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Distribution and hybridization of *Anguis fragilis* and *A. colchica* in Hungary

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Abstract. Slow worms (*Anguis* spp.) are widely distributed in Europe. Based on pronounced divergences in molecular markers the subspecies of the slow worm, *Anguis fragilis*, have been recently elevated to species level. In Hungary both *A. fragilis* and *A. colchica* are present in the mountainous areas with their range being separated by the Danube River with potential contact zones in the Danube valley. Based on morphology, hybridization of the two taxa has been described earlier from the Budai and Pilis Mountains. In order to reveal the exact distribution and confirm hybridization of *Anguis* taxa in Hungary we analyzed fragments of mitochondrial (ND2) and nuclear (Rag1) genes in 36 specimens from eight regions of Hungary and adjacent countries. The results confirmed the previously known distribution pattern with an east-west split along the Danube River and supported the morphological findings about hybridization in the Budai and Pilis Mountains.

Keywords: Carpathian basin, Danube River, mitochondrial DNA, nuclear DNA, slow worms.

Slow worms are widespread in the western Palearctic, inhabiting mostly undisturbed woodland habitats from the Iberian Peninsula to western Siberia, from southern Scandinavia to the Peloponnesus (Dely, 1981). Based on morphological traits, three subspecies (and several morphotypes) have been distinguished within what was considered for long as the polytypic species *A. fragilis* Linnaeus, 1758: the western nominotipical subspecies *A. f. fragilis* Linnaeus, 1758, the eastern *A. f. colchica* (Nordmann, 1840), and *A. f. peloponnesiacus* Stepánek, 1937 inhabiting the Peloponnesus. Using morphological characters, Grillitsch and Cabela (1990) suggested that *A. f. peloponnesiacus* is conspecific with *A. f.* var. *cephallonica* Werner, 1894, by thus elevating *Anguis cephallonica* Werner, 1894 to species level. These findings were confirmed by Mayer et al. (1991) based on protein electrophoretic studies.

To ascertain the taxonomic position and phylogenetic relationship of different slow worm forms, Gvoždík et al. (2010, 2013) surveyed genetic variation of various *Anguis* populations using both mitochondrial and nuclear markers. They found pronounced divergences in the mitochondrial lineages, with five distinct clades. Besides confirming *A. cephallonica* as a distinct species, they suggested to elevate the other four clades to species status, namely *A. fragilis* sensu stricto, *A. colchica*, *A. graeca* and *A. veronensis*.

Whatever the taxonomical status is, various *Anguis* forms have been long suggested to hybridize in several localities of their contact zones (Gvoždík et al., 2010, 2013). The contact zone between *A. fragilis* and *A. colchica* reaches from the Baltic Sea coast to the north-western Balkans (Petzold, 1971; Dely, 1981; Völkl and Alfermann, 2007). Sympatric occurrences of both forms and their intermediates have been reported several times from many points of this contact zone (Balkans: e.g. Musters and in den Bosch, 1982; Stojanov, 2001; Peloponnesus: e.g. Cabela and Grillitsch, 1989; Grillitsch and Cabela, 1990; Mayer et al., 1991; Czech Republic and Slovakia: e.g. Lác, 1967; Moravec, 1997; and Hungary: Dely, 1972). To date no genetic test was performed to confirm hybridization events deduced by morphology.

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Lying in the meeting point of different postglacial recolonisation routes and being an important extra-Mediterranean glacial refugium (Schmitt, 2007), the Carpathian Basin is a complex biogeographic area, located between the Carpathian Mountains and the western Alpine domain. Slow worms are common in the Carpathian Basin, occurring in most areas of Transdanubia, but inhabiting only undisturbed mountainous habitats east of the Danube (The Northern Middle Range, fig. 1). As described by Dely (1972, 1974a) based on morphological traits, both *A. fragilis* and *A. colchica* are present in the Carpathian Basin, with their range being separated by the Danube River. Yet, in their contact zone in the Danube valley (Budai and Pilis Mountains) slow worms show an intermediate morphology, suggesting that the two taxa hybridize in this region (Dely, 1972, 1974b).

The main objective of this study was to provide detailed information on the distribution of the two *Anguis* taxa in Hungary by identifying their mitochondrial lineages. Furthermore, by using single nuclear polymorphism (SNP) in the Rag1 nuclear gene, we attempted to examine hybridization between the two taxa in the Danube valley contact area.

Tissue samples from living or road-killed animals were collected from eight regions of Hungary and from surrounding countries (fig. 1, table 1). Given that the putative contact zone is located in the Budai and Pilis Mountains in the Danube valley, these regions were sampled more intensively. Tissue samples were preserved in 96% ethanol and kept at −20°C until processing. Total genomic DNA was extracted using DNeasy extraction kits following the manufacturer's protocols (Qiagen).

Two protein-coding gene fragments were amplified: 834 bp long fragment of the mitochondrial NADH dehydrogenase 2 gene (ND2), including a partial fragment of the Met tRNA, using the primers L4437n5 and AND2inR2 (Gvoždík et al., 2010), and a 1045 bp long partial exonic sequence of the nuclear recombination-activating gene 1 (Rag1), using the universal R13 and R18 primers (Groth and Barrowclough, 1999).

For both mitochondrial and nucler markers PCR was performed in a total volume of 50 μl using 1 U of Taq polymerase (Fermentas), 1.5 mM $MgCl_2$, 10 pmol dNTPs (Fermentas) and 5 pmol of the respective primer and approximately 50 ng of genomic DNA. ND2 amplifications were done with a profile of 95°C for 2 min, followed by 37 cycles of 95°C for 30 s, 60°C for 60 s and 72°C for 60 s, followed by a 7 min final extension at 72°C. Amplification of the Rag1 segment involved an initial 4 min denaturation at 94°C and 39 subsequent cycles of 94°C for 40 s, 63°C for 40 s and 72°C for 60 s, followed by a final extension step of 72°C for 7 min.

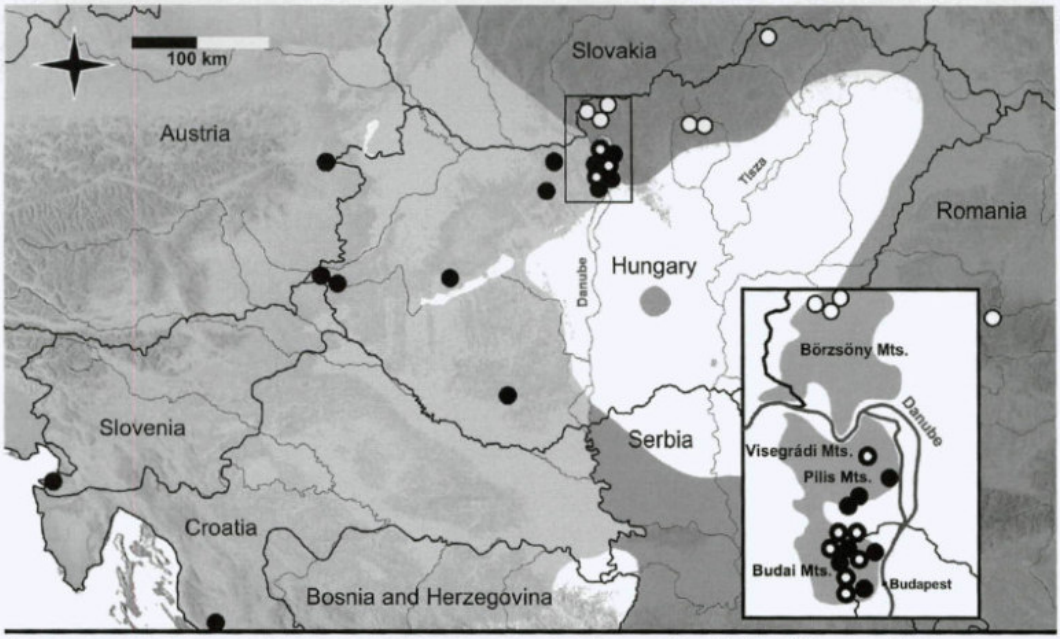
In each marker, we sequenced both strands following the ABI Prism BigDye Terminator Cycle sequencing protocol on an Abi 3130 Genetic Analyser (Applied Biosystems).

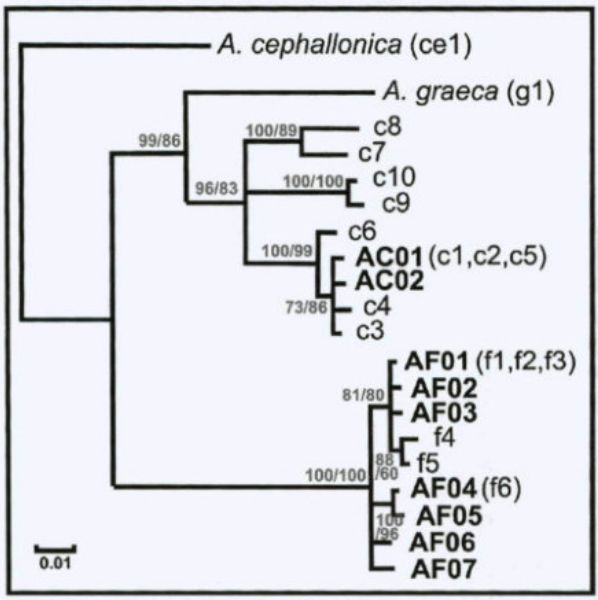
Sequences for the mitochondrial and nuclear gene regions were collapsed into haplotypes using Collapse 1.2 (Posada, 2011). These were aligned in ClustalW (Chenna et al., 2003) using the default settings for gap opening and extension penalties followed by limited manual correction of gap placement. In ND2 a three-basepair gap was detected in all samples belonging to *A. fragilis* clade, while this gap was absent in *A. colchica* samples. In nuclear Rag1 all sequences aligned perfectly, with no insertions or deletions. Heterozygous bases were detected in five positions. Variable positions were regarded as heterozygous when double peaks were detected in the trace file, and this double peak was re-detected after a second PCR and sequencing on the same sample.

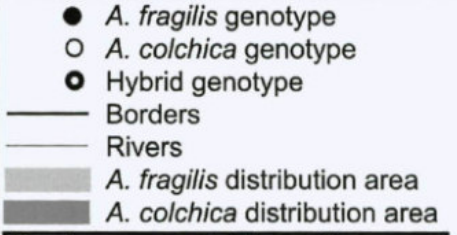
For the ND2 mtDNA data set, standard diversity indices (haplotype diversity and nucleotide diversity π) were calculated with DnaSP 5.0 (Rozas, 2009).

Phylogenies were constructed using maximum likelihood (ML) and Bayesian inference (BI) methods. Sampled *Anguis* sequences were aligned and analysed together with previously published *A. fragilis* (f1-f6, FJ666554-FJ666559) and *A. colchica* (c1-c10, FJ666576-FJ666585) haplotypes (Gvoždík et al., 2010), using *A. graeca* g1 (FJ666560) and *A. cephallonica* ce1 (FJ666586) as outgroup species (fig. 1). The appropriate model of sequence evolution was estimated using the Akaike Information Criterion (AIC) using the jModelTest 0.1.1 software (Posada, 2008). ML analyses were performed in phyML 3.0 (Guindon and Gascuel, 2003) using five initial random BioNJ trees and SPR branch swapping (pruning and regrafting algorithm; Hordijk and Gascuel, 2005) as tree topology search strategy, with options to optimize the topology and branch lengths. Bootstrap support values were calculated with 500 replicates. Bayesian infrence was performed using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) with two independent runs of Metropolis-coupled Markov chain Monte Carlo analyses, each with four incrementally heated Markov chains that were run for 1 million generations and sampled every 100 generations with the first 25% of samples excluded as burn-in. Posterior probabilities were obtained from the 50% majority rule consensus tree.

Mitochondrial DNA was analysed for a 765 bp length ND2 fragment. There was a significant split in haplotypes parallel to the geographic distribution of slow worms. Samples collected west of the Danube (Western Hungary, Austria, Slovenia, Croatia, Spain) formed







Rag1 genotype	variable positions 72	237	312	341	366	689	979	1003
AfR01	G	G	C	G	G	T	A	A
AfR02	G	G	C	G	C	T	A	A
AfR03	G	G	A	G	G	C	A	A
AfR04	G	G	M	G	G	Y	A	A
AfR05	G	G	M	G	G	C	A	A
AfR06	G	R	M	G	G	C	T	T
AfR07	R	R	M	G	G	C	A	A
AcR01	A	A	C	A	G	C	A	A
AcR02	A	A	C	R	G	C	A	A
AcR03	A	A	C	G	G	C	A	A

Figure 1. Distribution of *Anguis* species in Central Europe, sampling sites, ND2 Bayesian phylogram and Rag1 genotype assignment of Hungarian *Anguis* samples. The sampling location in Spain is not shown on the map. On the ND2 phylogram haplotypes found in samples of the present study are indicated as AF = *A. fragilis*, AC = *A. colchica*. Outgroup species (*A. cephallonica* and *A. graeca*) and other *A. fragilis/colchica* haplotypes (c1-c10, f1-f6) are retrieved from NCBI (Gvoždík et al., 2010). Bayesian posterior probabilities/ML bootstrap support values are given at the nodes. Shaded positions represent Rag1 diplotypes referring to hybrid origin. This figure is published in colour in the online version.

a distinct clade, together with haplotypes that were previously defined as *A. fragilis* (fig. 1, table 1). The other clade contained samples collected east of the Danube (North Hungary and Romania), grouping with *A. colchica* haplotypes from the NCBI database. In the *A. fragilis* clade, we detected 16 variable positions, defining seven haplotypes (AF01-AF07). In the *A.*

Table 1. Sampling locations, geographic coordinates, Genbank accession numbers and ND2 and Rag1 haplotypes/genotypes found in the *Anguis* samples collected in Hungary and adjacent countries. Hybrid genotypes are marked with asterisk. Haplotype sequences that were identical on 765 bp with haplotypes published by Gvoždík et al. (2010) are the followings: AC01 = c1 (FJ666576), c2 (FJ666577), c5 (FJ666580); AF01 = f1 (FJ666554), f2 (FJ666555), f3 (FJ666556); AF04 = f6 (FJ666559).

Country	Sampling locality	ND2	Acc. nr.	Rag1	Acc. nr.	S latitude	E longitude
Hungary	Aggtelek, Jósvafő	AC01	KF736829	AcR03	KF736840	48.48	20.58
Hungary	Bakony Mts, Nagyvázsony, Kab-hegy	AF04	KF736834	AfR05	KF736845	47.04	17.76
Hungary	Börzsöny Mts, Kemence	AC01	KF736829	AcR03	KF736840	48.02	18.91
Hungary	Börzsöny Mts, Kemence	AC01	KF736829	AcR03	KF736840	47.99	18.9
Hungary	Börzsöny Mts, Drégelypalánk	AC01	KF736829	AcR03	KF736840	48.05	19.03
Hungary	Budai Mts, Budapest	AF03	KF736833	AfR01	KF736841	47.49	18.99
Hungary	Budai Mts, Budapest	AF01	KF736831	AfR01	KF736841	47.53	18.99
Hungary	Budai Mts, Budapest	AF01	KF736831	AfR04	KF736844	47.54	18.95
Hungary	Budai Mts, Budapest	AF01	KF736831	AfR01	KF736841	47.53	19.00
Hungary	Budai Mts, Budapest	AF01	KF736831	**AfR06***	KF736846	47.54	19.00
Hungary	Budai Mts, Budapest	AF01	KF736831	AfR05	KF736845	47.54	18.94
Hungary	Budai Mts, Budapest	AF01	KF736831	AfR01	KF736841	47.54	18.95
Hungary	Budai Mts, Budapest	AF01	KF736831	–		47.53	18.96
Hungary	Budai Mts, Budapest	AF03	KF736833	**AfR07***	KF736847	47.58	18.96
Hungary	Budai Mts, Budapest	AF01	KF736831	AfR01	KF736841	47.53	18.96
Hungary	Budai Mts, Budapest	AF01	KF736831	**AfR07***	KF736847	47.57	18.93
Hungary	Budai Mts, Budapest, Normafa	AF01	KF736831	AfR01	KF736841	47.51	18.95
Hungary	Budai Mts, Budapest, Normafa	AF01	KF736831	**AfR07***	KF736847	47.52	18.96
Hungary	Budai Mts, Budapest, Normafa	AF03	KF736833	**AfR07***	KF736847	47.48	18.95
Hungary	Budai Mts, Remeteszőlős	AF01	KF736831	**AfR07***	KF736847	47.55	18.91
Hungary	Gerecse Mts, Zuppa-tető	AF02	KF736832	AfR05	KF736845	47.51	18.50
Hungary	Mátra Mts, Mátraháza	AC02	KF736830	AcR02	KF736839	47.82	19.97
Hungary	Mátra Mts, Mátrafüred	AC01	KF736829	AcR01	KF736838	47.87	19.98
Hungary	Mecsek Mts, Magyaregregy	AF01	KF736831	AfR01	KF736841	46.23	18.30
Hungary	Őrség, Szőce	AF01	KF736831	AfR01	KF736841	46.88	16.63
Hungary	Őrség, Felsőszőnök	AF01	KF736831	AfR01	KF736841	46.86	16.11
Hungary	Pilis Mts, Csobánka	AF04	KF736834	AfR01	KF736841	47.64	18.95
Hungary	Pilis Mts, Kiskovácsi	–		AfR01	KF736841	47.66	18.97
Hungary	Pilis Mts, Pilisszentlászló	AF01	KF736831	**AfR07***	KF736847	47.72	19.00
Hungary	Pilis Mts, Szentendre	AF01	KF736831	AfR01	KF736841	47.69	19.08
Hungary	Visegrádi Mts, Bajót	AF05	KF736835	AfR01	KF736841	47.72	18.57
Austria	Soproni Mts, Herrentisch	AF01	KF736831	AfR01	KF736841	47.66	16.42
Croatia	Velebit Mts, Prezid	AF01	KF736831	AfR01	KF736841	44.24	15.79
Slovenia	Piran, Fiesa	AF06	KF736836	AfR02	KF736842	45.52	13.58
Romania	Bihar Mts, Vartop	AC01	KF736829	AcR02	KF736842	46.51	22.66
Spain	Picos de Europa, Posada de Valdeón	AF07	KF736837	AfR03	KF736843	43.36	–4.86

colchica clade, our samples were less variable, with one single polymorpic position, defining two haplotyes (AC01 and AC02). