	0.0007	1.52	0.973

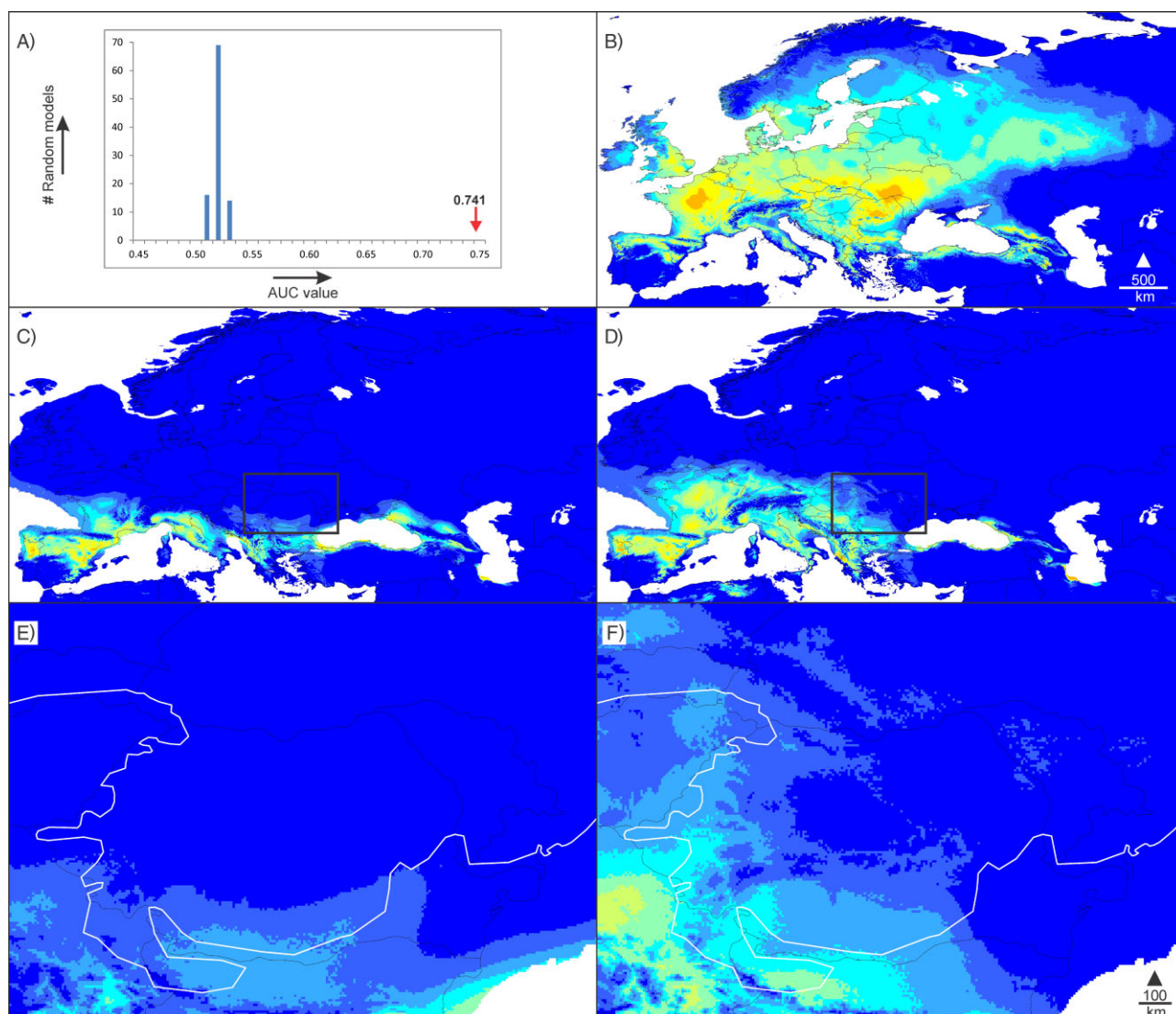
Individuals identified as introgressed were excluded. 'Total' refers to all populations and 'inside' and 'outside' to those inside and outside the Carpathians. h is number of haplotypes (with the number restricted to inside or outside the Carpathians in parentheses).  $\pi$  is nucleotide diversity. P values are provided for Fu's  $F_S$ , with those in bold and with an asterisk significant at the 0.05 level (this being a two-tailed test).

three mtDNA clades. Why did mtDNA clades 1 and 2 not spread into deglaciated Europe? The Danube River probably would have posed (and still poses) a physical barrier to the northward expansion of newts with clade 2 mtDNA (e.g. as the Tagus River does for the marbled newt *Triturus pygmaeus*; Espregueira Themudo & Arntzen, 2007). However, considering that the origin of the Iron Gates (dated to approximately 5.6 Mya; Suc *et al.*, 2011) – the gorge where the Danube runs through the current range of *T. cristatus* – predates the split between mtDNA clades 2 and 3, gene flow was evidently not impossible. The geographically limited syntopy of mtDNA clades 1 and 3 suggest that secondary contact was obtained after postglacial expansion. The presence of newts with mtDNA belonging to clade 3 could have hampered a wider postglacial spread of mtDNA clade 1 (or clade 2 for that matter) via high-density blocking (Waters, 2011; Waters, Fraser & Hewitt, 2013).

