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Insights into the phylogeny and phylogeography of the frog *Rana graeca* in the Balkan Peninsula (Amphibia: Anura)

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The genus *Rana* Linnaeus, 1758 includes Western Palearctic brown frogs; within this group eight species occur in Europe and 32 in Asia (Speybroeck et al. 2016, Yuan et al. 2016). The group underwent a basal post-Messinian radiation about 4 million years ago when they split into five major clades (Veith et al. 2003). Climatic changes with temperature oscillations that followed the Messinian crisis, restricted most brown frog species in their refugia with enough time to speciate, although the diversification into major clades was completed before the first Pleistocene glaciations (Veith et al. 2003). One of the three monotypic lineages that arose during the diversification, is the Stream Frog – *Rana graeca* Boulenger, 1891. Picariello et al. (2002) used S1 satellite DNA as a taxonomic marker to raise the former subspecies *R. graeca italica* (occurring in the Apennines) to the species level (Italian Stream Frog – *R. italica* Dubois, 1987), which restricted the distribution of *R. graeca* to the Balkans and Peloponnese in a continuous form (Fig. 1A). Phylogenetic analysis conducted so far (Veith et al. 2003, Yuan et al. 2016) show that the closest relatives to *R. graeca* are *R. latastei* Boulenger, 1879 and *R. dalmatina* Fitzinger, 1839 whereas the, apparently closest, *R. italica* revealed to be even a distinct evolutionary lineage (Veith et al. 2003). Afore mentioned *Rana* species are all Mediterranean endemics with the exception of *R. dalmatina* that is widely distributed over Europe (Speybroeck et al. 2016). The distribution extent of *R. graeca* might have been wider than today before the glaciations, since fossils are found outside their current distribution (Veith et al. 2003). Nowadays, in regard to other *Rana* species, *R. graeca* occurs in sympatry only with *R. dalmatina* and *R. temporaria* Linnaeus, 1758. The most marking characteristic that discriminates it from the latter two species is a mottled throat that has a pale central area resembling a white line dividing the marbled throat pattern in two equal parts (Lelo 2013). *Rana graeca* is specialized to canyons of fast flowing rivers, mountain streams and brooks (Speybroeck et al. 2016). More rarely, the species can be found near closed mountain lakes and estuaries or river deltas not far from the seaside (Šunje et al. 2018). The vertical distribution of the Stream Frog goes from 200 to 2,000 m a.s.l. (Džukić 1968).

Although the phylogenetic position of *R. graeca* is well studied in respect to other *Rana* species (Veith et al. 2003, Yuan et al. 2016), evolutionary relationships within *R. graeca* populations are completely unknown. This work aims to provide new insights to answer this question by analyzing a partial sequence of the cytochrome-b (cyt-b) of samples collected along the species range.

Sampling was done in the field and from the material deposited in various collections (Natural History Museum of Vienna, Hungarian National History Museum in Budapest, and private Zoological collection "Lelo" in Sarajevo). Field work was conducted in Bosnia and Herzegovina (BIH) and Montenegro (Table 1). Small toe clips were sampled and preserved in 95% ethanol. The material from the Zoological collection "Lelo" was stored in formalin, whereas the museum material was stored in ethanol prior to taking tis-

sue samples. From these collection samples, a piece of tissue from the hind leg was taken and preserved in 95% ethanol. In total, 22 samples, well spread over the entire range of *R. gracea*, were included in this study (Fig. 1B, Table 1). Additionally we obtained a sample of a *R. dalmatina* from the location Vrelo Bosne (VB, Table 1) during field work in BIH.

Total genomic DNA was extracted manually from the toe clips using the Qiagen DNeasy Blood and Tissue Kit (Quiagen) and from the museum samples using the QIAamp DNA Micro Kit (Qiagen), following the manufacturer's instructions. The samples taken from museum specimens were kept in 1 ml of PBS buffer for 24 hours prior to DNA extraction to enhance the purity of tissues. The quality and amount of extracted DNA was measured using a NanoDrop machine (Thermo Fisher Scientific) and afterwards diluted to a working concentration of 10ng/µl.

