

MITOCHONDRIAL DNA DIVERSITY OF THE ALPINE NEWT  
(*ICHTHYOSAURA ALPESTRIS*) IN THE CARPATHIAN BASIN:  
EVIDENCE FOR MULTIPLE CRYPTIC LINEAGES  
ASSOCIATED WITH PLEISTOCENE REFUGIA\*

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The phylogeography and molecular taxonomy of the Alpine newt, *Ichthyosaura alpestris*, has been intensively studied in the past. However, previous studies did not include a comprehensive sampling from the Carpathian Basin, possibly a key region in the evolution of the species. We used a 1422 bp long fragment of the mitochondrial genome to infer the species' evolutionary history in central-eastern Europe by assigning isolated Carpathian Basin populations from 6 regions to previously defined mtDNA lineages. We also revised the morphology-based intraspecific taxonomy of the species in the light of new genetic data. Alpine newt populations from the Carpathian Basin represented two different mitochondrial lineages. The Mátra, Bükk and Zemplén Mts populations can be assigned to the Western lineage of the nominotypical subspecies. Bakony and Órség populations showed high haplotype diversity and formed a separate clade within the Western lineage, suggesting that the Carpathian Basin might have provided cryptic refugia for Alpine newt populations in their cold-continental forest-steppe landscapes during the younger Pleistocene. Newts from Apuseni Mts were related to the Eastern lineage but formed a distinct clade within this lineage. Considering the morphological and genetic differentiation of the Bakony and Órség populations, consistent with a long independent evolutionary history, we propose these populations be referred to as *Ichthyosaura alpestris bakonyiensis* (Dely, 1964). We provide a redescription of this poorly known subspecies.

Keywords: Caudata, Salamandridae, phylogeography, molecular taxonomy, genetic diversity, Pleistocene refugia, alpine newt, *Ichthyosaura alpestris*.

## INTRODUCTION

The Alpine newt (*Ichthyosaura alpestris* (Laurenti, 1768)) is widely distributed in the temperate and Mediterranean regions of Europe, showing exten-

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\* This paper is dedicated to the memory of our dear colleague, teacher and advisor, Dr. László Papp, taxonomist, dipterologist, who passed away recently.

sive morphological and ecological variation (RECUERO & MARTÍNEZ-SOLANO 2002, ROČEK *et al.* 2003, ANDREONE & TRIPEPI 2006, SOTIROPOULOS *et al.* 2008). Prior to the molecular era, the taxonomic status of certain populations, often isolated, was long debated. Moreover, several subspecies of doubtful validity were described based on morphology (body proportions and colouration) or life-history traits (e.g. DELY 1959, HERRERO & ARANO 1986, ZUIDERWIJK 1997, DUBOIS & RAFFAELLI 2009, SPEYBROECK *et al.* 2010, LUŽNIK *et al.* 2011, SPEYBROECK *et al.* 2020). With the advance of molecular technologies, multilocus datasets helped clarify the species' evolutionary history and phylogeography. Using mtDNA data, SOTIROPOULOS *et al.* (2007) defined five clades (A–E), divided into three highly divergent lineages (the Vlašina lineage, restricted to a small area in the Balkans, a very diverse eastern lineage with samples from the rest of the Balkan Peninsula, and a more genetically homogenous western lineage including populations from South-western and Central Europe). These results were refined and partly confirmed by a time-calibrated multilocus phylogeny by RECUERO *et al.* (2014). This study showed that the Vlašina mtDNA lineage was not deeply divergent in nuclear DNA, and delineated four major lineages that are somewhat discordant with the taxonomical status of some formerly described subspecies (*I. a. veluchiensis* (Wolterstorff, 1935) in Greece, *I. a. reiseri* (Werner, 1902) and *I. a. montenegrina* (Radovanovic, 1951) in Eastern Europe and the Balkans, *I. a. apuana* (Bonaparte, 1839) + *I. a. inexpectata* (Dubois & Breuil, 1983) on the Apennine Peninsula and *I. a. alpestris* + *I. a. cyreni* (Wolterstorff, 1932) in Western and Central Europe and northern Spain; Fig. 2A). The major events in the evolutionary history of the species according to this study were: 1) the split of Western and Eastern lineages in the late Miocene associated with Paratethys sea oscillations, 2) further parapatric diversifications due to local isolation events in higher temperature periods in the Miocene and Pliocene, and 3) distribution range shifts during the glacial cycles in the Pleistocene with local extinctions, recolonisation and secondary contact and admixture events.

While *I. alpestris* is very common in the surrounding Alps and Carpathians, its occurrence in the Carpathian Basin – an area defined by the Carpathians in the north, east and southeast, the Dinaric Alps from the southwest and the eastern Alpine domain in the West – is highly fragmented because of the lack of suitable habitats (they are restricted to the mountains at a minimum of 400 m elevation in that area). Some populations in the Carpathian Basin can be regarded as marginal in contrast with the continuously distributed Alpine and Carpathian populations (Őrség Region in the foothills of the Eastern Alps, Zemplén Mts in the Northern Mountain Range), while the remaining populations (Bakony Mts in Transdanubia, Mátra and Bükk Mts in the Northern Mountain Range and Apuseni Mts in Romania) represent geographical isolates (Figs 1 & 3). The fragmented occurrence of Alpine newts in this region has long intrigued taxonomists.

Four subspecies of *Ichthyosaura alpestris* were described by Dely as that of *Triturus alpestris* (DELY 1959, 1964, 1967) based on populations from the Carpathian Basin. The descriptions were based on 26 morphometric and 24 osteological characters, which diagnosed *Triturus alpestris bükkiensis* in the Bükk Mts, *T. a. satoriensis* in the Zemplén Mts (both from the North Hungarian Mountains) (DELY 1959), *T. a. carpathicus* in the Southern Carpathians in Romania (DELY 1959), and *T. a. bakonyiensis* in the Bakony Mts in Transdanubia (DELY 1964). Several authors rejected the distinctiveness of three of the four subspecies (*bükkiensis*, *satoriensis* and *carpathicus*) on methodological grounds (ROČEK 1974, DENOËL 1994, RAFFAËLLI 2018). However, the status of *T. a. bakonyiensis*, described separately in 1964 in Dely's dissertation and then in 1967 in the book series *Fauna Hungariae* (only in Hungarian language), remains uncertain.

Since previous phylogeographic surveys (SOTIROPOULOS *et al.* 2007, RECUEIRO *et al.* 2014) did not include a comprehensive sampling of the Carpathian Basin, possibly a key region in the evolution of the species, our main goals in this study were (1) to describe the genetic diversity of *Ichthyosaura alpestris* in the Carpathian Basin based on mitochondrial data, (2) to infer the evolutionary history of the species in central-eastern Europe region by assigning isolated Carpathian Basin populations to previously defined mtDNA lineages, and (3) to revise the previously published morphology-based intraspecific taxonomy of the species in the light of new genetic data.

