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A multimarker phylogeography of crested newts (*Triturus cristatus* superspecies) reveals cryptic species

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ABSTRACT

The crested newt *Triturus cristatus* superspecies is composed of five recognized species. One of these, *T. karelinii* sensu lato, comprises three geographically structured mitochondrial DNA lineages: 'eastern', 'central' and 'western *T. karelinii*'. Genetic divergence among these lineages is comparable to that of recognized crested newt species, but morphologically they are indistinguishable. Here, we conduct a multimarker phylogeographical survey to explore the evolutionary independence of these mitochondrial DNA lineages and we include representatives of the other species to guide our interpretation of the results. All markers show distinct patterns when analyzed singly (as a phylogeny or haplotype network) and none of them sort haplotypes fully in line with species or mitochondrial DNA lineage. A multilocus approach (BAPS and *BEAST) on the other hand shows that not only the recognized species, but also the three mitochondrial DNA lineages represent discrete nuclear DNA gene pools. A mismatch is found in the northwest of Asiatic Turkey, where several populations identified as 'central *T. karelinii*' based on nuclear DNA possesses 'western *T. karelinii*' mitochondrial DNA. We invoke asymmetric mitochondrial DNA introgression to explain this pattern and support this with a historical biogeographical scenario. The three spatial groups in *T. karelinii* sensu lato should be regarded as distinct species.

1. Introduction

The crested newt (*Triturus cristatus*) superspecies comprises five parapatric species (Fig. 1). In a previous phylogeographical survey of the species traditionally referred to as '*T. karelinii*' (hereafter *T. karelinii* sensu lato), we uncovered three geographically structured mitochondrial DNA lineages. In terms of mitochondrial DNA divergence, these three lineages are as distinct from each other as recognized (non-*T. karelinii*) crested newt species are (Wielstra et al., 2010). The recognized crested newt species are known to differ based on morphological features and a battery of allozymes markers (Arntzen et al., in preparation). On the other hand, range-wide allozyme data are not available for *T. karelinii* sensu lato (Arntzen and Wielstra, 2010), and analysis of morphological features known to discriminate the recognized species did not reveal unequivocal differences among the three mitochondrial DNA lineages (Arntzen, 2003; Ivanović et al., in press). However, in terms of niche differentiation the three lineages are as distinct as recognized species (Wielstra et al., 2012).

We explore whether the three mitochondrial DNA lineages forming *T. karelinii* sensu lato represent independent evolutionary trajectories. To this aim, we analyze three nuclear DNA markers, using both single and multilocus analytical approaches. Throughout the present paper we refer to the three *T. karelinii* sensu lato candidate species as 'eastern,' 'central' and 'western *T. karelinii*' and acknowledge that their true distribution (and distinction) based on the nuclear genome might be different than suggested by mitochondrial DNA. To guide our interpretation, we compare the situation in *T. karelinii* sensu lato with that shown by the recognized crested newt species.

2. Materials and methods

2.1. Sampling strategy, laboratory methods and data preparation

We included 335 crested newts from 113 populations (Fig. 1, Appendix S1). Our dataset comprised a dense geographical sampling for the representatives of the three mitochondrial DNA lineages constituting *T. karelinii* sensu lato as well as for all the recognized non-*T. karelinii* crested newt species. For each crested newt species and *T. karelinii* lineage we sampled in the core of the range and near the contact zones with other species or lineages.

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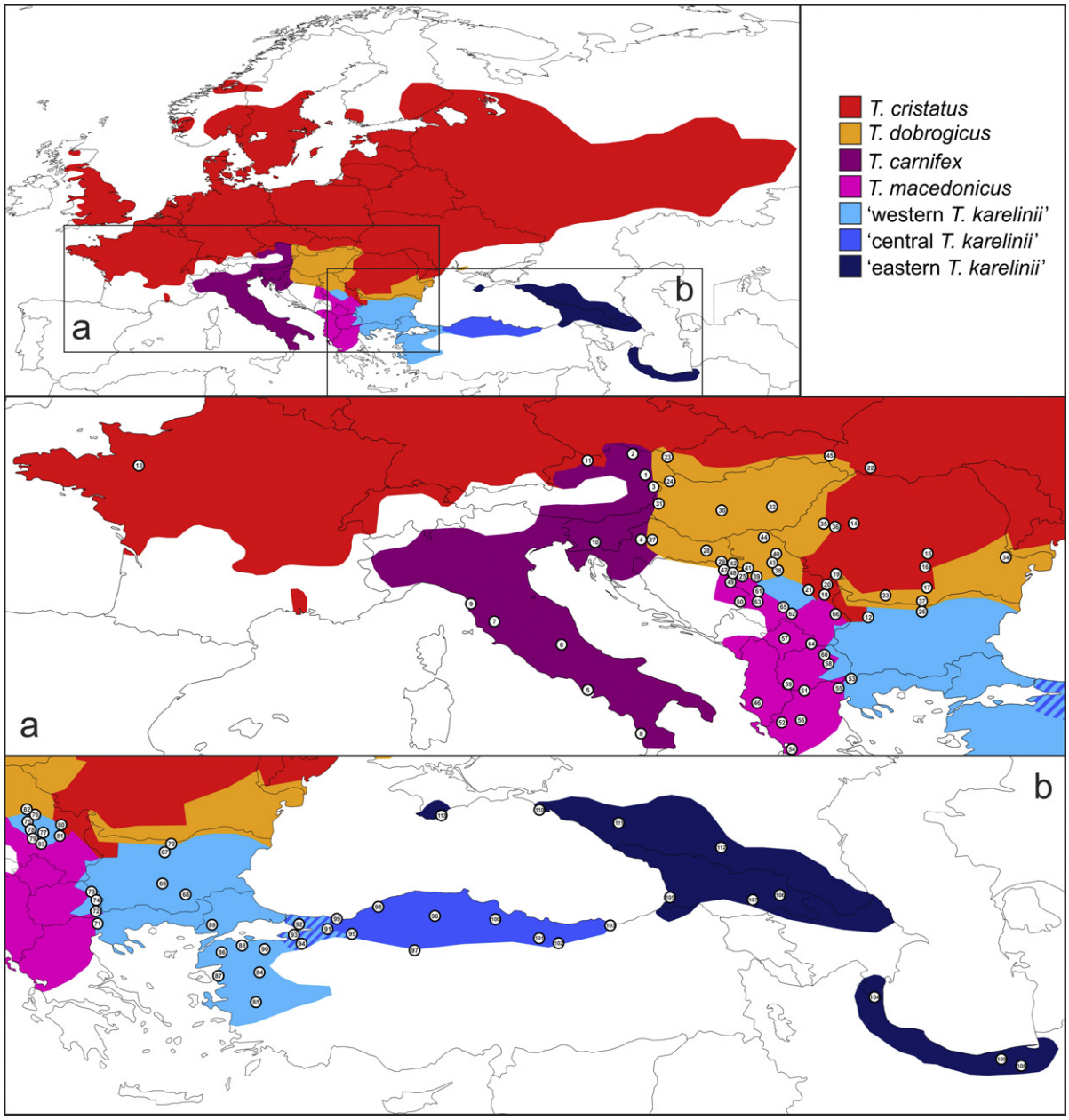

