

RECENT RANGE EXPANSION OF *POMATIAS RIVULARE*  
(EICHWALD, 1829) (MOLLUSCA: POMATIIDAE)  
IN CENTRAL-EASTERN EUROPE

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We have investigated the diversity of mitochondrial COI gene sequences of *Pomatias rivulare* populations, originated from the western edge of the species' area. COI gene has not shown any variability, the same haplotype was found in all of the examined Hungarian, Romanian, Serbian and Montenegrin populations, which arises most probably from a rapid range expansion. Based on it, we can rule out that Hungarian populations are relicts and we can conclude that the Pannonicum, the Southern Carpathians and the Eastern Dinarids were colonized very recently, most probably in the Holocene.

Key words: *Pomatias*, biogeography, phylogeography, Pannonicum, Carpathians, Balkans

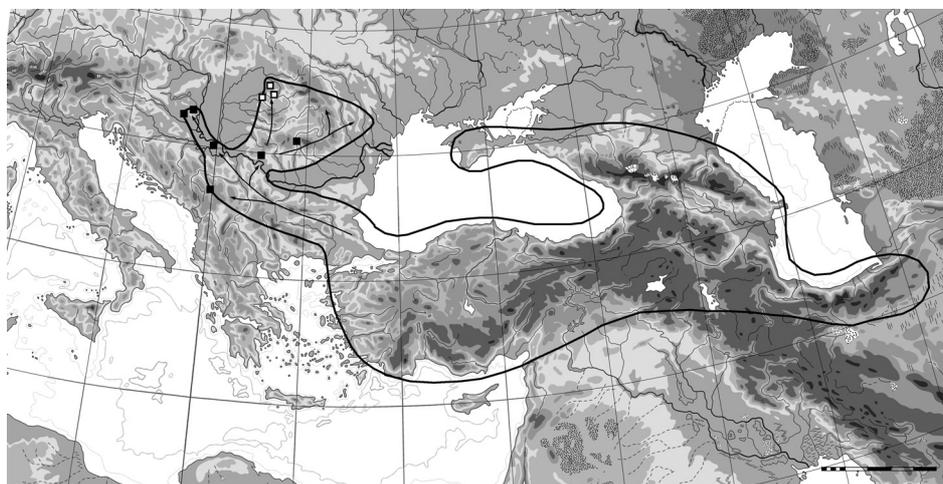
## INTRODUCTION

*Pomatias rivulare* (EICHWALD, 1829) is a prosobranch land snail species of Pontic origin. The centre of its distribution is in Turkey from Asia Minor to East Anatolia (SCHÜTT 2001), it reaches the Crimea northwards, the Caucasus and North Iran eastwards (LIKHAREV & RAMMELMEIER 1952), Syria and Palestina southwards (SCHÜTT 2001) and East Europe westwards. In Europe, it can be found in Bulgaria (DAMJANOV & LIKHAREV 1975), in Rumania (GROSSU 1986), in Serbia, in Montenegro (ŠTAMOL & JOVANOVIĆ 1990) and in Hungary. Its earliest known, and for a long time the only Hungarian occurrence is in Bátorliget, which was generally considered subfossil before the 1990s (e.g. SOÓS 1943). Despite NYILAS and SÜMEGI (1991) reported to find one living specimen there, the recent occurrence of the species in Bátorliget is discredited after all by PINTÉR (1991). Later faunistical investigations failed to detect any living specimens (DELI 2008), which seems to support PINTÉR's view. In the Transdanubia (Szekszárd Hills, Mecsek Mountains), however, undeniably living populations was discovered recently (MAJOROS 1987, UHERKOVICH & TÓTH 2001), which assign the westernmost occurrences of the species (Fig. 1). Due to its rarity, *P. rivulare* is protected in Hungary and listed by the Hungarian Red Data Book as a threatened species

(RAKONCZAY 1989) and due to the above mentioned distribution pattern, it was found to be an important character species in the quantitative biogeographic characterization of Hungary (SÓLYMOS 2008). Paradoxically, it occurs in some disturbed, dry woods of the Mecsek Mountains, whereas absent in the most diverse, natural forests nearby, thus high proportion of known Hungarian populations occur out of protected areas. This factor, accompanied by rarity, is the reason why *P. rivulare* is ranked as the land snail species of the third highest conservation priority in Hungary (SÓLYMOS 2006).

In the lack of fossil records, there are no hypothesis on the origin and distribution history of the Transdanubian populations. In Bátorliget, however, WILLIS *et al.* (1995) and SÜMEGI & DELI (2004) have found *P. rivulare* to enter the fossil records ca. 7000 years before present, thus they have concluded that the Carpathian Basin was colonized by this species in the early postglacial. In contrast with this, VARGA (2003) considers this species a Tertiary relict in the Pannonicum. If the latter was true, it would increase the conservation biological importance of this species to a large extent.

