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Phylogenetic Affinity and Genetic Structure of the Southern Crested Newt *Triturus karelinii* Strauch, 1870 (Amphibia: Caudata) in Iran Inferred from mtDNA Sequences

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Abstract: The southern crested newt *Triturus karelinii* Strauch, 1870, is widely distributed on the northern Iran plateau, where it is one of the most common newts. Mitochondrial DNA sequences (the genes cyt b and ND4) were used to determine the genetic diversity and phylogenetic position of Iranian *Triturus.* The results corroborate the monophyly of *T. karelinii* and confirm that all Iranian and Azerbaijan populations belong to the same species. Genetic distances (K2-p) between Iranian *T. karelinii* and other *Triturus* species was relatively high, (7–25% for ND4 and 8–19% for cytb). However, infraspecific genetic diversity among the Iranian populations was very low, which may indicate strong gene flow between populations.

Key words: Phylogeny; *Triturus karelinii*; cyt b; ND4; gene flow; Iran

Introduction

The genus *Triturus* Rafinesque, 1815 comprises the marbled and crested newts (Rancilhac et al. 2021). The marbled newt group includes *T. marmoratus* (Latreille, 1800) and *T. pygmaeus* (Wolterstorff, 1905). The crested newt group includes *T. anatolicus* Wielstra and Arntzen, 2016, *T. carnifex* (Laurenti, 1768), *T. cristatus* (Laurenti, 1768), *T. dobrogicus* (Kiritzescu, 1903), *T. ivanbureschi* Arntzen and Wielstra, 2013, *T. karelinii* (Strauch, 1870) and *T. macedonicus* (Karaman, 1922). These are distributed in Europe and adjacent Asia (Wielstra et al. 2019). During recent decades, several attempts have been made to clarify the taxonomy of this genus based on molecular and ecological data (Arntzen et al. 2007, Litvinchuk & Borkin 2009, Wielstra et al. 2012, 2016, 2020, Ivanović et al. 2013). The *Triturus karelinii* species complex is distributed from the southern Balkans in the west to the Caucasus area and southern shore of the Caspian Sea in the east. This species complex was first split into *T. karelinii* in the east and *T. arntzeni* in the west (Arntzen et al. 2010).

Later, the name of the western populations was changed from *T. arntzeni* to *T. ivanbureschi* (Wielstra et al. 2013). However, recently, a multilocus phylogeographic study provided compelling evidence that *Triturus ivanbureschi* comprises two distinct gene pools (Wielstra & Arntzen 2016). These gene pools were subsequently recognised as two distinct species, *T. ivanbureschi* of western Asiatic Turkey and the south-eastern Balkan Peninsula and *T. anatolicus* of northern Asiatic Turkey (Wielstra & Arntzen 2016). It is now widely accepted that *Triturus karelinii* is endemic to the Pontocaspian Region and occupies three regions: Crimea,

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Caucasus and the Southern shore of the Caspian Sea (Ivanović et al. 2013, Wielstra & Arntzen 2020). The distribution range of this species in Iran includes the Mazandaran, Guilan, Golestan, East Azerbaijan and West Azerbaijan Provinces (Safaei-Mahroo et al. 2015, Pourhallaji et al. 2021).

In the present study, we focused on the genetic variation among the populations of *Triturus karelinii* from northern Iran and investigated the phylogenetic position of these populations within the genus using mitochondrial cyt b and ND4 genes sequences. These markers are traditionally used for assessing both intraspecific variability within species and relationships among different species and genera. The assessment of the Iranian populations may help not only uncovering biogeographical history of the genus *Triturus* but also provide a reliable model revealing the evolutionary history of biodiversity within this region.

Materials and Methods

Sampling

A total of 23 individuals of the *T. karelinii* were studied from three populations distributed in northern Iran. Blood and tail tip samples were collected and the animals were released in their natural environment again (Table 1, Fig. 1). The tissue samples have been deposited in Sabzevar University Herpetological Collection (SUHC).

