COI Based Phylogeography and Intraspecific Genetic Variation of *Rana dalmatina* Populations in the Vicinity of the Carpathians

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ABSTRACT

In the herein study we investigated the phylogeographical structure and genetic variability of the agile frog (*Rana dalmatina*) populations in the vicinity of the Carpathian Mountains, including the Transylvanian plateau and Northern Dobruja. The agile frog being on the IUCN Red List needs a special attention regarding its genetic variability. We have analyzed a 503 bps long sequence from the cytochrome oxidase subunit I (COI) gene from 62 individuals sampled from 7 geographical regions in Romania. In total 9 haplotypes were identified that differed from each other only in singletons. All the nucleotide substitutions were synonymous. Samples taken from the Olt River valley and Southern Carpathians represent the highest allelic diversity with 5 different alleles and the highest values for the pairwise Nei genetic distances. The AMOVA test also indicated population differentiation with significant p-value for this population. We predict a possible cryptic refugium in this region.

Keywords: *Rana dalmatina*, COI, phylogeography, cryptic refugium, genetic variability, population differentiation

INTRODUCTION

Molecular phylogeography is the concept that links biogeography with population genetic structure (Avise et al. 1987) and it is based on comparison of genotype information often derived from mitochondrial DNA sequences. Amphibians represent an advantageous tool to test phylogeographical models and vicariance hypotheses, because they generally exhibit low mobility and high philopatry to natal sites (Beebee 1996), accumulating the signals that

generated current species distribution (Zeisset & Beebee 2008). Unlike the semi aquatic water frogs the brown frogs such as *Rana dalmatina, Rana temporaria* and *Rana arvalis* are largely terrestrial and cold adapted.

The phylogeny of the Western Palearctic brown frogs has recently been studied by Veith et al. 2002 using the 16S rRNA and the cytochrome b mitochondrial markers, indicating that speciation of these species was triggered by the onset of glacials with cycles of repeated cold and warm periods. The glacials of the Pleistocene and early Holocene explains the current distribution of many European species. The most well known regions that provided refugia for various animal and plant species are the Mediterranean Peninsulas and the Caucasus (Taberlet et al. 1998). However it seems that recent glacials could have been survived in more northern cryptic refugia as in case of the cold tolerant *Rana arvalis*, which species shows a high diversity in the Carpathian Basin (Babik et al. 2004). Local refugium was identified with genetically distinct haplotypes for the *Rana temporaria* in the south west of Ireland (Teacher et al. 2009).

From the three species of brown frogs the *Rana dalmatina* has the southernmost distribution in Europe (Fig. 1).The Balkan Peninsula may have served for this species as refugium, for postglacial colonization. Considering the presence of cryptic refugia for the *Rana temporaria* and *Rana arvalis* outside the Mediterranean Peninsulas, the goal of our study was to search for possible cryptic refugia of the *Rana dalmatina* species in the vicinity of the Carpathian Mountains and in the Transylvanian Plateau.