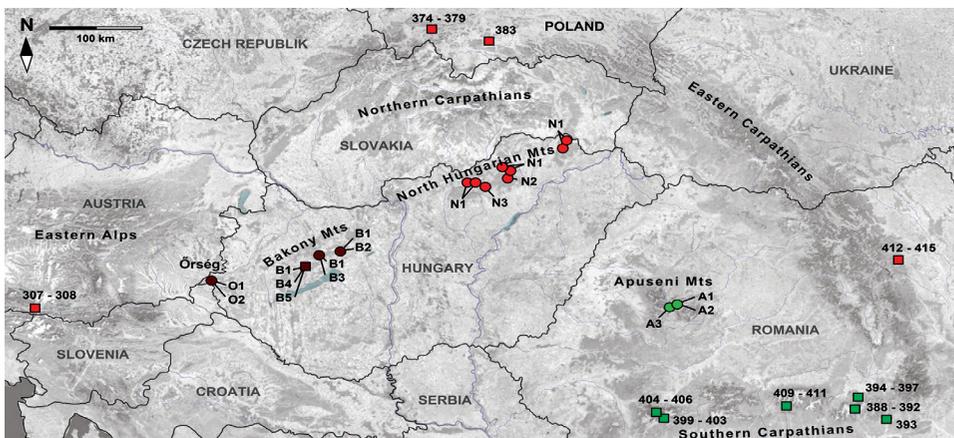
## MATERIAL AND METHODS

Tissue samples were collected by tail clipping or swabbing from 24 living animals from 13 localities in 6 regions in the Carpathian Basin (Órség Region and Bakony Mts in western Hungary, Mátra, Bükk and Zemplén Mts in the North Hungarian Mountains, and Apuseni Mts in Romania) during the breeding seasons of 2010–2012 (Fig. 1, Table 1). Tissue samples were preserved in 96% ethanol and kept at  $-20^{\circ}\text{C}$  until processing. Whole genomic DNA was extracted either using DNeasy Blood&Tissue Kit (tails) or QIAmp DNA Mini Kit (swabs) extraction kits following the manufacturer's protocols (Qiagen, Hilden, Germany). Two mitochondrial gene regions were amplified by PCR: a 553 bp long partial sequence of the ribosomal 16S RNA gene (16S), using the primer pairs 16Sar-16Sbr (PALUMBI *et al.* 1991) and a 689 bp long fragment of the mitochondrial NADH dehydrogenase 4 gene (ND4), together with a 191 bp fragment of the adjacent tRNAs (Ile, Leu, and partially Met) using the primer pairs ND4-Leu (ARÉVALO *et al.* 1994). PCRs were performed in a total volume of 50  $\mu\text{l}$  using 1 U of Taq polymerase (Thermo Fisher Scientific, Waltham, USA), 1.5 mM  $\text{MgCl}_2$ , 10 pmol dNTPs (Thermo Fisher Scientific, Waltham, USA), 20 pmol of both primers and ca. 50 ng of genomic DNA with the following profiles:  $95^{\circ}\text{C}$  for 2 min, followed by 38 cycles of  $95^{\circ}\text{C}$  for 30 sec, 50 or  $56^{\circ}\text{C}$  for 60 sec (16S and ND4, respectively) and  $72^{\circ}\text{C}$  for 60 sec, followed by a 7 min final extension at  $72^{\circ}\text{C}$ . Amplified double-strand products were purified by using a High Pure PCR Product Purification Kit (Sigma-Aldrich, St. Louis, USA) and directly sequenced from both directions following the ABI Prism BigDye Terminator Cycle sequencing protocol on an ABI 3130 Genetic Analyser (Applied Biosystems, Foster City, USA).

Sequences were collapsed into haplotypes using COLLAPSE 1.2 (POSADA 2011). These were aligned in CLUSTALW (CHENNA *et al.* 2003) using the default settings for gap opening and extension penalties followed by limited manual correction of gap placement. Sampled sequences were aligned and analysed together with previously published overlapping *I. alpestris* sequences from different lineages, covering the entire distribution range of the species (altogether 136 specimens, collapsed into 54 haplotypes; RECUERO *et al.* 2014). ND4 sequences aligned perfectly, with no insertions or deletions. Due to alignment uncertainty in the tRNA sequences, only 1422 bp of the dataset were used in the analyses.

In the Carpathian Basin samples, standard diversity indices (haplotype diversity and nucleotide diversity  $\pi$ ) were calculated for the entire mtDNA dataset with DNASP 5.0 (LIBRADO & ROZAS 2009). Relationships among mtDNA haplotypes in the Carpathian Basin were displayed by a median-joining network (BANDEL *et al.* 1999) using the software POPART 1.7 (<http://popart.otago.ac.nz>). The evolutionary divergence over sequence pairs between subspecies/evolutionary lineages was estimated using the software MEGA version 6. (TAMURA *et al.* 2013). The analysis involved 147 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1422 positions in the final dataset.

Phylogenetic relationships were inferred by constructing Bayesian inference (BI) phylograms in MRBAYES 3.1 (RONQUIST & HUELSENBECK 2003). *Lissotriton vulgaris* was selected as an outgroup (collected in Telkibánya, Zemplén Mts, Hungary; 48.45°N, 21.35°E). The optimal partitioning strategy of the analysed dataset and the appropriate model of sequence evolution for each partition were estimated under the Bayesian Information Criterion (BIC) in the software PARTITION FINDER 1.1 (LANFEAR *et al.* 2012). Five independent partitions were defined as the best partitioning scheme: HKY+G for 16S sequences, three separate models for the ND4 gene (K80 + I for 1st, TrN + I for 2nd, TrN+G for the 3rd codon positions) and K81uf+G for the tRNAs. MRBAYES analyses consisted of two independent Metropolis-coupled Markov chain Monte Carlo runs, each with six incrementally heated



**Fig. 1.** Sampling sites and haplotypes found in and around the Carpathian Basin. Samples collected in this study are marked with circles and haplotype codes, and previously published samples are marked with squares and their codes as described in RECUERO *et al.* (2014). Colours refer to different mtDNA lineages (see Figs 2 and 3)