Fig. 1. Distribution map of crested newts. The inset shows the distribution of the recognized crested newt species and of the 'eastern,' 'central' and 'western *T. karelinii*' mitochondrial DNA lineages. Cut-out a shows the sampling for the recognized species and cut-out b for *T. karelinii* sensu lato. The hatched area in the cut-outs shows a region where 'central *T. karelinii*' newts as identified based on nuclear DNA contain 'western *T. karelinii*' mitochondrial DNA. Population numbers correspond to Appendix S1. This map is based on Wielstra and Arntzen (2011) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

We obtained sequence data for three nuclear introns: β-Fibrinogen intron 7 (βfib*int*7), Calreticulin intron C (*Calint*C) and Platelet-derived growth factor receptor α intron 11 (Pdgfrα). See Espregueira Themudo et al. (2009) for details on primers and laboratory methods. Furthermore, we include one mitochondrial protein-coding gene: subunit 4 of the NADH dehydrogenase gene complex (ND4). All ND4 data were taken from other studies (Wielstra and Arntzen, 2012; Wielstra et al., 2010; Arntzen et al., in preparation; Wielstra et al., in preparation).

For the nuclear markers, individuals possess two alleles, which may or may not be identical. We managed to retrieve the two alleles for each individual from direct sequencing products. If alleles were identical or differed only by one substitution, identification was straightforward. Diploid individuals containing alleles of different length were recovered following the method outlined in Flot et al. (2006). For alleles of identical length but differing at more than one base pair, allele-specific walking primers were developed (cf. Baird et al., 2009). For GenBank accession numbers, see Appendix S2. Sequences were manually aligned and identical ones merged into haplotypes using MacClade 4.08 (Maddison and Maddison, 2005).

2.2. Data analyses

Following the recommendations of Lemmon et al. (2009), we excluded indels from the analyses. Data matrices are available from TreeBASE (study ID S13327). We analyzed the nuclear DNA data using single and multilocus analytical approaches. First, for each of the three nuclear DNA markers (and the mitochondrial

DNA data) we conducted a Neighbor Joining analysis with 1000 bootstrap replications in MEGA 5.05 (Tamura et al., 2011). We included the marbled newt *T. marmoratus* to function as an outgroup (see Appendix S1 for sampling details). Furthermore, for each nuclear marker, a minimum spanning haplotype network was created with HapStar 0.5 (Teacher and Griffiths, 2011), based on distance matrices produced with Arlequin 3.5 (Excoffier and Lischer, 2010).

Secondly, we used a Bayesian analysis of population structure with the program BAPS v.5.3 (Corander et al., 2009). BAPS assigns individuals to distinct gene pools probabilistically, based upon multilocus genetic data, where each individual allele is coded as a haplotype (two alleles per marker, which may or may not belong to the same haplotype). 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 enforced BAPS to partition the individuals in seven groups ($k = 7$), as we are dealing with four recognized species and three candidate species. Then we let BAPS determine the most probable number of distinct gene pools, evaluating k over a $1 \leqslant k \leqslant 113$ range. For both searches we used ten replicates.

Thirdly, we conducted Bayesian inference of the species tree using ∗BEAST (Heled and Drummond, 2010), a multi-species coalescent model available in BEAST 1.7 (Drummond et al., 2012). We conducted two 500 million generation runs in ∗BEAST, applied a sampling frequency of 0.0001 and discarded the first half of generations as burn-in. We determined the most appropriate model of sequence evolution based on the Akaike Information Criterion with MrModeltest 2.2 (Nylander, 2004) for each marker: GTR + G for βfib*int*7 and Pdgfrα and HKY + G for *Calint*C. We applied a Yule process species-tree prior, a piecewise linear and constant root population-size model, a strict molecular clock and a random starting tree. Tracer 1.5 (Rambaut and Drummond, 2007) was used to make sure that runs had converged and effective samples sizes were at least 200. ∗BEAST requires individual sequences to be appointed to operational taxonomical units *a priori*. However, when testing for the presence of candidate species, such classification is not straightforward (and in the present case should be strictly independent from the mitochondrial DNA signal). To deal with this limitation, we used the BAPS groups identified under a search for the optimal k as operational taxonomical units in ∗BEAST. Thus, for each marker, we partitioned haplotypes according to BAPS group, and the same haplotype could be present in more than one BAPS group.

3. Results

For the distribution of haplotypes among the included individuals, see Appendix S1 and S3. The mitochondrial DNA sorts into seven distinct lineages, corresponding to the four recognized crested newt species and the three mitochondrial DNA lineages forming *T. karelinii* sensu lato (Fig. 2). Mismatches between morphology and mitochondrial DNA type in the recognized species are generally restricted to the contact zones (see Appendix S1), though *T. macedonicus* contains 'western *T. karelinii*' mitochondrial DNA over a more extensive area. The phylogenies and haplotype networks of the different nuclear genetic markers show dissimilar patterns (Fig. 3). Species/mitochondrial DNA lineages are not reciprocally monophyletic and haplotypes are shared among them.