Partial sequences of cyt-b were amplified using primer sequences (Rana-Cytb-F2, and Rana-Cytb-R2) and temperature profiles given in Vences at al. (2013). The cyt-b was amplified in a total volume of 20 µl which included 0.8 µl of 0.2 µM of each primer, 2 µl of each of the: 200 µM of dNTP mix, 1 × Buffer, and 2.5 mM $MgCl_2$. Additionally, 0.08 µl of 0.5 U of Taq DNA polymerase, and 7.3 µl of MiliQ Water were finally added to 5 µl template DNA. For collection samples, 2 µl of 1 × Buffer containing $MgCl_2$ (instead of separately adding $MgCl_2$) was used for the PCR reaction together with 2 µl of 1 × BSA buffer. For all amplifications, contamination was checked using negative controls on gel electrophoresis.

Before sequencing, PCR products were purified using the high pure PCR product purification kit (Roche) following the manufacturer's protocol. DNA sequences of forward strands were obtained for all PCR products following the ABI Prism Big-Dye Terminator Cycle Sequencing standard protocol. The sequencing reaction products were run on an ABI 3130 Genetic Analyzer (Applied Biosystems). The resulting sequences were edited using CodonCodeAligner v.7 (CodonCode Corporation). Identified variable sites were checked by eye on the original chromatograph file in BioEdit (Hall 1999). Sequences were aligned in BioEdit (Hall 1999) with Clustal W (Thompson et al. 1994). Cyt-b was translated into amino acids for authentication on Ex-