#### NUCLEAR DNA INTRONS

For the three nuclear DNA introns, genetic bottlenecking in postglacially colonized area seems less severe than for mtDNA in that more of the haplotypes found are shared with the Carpathians. Furthermore, although the geographical distribution of the BAPS groups under  $k = 3$  generally overlaps with the three mtDNA clades, there is some deviation and the geographical distribution of the BAPS groups appears less well demarcated than those based on mtDNA. This can be explained by differences in characteristics

of the two genomes. The four-fold smaller effective population size of mtDNA (haploid, matrilineal inheritance) compared with nuclear DNA makes mtDNA more susceptible to genetic impoverishment owing to an increased influence of genetic drift (Ballard & Whitlock, 2004). In effect, genetic diversity is lost faster for mtDNA than for nuclear DNA during the serial colonization that embodies postglacial range expansion (Excoffier *et al.*, 2009). Additionally, compared with nuclear DNA, mtDNA in many species shows less intraspecific gene flow and hence distinct mtDNA clades show less geographical mixing upon secondary contact and the geographical position of secondary contact zones is retained for longer (Petit & Excoffier, 2009). In line with this, the source population(s) contributing to postglacial expansion would be expected to possess a higher fraction of the total genetic diversity present in the glacial refugial area for nuclear DNA than for mtDNA. Meanwhile, the relatively fast mutation rate of mtDNA allows more new haplotypes to arise, derived from the subset of haplotypes being spread in the newly colonized area (Excoffier *et al.*, 2009). The positive Fu's  $F_S$  values for nuclear introns outside the Carpathians reflect a deficiency of haplotypes compared with equilibrium neutral expectations, in line with a genetic bottleneck. On the other hand, the negative Fu's  $F_S$  values for mtDNA reflect an excess of haplotypes in line with newly arisen haplotypes during and after population expansion. Thus, these seemingly contradictory signals are actually concordant and the apparent discrepancy may be explained by the differences in mutation rates between the two classes of markers.



**Figure 5.** Results of the species distribution modelling for *Triturus cristatus*. The performance of the model is shown in comparison with 99 random models (A). Shown are the current distribution (B), and the predicted distribution during the Last Glacial Maximum based on the CCSM climate simulation (C) and the MIROC climate simulation (D). The cut-out of the Carpathian region, with the current range limit of *T. cristatus* superimposed in white, shows the predicted Last Glacial Maximum distribution based on the CCSM climate simulation (E) and the MIROC climate simulation (F) in more detail. In B–F, the warmer the colour of a grid cell, the higher its predicted suitability.

#### MHC DATA

Only a single allele (*Trcr-DAB\*01*) is present outside the Carpathians, whereas all 37 identified alleles are present inside the Carpathians. So, compared with the three nuclear introns, MHC shows a stronger pattern of ‘southern richness vs. northern purity’ (Hewitt, 2000), even though all four nuclear markers have the same effective population size. This striking pattern of genetic richness inside, and genetic depletion outside, the Carpathians substantiates the findings of Babik *et al.* (2009), based on a much smaller sampling. Balancing selection in a demographically

stable population explains the high genetic diversity within the Carpathians (Babik *et al.*, 2009). The genetic depletion outside the Carpathians is surprising, as a higher MHC diversity is generally considered to be beneficial to fitness and a strong balancing selection pressure to retain genetic diversity would be expected to operate (Spurgin & Richardson, 2010). However, the occurrence of selective sweeps in MHC has been proposed (e.g. De Groot *et al.*, 2008) and balancing selection can be overwhelmed by genetic drift associated with postglacial expansion (Ejsmond & Radwan, 2011). It is difficult to distinguish between

these explanations, but the loss of variation during expansion documented in other genomic regions presumed to be selectively neutral makes genetic drift more parsimonious. Either way, despite the observed MHC impoverishment, *T. cristatus* managed to greatly increase its distributional range and global population size postglacially.

#### SPECIES DISTRIBUTION MODEL

The species distribution model supports a pattern of postglacial expansion as inferred from the genetic data: most of the current range of *T. cristatus* is predicted as unsuitable at the Last Glacial Maximum. However, the extent of the area predicted as suitable in the Carpathians is particularly small: only a small region in the southern part of the Carpathians, where Romania, Serbia, and Bulgaria meet, is predicted to have been suitable at the Last Glacial Maximum. Hence, the species distribution model appears to underestimate the Last Glacial Maximum glacial refugium in the Carpathians predicted from the genetic data. The potential failure of species distribution models to predict glacial refugia supported by genetic data is increasingly recognized (Worth *et al.*, 2014). Furthermore, the species distribution model 'over-predicts' in continental Western Europe at the Last Glacial Maximum, suggesting a suitable area, whereas there is no support for a glacial refugium from the genetic data. Over-prediction is a more widely recognized issue for species distribution modelling (Elith *et al.*, 2011; Worth *et al.*, 2014).