With BAPS enforcing seven groups, 16 out of 165 non-*T. karelinii* crested newts do not cluster according to the species they were appointed based on phenotype (Appendix S1). Nine of the 16 instances where BAPS appoints individuals to a different species concern individuals from a contact zone (Appendix S1). Furthermore, the seven *T. carnifex* individuals from outside Italy (from Austria and the northern Balkans) are clustered with *T. cristatus*. The 170 *T. karelinii* sensu lato individuals generally group according to mitochondrial DNA lineage, but four populations (localities 91–94 in the hatched area in Fig. 1), despite containing 'western *T. karelinii*' mitochondrial DNA (haplotypes in bold in Fig. 2), cluster with 'central *T. karelinii*' based on nuclear DNA. There is one exception: an individual from locality 92 is placed in 'western *T. karelinii*' (but see below). Two 'western *T. karelinii*' newts from the contact zone are classified as *T. macedonicus* by BAPS.

With BAPS allowing the optimal value of k, 38 genetic clusters are resolved, generally conforming to species identification, or, in the case of *T. karelinii* sensu lato, to 'eastern,' 'central' and 'western *T. karelinii*' as delimited by BAPS with $k = 7$. One BAPS group belongs to *T. carnifex*, six BAPS groups belong to *T. cristatus*, seven to *T. dobrogicus*, eight to *T. macedonicus*, six to 'western *T. karelinii*', seven to 'central *T. karelinii*' (including localities 91–94, see above) and three to 'eastern *T. karelinii*' (Appendix S1). Again nine individuals belonging to the recognized species and originating from contact zones are clustered with a different species than their phenotype would suggest, although one individual has been swapped. The seven *T. carnifex* that clustered with *T. cristatus* under $k = 7$ are now placed in a separate group, together with a single *T. cristatus* individual from the contact zone with *T. carnifex*. The one individual from locality 92 in western Asiatic Turkey that clustered with 'western *T. karelinii*' with $k = 7$ is placed in a 'central *T. karelinii*' cluster with $k = 38$. A third 'western *T. karelinii*' newt from the contact zone with *T. macedonicus* is classified as that species with $k = 38$ (next to the two 'western *T. karelinii*' individuals classified as *T. macedonicus* with $k = 7$).

The species tree obtained with ∗BEAST is shown in Fig. 4a. Monophyly of most (candidate) species is suggested, but support is not high: posterior probability (pp.) = 0.77 for *T. cristatus*, pp. = 0.90 for *T. dobrogicus*, pp. = 0.85 for 'central *T. karelinii*, and pp. = 0.93 for 'eastern *T. karelinii*'. Italian *T. carnifex* comprises only a single BAPS group (i.e. pp. is not applicable); the BAPS group comprising the *T. carnifex* individuals from outside of Italy is nested within *T. cristatus* (as already alluded to by BAPS itself when enforcing $k = 7$). Another peculiar result is the placement of *T. macedonicus*: most of the individuals are grouped with 'western *T. karelinii*' in a relatively highly supported clade (pp. = 0.89); however, support for the monophyly of *T. macedonicus* and 'western *T. karelinii*' within this group is low (pp. = 0.50 and 0.49). The remaining *T. macedonicus* BAPS group is nested in *T. dobrogicus* (and contains two newts from the contact zone with that species).

Fig. 4b shows the same analysis, but excluding those newts that show signs of genetic admixture, because they cluster with a different species than would be expected based on morphology and allozymes and/or they contain mitochondrial DNA typical of another (candidate) species (see Appendix S1 for details; note that a number of BAPS groups were excluded completely for this reason). Support values slightly differ in most cases, increasing to pp. = 0.81 for *T. cristatus* and pp. = 0.99 for *T. dobrogicus* and decreasing to pp. = 0.78 for 'central *T. karelinii* and pp. = 0.83 for 'eastern *T. karelinii*'. A more obvious change is observed for *T. macedonicus* and 'western *T. karelinii*', for which support values increase to pp. = 0.79 and pp. = 0.71. The two are still placed together with relatively high support (pp. = 0.88).

There is no strong support for phylogenetic structure within the (candidate) species according to ∗BEAST (Fig. 4). Furthermore, support for phylogenetic relationships among the recognized crested newt species is low. On the other hand, *T. karelinii* sensu lato shows reasonable support for phylogenetic structure among the three candidate species: 'eastern' and 'central *T. karelinii*' are sister taxa (pp. = 0.94 or 0.99, based on the entire or reduced dataset). Based on the analysis of the complete dataset this clade is the sister taxon (pp. = 0.89) to the group containing 'western *T. karelinii*' and *T. macedonicus*, but support drops when considering the reduced dataset (pp. = 0.46).