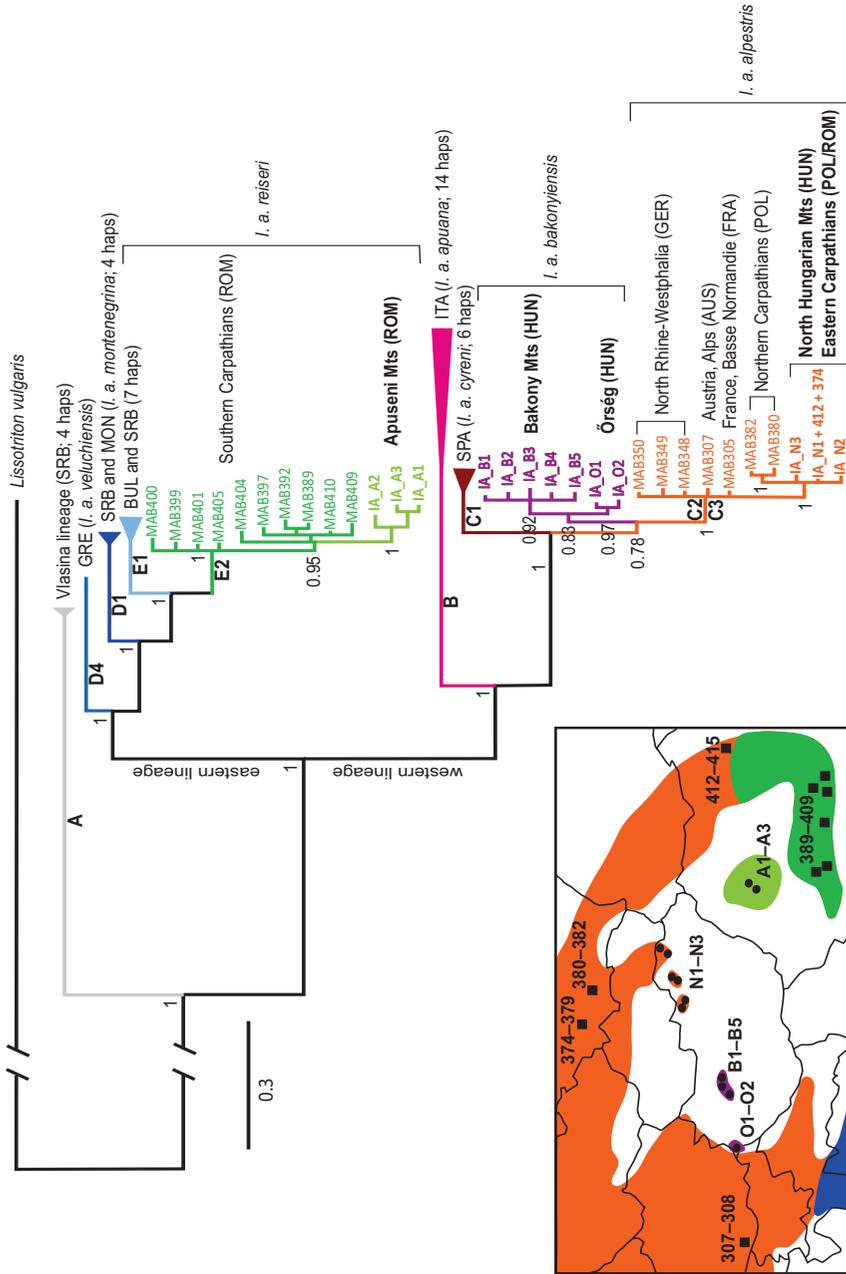
**Table 1.** MtDNA haplotypes found in sampled populations of *I. alpestris* in and around the Carpathian Basin, with GenBank accession numbers (16s and ND4), variable positions, number of polymorphic sites and diversity indices (mean and standard deviation of gene diversity  $H$ , nucleotide diversity  $\pi$ , and  $\Theta$  averaged for each population). Populations sampled by RECUERO *et al.* (2014) are marked with an asterisk. Carp = Carpathians, NHM = North Hungarian Mountains.

Haplotype	Apuse- ni Mts	Bakony Mts	Őrség	NHM	*NW Carp	*S Carp	Genbank accession no.
IA_A1	1	–	–	–	–	–	MW685537, MW685550
IA_A2	1	–	–	–	–	–	MW685538, MW685551
IA_A3	1	–	–	–	–	–	MW685539, MW685552
IA_B1	–	7	–	–	–	–	MW685540, MW685553
IA_B2	–	3	–	–	–	–	MW685541, MW685554
IA_B3	–	1	–	–	–	–	MW685542, MW685555
IA_B4	–	2	–	–	–	–	MW685543, MW685556
IA_B5	–	1	–	–	–	–	MW685544, MW685557
IA_O1	–	–	2	–	–	–	MW685545, MW685558
IA_O2	–	–	1	–	–	–	MW685546, MW685559
IA_N1	–	–	–	7	10	–	MW685547, MW685560
IA_N2	–	–	–	1	–	–	MW685548, MW685561
IA_N3	–	–	–	1	–	–	MW685549, MW685562
N	3	14	3	9	10	20	
Hapl. num	3	5	2	3	1	10	
Var. sites	7	5	1	2	0	12	
$H$	1 (0.27)	0.72 (0.1)	0.67 (0.31)	0.41 (0.19)	0	0.82 (0.08)	
$\pi \times 10^{-2}$	0.32 (0.00)	0.074 (0.02)	0.05 (0.02)	0.031 (0.02)	0	0.15 (0.02)	
$\Theta \times 10^{-2}$	0.32 (0.22)	0.11 (0.06)	0.05 (0.00)	0.052 (0.04)	0	0.23 (0.1)	

Markov chains, run for 10 million generations and sampled every 1000 generations with the first 25% of samples excluded as burn-in. Convergence was assessed by examining the standard deviation of split frequencies, which was well below the recommended thresholds. A consensus phylogram was computed after discarding trees reconstructed during the default burn-in period.

## RESULTS

Altogether 13 haplotypes were found in the 6 sampled localities of the Carpathian Basin (11 from our samples, two more from RECUERO *et al.* 2014;