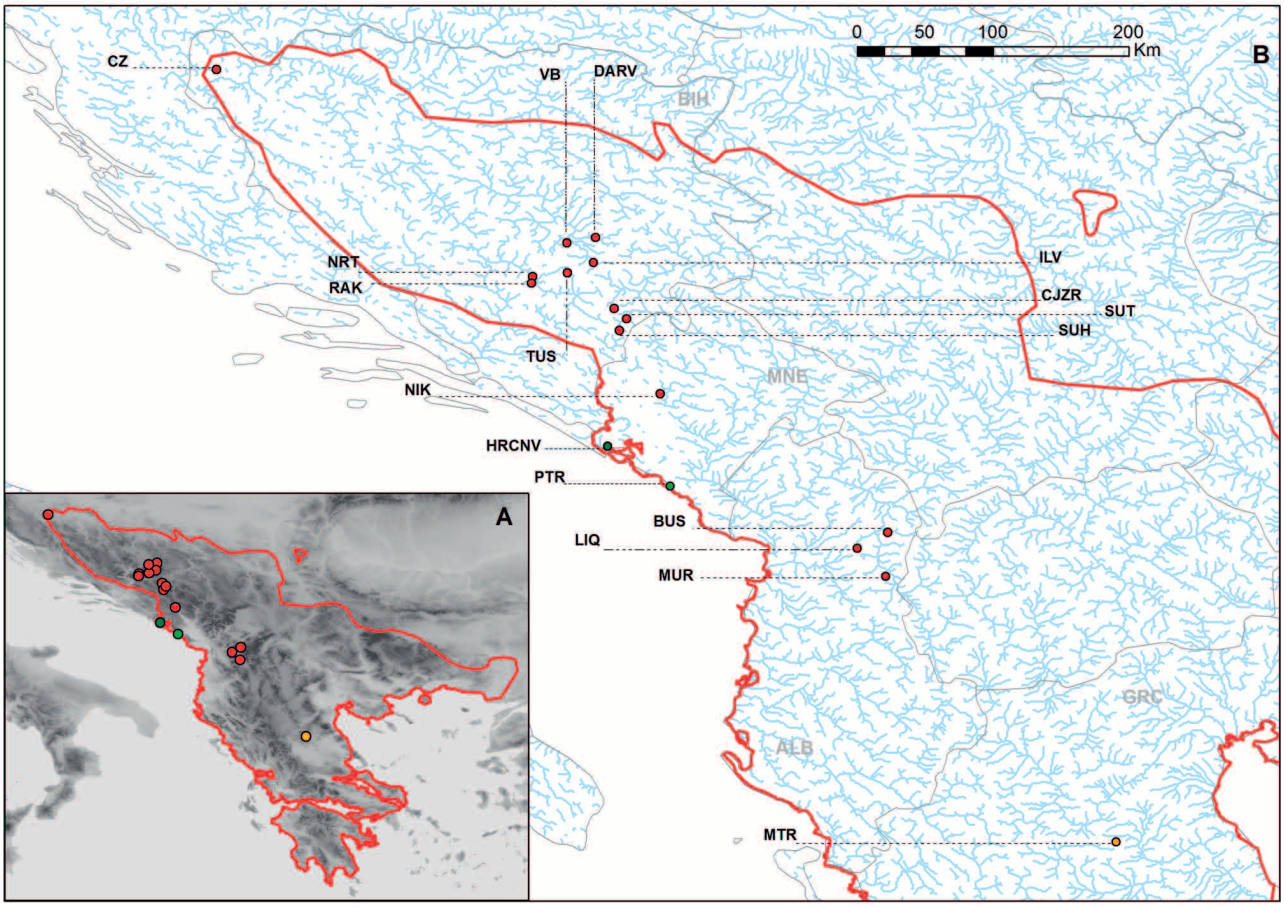

Figure 1. (A) Sampling locations of *Rana graeca* along the species range (taken from Šunje et al. 2018). The color of each point indicates the haplotype inferred from the respective sample code (N = 4). The grayscale map is drawn from the NASA elevation model SRTMGL1 (Farr & Kobrick 2000). (B) Zoomed area with sampling codes, respective locations and countries (cf. Table 1). The baseline map is showing river flows and tributaries in light blue (www.sciencebase.gov/catalog/item/537f6c6be4b021317a87279a).

Table 1. Sampling codes and locations with geographic coordinates in WGS84 format. BIH – Bosnia and Herzegovina, MNE – Montenegro, ALB – Albania, GRC – Greece. PZC – private Zoological collection, NHMW – Natural History museum of Vienna, HNHM – Hungarian Natural History Museum. The code in the parenthesis, following the collection/museum abbreviation, indicates its registration number in the respective collection. Nb – Number of samples (total Nb = 22). H – Code of inferred haplotype. EMBL An – GenBank accession number.

Code	Location	N	E	Source	Nb	H	EMBL An
CZ	Cazin, BIH	44.96	15.94	PZC: „Lelo“ (Rg9L)	1	1	MH607444
VB	Vrelo Bosne, BIH	43.81	18.26	Field work	1	1	MH607446
DARV	Dariva, Bentbaša, BIH	43.85	18.45	Field work	1	1	MH607445
ILV	Ilovice, BIH	43.68	18.44	NHMW (30428/1)	1	1	MH607447
NRT	Neretva - Đaići, BIH	43.59	18.04	Field work	1	1	MH607450
RAK	Rakitnica - Kašići, BIH	43.55	18.03	Field work	4	1	MH607451-54
TUS	Tušilačka rijeka - Visočica, BIH	43.62	18.27	Field work	1	1	MH607460
CJZR	Crno jezero - Zelengora, BIH	43.38	18.58	Field work	1	1	MH607443
SUH	Suha - Čemerno, BIH	43.23	18.61	NHMW (30428/5)	1	1	MH607459
SUT	Sutjeska, BIH	43.31	18.66	Field work	3	1	MH607455-57
NIK	Nikšić, MNE	42.81	18.88	EMBL (Yuan et al. 2016)	1	1	KX269345.1
HRCNV	Herceg Novi, MNE	42.47	18.54	Field work	1	2	MH607461
PTR	Petrovac, MNE	42.20	18.95	Field work	1	3	MH607463
BUS	Bushtrice, ALB	41.90	20.39	HNHM (2358)	1	1	MH607458
MUR	Murres, ALB	41.60	20.38	HNHM (2357)	1	1	MH607449
LIQ	Liqeni, ALB	41.79	20.19	HNHM (2355)	1	1	MH607448
MTR	Meteora, GRC	39.84	21.91	NHMW (29072/1)	1	4	MH607462

pasy server (https://web.expasy.org/translate/). Sequences are deposited in the EMBL database (Table 1).