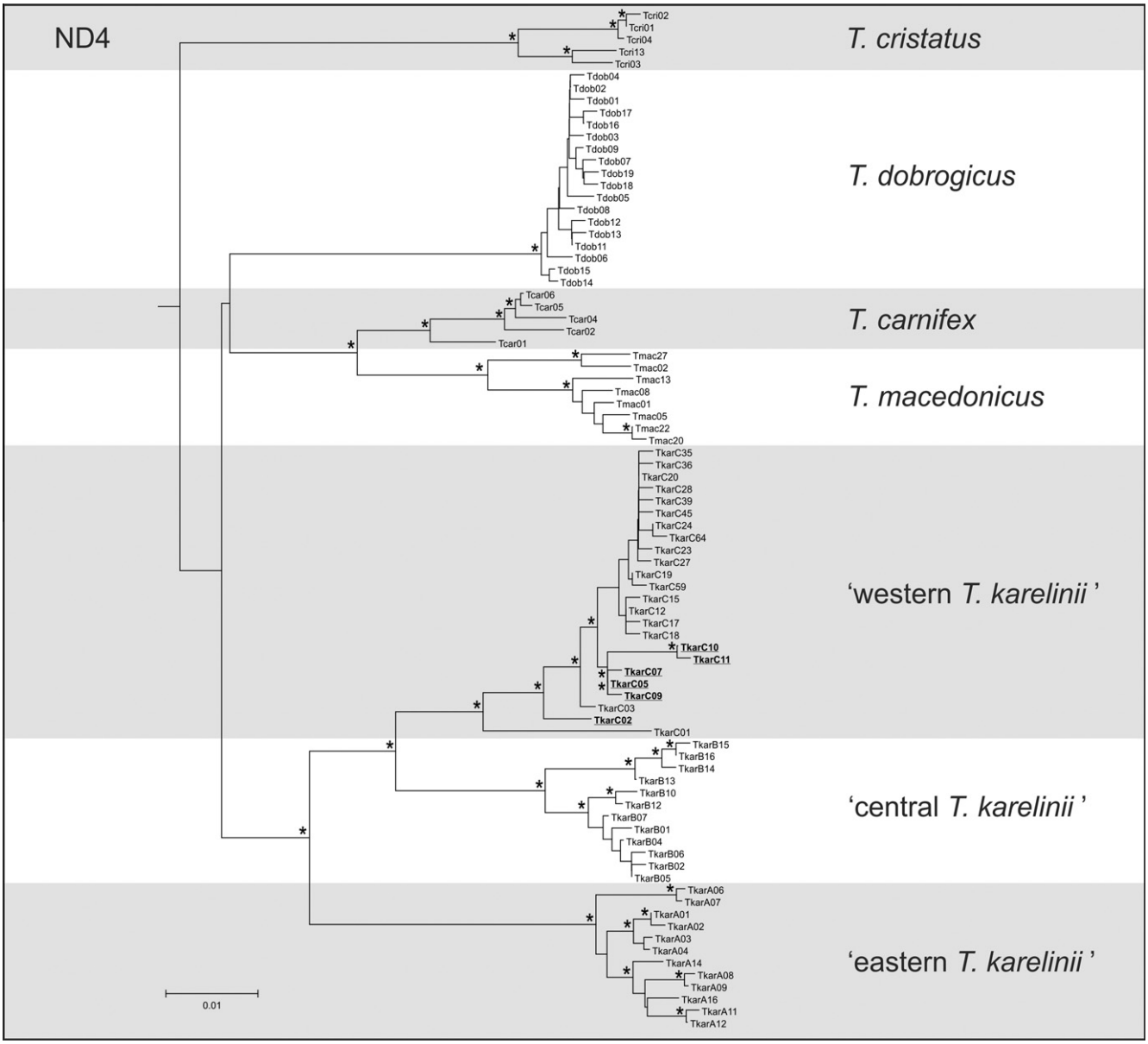

Fig. 2. Neighbor-joining tree for the ND4 haplotypes. Branches supported with a bootstrap over 70 are marked with an asterisk. The *T. marmoratus* outgroup is not shown. Haplotype codes correspond to Appendix S1 and S3. 'Western *T. karelinii*' mitochondrial DNA haplotypes found in 'central *T. karelinii*' as delineated by nuclear DNA are shown in bold and underlined.

4. Discussion

4.1. A single-locus analysis suggests reticulated evolution

The different genetic markers reveal distinct patterns. Whereas the mitochondrial gene tree shows geographically distinct clades, this is not the case for the nuclear gene trees (Figs. 2 and 3). All three nuclear gene trees differ from each other and none of them shows reciprocally monophyletic groups corresponding to units identifiable based on other data such as morphology, mitochondrial DNA or allozymes (Arntzen et al.,in preparation). The complex situation becomes clearer when the data are analyzed as haplotype networks (Fig. 3). Haplotypes are regularly shared among several species, and those haplotypes found in the same species are often scattered throughout the network. It should be stressed that this concerns not only the three mitochondrial DNA lineages within *T. karelinii*, but also the four recognized crested newt species.

The results indicate the importance of including the recognized species when assessing evolutionary independence of the three *T. karelinii* sensu lato candidate species. Without putting the pattern shown in *T. karelinii* sensu lato into the context of the situation shown by the recognized species, we might have wrongly interpreted allele sharing and non-monophyly as evidence for the lack of a barrier to gene flow. Two phenomena can explain the confusing pattern shown by the individual markers. Firstly, the sharing of alleles can be explained partially by ancestral polymorphism, retained from before the time that the different species split. Secondly, the low frequency presence of haplotypes in one species, typically restricted to the contact zone with a neighboring species in which this haplotype is more abundant, suggests that gene flow