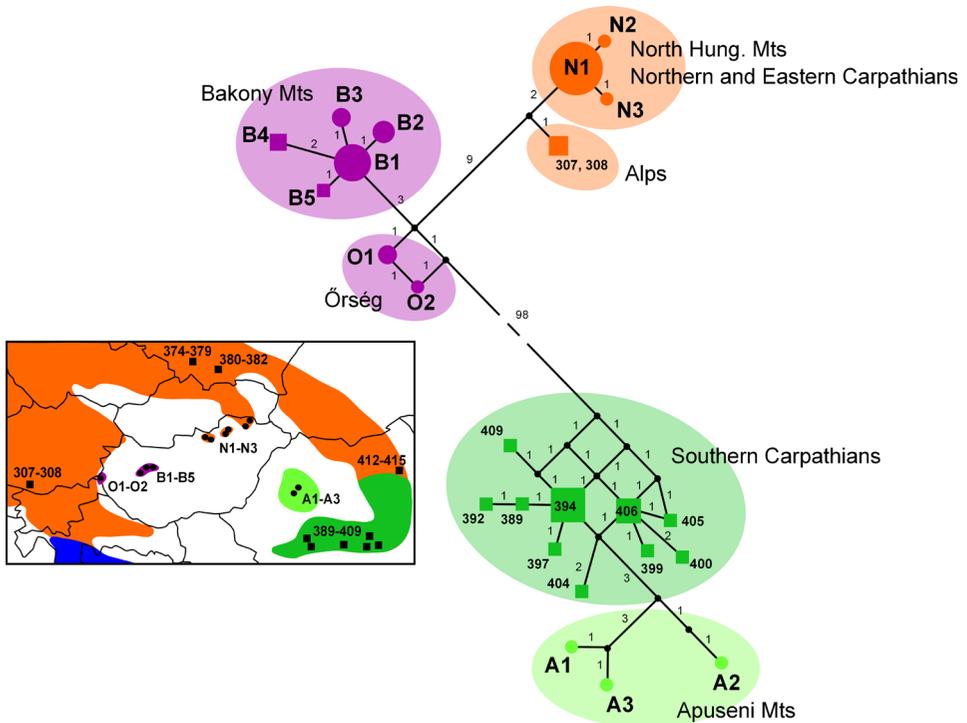
**Fig. 2.** Bayesian phylogenetic tree of the combined mtDNA haplotypes found in and around the Carpathian Basin (inferred with MrBayes 3.1). Inset: distribution of *Ichthyosaura alpestris* in Europe. Colours refer to different mtDNA lineages (based on RECUERO *et al.* 2014). The main lineages are marked in the phylogenetic tree following SOROKOPOULOS *et al.* (2007) (A-E2). Subspecies assignment is based on RECUERO *et al.* (2014) and the new results from this study. Bayesian posterior probabilities are given at the nodes

**Table 2.** Sampling locations, mtDNA haplotypes and geographic coordinates of the samples of *I. alpestris* analysed in this study. Samples marked with an asterisk were published by RECUERO *et al.* (2014). RO = Romania, HU = Hungary

Region, country	Locality	Sample name	Haplotype	Latitude	Longitude
Apuseni Mts, RO	Vartop	IA_202	IA_A1	46.51	22.66
Apuseni Mts, RO	Padis	IA_211	IA_A2	46.59	22.71
Apuseni Mts, RO	Padis	IA_212	IA_A3	46.59	22.71
Bakony Mts, HU	Isztimér	IA_401	IA_B1	47.26	18.11
Bakony Mts, HU	Isztimér	IA_402	IA_B2	47.26	18.11
Bakony Mts, HU	Isztimér	IA_403	IA_B2	47.26	18.11
Bakony Mts, HU	Isztimér	IA_404	IA_B2	47.26	18.11
Bakony Mts, HU	Bakonybél	IA_201	IA_B1	47.26	17.67
Bakony Mts, HU	Bakonybél	IA_209	IA_B1	47.26	17.67
Bakony Mts, HU	Bakonybél	IA_301	IA_B3	47.26	17.67
Bakony Mts, HU	Bakonybél	IA_302	IA_B1	47.26	17.67
Bakony Mts, HU	Bakonybél	IA_303	IA_B1	47.26	17.67
Bakony Mts, HU	Ajka	MAB309*	IA_B4	47.04	17.65
Bakony Mts, HU	Ajka	MAB310*	IA_B1	47.04	17.65
Bakony Mts, HU	Ajka	MAB311*	IA_B1	47.04	17.65
Bakony Mts, HU	Ajka	MAB312*	IA_B5	47.04	17.65
Bakony Mts, HU	Ajka	MAB313*	IA_B4	47.04	17.65
Órség, HU	Szakonyfalu	IA_206	IA_O1	46.91	16.23
Órség, HU	Szakonyfalu	IA_207	IA_O1	46.91	16.23
Órség, HU	Szakonyfalu	IA_215	IA_O2	46.91	16.23
NHM (Bükk Mts), HU	Kemesnye	IA_208	IA_N2	48.13	20.50
NHM (Bükk Mts), HU	Bükkszentkereszt	IA_213	IA_N1	48.07	20.59
NHM (Bükk Mts), HU	Répáshuta	IA_214	IA_N1	48.04	20.55
NHM (Mátra Mts), HU	Parádsasvár	IA_001	IA_N1	47.88	19.95
NHM (Mátra Mts), HU	Mátraszentimre	IA_002	IA_N1	47.93	19.86
NHM (Mátra Mts), HU	Parád	IA_003	IA_N3	47.88	20.05
NHM (Zemplén Mts), HU	Telkibánya	IA_217	IA_N1	47.45	21.35
NHM (Zemplén Mts), HU	Telkibánya	IA_218	IA_N1	47.45	21.35
NHM (Zemplén Mts), HU	Hollókő	IA_219	IA_N1	48.56	21.40

Fig. 1, Table 1). Newly found haplotype sequences were deposited in GenBank. Haplotype composition and diversity varied considerably among regions.

Populations in the western part of the Carpathian Basin proved to be highly polymorphic (2 haplotypes from 3 samples in the Órség and 5 haplotypes from 14 samples in the Bakony Mts), whereas samples from the North Hungarian Mountains had only three closely related haplotypes. However, two unique haplotypes were found in this region (N2 in the Bükk Mts and N3 in the Mátra Mts; Fig. 1). The Apuseni Mts population was highly diverse (3 haplotypes in 3 individuals, one of them differing by 6 substitutions from the other ones; Fig. 3). There were no shared haplotypes between geographic regions. For more detailed data on diversity indices, see Table 1. Genetic distances between subspecies/evolutionary lineages are shown in Table 3.



**Fig. 3.** Median-joining network of the combined mtDNA haplotypes found in and around the Carpathian Basin (drawn with PopArt 1.7). Inset: distribution of *Ichthyosaura alpestris* in this region. On the network, circles with haplotype names mark haplotypes found in this study, and squares with numbers mark haplotypes from RECUERO *et al.* (2014). Nucleotide substitutions are marked with grey numbers. Colours refer to different mtDNA lineages (based on RECUERO *et al.* 2014)

**Table 3.** Estimates of evolutionary divergence over sequence pairs between subspecies/evolutionary lineages. The number of base differences per sequence from averaging over all sequence pairs between groups are shown. Standard error estimate(s) are shown above the diagonal.