The number of haplotypes, haplotype diversity, number of polymorphic sites (singletons and parismony informative) as well as nucleotide diversity were computed with DnaSp (Librado & Rozas 2009). The same software was used to assess Strobeck's S, Fu and Li's statistics and Tajima D test. Due to the fact that the number of samples from same sampling locations was overall too low (Table 1), it was not considered meaningful to define distinct populations which would allow additional population genetic tests.

For the phylogenetic analyses we downloaded the single available *R. graeca* sequence longer than 500 bp available in EMBL Genbank database (accession number: KX269345.1, Yuan et al. 2016). We included the sequence of *R. dalmatina* from this study (accession number: MH607464) in the analyses and specified it as outgroup following the results of previous molecular analyses that included different *Rana* species (Vences et al. 2013, Yuan et al. 2016). Phylogenetic relationships were estimated using Maximum Likelihood (ML) and Bayesian Inference (BI). Partition of the dataset was carried out in DAMBE (Xia 2001). JModelTest 2.1.1 (Darriba et al. 2012) was used to infer the best evolutionary model under the AIC criterion for each partition separately and for the original alignment. To test if the partitioned dataset is of good quality for phylogenetic analysis we performed the substitution saturation test in DAMBE (Xia et al. 2003) for each partition separately. ML analyses were done in MEGA 7 (Kumar et al. 2015) using the bootstrap method (1,000 times) under the Tamura-Nei model, as suggested from the results of JModelTest. BI was done in MRBAYES 3.2.6 (Huelsenbeck & Ronquist 2001); for each partition separately, we specified respective model parameters based on the results obtained in JModeltest. Bayesian analysis were done for two independent runs of 2 million generations, each with four Markov Chains, discarding a 10% as burn-in, and sampled every 1,000 generations. The tree was visualized and edited with FigTree v. 1.3.1 (Rambaut 2009).

Divergence and geographical distribution of the haplotypes were analyzed by means of TCS networks (Clement et al. 2000), using POPART 1.7 (Leight & Brian 2015).

The amplified cyt-b was 554 bp in length. Due to missing data and gaps (N = 66), 488 sites were included in the analysis. In total, eight sites revealed to be polymorphic; all were singletons with no parsimony informative sites. The nucleotide diversity (Pi) was 0.00149 and the average number of nucleotide differences (k) was 0.727. Four (4) distinct haplotypes were revealed in this study (Table 1, Fig. 1 and Fig. 2) showing a haplotype diversity of 0.26 (sd = 0.120). Strobeck's S statistic (0.861, P = 0.207) confirmed that the number of haplotypes is lower or equal to four. Fu and Li's statistic proved that all mutations are not selectively neutral (D = -3.32, P < 0.02; F = -3.19, P < 0.02), whereas the Tajima's D suggested a recent population expansion (D = -2.19, P < 0.01).

The substitution saturation test showed that the sequences have experienced little substitution saturation (P = 0.000 for all partitions), and therefore are suitable for phylogenetic analysis. Both ML and BI phylogenetic trees of the haplotypes were consistent in topology and confirmed that *R. graeca* is monotypic with respect to *R. dalmatina*. Based

on the phylogenetic tree (Fig. 2), all sequences seem to be part of one clade but the haplotype network (Fig. 2) suggests that the sample from Greece might belong to another lineage differentiated from the rest of the populations.

Overall, a low variation of mtDNA has been revealed in this study. The largest number of individuals represented a single haplotype that is present across the entire northern part of the species' range (from BIH to Albania – Table 1, Figs 1–2). The sample from the southern part of the species' range is represented by a more diverged mitochondrial haplotype (Fig. 2) suggesting a different colonization origin of these populations possibly located in Greece. Glacial refugia and colonization events that started from Greece are reported for several other taxa, such as plants (Brus 2010), amphibians (Pabijan et al. 2014), reptiles (Lenk et al. 1999, Poulakakis et al. 2005) and birds (Griswold & Baker 2002, Brito 2005). The area of Montenegro harbors two haplotypes and is characterized by higher genetic variation, which suggests that this region could have been a diversification center for the northern populations. Ursenbacher et al. (2008) also report the area of Montenegro as an important diversification center explaining the phylogeography of the nose horned Viper (*Vipera ammodytes* Linnaeus, 1758). The existence of multiple glacial refugia in the Balkans has been reported by many authors (e.g. see afore mentioned) but well showed in Vences et al. (2013) where the authors assessed these using environmental niche modelling.

During the last glacial maxima the Balkans were mostly free of ice (Fig. 1B), but the glaciation has made a marked

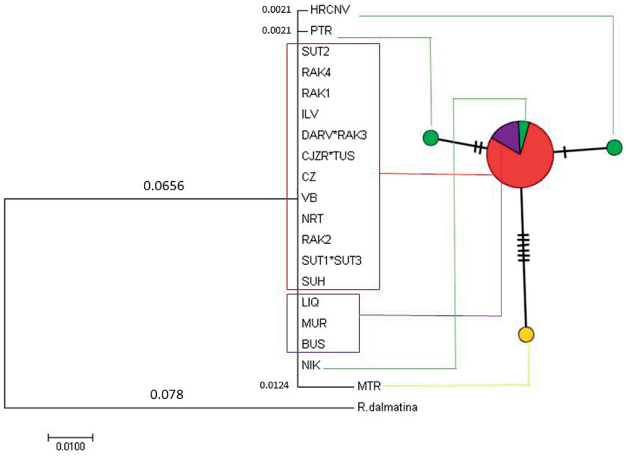

Figure 2. Phylogenetic tree and haplotype network of 22 individuals sampled along the species' range. In the phylogenetic tree, samples with identical sequences are separated with an asterisk (*); labels are as in Table 1 and Figure 1B. Values on nodes correspond to number of substitution per site (the absence of a number in front of a sequence indicates no substitution – 0.000). In the haplotype network, each identified haplotype (N = 4) is a separate chart pie; colors correspond to countries of origin (red = Bosnia and Herzegovina, green = Montenegro, purple = Albania, yellow = Greece); the minimum number of substitutions between haplotypes corresponds to the number of bars on the branches of the haplotype network.

effect on the Mediterranean climate that might have been responsible for a retreat of brown frogs within areas of their distribution range (Veith et al. 2003, Šunje et al. 2018). Possibly, at that time, the dispersal events of *R. graeca* were ongoing to a certain extent during warm phases but were probably restrained by climatic oscillations and the existence of several geographic barriers (e.g. mountain glaciers). These might have influenced a spread of populations from North to South and vice versa.

A lower diversity characterized by homogenous and invariable sequences was detected also in *R. dalmatina* when compared to *R. temporaria*, which is also explained by the more narrow distribution of the former when compared to the latter (Veith et al. 2003). The distribution of *R. graeca* is even narrower when compared to *R. dalmatina*, therefore the observed low variation revealed in this study may not be surprising. A more shallow and derived lineages and low variation is characteristic to areas of recent range expansion (Veith et al. 2013) which seems also to be the case of *R. graeca* as showed by the Tajima test. Additionally, only limited genetic variation and a small number of haplotypes occur in recently (re)colonized areas due to range expansions that are associated with an overall reduction of variation (Excoffier et al. 2009). Based on these findings, our result suggest that *R. graeca* is expanding northwards since a single haplotype is present across the entire northern part of the species' range. Fast postglacial range expansions associated with genetic uniformity have been reported also for other amphibian species (e.g. see Kuchta & Tan 2005, Makowsky et al. 2009). The rich river net along the range consisting of interconnected rivers, streams, and tributaries (Fig. 1B) seem to be facilitating dispersal events and expansion. Although dispersal abilities of *R. graeca* are unknown, observations far from water sources have been reported (see Bolkay 1919, Džukić 1968) which pose the question of the species' plasticity. The registered presence of the species in unconventional habitats in the northern part of the range (Šunje et al. 2018) suggests a still ongoing expansion and the possible existence of traits that favor species persistence and increase its potential to occupy novel habitats. By unconventional habitats we refer to karstic fields with (sinking) rivers, the beds of which are armored with grass rather than rocks, which is atypical when compared to rocky canyons of fast flowing rivers that are common to *R. graeca* (Džukić 1968). The (adaptive) variability in ecological, morphological, behavioral, and physiological traits remains still to be studied to confirm this speculation. A wider sampling, together with additional genetic markers are needed to further clarify the evolutionary relationships within *R. graeca*.

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