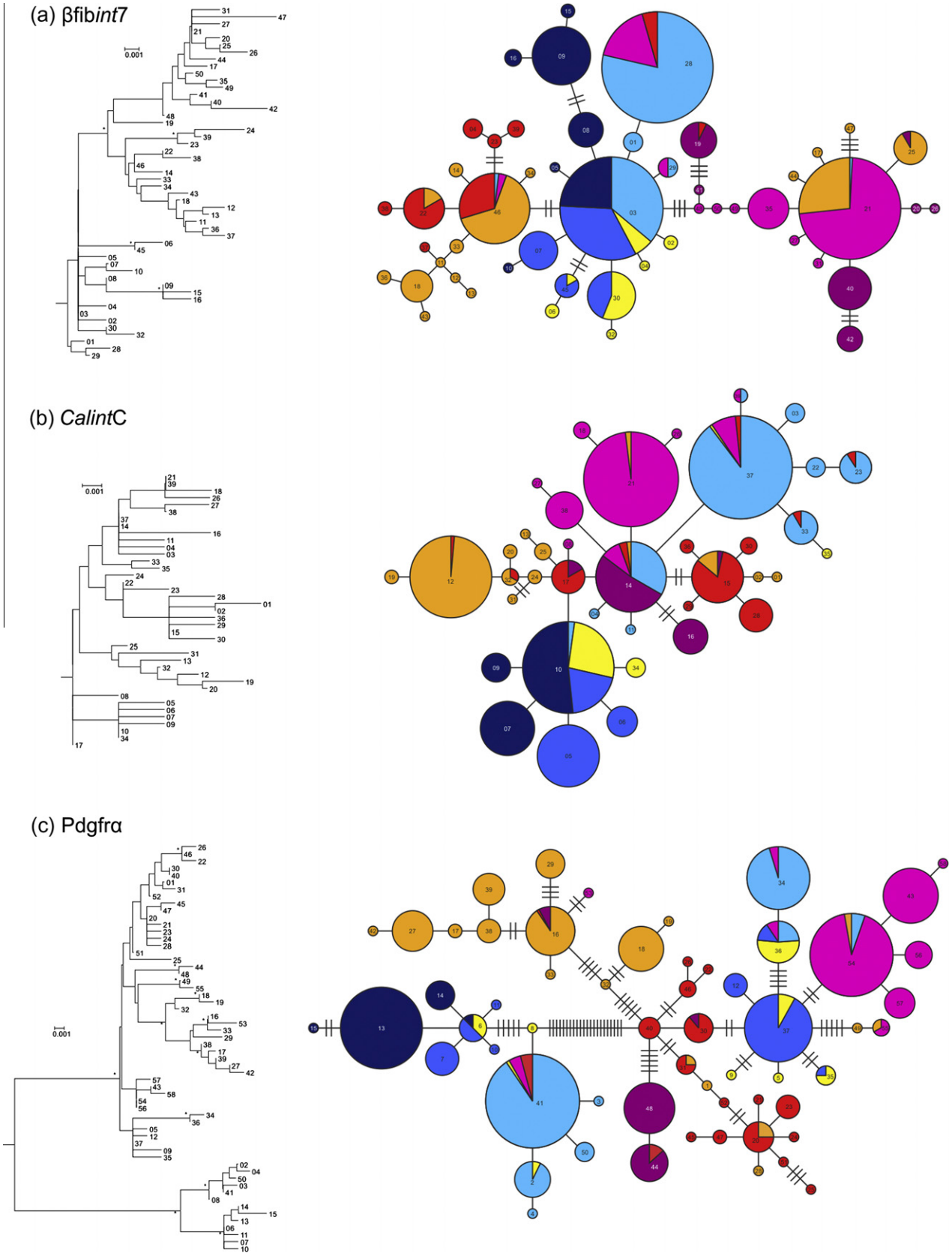

Fig. 3. Neighbor-joinging trees and haplotype networks for each of the three nuclear markers. The *T. marmoratus* outgroup is not shown in the neighbor-joining trees. Branches supported with a bootstrap over 70 are marked with an asterisk. For the networks, the frequency is expressed by the diameter of the circles (see Appendix S3 for details). Substitutions along branches are represented by bars if more than one. Colors (corresponding to Fig. 1) reflect the species in which each haplotype occurs or, in the case of *T. karelinii* sensu lato, which mitochondrial DNA lineage they possess. Newts identified as 'central *T. karelinii*' based on nuclear DNA but containing 'western *T. karelinii*' mitochondrial DNA are colored yellow. Haplotype codes correspond to Appendix S1 and S3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