	<i>I. a. alpestris</i>	<i>I. a. apuana</i>	<i>I. a. bako- nyiensis</i>	<i>I. a. cyreni</i>	<i>I. a. monte- negrina</i>	<i>I. a. reiseri</i>	<i>I. a. veluchiensis</i>
<i>I. a. alpestris</i>		8.872	3.150	4.570	10.196	9.145	9.678
<i>I. a. apuana</i>	89.280		8.609	9.012	9.790	9.035	9.713
<i>I. a. bakonyiensis</i>	10.843	86.745		3.688	10.210	9.316	10.011
<i>I. a. cyreni</i>	20.238	91.145	17.659		10.487	9.634	9.897
<i>I. a. montenegrina</i>	97.030	95.619	95.765	103.160		5.997	7.606
<i>I. a. reiseri</i>	92.169	93.646	91.189	99.639	48.273		7.642
<i>I. a. veluchiensis</i>	88.152	95.190	89.353	95.520	67.000	68.068	

Despite their geographic proximity, Alpine newt populations in the Carpathian Basin represent two different mitochondrial lineages. The North Hungarian Mountain localities (Mátra, Bükk and Zemplén Mts) are very closely related to each other and can be assigned to the Western lineage (C3 mtDNA subclade both in SOTIPOULOS *et al.* 2007 and RECUERO *et al.* 2014; Fig. 2), most of them bearing the same N1 haplotype as each individual found in the NE Carpathians (Poland and Romania; Fig. 3). Bakony and Órség haplotypes formed a separated clade within the Western lineage (C2–C3 subclade; Fig. 2), differing by 11–18 substitutions from samples found in the Alps and the NE Carpathians (Fig. 3). Newts from Apuseni Mts are related to the Eastern lineage (E2 sublineage) but form a distinct clade within this lineage (Fig. 2), differing by 7–10 substitutions from the haplotypes found in the Southern Carpathians (Fig. 3). We found no evidence of admixture between the two main eastern and western lineages of the Alpine newt.

### DISCUSSION

Due to its transitional biogeographical position, the Carpathian Basin is one of the regions with the highest biodiversity in Europe (WILLIAMS *et al.* 1999, VARGA 2010, 2019). While Pannonian, Illyrean, and Alpine influences overlap in the western and south-western parts of Transdanubia, Pannonian, Carpathian, and Dacian

influences overlap in the north-eastern edges, and different Mediterranean elements (Holo and Ponto-Mediterranean, Balkan and Anatolian) enrich the Hungarian Middle Range's low elevation areas (VARGA 1995). The Alpine newt's evolutionary history in the Carpathian Basin is a testimony of this rich biogeographic history, with three different mitochondrial lineages meeting in this region and showing high levels of genetic diversity. Two lineages are associated with the major western lineage in *Ichthyosaura alpestris*: one in the Northern Hungarian Mountains and a second one in the Bakony Mts and Órség area. Besides, a third lineage associated with the eastern lineage is present in the Apuseni Mts. This diversity mirrors patterns of genetic structure in numerous co-distributed taxa. For instance, an east-west genetic split has been documented in the Alpine and Carpathian lineages of *Bombina variegata* and *Salamandra salamandra* (VÖRÖS *et al.* 2006, VÖRÖS *et al.* 2016b), in *Anguis fragilis* and *A. colchica* inhabiting Transdanubia and the North Hungarian Mountains (SZABÓ & VÖRÖS 2014), and in the Carpathian and Balkan clades of the adder, *Vipera berus* (URSENBACHER *et al.* 2006). Similar patterns of genetic differentiation have also been reported in some butterfly species in the Carpathian Basin. The genetic split, with or without admixture, between the south-western (Transdanubian) and north-eastern (sub)Carpathian populations has been observed e.g. in *Parnassius mnemosyne* (PECSENYE *et al.* 2015), *Phengaris nausithous* (HOLLÓS *et al.* 2012) and *Erebia medusa* (SCHMITT & SEITZ 2001). Recently, differentiation between the Órség region and Bakony Mts was also found in populations of the Marsh fritillary *Euphydryas aurinia* (PECSENYE *et al.* 2018); both populations form a sub-clade within European populations, highly differentiated from the Transylvanian ones.

RECUERO *et al.* (2014) hypothesised that the ancestor of *Ichthyosaura alpestris* was already widespread in the late Miocene. Oscillations of the Paratethys Sea level would have promoted the two major lineages' split during the Tortonian: the eastern lineage in the Balkans and the western lineage in the Alps. The Carpathian Basin might have been colonised by the two *I. alpestris* lineages along two major routes: the western lineage from the Alps to Transdanubia and from the Northeast Carpathians to the North Hungarian Mountains, and the eastern lineage would have colonised the Apuseni Mts from the Southern Carpathians.

Higher temperatures in Europe during the Miocene and Pliocene probably promoted the isolation of population groups into different mountain ranges, with the ranges of the different lineages further shifting during the Pleistocene glaciations. The Carpathian Basin might have provided cryptic refugia for Alpine newt populations in their cold-continental forest-steppe landscapes during the younger Pleistocene (WILLIS *et al.* 1997, WILLIS & ANDEL 2004, VARGA 2010, SCHMITT & VARGA 2012), which is supported by the high haplotype diversity and the genetic differentiation of newts from the Bakony

Mts and Órség area compared to the rest of the western lineage of *I. a. alpestris*. Using 35 loci ARANO and ARNTZEN (1987) investigated the inter-population genetic differentiation and evolutionary relationships within *I. alpestris* subspecies. They used four samples from Bakony Mts (Ajka) which nested within the *I. alpestris alpestris* clade of populations from France, Germany, Yugoslavia (that time), Austria and Bulgaria. RECUERO *et al.* (2014) included five specimens from Bakony Mts in their comprehensive molecular study (locality Ajka, specimen IDs: MAB309-313). Their Bayesian consensus phylogram based on mtDNA data provided high statistical support for two main clades within *I. a. alpestris*, with the clade comprising samples from the Bakony Mts population being sister to a clade containing the rest of the nominotypical subspecies. The split of the two *I. a. alpestris* clades was estimated to occur in the Pleistocene, less than 1.6 Mya. The distinctiveness of this population was confirmed by the addition of new samples in our study.

The existence of cryptic refugia in the Carpathian Basin has been documented in several studies (see SCHMITT & VARGA 2012). In addition to the Alpine newt, three lineages of the Moor frog (*Rana arvalis*) (BABIK *et al.* 2004), one lineage of the Adder, *Vipera berus* (URSENBACHER *et al.* 2006), and two lineages of the Danube crested newt, *Triturus dobrogicus* (VÖRÖS *et al.* 2016a), may have survived the last glacial maximum in extra-Mediterranean, northerly refugia in the Carpathian Basin.