DNA extraction, PCR and sequencing

Genomic DNA was extracted from tissue samples using the salt-based method, proteinase K digestion followed by ammonium acetate extraction (Kapli et al. 2013). We performed PCR reactions to amplify partial regions of protein coding ND4 (719 bp) and the protein-coding gene cytochrome b (Cytb, 820 bp). The PCR conditions for both genes were as follows: 4 minutes at 94°C for denaturation, followed by 36 cycles of 40 seconds at 94°C, 40 seconds at 56°C for annealing and 1:30 minutes at 72°C for primer extension, then a final 10 minutes incubation at 72°C. Amplification of the ND4 marker was done using the primers ND4 (5′-CACCTATGACTACCAAAAGCTCATGTAGAAGC -3′; Arévalo et al. 1994) andND4 (5′-CCCTGAAATAAGAGAGGGTTTAA-3′; Arntzen et al. 2007), and cytochrome b (cytb) with TRIcytb-f (5′- CCTCACAGGCCTATTCCTAGC -3′)and TRIcytb-r (5′- TAGAAGAGATACCTGTTGGGT -3′; Maletzky et al. 2008). The alignment comprised 23 partial sequences of ND4 and Cytb. The amplified fragments were sequenced on an automated sequencer ABI 3730XL (Macrogen, Seoul, South Korea).

Phylogenetic analyses

A total of 41 sequences from two mitochondrial markers (cytochrome *b* and nd4) were analysed, including the 23 novel sequences produced in this study and 18 sequences retrieved from NCBI (details are presented in Table 1). *Calotriton asper* was chosen as outgroup (accession numbers are presented in Table 1). Clustal W, as implemented in BioEdit sequence alignment editor v.7.0 (Hall 1999), was used to align the sequences, with default parameters. Mega 7 (Kumar et al. 2016) was used to check for stop codons, align reading frames in both genes as well as calculating genetic distances (K2-p-distance) for each marker.

Phylogenetic analysis was performed using Bayesian Inference (BI). For extracting the best-fitting evolutionary model with Akaike Information Criterion (AIC; Akaike 1974) the program JModeltest v.2.1.3 was used (Posada 2008). The best fit model for the combined dataset (cytb, ND4) was TVM+I+G. The BI analysis was done using MrBayes 3.2.5 (Ronquist et al. 2012) for the combined dataset for 10^6 generations with a sample frequency of 0.001 with 25% of the initial samples discarded as burn-in. The final trees were combined as a 50% majority-rule consensus tree to calculate posterior probabilities (PPs) for each node.

Results

Phylogenetic analyses

Seven major clades were revealed in Bayesian inference phylogenetic tree (Fig. 2). Clade A comprised specimens of *T. karelinii* from Azerbaijan and northern Iran (Golestan, Mazanderan, Gilan and Ardabil provinces). Clade B comprised the specimens from Turkey, previously identified as *T. karelinii* but now Turkish specimens are recognized as two distinct species, *T. ivanbureschi and T. anatolicus* (Wielstra et al. 2013, 2016). Clades C, D, E, F and G comprise *Triturus* species from Europe: *T. macedonicus, T. carnifex, T. cristatus, T.marmoratus* and *T.pygmaeus*.

The Iranian and Azerbaijan populations (clade A) and the populations from Turkey (clade B) were sister clades but with a low bootstrap value of 0.57). The calculated genetic divergence (*p*-distance) varied from 0.057 to 0.186 in cyt*b* and from 0.051 to 0.250 % in ND4 sequences (Table 2). The genetic distance showed that *T. karelinii* has the least genetic difference to the species of Turkey (Fig. 2).