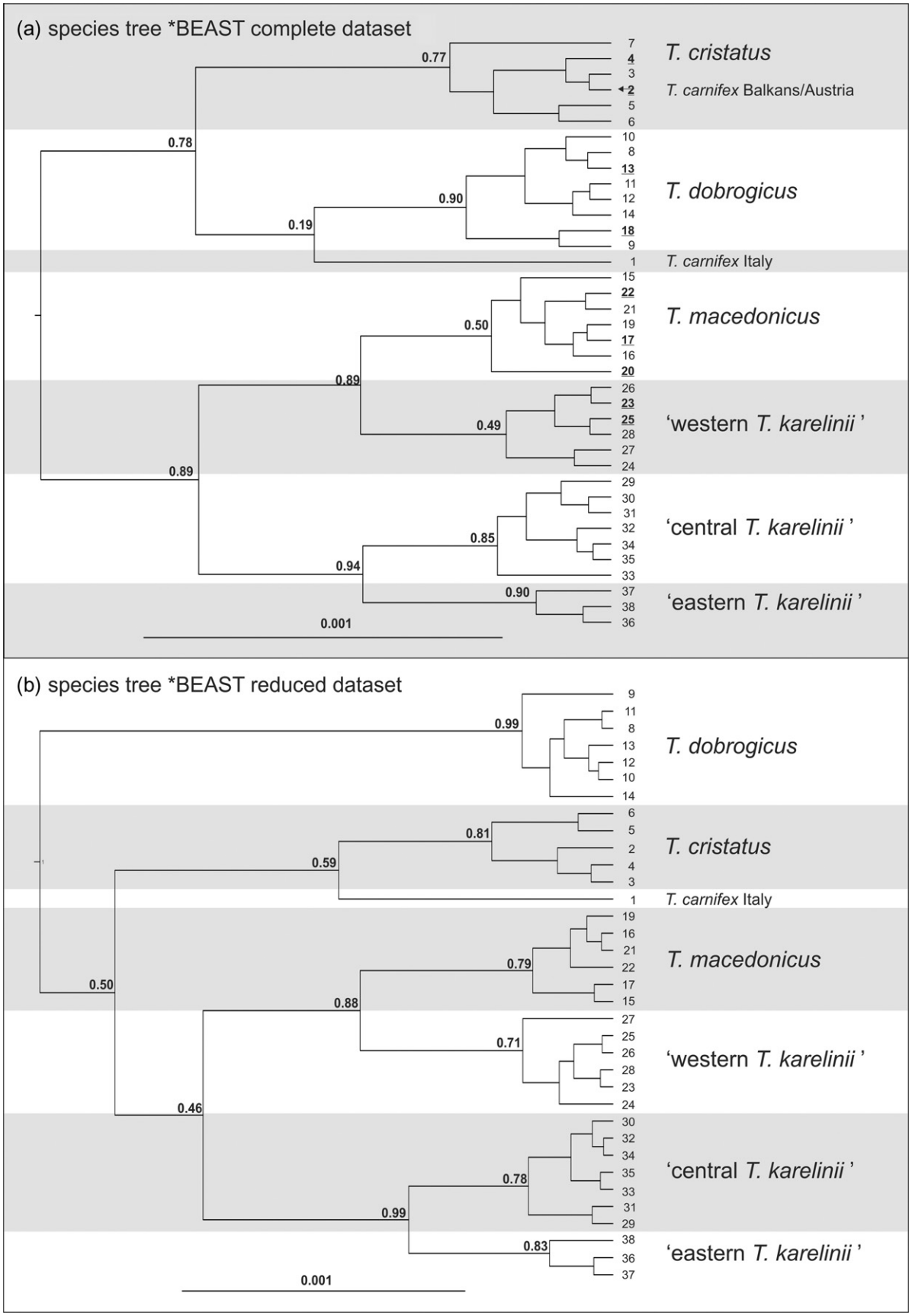

Fig. 4. Species tree resulting from the ∗BEAST multi-species coalescent analysis based on the three nuclear DNA markers using the complete dataset (a) and a reduced dataset (b) aiming to minimize the adverse effects of gene flow (see text for details). Only posterior probabilities for (candidate) species and the relationships among them are shown; posterior probabilities within (candidate) species are <<0.50. Note that ∗BEAST does not require an outgroup. The numbers at the tips refer to BAPS groups and correspond to Appendix S1; bold, underlined numbers (in Fig. 4 a only) reflect BAPS groups that contain newts belonging to another species (see text).

due to hybridization also occurred during crested newt evolution. To unravel such a pattern of reticulated evolution requires a multilocus approach.

4.2. Classification based on multilocus data sorts out crested newt (candidate) species

The BAPS analysis helps to clarify the complicated evolutionary pattern revealed by the single-locus approach. When we force BAPS to partition the individual crested newts into seven groups (considering we have four recognized and three candidate species), BAPS assigns most newts to their previously determined species or mitochondrial DNA lineage. When BAPS is allowed to choose the optimal number of gene pools, the identified clusters again mostly can be neatly grouped into the seven (candidate) species. Most mismatches are, as expected, close to the contact zone with other species, suggesting gene flow due to hybridization (Arntzen et al., in preparation).

There is one peculiar finding: the *T. carnifex* populations outside Italy, from the northern Balkan Peninsula and east of the Alps, are clustered with *T. cristatus*. Allozyme and morphological data show that these newts are *T. carnifex* (Arntzen and Wallis, 1999; Arntzen et al., in preparation). When BAPS is allowed to determine the optimal number of gene pools, these newts are clustered in a unique group. However, the inclusion of one *T. cristatus* individual from the contact zone in this group and its placement in the species tree (see below) would suggest genetic influence of *T. cristatus* during the evolutionary history of this *T. carnifex* stock. An analysis employing a much larger number of nuclear markers is required to test this explanation.

In contrast to the nuclear phylogenetic trees and haplotype networks, BAPS supports the presence of three distinct gene pools in *T. karelinii* sensu lato. These groups almost fully correspond to the geographical pattern suggested by mitochondrial DNA. The only exception is that 'central *T. karelinii*' contains 'western *T. karelinii*' mitochondrial DNA in the westernmost part of its range (Fig. 1, further discussed in Sections 4.4 and 4.5).

4.3. A species tree for Triturus based on multilocus data

Our approach to treat BAPS groups as operational taxonomical units circumvents the arbitrary *a priori* defining of 'species' required by *BEAST. The different BAPS groups generally cluster in (candidate) species as expected in the species tree produced by *BEAST. Support for the monophyly of each is suggestive, but in most cases not statistically significant. It should be emphasized that the recognized crested newt species are supported as discrete nuclear gene pools by allozyme data and we thus do not doubt their status as distinct species (Arntzen et al., in preparation, whose sampling of individuals from recognized crested newt species largely overlaps with the current paper). Support for the monophyly of the three candidate species is in the same range as that for the recognized species.

To a certain extent low support might reflect a lack of resolution in the data, but we suspect gene flow due to hybridization (cf. Section 4.1.) also influences our results. *BEAST assumes that there is no horizontal gene flow between 'species' (Heled and Drummond, 2010). However, this assumption is likely to be violated for recently formed species. Note that such groups would also be the ones that particularly benefit from the application of a multi-species coalescent approach, as they are more likely to show ancestral polymorphism. The current inability to deal with gene flow directly is thus a considerable limitation and an important issue to be resolved in future development of multispecies coalescent models. BAPS groups containing a combination of alleles derived from different species can be expected to be difficult to place in a species tree. Therefore, we tried to correct for gene flow by excluding individuals that show signs of genetic admixture. This reduced dataset excluded those individuals that clustered with a different species than would be expected based on morphology and allozymes and/or that contain mitochondrial DNA typical of another (candidate) species.

The placement of *T. macedonicus* with 'western *T. karelinii*' is peculiar. Note that evidence for a sister-species relationship of *T. macedonicus* and *T. carnifex* (instead of 'western *T. karelinii*') is strong, based on morphology, mitochondrial DNA and allozymes (Arntzen et al., 2007). Also, based on allozyme data, it is clear that *T. macedonicus* and 'western *T. karelinii*' constitute distinct gene pools, i.e. behave as real species (Arntzen and Wielstra, 2010; Arntzen et al., in preparation). Although BAPS manages to separate *T. macedonicus* and 'western *T. karelinii*', the two cluster together in the species tree, and support for their reciprocal monophyly is low. It has been hypothesized that *T. macedonicus* has displaced 'western *T. karelinii*', meaning a large part of its current range was formerly occupied by 'western *T. karelinii*' (Wielstra and Arntzen, 2012). This process co-occurred with introgressive hybridization, reflected by the presence of 'western *T. karelinii*' mitochondrial DNA in *T. macedonicus*. Coinciding asymmetric introgression of neutral nuclear DNA could explain the clustering of *T. macedonicus* with 'western *T. karelinii*' (cf. Currat et al., 2008). Analysis of our reduced dataset, excluding all *T. macedonicus* containing introgressed 'western *T. karelinii*' mitochondrial DNA, partially agrees with this hypothesis, in the sense that support for reciprocal monophyly of the two increased and support for the placement of *T. macedonicus* within *T. karelinii* sensu lato decreased. However, contrary to what would be expected, *T. macedonicus* still clusters with 'western *T. karelinii*'. A possible explanation could be positive selection having 'dragged' nuclear DNA into regions outside of the area where *T. macedonicus* displaced 'western *T. karelinii*', perhaps even from *T. macedonicus* into 'western *T. karelinii*' again. To test this explanation, a much larger number of nuclear DNA markers should be analyzed. Note that, in contrast, clustering with 'western *T. karelinii*' is not shown by the 'central *T. karelinii*' that contain asymmetrically introgressed 'western' mitochondrial DNA.