We have investigated the molecular diversity of some *P. rivulare* populations, originated from the western edge of the geographic range of the species, in order to (i) confirm or deny that Hungarian populations are relicts, (ii) reveal the species' distribution history in the Pannonian-Carpathian region and as a consequence of these (iii) draw conservation biological conclusions.



**Fig. 1.** Distribution area of *Pomatias rivulare* according to SCHÜTT (2001), LIKHAREV and RAMMELMEIER (1952), DAMJANOV and LIKHAREV (1975), GROSSU (1986) and ŠTAMOL and JOVANOVIĆ (1990). Filled squares indicate sampling sites of examined material, empty squares indicate subfossil occurrences in the Pannonian region according to BERTALAN *et al.* (1995), arrows indicate hypothetical directions of the species' European expansion. Scalebar indicates 500 km

## MATERIAL AND METHODS

In total, seven *Pomatias rivulare*, seven *Pomatias elegans* and one *Pomatias olivieri* specimens were studied from different localities, which are listed in Table 1. Examined materials are deposited in the Mollusca Collection of the Hungarian Natural History Museum, Budapest (HNHM).

After cracking shells, collected animals were fixed and preserved in 96% ethanol. Between 15 and 35 mg of foot and/or head tissue was used to extract genomic DNA using a CTAB protocol widely used for gastropods (WINNEPENINCKX *et al.* 1993). A 658 bp fragment of the mitochondrial gene cytochrome oxidase subunit I (COI) was amplified by polymerase chain reaction (PCR) using the primers LCO 1490 and HCO 2198 (FOLMER *et al.* 1994). PCR reactions were carried out in 25  $\mu$ l reaction volumes using a PTC-200 (MJ Research) thermocycler machine. PCR reactions consisted of approximately 100 ng of template genomic DNA, 0.5  $\mu$ M of each primer, 0.2 mM dNTPs, 2 mM MgCl<sub>2</sub>, 1 unit of Taq DNA polymerase (Fermentas) and the 10X Taq buffer in a final volume of 25  $\mu$ l. All PCR reactions were run for 36 cycles with the following cycling parameters: initial 2 min. denaturation at 94 °C; then cycled at 94°C for 30 sec., 50 °C for 90 sec., and 72 °C for 90 sec. Reactions were completed with a 10 min. final extension step at 72 °C. PCR products were checked on 1% agarose gel, stained with ethidium bromide. Successfully amplified products were purified from agarose gels using Millipore Ultrafree-DA PCR Purification columns, or digested with Shrimp Alkaline Phosphatase (Fermentas) and *E. coli* Exonuclease-I (Fermentas), according to the manufacturer's protocol. Purified PCR products were then sequenced on an ABI 3100 Genetic Analyzer sequencer machine (Applied Biosystems), using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) chemistry.

Sequences are deposited in the GenBank (EU239235-EU239241, Table 1). As an outgroup, one sequence of *Littorina plena* was downloaded from the GenBank (AJ622948). Sequences were aligned by ClustalX version 1.83 (THOMPSON *et al.* 1997) using default settings for multiple alignment. Standard DNA polymorphism measures such as base content (%), number of polymorphic sites, intraspecific nucleotide diversity ( $\pi$ ) and average number of JC corrected nucleotide substitutions per site ( $D_{xy}^{(JC)}$ ) were estimated by DnaSP version 4.10.9 (ROZAS *et al.* 2003). Appropriate model of the nucleotide substitution was selected by Modeltest version 3.7 (POSADA & CRANDALL 1998) using Bayesian Information Criterion (BIC). Bayesian analysis was performed by MrBayes version 3.0 (HUELSENBECK & RONQUIST 2001) using the following settings: HKY model with a gamma distribution of rate variation across sites, and a proportion of invariant sites for nucleotide substitution, unconstrained topology and branch length specifying flat Dirichlet-prior, four-chain (one cold, three heated; T = 0.2) Markov Chain Monte Carlo (MCMC) analysis run for 1.000.000 generations, after a burn-in of 200.000 generations every 100th tree was sampled and used to calculate posterior probabilities. Sufficient convergence was achieved according to the MrBayes Manual.