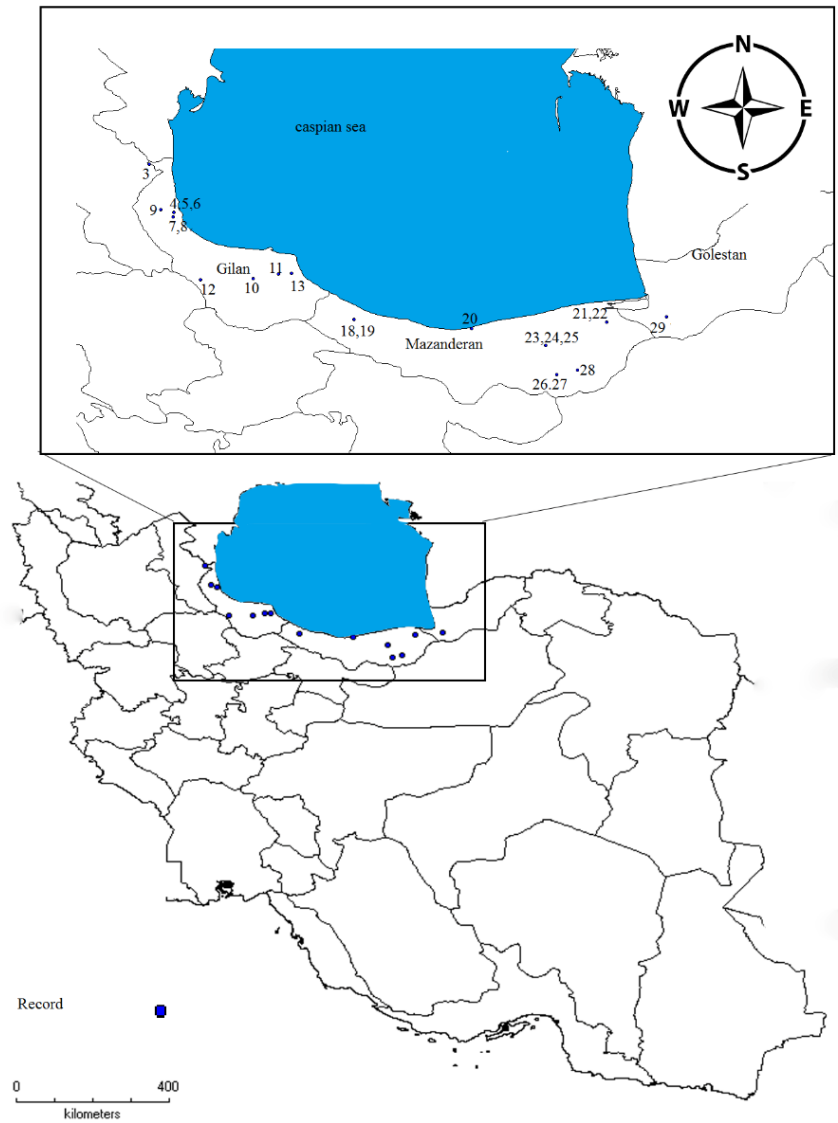

Fig. 1. Map of Iran with sampling locations included in this study.

Discussion

According to our Bayesian phylogeny, species within the genus *Triturus* can be divided into two groups (Fig. 2). The first group includes the marbled newts *T. marmoratus* and *T. pygmaeus*. The second group includes the crested newts *T. macedonicus, T. carnifex, T. cristatus* and the *T. karelinii* group. Our results confirm the monophyly of *T. karelinii* and its position within the genus *Triturus*. The Iranian clade is genetically distinct and well-supported by mitochondrial sequences. According to Wielstra et al. (2020), *T. karelinii* is widely distributed throughout the Pontocaspian area and comprises three allopatric sections: The Crimean, Caucasian and Caspian. The Caspian populations are inhabited in the lowland between the Caspian Sea in the north and the Elburz Mountain range in the south. Nuclear DNA data reveal little genetic differentiation between the three range sections; there is extensive gene flow between the Caspian and Caucasian range sections (Wielstra et al. 2020).

According to our analyses (Fig. 2), clade A, including populations from Iran and Azerbaijan, shows very little genetic difference. Two hypotheses could explain this pattern: (1) The Iranian populations originated from the Caucasus populations and invaded north Iran relatively recently, or (2) There exist strong gene flow between Iranian and Azerbaijan populations due to the lack of a significant geographical barriers.

The Balkan Peninsula has been recognised as the centre of origin for crested newt diversity (Crnobrnja-Isailović et al. 1997). According to Arntzen et al.

Table 1. List of material used in this study with GenBank accession numbers locality codes.