The *BEAST species tree constructed here does not provide insight into the phylogenetic relationships among the recognized crested newt species. It does so for the three candidate species forming *T. karelinii* sensu lato. However, the suggested phylogeny differs from the one based on mitochondrial DNA (Fig. 2; cf. Wielstra and Arntzen, 2011; Wielstra et al., 2010). Whereas mitochondrial DNA suggests 'central' and 'western *T. karelinii*' are more closely related to each other than each of them is to 'eastern *T. karelinii*', the *BEAST species tree clusters 'central' together with 'eastern *T. karelinii*'. Furthermore, the *BEAST species tree does not support the monophyly of *T. karelinii* sensu lato because, as discussed above, 'western *T. karelinii*' clusters with *T. macedonicus*. Considering that low phylogenetic signal and gene flow hamper our estimation of the *Triturus* species tree here, we for now prefer the phylogenetic hypothesis for *Triturus* as derived from mitochondrial DNA. We recommend an approach using many more (i.e. at least several tens of) nuclear DNA markers to test the crested newt phylogeny and to explore clinal transition of individual markers across hybrid zones, especially for the 'western *T. karelinii*' – *T. macedonicus* case.

*BEAST is typically applied to recognized species known to be closely related (Heled and Drummond, 2010). The program has up to now been only sparsely applied in a phylogeographical setting, densely sampling taxa potentially containing multiple species (Hung et al., 2012; Jackson and Austin, 2012; Tavares et al., 2010; this study). *BEAST appears to perform reasonably well for the identification of distinct gene pools in multilocus datasets, and the program is thus a welcome addition to the phylogeographer's toolkit.

4.4. The current (lack of) genetic interaction between T. karelinii sensu lato lineages

'Eastern' and 'central *T. karelinii*' show no signs of genetic admixture. This is in line with their apparent current allopatry (cf. Fig. 1; Wielstra et al., 2010). Historical records are from the Turkish side of the border with Georgia but could not be confirmed during recent fieldwork (reviewed in Wielstra et al., 2010). We suspect these historical records concern 'eastern *T. karelinii*', considering the geographical proximity to known 'eastern *T. karelinii* localities' (locality 105 in Fig. 1); the distance to the nearest known 'central *T. karelinii* locality' (locality 103 in Fig. 1) is considerably larger. If and when crested newts are re-discovered on the Turkish sides of the border, their identity can easily be determined using the dataset and procedure presented in this paper.

'Western' and 'central *T. karelinii*' are parapatric (cf. Fig. 1). The two represent distinct, geographically coherent nuclear gene pools. The one exception found by BAPS when a fixed *k* value is enforced (concerning a single individual from locality 92 clustering with 'western *T. karelinii*'), is 'correctly' resolved when BAPS determines the optimal *k* value. Past genetic interaction between the two is reflected by introgression of 'western' mitochondrial DNA into 'central *T. karelinii*'. Note that the alternative, introgression of 'central' nuclear DNA into 'western *T. karelinii*', is not in line with the mitochondrial DNA pattern: introgressed mitochondrial DNA is firmly nested within the 'western' mitochondrial DNA gene tree and dissimilar from that found in pure 'western'. This suggests long term evolution of 'western' mitochondrial DNA in situ, before it was captured by 'central *T. karelinii*' (cf. Fig. 2). An earlier attempt to locate the contact zone between 'western' and 'central *T. karelinii*' (Arntzen and Wielstra, 2010) similarly showed that 'central *T. karelinii*' was distributed more westerly than would be expected based on mitochondrial DNA. However, a more convoluted shape for the contact zone was suggested, with individuals identified as 'western *T. karelinii*' present close to our locality 98 (Fig. 1). The study by Arntzen and Wielstra (2010) suffered from a rather limited sampling of 'central *T. karelinii*' (and 'eastern *T. karelinii*' was not included at all). Therefore, we put more confidence in the present findings. However, we do suggest a detailed hybrid zone analysis to be conducted, using a denser sampling in terms of more populations, more individuals per population and more nuclear DNA markers, to clarify the transition between 'central' and 'western *T. karelinii*'.

4.5. Different newts, comparative phylogeographical pattern

There is a striking similarity between the 'central *T. karelinii*' – 'western *T. karelinii*' and the *Lissotriton kosswigi* – *L. vulgaris* contact zones (Nadachowska and Babik, 2009). Not only is the contact zone between the two pairs of newts positioned in the same region, but *L. kosswigi* also possesses *L. vulgaris* mitochondrial DNA in the same area where 'central *T. karelinii*' contains 'western *T. karelinii*' mitochondrial DNA. Furthermore, the temporal estimation of the split between both species pairs is similar (around 5.5 Ma; Nadachowska and Babik, 2009; Wielstra et al., 2010). We propose the following shared historical biogeographical scenario underlying the pattern shown by the two pairs of newts.

The reconnecting of the Black Sea with the Aegean Sea at the conclusion of the Messinian Salinity Crisis, around 5.33 Ma (Krijgsman et al., 1999), caused the initial split between both pairs of newts. The route of this marine connection was re-ordered extensively on several occasions due to tectonic developments. Although the intermediate stages are not yet fully understood, the initial route across the Balkans (Suc et al., 2011) shifted and by the start of the Pleistocene (2.6 Ma) incorporated the Marmara Sea (Elmas, 2003). Until the beginning of the Holocene (11.7 Ka), a sea straight called the İzmit Gulf – Lake Sapanca – Sakarya Valley waterway connected the Black Sea with the Sea of Marmora (Elmas, 2003). Although the re-ordering of the Aegean Sea - Black Sea connection may have facilitated periodic geographical contact between the two pairs of newts prior to the Pleistocene, no ancient introgression could be identified.