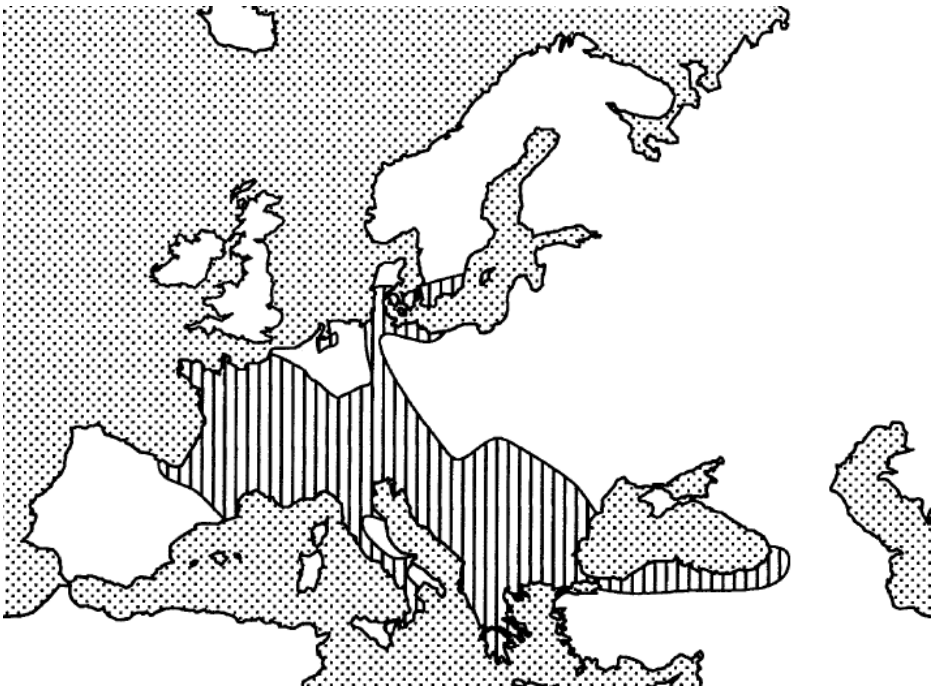

Figure 1: European distribution map of *Rana dalmatina* (after the IUCN Red List of Threatened Species, Kaya et al. 2008).

The second aim of our study was to elucidate the genetic variability of the Rana dalmatina populations in Romania. The genetic marker we applied for this purpose was a fragment of the cytochrome oxidase subunit I gene (COI) that is also used for species barcoding (Smith et al. 2008).

We consider the COI fragment is more suitable to determine population differentiation and phylogeography than the 16S rRNA gene or cytochrome b gene fragment. The COI sequence provides better resolution since it accumulates more variability (Marosi et al. unpublished data).

The scientific contributions of this paper are: 1) Estimating the genetic variability of the *Rana dalmatina* species in Romania. 2) Identification of a possible cryptic refugium of the species. 3) Understanding the variability and distribution of this species helps to elaborate the proper conservation management. 4) Better understanding the post glacial colonization phenomenon of amphibian species.

MATERIALS AND METHODS

Sample collection

Frog specimens were sampled from 7 geographical regions of Romania (Fig. 2). The regions were marked with letters from A → G.

Samples belonging to the region A (n = 31) were from the Transylvanian Plateau. They were regarded as one metapopulation since there was no considerable geographical barrier that could split the populations.

The region B was west to the Apuseni Mountains and south to the Criș River with sample size of n = 5.

The region marked with C was near the southeast part of the Danube River with n = 5 samples.

The region D was bordered to the east by the Olt River valley and to the north by the Southern Carpathians with sample size n = 6.

The region E included the undulating area in the southeast of the Carpathians with sample size n = 6.

From the region marked with F in the vicinity of the Siret River valley we could only collect n = 2 specimens.

The region marked with G was the Plateau of Dobruja with n = 7 samples.

Beside the 62 samples from Romania we analyzed sequences from specimens received from other parts of Europe: n = 1 sample from Spain (north west of the Pyrenees), n = 2 samples from Croatia (south of the Drava River), n = 4 samples from Slovakia, n = 1 sample from Austria and n = 2 samples from Hungary. These sequences were not included in the population structure analyses, only in the construction of phylogenetic tree.

Figure 2: Sample collection sites.

DNA extraction and target sequence amplification

In case of adults the DNA was extracted from toe clips while in case of tadpoles from the tail clips, using the Nucleospin®Tissue Kit (MACHEREY NAGEL, Düren, Germany) standard protocol for DNA extraction.

In order to obtain the target partial COI gene sequence we used the Rana species specific primers: RanaCOIF: 5'TTCTCTACTAACCACAAAGACATTGG3' and RanaCOIR: 5'TAGACTTCTGGGTGGCCGAAAAATCA3' (Marosi et al. 2010).

The PCR was carried out in 50µl solution, using the GoTaq® Flexi DNA Polymerase Kit (Promega, USA) at 1.5 mM $MgCl_2$ concentration with an initial denaturation at 94°C for 5 mins; followed by 35 cycles each of a denaturation at 94°C for 40 sec, primer annealing at 55° C for 40 sec and extension at 72°C for 60 sec; and finished with a single extension for 5 mins at 72°C.

Amplification on genomic DNA yielded products of 710 bps that were subsequently sequenced at Macrogen Inc. (Seoul, Korea) using the RanaCOIF primer. After direct sequencing the length of sequences was around 600 bps. The sequences overlapping in all individuals and included in analyses were 503 bps long.