## RESULTS

Of 125 variable sites of the examined *Pomatias* samples, nine resulted in a nonsynonymous substitution. Base contents by species are given in Table 2. According to the average numbers of inter-specific nucleotide substitutions, *P. olivieri* seems to be the sister species of *P. rivulare*, their  $D_{xy}^{(JC)}$  value was 0.10858, whereas that of *P. elegans* and *P. olivieri* was 0.17105, and that of *P. elegans* and

**Table 1.** Collecting localities, GenBank numbers, haplotype codes and museum voucher numbers of the material used in this study.

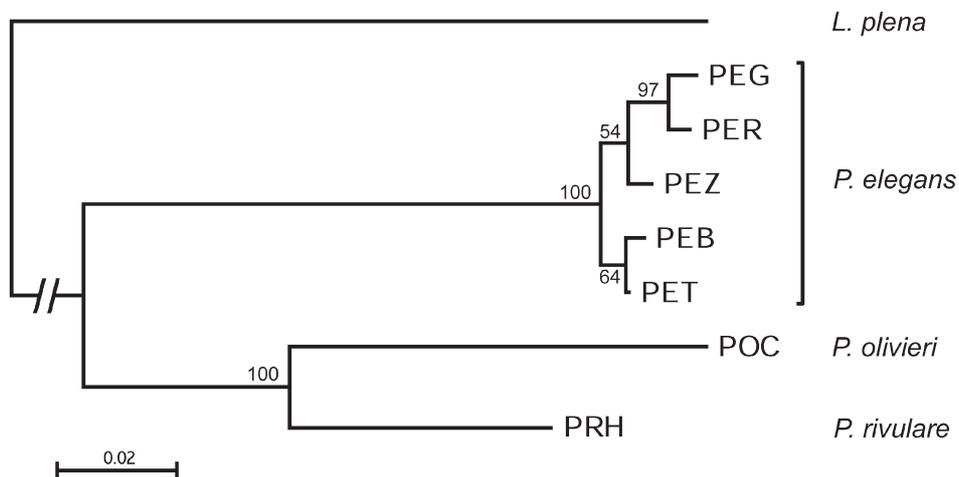
Species	Haplotype	GenBank no.	Voucher	Locality	Collected by	Date
<i>P. elegans</i>	PEG	EU239237	HNHM 96481	Albania, Gjirokaštër (40°04'N 20°08'E)	FEHÉR, KONTSCHÁN, MURÁNYI	13.10.2004.
	PEZ	EU239240	HNHM 95720	Hungary, Zákány (46°16'N 16°56'E)	FEHÉR, UHERKOVICH	29.04.2005.
	PEB	EU239239	HNHM 95646	Hungary, Béraltavár (47°01'N 16°58'E)	FEHÉR	29.04.2005.
	PET	EU239241	HNHM 95648	Hungary, Órtilos (46°17'N 16°54'E)	FEHÉR, UHERKOVICH	29.04.2005.
			HNHM 94993	Hungary, Tihany (46°55'N 17°53'E)	FEHÉR	15.05.2005.
			HNHM 94971	Croatia, Velebit Mts, Zidine (44°18'N 15°28'E)	MURÁNYI	15.05.2005.
<i>P. rivulare</i>	PER	EU239238	HNHM 94252	Macedonia, Raec gorge near Prilep (41°26'N 21°49'E)	ERŐSS, FEHÉR, HUNYADI	05.04.2004.
	PRH	EU239235	HNHM 95721	Hungary, Szekszárd (46°20'N 18°38'E)	FEHÉR	28.04.2005.
			HNHM 95744	Hungary, Mecsek Mts, Szászvár (46°16'N 18°21'E)	FEHÉR, TÓTH, UHERKOVICH	28.04.2005.
<i>P. olivieri</i>			HNHM 95742	Hungary, Mecsek Mts, Máza (46°15'N 18°23'E)	FEHÉR, TÓTH, UHERKOVICH	28.04.2005.
			HNHM 95803	Montenegro, 4 km S of Brodarevo (43°13'N 19°46'E)	DELI, ERŐSS, FEHÉR, MURÁNYI	03.10.2005.
			HNHM 96482	Romania, Domogled Mts, Valei Cernei (45°01'N 22°34'E)	DELI, DOMOKOS, KÓRA	19.06.2006.
			HNHM 96483	Romania, Făgăraș Mts, Tâlmăciu (45°40'N 24°18'E)	DELI, DOMOKOS, KÓRA	20.06.2006.
			HNHM 95802	Serbia, Fruška Gora Mts, 5 km S of Petrovaradin (45°10'N 19°50'E)	DELI, ERŐSS, FEHÉR, MURÁNYI	03.10.2005.
			HNHM 89792	Turkey, Çevlik (36°07'N 35°55'E)	ERŐSS, FEHÉR, HUNYADI	25.03.2002.