We suggest that the İzmit Gulf – Lake Sapanca – Sakarya Valley waterway separated the geographical ranges of both pairs of newts (cf. Fig. 5). After this waterway closed, 'central *T. karelinii*' and *L. kosswigi* expanded their ranges westwards, at the expense of 'western *T. karelinii*' and *L. vulgaris*. This displacement coincided with hybridization, and mitochondrial DNA introgressed in the process (cf. Currat et al., 2008). Both 'western' and 'central' '*T. karelinii*'

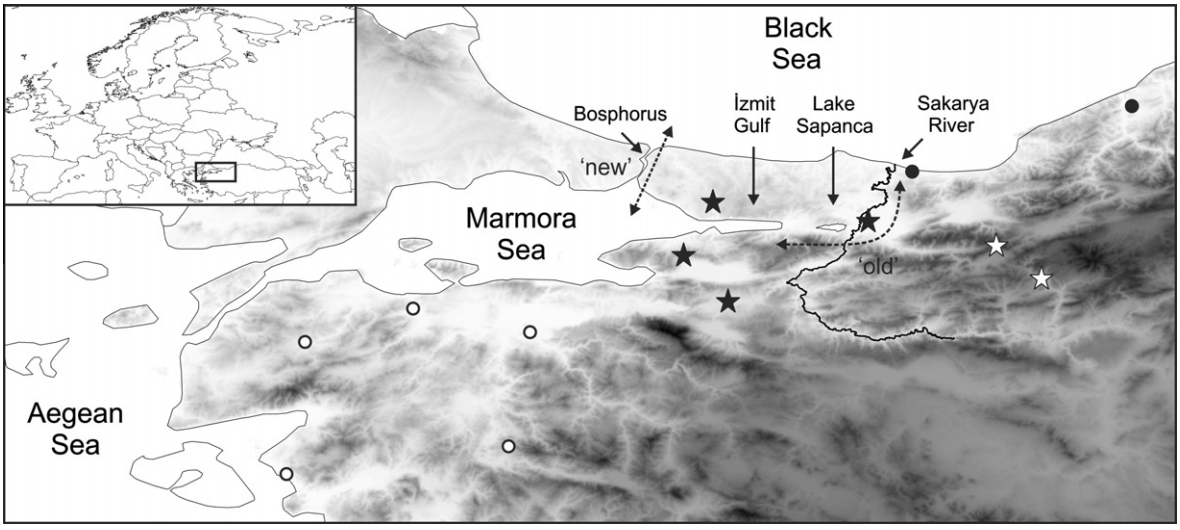

Fig. 5. Map showing (paleo)geological features mentioned in the biogeographical scenario explaining the asymmetrical introgression of 'western *T. karelinii*' mitochondrial DNA into 'central *T. karelinii*'. Closed circles reflect 'central *T. karelinii*' containing 'central' mitochondrial DNA and open circles 'western *T. karelinii*' containing 'western' mitochondrial DNA; closed stars reflect 'central *T. karelinii*' containing 'western' mitochondrial DNA. The white stars represent localities where both mitochondrial DNA types were found in syntopy in another study (Wielstra et al., in preparation). Elevation (only shown for Turkish territory) is expressed as a continuous scale, running from light (low elevation) to dark (high elevation). Up to recently the Marmara Sea was connected to the Black Sea via an 'old' waterway, incorporating the İzmit Gulf, Lake Sapanca and the Sakarya Valley. At the beginning of the Holocene, a 'new' waterway, the Bosphorus, took over. With the former sea strait gone, 'central *T. karelinii*' expanded westwards, at the expense of 'western *T. karelinii*' and took up 'western' mitochondrial DNA in the process via introgressive hybridization (see text for details).

mitochondrial DNA are currently in syntopy at locality 91 (Fig. 1, Appendix S1), close to where the waterway was positioned. We predict that a denser sampling in *L. kosswigi* along the route of the ancient waterway will also reveal syntopy of *L. kosswigi* and *L. vulgaris* mitochondrial DNA. With the formation of the Bosporus in the Holocene (11.7 Ka to present), an alternative connection between the Marmara and Black Seas arose (Elmas, 2003; Kerey et al., 2004), which prevented 'central *T. karelinii*' and *L. kosswigi* to colonize Europe (cf. Fig. 5).

South of the İzmit Gulf – Lake Sapanca – Sakarya Valley waterway, the arid, high altitude Anatolian Plateau would have acted as a barrier, contributing to the isolation of 'western' and 'central *T. karelinii*'. However, Wielstra et al. (in preparation), employing a dense sampling of mitochondrial DNA, found both 'western' and 'central *T. karelinii*' mitochondrial DNA at and approximately 30 km. southeast of locality 95 (cf. Fig. 5). This finding suggests that interaction between 'western' and 'central *T. karelinii*' was not completely restricted by the İzmit Gulf – Lake Sapanca – Sakarya Valley waterway. Evidently, the extent of such interaction must have been limited. How 'central *T. karelinii*' and *L. kosswigi* managed to outcompete 'western *T. karelinii*' and *L. vulgaris* after the İzmit Gulf – Lake Sapanca – Sakarya Valley waterway closed and whether their current contact zones are in equilibrium or still shifting are promising prospects for future research.

5. Conclusions

Based on a multimarker phylogeographical survey and a combined use of two multi-locus analytical approaches, we have shown that the three mitochondrial DNA lineages we previously identified in *T. karelinii* senu lato (cf. Wielstra et al., 2010) are also differentiated from the perspective of nuclear DNA. The divergence among the three *T. karelinii* sensu lato lineages is on par with that between recognized *Triturus* species, suggesting *T. karelinii* comprises three distinct species. A taxonomical treatment will be presented elsewhere. No morphological characters are currently known to discriminate the three *T. karelinii* sensu lato species, suggesting they represent cryptic species. However, we hope our new findings will stimulate further morphological investigation, putting the cryptic species hypothesis to the test.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.01.009.

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