Data analysis

We used the Vector NTI10 software to edit our sequences. For the alignment of the sequences (nucleotide and amino acid) we used MEGA version 5.0 (Tamura et al. 2011). Population analyses were calculated in GenAlEx6.4 (Peakall & Smouse, 2006).

For the Romania samples we calculated the Nei genetic distances, Pairwise Population Mean Haploid Genetic distances and Haplotype Diversities by populations.

We used Mantel test to see the correlation between genetic distance and geographic distance and AMOVA (analysis of molecular variance) to test for geographic structuring and population differentiations.

We tested the hypotheses: 1) The Carpathian Mountains act as geographical barriers between 2 metapopulations. 2) The samples collected from the 7 geographical regions can be regarded as different populations.

PhiPT values probabilities were calculated after 999 permutations.

RESULTS

From the analyzed 72 *R. dalmatina* specimens in total we have identified 13 variable nucleotide positions. All the nucleotide positions were synonymous; they did not alter the amino acid of the translated protein. The identified sequences differed only in singletons. In the Romanian samples we found 9 different alleles (Table 1).

Table 1: List of the identified alleles. Numbers from 1 – 13, mark the different alleles. Alleles identified infrom samples collected fromin Romania are presentedmarked in rows with grey background. The nucleotide positions are numbered starting with the translation initiation site. Abreviations: pos: position, Syn: synonymous

	variable sites												
Alleles	1	2	3	4	5	6	7	8	9	10	11	12	13
	pos.171 T/C	pos.222 C/T	pos.258 C/T	pos. 357 A/C	pos. 381 T/C	pos. 465 A/G	pos. 474 A/G	pos. 477 A/G	pos. 510 A/G	pos. 513 G/A	pos 579 A/T	pos. 627 T/C	pos. 666 G/A
1	*	*	*	*	*	*	*	*	*	*	*	*	*
2	*	*	*	*	*	*	*	*	*	*	*	*	G
3	*	*	*	*	*	*	*	*	*	*	A	*	*
4	*	*	*	A	*	*	*	*	*	*	*	*	*
5	*	*	C	*	*	*	*	*	*	*	*	*	*
6	*	*	*	*	T	*	*	*	*	*	*	*	*
7	*	*	*	*	*	*	A	*	*	*	*	*	*
8	*	*	*	*	*	*	*	*	A	*	*	*	*
9	T	*	*	*	*	*	*	*	*	*	*	*	*
10	*	*	*	*	*	A	*	*	*	*	*	*	*
11	*	C	*	*	*	*	*	*	*	G	*	*	*
12	*	*	*	*	*	*	*	*	*	*	*	T	*
13	*	*	*	*	*	*	*	A	*	*	*	*	*
mutation type	Syn (Val)	Syn (Ile)	Syn (Ile)	Syn (Ala)	Syn (Val)	Syn (Ser)	Syn (Leu)	Syn (Gly)	Syn (Met)	Syn (Lys)	Syn (Leu)	Syn (Lys)	Syn (Ala)

The haplotype diversity was the highest for samples of the D region (Olt River valley near the Southern Carpathians) comprising 5 alleles. In the region A there were 3 alleles and 2 alleles for the B region. In region F we also found 2 alleles from the 2 specimens but due to this low number of the specimens, haplotype diversity must be treated with caution in this case. For the regions C, G, E, we only found the same common allele (Table 2).

Table 2: Haplotype diversity of the 7 sampled regions; Abreviations: h: haplotype diversity, uh: unbiased haplotype diversity. Highest value obtained for the D region.

Pop	h	uh
C	0.000	0.000
D	0.778	0.933
A	0.231	0.239
B	0.320	0.400
G	0.000	0.000
E	0.000	0.000
F	0.500	1.000

The Pairwise Nei genetic distances were the highest for the samples from the region D (Table 3). This means that the population form this region is well differentiated from all the other populations. The genetic distance among the other populations is less.