Species	Codes	Coordinates (N, E)	GenBank acc. no.
Triturus karelinii	03	Astara, Gilan Province, 38.37627 48.6151	non
Triturus karelinii	04, 05, 06	Saragah, Gilan Province, 37.8240 48.8767	non
Triturus karelinii	07, 08	Saragah, Gilan Province, 37.835 48.879	non
Triturus karelinii	09	Basak, Gilan Province, 37.8785 48.74391	
Triturus karelinii	10	Rasht, Gilan Province, 37.1290 49.7478	
Triturus karelinii	11	Sustan, Gilan Province, 37.1806 50.02210	
Triturus karelinii	12	Rodkhan Road, Gilan Province, 37.11778 49.1729	
Triturus karelinii	13	Langarud, Gilan Province, 37.184 50.163	
Triturus karelinii	18, 19	Tonekabon, Mazandaran Province, 36.68181 50.84058	
Triturus karelinii	20	Rostam Rud, Mazandaran Province, 36.5828 52.1215	
Triturus karelinii	21, 22	Behshahr, Mazandaran Province, 36.65585 53.58986	
Triturus karelinii	23, 24, 25	Qaemshahr, Mazandaran Province, 36.39979 52.92324	non
Triturus karelinii	26, 27	Shur Mast, Mazandaran Province, 36.08693 53.04258	non
Triturus karelinii	28	Sari, Mazandaran Province, 36.1329 53.2734	non
Triturus karelinii	29, 30	Kordkuy, Golestan Province, 36.71111 54.23969	non
Triturus karelinii		Azerbaijan: Avyarud, 38.30 N 48.36 E	HQ697276
Triturus anatolicus		Turkey: Trabzon, 39.86 N 40.86 E	HQ697275
Triturus ivanbureschi		Turkey: Keşan, 26.38 N 40.9166 E	HQ697277
Triturus anatolicus		Non	NC_015792
Triturus macedonicus		Greece: Asprangeli, 39.50 N 20.44 E	HQ697278
Triturus macedonicus		Non	NC_015794
Triturus carnifex		Italy: Fuscaldo, 39.25 N 16.02 E	HQ697272
Triturus carnifex		non	NC_015788
Triturus cristatus		England: Kent, 51.17 N 00.32 E	HQ697273
Triturus cristatus		non	Nc_015790
Triturus cristatus		non	EU880336
Triturus dobrogicus		non	MN122819
Triturus marmoratus		France: Confolens, 46.01 N 00.40 E	HQ697279
Triturus marmoratus		non	NC_015795
Triturus marmoratus		non	EU880337
Triturus pygmaeus		Spain: Venta del Charco, 38.12 N 04.16 E	HQ697280
Triturus pygmaeus		non	NC_015796
Calotriton asper		non	EU880307p

(2007), the onset of this radiation is dated to about 10-11 My BP (Millions of years before present). This scenario involves the simultaneous origin of species of crested newts through large-scale vicariance events, supported by palaeogeographical reconstructions for the region at the end of the Middle Miocene)Wielstra et al. 2010). The ancestor of the *T. karelinii* complex, currently represented by *T. ivanbureschi* in the Balkans and Turkey, *T. anatolicus* in Turkey and *T. karelinii* in the Crimea, Caucasus and Iran, diverged in the area that covers present day Turkey.

Predictive distribution predictive maps shows that the Golestan, Mazandaran, Guilan and Ardabil Provinces in Iran contain suitable habitat for *T. karelinii* (Pourhallaji et al. 2021). In other words, the Caspian region in north Iran is suitable for this taxon, which is highly dependent on water and moisture. The single individual from locality 29 is genetically relatively distinct. The genetic distance with other Iranian specimens is 0.004 in ND4 and 0.008 in cyt b. This specimen was sampled in the forest area of Kiasar. At an altitude of 1200 m above

Table 2. Pairwise uncorrected genetic divergence (*p*-distance) among major clades of the genus *Triturus* from the mitochondrial markers for cyt*b* (upper-right in bold-italic) and ND4 (lower-left in regular).

	***T. karelinii* (Azerbaijan and Iran)**	***Triturus* (Turkey)**	***T. cristatus***	***T. carnifex***	***T. macedonicus***	***T. marmoratus***	***T. pygmaeus***	***Calotriton asper***
Clade A: *T. karelinii* (Azerbaijan and Iran)		***0.080***	***0.086***	***0.090***	***0.096***	***0.096***	***0.179***	***0.194***
Clade B: *Triturus* (Turkey)	0.076		***0.085***	***0.091***	***0.099***	***0.099***	***0.171***	***0.210***
Clade C: *T. cristatus*	0.146	0.144		***0.057***	***0.099***	***0.099***	***0.176***	***0.198***
Clade D: *T. carnifex*	0.146	0.129	0.086		***0.090***	***0.180***	***0.186***	***0.212***
Clade E: *T.macedonicus*	0.171	0.157	0.087			***0.094***	***0.180***	***0.230***
Clade F: *T.marmoratus*	0.250	0.250	0.198	0.239	0,191		***0.042***	***0.184***
Clade G: *T.pygmaeus*	0.250	0.246	0.188	0.228	0.188	0.051		***0.188***
Outgroup: *Calotriton asper*	0.311	0.297	0.239	0.285	0.222	0.208	0.208	