The split of the distinct clade of the Apuseni Mts is less than 2.5 Mya (when the E1 and E2 clades split). The Apuseni Mts is a detached lower range of the Southern Carpathians, spatially isolated from the main Carpathian Arc since the Tertiary (MRÁZ & RONIÉK 2016). This old divergence is associated with the high species and endemic richness of the region, making Apuseni Mts one of the major biodiversity hotspots in the Carpathians. The distinctiveness of the Apuseni Mts *Ichthyosaura alpestris* population underpins the importance of these mountains as a biogeographically distinct region with a high number of endemic taxa, including earthworms (POP *et al.* 2010), aquatic insects (BÁLINT *et al.* 2011), and a distinct *Salamandra salamandra* lineage (VÖRÖS *et al.* 2016b).

### *Taxonomic implications*

In his dissertation, DELY (1964) presented a series of keys to identify all subspecies of *Triturus alpestris* (= *Ichthyosaura alpestris*) recognised at that time, including *T. a. bakonyiensis*. The key features include palatal teeth structure, head shape, snout shape, forelimb length, and overlap/no overlap of limbs when folded along the body. For comparison of *T. a. bakonyiensis* and *T. a. alpestris* (translated from DELY 1964), see Table 4 and Figs 5–8. Altogether, he used 26 morphometrical and 24 osteological characters to compare the subspecies.

MOLNÁR (2001), in his PhD dissertation, surveyed *Ichthyosaura alpestris* populations from Hungary and used four diagnostic indices (L/LCd, L/Ltc, L/PA, L/Lie, where L = length of body, LCd = length of tail, Ltc = width of head, PA = length of forelimb, Lie = distance between fore and hind limb) introduced by ROČEK (1974) to compare Hungarian *I. alpestris* samples with other populations and assess the morphometric similarity/differentiation concerning the nominotypical subspecies. This study provided evidence that Alpine newts from Órség and Bakony Mts significantly differ from the rest of the populations (L/Ltc and L/Lie for both males and females, and L/PA for males), while newts from Mátra, Bükk and Zemplén Mts were similar to *I. a. alpestris*. However, these results should be taken with caution because they are based on the comparison of measurements in live specimens with formalin-fixed ones.

Considering the levels of morphological differences found by DELY (1964) and MOLNÁR (2001) coupled with the genetic differentiation and the long independent evolutionary history of the Bakony + Órség clade, these populations may be referred to as *Ichthyosaura alpestris bakonyiensis* (Dely, 1964), and we call for additional studies on patterns of morphological and nuclear DNA variation in this taxon. In order to formally reconstitute *Ichthyosaura alpestris bakonyiensis* as a valid subspecies, below we provide the full description translated from DELY (1964).

*Ichthyosaura alpestris bakonyiensis* (Dely, 1964)  
(Figs 4–8)

Synonym: *Triturus alpestris bakonyiensis* Dely, 1964

Holotype: HNHM-HER-61.27.1., adult female, collected between 7–12 of May, 1961 by O. Gy. Dely. Type locality: Ajka-Felsőcsinger, Bocskor Hill, Bakony Mountains, Veszprém County, Hungary (Fig. 4).

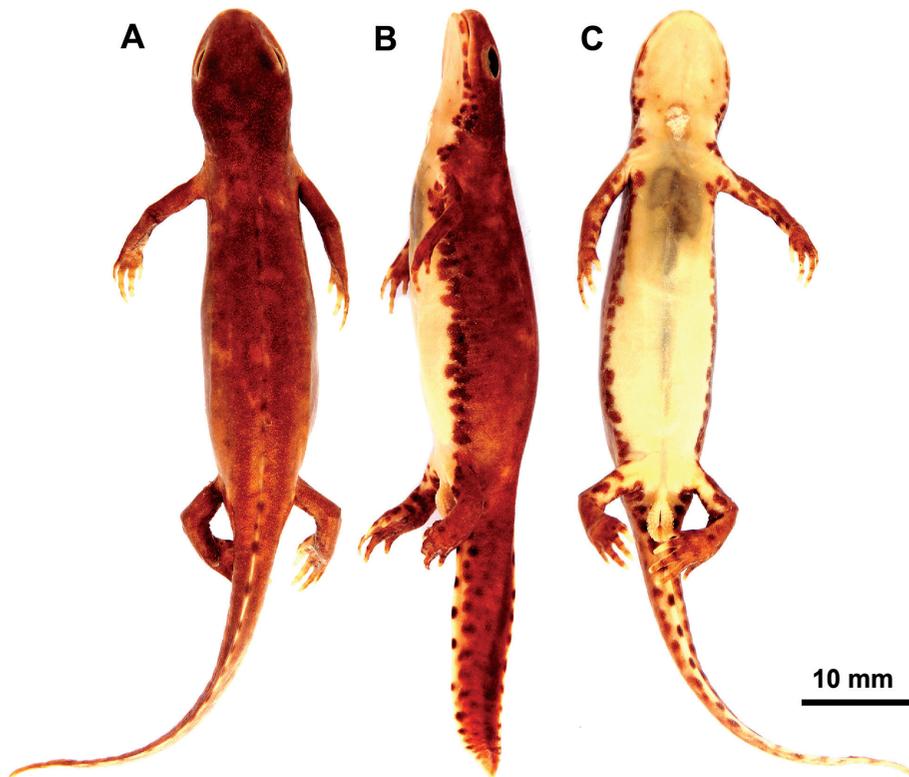
Paratypes: one adult female (HNHM-HER-2002.45.2.) collected between 7–12 of May, 1961, and two adult males (HNHM-HER-2002.45.1., HNHM-HER-2002.45.3.) collected between 9–12. of May, 1961 by O. Gy. Dely. at the same location as the holotype.

Redescription and diagnosis: Body more or less short and robust. Total length is between 70.6–77.9 mm (mean = 73 mm) (males) and 78.3–95.0 mm (mean = 85.43 mm) (females), body length is 41.5–46.3 mm (mean = 42 mm) (males) and 46.2–56.3 mm (mean = 49.15 mm) (females). Head robust and wide (especially at the corner of eyes) (Fig. 6). Snout longish, abruptly descending until the corner of mouth. Tip of snout is widely (males) or less widely (females) rounded until the outer edge of nares. Nares are positioned in the lateral corner of snout. Upper jaws form a semicircle from the nares running to the caudal direction and reach their widest point in the last third of the head, from where they continue slightly more narrow (males), or they diverge gradually until the caudal end of eyes where they reach the maximum width. From here, they continue nearly parallel (females). Lips are weakly developed. Palatal teeth start at the lower end of choanae, forming V-shape, with caudal ends slightly bending outward.

Body is robust, limbs are short. When bending the forelimb forward, 1st finger stretches until between frontal end of eyes and nares, 2nd finger slightly outreaches tip of snout, while 3rd finger far outreaches tip of snout. When bending forelimb backwards and hindlimb forward along the body, 3rd finger of forelimb touches the tubercle of hindlimb. The length of the forelimb is 56.95–66.52% (mean = 60.5%) (males) and 48.46–63.08% (mean = 55%) (females) of the distance between forelimb and hindlimb.

Dorsal surface of males is greenish brown or greenish purple. The low mid-dorsal crest runs from the end of the head to the end of the tail, is yellow with black spots, and is 1–2 mm high. Body flank is ornated with a longitudinal band consisting of black spots with greyish-white margins. Below the band there is a bright blue stripe. Same stripe stretches on the sides of the head from the snout to the shoulders. Tail is a bit lighter or darker than body, with smaller black spots; ventral edge is yellow with black spots. Ventral surface is reddish orange, throat is light yellow with small grey spots. Limbs and cloaca are ornated with black spots.

Dorsal surface of females is greyish-green with reddish-brown marble pattern. A light stripe running from the end of the head to the end of the tail. The edge of the dorsal and ventral surfaces is densely covered with small black spots. Similar pattern is found between the tip of snout and the shoulders. Tail has the same colour as the dorsal surface of body, covered with marbled pattern and small black patches; ventral edge is yellow with



**Fig. 4.** Holotype of *Triturus* (= *Ichthyosaura*) *alpestris bakonyiensis* (Dely, 1964) (HNHM-HER-61.27.1.) from dorsal (A), lateral (B) and ventral (C) view

several small black dots above. Ventral surface is orange. Upper parts of limbs and fingers are with small black patches. Cloaca has the same colour as dorsal surface of the body.

Skull is medium size, slim and flat, sharply chopped in the middle. Length of skull is 9.2–9.3 mm (mean = 9.25 mm) (males) and 9.8–10.6 mm (mean = 10.12 mm) (females). Length of skull is 12.9–13.1% (mean = 13%) (males) and 11.2–12.5% (mean = 11.8%) (females) of total body size. Width of skull is 57.6–62.3% (mean = 59.9%) (males) and 61.2–66% (mean = 63.7%) (females) of skull length. Width of quadratum is 76.0–78.4% (mean = 77.2%) (males) and 74–78.3 % (mean = 77%) (females) of the skull length. Snout more (males) or less (females) rounded, maxillariae form a semicircle running to caudal direction. Processus posteriores of the maxillariae are long, located close to the anterior ends of ptegygoideum, overreaching them and positioned a bit inwards compared to the quadratae. Cavum internasale do not reach upper edge of nares. Nasaliae are elongated (Fig. 8).

The index of regio otica compared to the condylobasal distance in anteroposterior direction is 29–29.3% (mean = 29.1%) (males) and 27.3–30% (mean = 29.1%) (females). The width index of regio otica is 103–111% (mean = 107%) (males) and 103–124% (mean = 111.5%) (females). Buccal sacs are small and quadrangular. The anterosuperior ends of tympanica do not overlap with regio orbitale. Paraoccipital corners are moderate. The neu-

**Table 4.** Major keys to differentiate between *I. a. alpestris* (based on populations of Switzerland – Neuchatel and/or Germany – Harz Mts, Rosenthal, Hagenbach, Kassel, or Marburg) and *I. a. bakonyiensis* (based on populations of Bakony Mts – Ajka-Felsőcsinger, Némethánya, and/or Úrkút), translated from Hungarian, presented in DELY (1964).

	<i>I. alpestris alpestris</i>	<i>I. alpestris bakonyiensis</i>
Palatal teeth structure	Palatal teeth getting wider in the last third (shape of a sugar tong) and the caudal ends are more or less bending inward.	Palatal teeth V shaped, frontal ends form sharp angle, caudal ends are slightly bending outward.
Head shape	Head longer than wide, oval shape, never circular from dorsal view.	Head robust, approximately as wide as the width of the body at the axillary region. It reaches its narrowest width behind the eyes.
Snout shape	Snout long, tip of snout projecting forward or slightly broadened (males).	Snout longish, tip of snout is widely (males) or less widely (females) rounded until the outer edge of nares.
Forelimb length	When bending the forelimb forward, 1st finger outreach frontal end of eyes, but never reaches the nares, while 2nd finger reaches tip of snout.	When bending the forelimb forward, 1st finger stretches until between frontal end of eyes and nares, while 2nd finger outreaches tip of snout.
Position of limbs when folded along the body	When bending forelimb backwards and hindlimb forward along the body, feet overlap (males) or longest fingers just or do not touch (females).	When bending forelimb backwards and hindlimb forward along the body, 3rd finger of forelimb touches the tubercle of hindlimb.

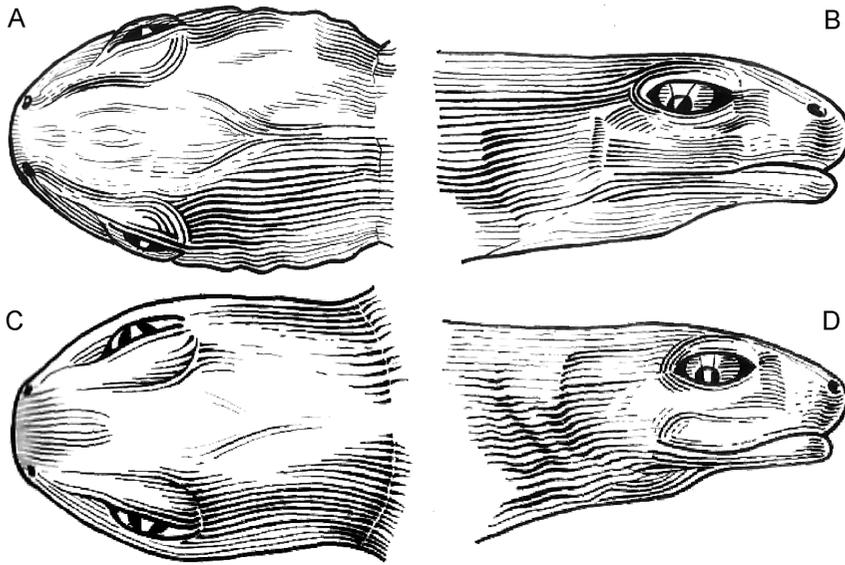


Fig. 5. Original drawings by O. Gy. Dely: male (A, B) and female (C, D) *Ichthyosaura alpestris alpestris* head shape from dorsal (A, C) and lateral (B, D) view

rocranium is 62.5–63.5% (mean = 63%) (males) and 61.4–71.9% (mean = 64.4%) (females) of the visceral cranium.