**Table 2.** Base content [in %] of the COI genes of examined *Pomatias* samples.

	T	C	A	G
<i>P. elegans</i>	42.8–43.4	13.7–14.2	19.6–19.2	23.4–23.7
<i>P. olivieri</i>	42.1	15.4	16.0	26.5
<i>P. rivulare</i>	43.6	14.3	17.3	24.8

*P. rivulare* was 0.14368. In Bayesian analysis monophyly of the *rivulare-olivieri* clade was supported by high posterior probability (Fig. 2). Among the *P. elegans* samples, originated from seven localities, twelve variable sites and five different haplotypes were detected, the largest difference between two haplotypes was 7 bp (PEG – PEB and PEG – PEZ) and intraspecific nucleotide diversity ( $\pi$ ) is  $0.00575 \pm 0.00144$ . It is noteworthy, that relatively large difference (4 bp) was found between those populations which are nearest of each others among the examined ones (Zákány – Órtilos: 3.1 km). The whole *P. elegans* clade is supported by high posterior probability, but owing to the small sample size and the relatively low sequence variability, its intraspecific relationships are not resolved clearly by Bayesian inference (Fig. 2).

Sequences of *P. rivulare* specimens from seven different localities differ only in ambiguous sites, therefore intraspecific polymorphism could not be estimated.



**Fig. 2.** Bayesian tree of COI genes of the examined *Pomatias* samples. *Littorina plena* (AJ622948) was used as an outgroup. Numbers at the branches indicate Bayesian posterior probabilities. Scalebar indicates substitutions per site

## DISCUSSION

Based on the pattern of its geographic distribution, the Pontic origin of *Pomatias rivulare* was beyond doubt (EHRMANN 1956), it was hardly known, however, how long its European distribution history reaches back in time. Mitochondrial protein coding genes, due to their relatively high mutation rates, are widely used to reveal intra- and inter-specific relationships, as well as for estimation of time of phylogenetic events. For the latter, there is a wide-spread assumption, that mitochondrial protein coding genes are mutating with the speed of 1–2% per million years and thus they can be used to estimate divergence times also in the lack of calibration points (e.g. ALBRECHT *et al.* 2006, AVISE 2000). Some authors, however, have drawn attention that this is very different on a timescale, shorter than 1–2 My before present: the shorter is the investigated period, the higher is the rate found (see HO *et al.* 2005 for a review). This warns us, that using “traditional” mutation rate of 1–2% on short timescales for divergence time estimation can cause an overestimation bias of one or even two orders of magnitude, and we should be very cautious when intra-specific and congeneric events are estimated without any fossil calibration point. This is the reason why we do not attempt to estimate neither the divergence of *Pomatias* species nor the divergence of *P. elegans* lineages on the basis of available data.

In the same time, invariability of the COI gene sequences within *P. rivulare* samples is quite informative. Although alternative explanations can not be fully excluded (see BALLARD & WHITLOCK 2004 for a review), it seems to be a clear indication of a very recent bottleneck, i.e. all of the examined specimens are descendents of the same ancestor. Distribution of *P. rivulare* is very disjunct in the sampled area, Transdanubian, Southern Carpathian and Montenegrin populations are isolated by gaps of several hundred kilometers, any migration and thus any gene flow between these remote localities are quite unrealistic. The most probable explanation for their presence in remote localities is the rapid range expansion.

Therefore, we can conclude, that not only the Pannonicum, but the Southern Carpathians, the Fruška Mountains and the Eastern Dinarids were also colonized very recently, most probably in the Holocene. On the grounds, that similarly to the sampled area, the species’ distribution is disjunct in the East Balkans too (Stara Mountains, Rhodope Mountains, Strandzha Mountains), we can even hypothesize that colonization of the whole European range happened recently. In order to prove this hypothesis, a broader area should be sampled, but this was out of the scope of the present study.

In principle, fragmentation of a previously contiguous range can account for a disjunct distribution pattern. Considering the shortness of the dispersal time how-

ever, jump dispersal, which is not unusual among landsnails (see e.g. GITTENBERGER *et al.* 2006), is a more likely reason for the case of *P. rivulare*. The phenomenon, that it does not seem to be particular about the habitat, is also a feature of a spreading rather than a declining species, although its spreading, unlike those of the most aggressive invaders, is not perceptible on the human scale.

The high conservation priority index of *P. rivulare* (SÓLYMOS 2006) is due to the fact, that beside the rarity of the species, a high proportion of its populations occur out of protected areas. But the species is rare only because it is a newcomer in the Pannonian fauna, and its paradoxical habitat preference is simply due to the randomness of jump dispersal, therefore it would be a mistake to overemphasize its conservation biological importance.

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