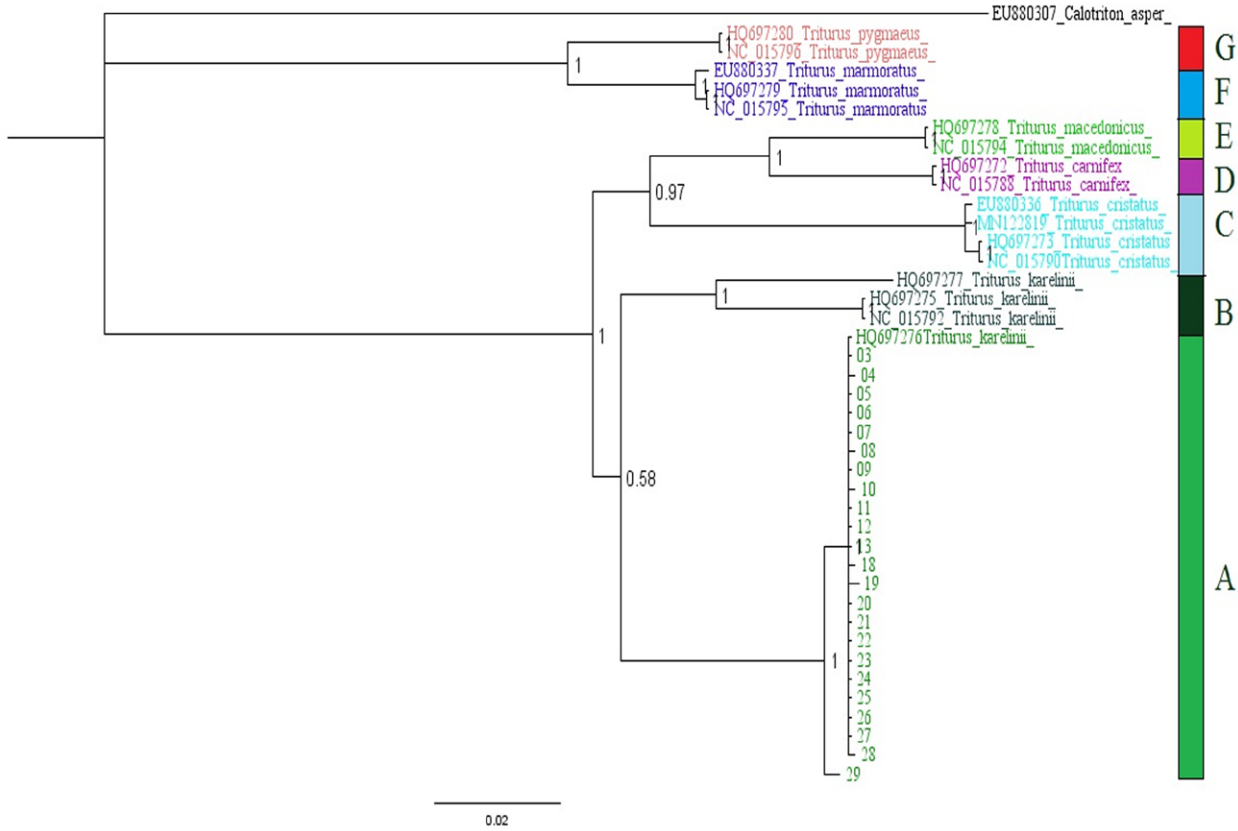

Fig. 2. Bayesian inference phylogenetic tree based on 1540 base pairs of the concatenated cytochrome *b* and ND4 sequence data set. The numbers next to the nodes indicate clade credibility in the Bayesian analysis.

sea level, this population appears to be relatively isolated. Wider sampling in the area is needed to better understand the situation.

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