Although niches evolve over time (Wiens & Graham, 2005), for the relatively short time period between the Last Glacial Maximum and the present day, niche conservatism is considered a reasonable assumption (Peterson, 2011). Recent niche shifts have, however, been proposed and could explain both under- and over-prediction of species distribution models (Pearman *et al.*, 2008; Worth *et al.*, 2014). Under the assumption of a stable niche, under-prediction could suggest that the climate layers used to create the species distribution model do not properly reflect the factors limiting the distribution of *T. cristatus*, or, if they do, that *T. cristatus* is currently not in equilibrium with its environment or that non-analogue conditions occurred at the Last Glacial Maximum (Elith *et al.*, 2011; Worth *et al.*, 2014). Over-prediction could likewise suggest a suboptimal model or reflect a suitable area that could not be colonized owing to dispersal constraints (Elith *et al.*, 2011). Finally, the accuracy and resolution of the global circulation models underlying the climate layers for the Last Glacial Maximum might be insufficient for reliable predictions at a fine resolution (Tzedakis *et al.*, 2013; Worth *et al.*, 2014).

The species distribution models suggest that at the Last Glacial Maximum, *T. cristatus* had a glacial

refugium south of its current range, in the traditional Balkan Peninsula glacial refugium. The *Triturus* species currently occurring south of the range of *T. cristatus* (Supporting Information, Appendix S2) only colonized their northern Balkan range after the Last Glacial Maximum (Wielstra *et al.*, 2013b). This means that *T. cristatus* would not face competitive exclusion in the area south of its present range as it does at present. However, two lines of evidence refute *T. cristatus* having had its entire glacial refugium south of the Danube River. First, *T. cristatus* mtDNA haplotypes found south of the Danube River belong exclusively to mtDNA clade 2, whereas mtDNA clades 1 and 3 are found only north of the Danube River and hence must have had a more northerly glacial refugium. Second, the *T. ivanbureschi* mtDNA asymmetrically introgressed into *T. cristatus* in the part of the range south of the Danube River is suggestive of postglacial expansion of *T. cristatus* here at the expense of *T. ivanbureschi* (Wielstra & Arntzen, 2012).

#### A NOTE ON THE *TRITURUS* FOSSIL RECORD

Fossils can be used as independent data to deduce the location of glacial refugia (Martínez-Meyer, Townsend Peterson & Hargrove, 2004; Martínez-Meyer & Peterson, 2006). However, their usefulness depends on the confidence with which the species involved can be identified. Reliable identification is particularly difficult for morphologically similar members of groups of closely related species, such as crested newts. For the different crested newt species there are no discrete skeletal differences in the vertebrae (M. Slijepević and A. Ivanović, unpubl. data) or the skull (Ivanović & Arntzen, 2014). Hence, we cannot fully rely on fossils as a third, independent data set to predict the glacial refugium for *T. cristatus*.

#### CONCLUSION

Our data show that *T. cristatus* recolonized temperate Eurasia during the current interglacial period, exclusively from a Carpathian glacial refugium. We found a considerably higher degree of genetic diversity within the Carpathians than outside, although the extent of genetic depletion was stronger for mtDNA and (strikingly) for MHC than for three nuclear DNA introns. Species distribution models agree that the area with conditions suitable for *T. cristatus* was much reduced at the Last Glacial Maximum, but the extent of suitable area in the Carpathians appears to be under-predicted.

The possession of a relatively northern glacial refugium offers a strategic advantage over competing species: colonizing terrain that has become suitable after alleviation of glacial conditions partially works



on a first-come, first-served basis, where the 'early bird' can exclude latecomers by high-density blocking rather than by having an adaptive advantage (Waters, 2011; Waters *et al.*, 2013). Although *T. cristatus* is the only crested newt species that managed to colonize temperate Eurasia after the last glaciation, introduced populations of *T. carnifex* have been shown to be able to (at least locally) outcompete native *T. cristatus* (Arntzen & Thorpe, 1999; Brede *et al.*, 2000).

Our genetic data suggest that *T. cristatus* maintained multiple geographically distinct populations within the Carpathians during glacial cycles, providing further support for a 'refugia within refugia' scenario (Gómez & Lunt, 2007) applying to the Carpathians, as previously suggested for several amphibian and plant species (Fijarczyk *et al.*, 2011; Ronikier, 2011; Zieliński *et al.*, 2013). For *T. cristatus*, one of these Carpathian glacial refugia was the source of colonization of temperate Eurasia after the Last Glacial Maximum. Our study, combining multilocus phylogeography and species distribution modelling, is the most detailed exploration yet of the Carpathian glacial refugium.

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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:

**Appendix S1.** Online additional tables.

**Appendix S2.** Online additional figures.

## SHARED DATA

Data deposited in the Dryad digital repository (Wielstra, Babik & Arntzen, 2015).