The intermaxillare reach the midline of vomero-palatine apparatus. Palatal teeth start at the inferior ends of choanae. From the starting point the two rows run apart caudal di-

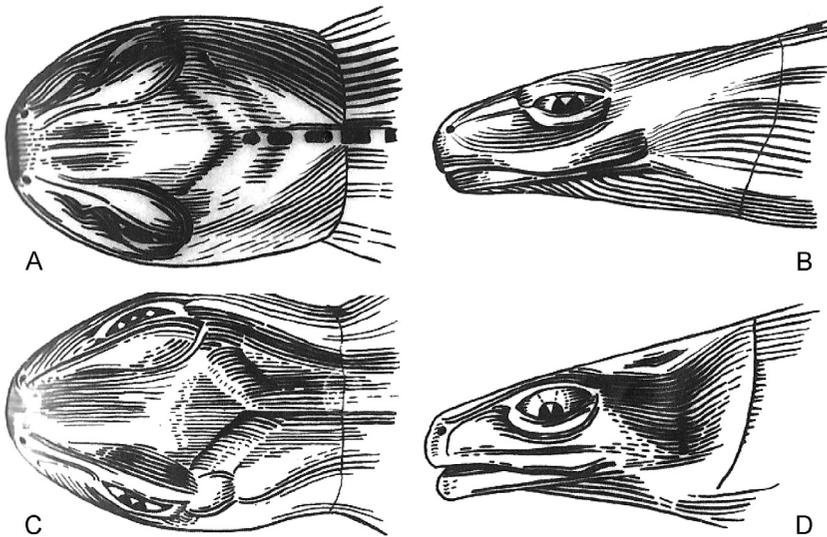
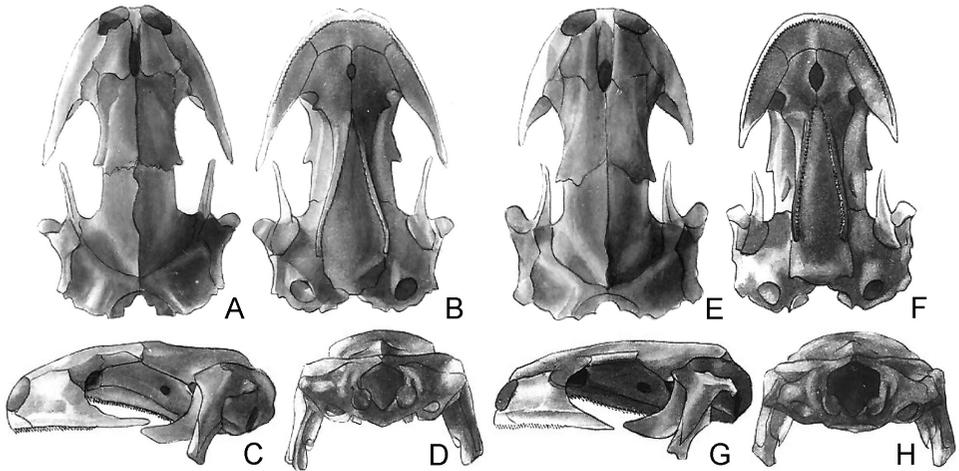


Fig. 6. Original drawings by O. Gy. Dely: male (A, B) and female (C, D) *Ichthyosaura alpestris bakonyiensis* head shape from dorsal (A, C) and lateral (B, D) view

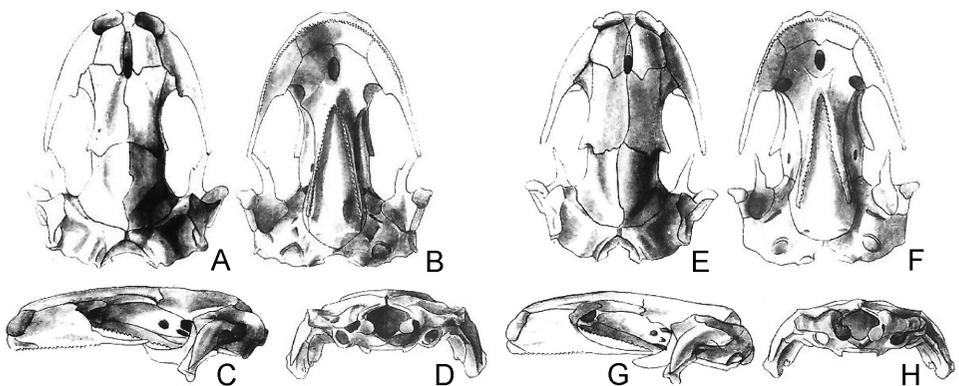


**Fig. 7.** Original drawings by O. Gy. Dely: female (A–D) and male (E–H) *Ichthyosaura alpestris alpestris* skull from dorsal (A, E), ventral (B, F), lateral (C, G) and posterior (D, H) view

rection and end far before the dorsal edge of the quadratum. The two rows of the V-shaped teeth are straight, caudal ends are slightly bending outward.

From lateral view the skull is flat. It starts inclined, and from the upper edge of the nostril it ascends until the caudal end of choanae; from here it runs straight until the arch above the foramen magnum; from here it abruptly (males) or gradually (females) descends until the upper edge of foramen magnum.

From posterior view the skull is flat and wide. The foramen magnum is quadrangular; the arch above is present mostly at males. Buccal sacs are depressed, canalis semicircularis is developed. The dorso-occipital line in lateral direction is either straight (males) or slightly cascading (females). Both fenestra ovalis are entirely visible (Fig. 8).



**Fig. 8.** Original drawings by O. Gy. Dely: female (A–D) and male (E–H) *Ichthyosaura alpestris bakonyiensis* skull from dorsal (A, E), ventral (B, F), lateral (C, G) and posterior (D, H) view

*Ichthyosaura alpestris bakonyiensis* differs from *I. a. alpestris* in head shape, shape of vomero-palatinal teeth, shape and structure of skull, and some morphometric parameters such as full body length, tail length, forelimb length, length between tip of snout and gular fold, and distance between anterior corner of eye and forelimb (in males) or forelimb length and distance between anterior corner of eye and forelimb (in females).

Known range: Bakony Mountains and Őrség-Vendvidék Region, Hungary.

Protection status and threats: *I. alpestris* is strictly protected in Hungary (Decree 100/2012 (IX. 28.) VM). The most important threats to the species are destruction and desiccation of breeding sites, an increasing number of predators (e.g. wild boar; fish), improper wildlife management and use of forests, tourism, the spread of technical sports (Vörös *et al.* 2014) and infectious diseases such as chytridiomycosis (Vörös *et al.* 2018) or ranaviruses (Vörös *et al.* 2020).

\*

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We dedicate this publication to the memory of the brilliant taxonomist Dr. László Papp, head of Diptera Collection in the Hungarian Natural History Museum, who passed away on the 28th of March, 2021. He was not only a great scientist and an amazing person, he was an advisor for the first author of this paper, following and supporting her academic path. Moreover, as a lecturer of taxonomy he introduced this amazing science to hundreds of students including the first and last authors of this paper.

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