Table 3: Nei genetic distance (GD) and Nei genetic identity (ID) values for the 7 sampled regions

Pop1	Pop2	Nei GD	Nei ID
C	D	0.347	0.707
C	A	0.007	0.993
D	A	0.353	0.702
C	B	0.030	0.970
D	B	0.377	0.686
A	B	0.037	0.964
C	G	0.000	1.000
D	G	0.347	0.707
A	G	0.007	0.993
B	G	0.030	0.970
C	E	0.000	1.000
D	E	0.347	0.707
A	E	0.007	0.993
B	E	0.030	0.970
G	E	0.000	1.000
C	F	0.347	0.707
D	F	0.693	0.500
A	F	0.353	0.702
B	F	0.377	0.686
G	F	0.347	0.707
E	F	0.347	0.707

The Mantel test between the genetic distance (GD) and geographic distance (GGD) indicate a weak negative correlation Rxy = -0.108 with a no significant P = 0.07 value.

AMOVA test did not indicate population differentiation between 2 metapopulation separated by the Carpathian Mountains (Hypothesis nr.1). However the test indicated population differentiations when the 7 regions were analysed separately (Table 4). The highest and significant pairwise PhiPT values were obtained for the D region samples confirming that this population is well differenciated from the others.

Table 4: Pairwise population PhiPT and linearized PhiPT values estimation

Pop1	Pop2	PhiPT	LinPhiPT	P(rand >= data)
C	D	0.261	0.352	0.060
C	A	0.000	0.000	0.220
D	A	0.337	0.508	0.010
C	B	0.000	0.000	0.010
D	B	0.075	0.082	0.160
A	B	0.000	0.000	0.110
C	G	0.000	0.000	1.000
D	G	0.333	0.500	0.010
A	G	0.000	0.000	0.130
B	G	0.073	0.079	0.440
C	E	0.000	0.000	1.000
D	E	0.300	0.429	0.050
A	E	0.000	0.000	0.300
B	E	0.040	0.042	0.560
G	E	0.000	0.000	1.000
C	F	0.474	0.900	0.330
D	F	0.000	0.000	0.520
A	F	0.329	0.490	0.070
B	F	0.015	0.015	0.540
G	F	0.588	1.429	0.260
E	F	0.538	1.167	0.270

DISCUSSION

Our results provide insight into the phylogeography and genetic variability of the R. *dalmatina* population located around the Carpathian Mountains, Transylvanian Plateau and Dobruja Plateau in Romania. According to the IUCN reports (Kaya et al. 2008) the species was encountered in the above mentioned locations; however it lacks from the southern and eastern lowlands of Romania. This is in concordance with our finding during sample collection.

We have found 9 alleles in the examined populations from Romania and 13 different alleles from total analyzed samples. A relatively low genetic variation was already indicated in the European *Rana arvalis* populations (Rafinski & Babik 2000). Except for the metapopulation of the Transylvanian Plateau the number of analyzed individuals used per region was relatively few but enough for statistical analyses.

The highest haplotype diversity was found in the region marked with D. It is positioned on the undulating sites south to the Southern Carpathians and west to the Olt River valley.

The rest of the sampled regions had only one or no unique allele, beside the most common allele. The common allele could be found in all the populations and in the homogenous populations it was the only allele.

We have tested the hypothesis with AMOVA whether the Carpathian Mountains act as geographical barrier for two metapopulations of *R. dalmatina*, but the test did not indicate population differentiation.

The Mantel test did not reveal significant correlation between geographic distance and genetic distance, thus the isolation of populations caused by geographic distance can be excluded.

Treating our samples grouped in the 7 geographical regions the AMOVA indicated population differentiation with significant p-values for region D and A (P = 0.01), D and G (P = 0.01) and D and E (P = 0.05) (Table 4).

Our explanation for this high variability experienced in the population D is possible cryptic refugia of the species. Babik et al. 2004 indicated high allelic diversity for the *Rana arvalis* in the Carpathian Basin and presumed there cryptic refugia of the species. Teacher et al. (2009) identified local refugia for the *Rana temporaria* species in South Ireland. From these 3 brown frog species the *Rana dalmatina* is the less cold tolerant but since the brown Rana species are closely related, emerging as different species during the repeated glacial cycles (Veith et al. 2002) they followed similar pattern of dispersion and colonization.

In conclusions, considering also results from the previous studies; we predict that our study species had a glacial refugium near the Southern Carpathian Mountains. This idea is supported by the fact that the population from region D had the highest allelic diversity and it was significantly differentiated from the rest of the populations. The rest of the populations have low genetic variability. This may be the result of recent colonization and founder effect after the last cold period. The fact that the Olt River valley has higher genetic diversity involves special attention in elaborating the conservation plan of this species.

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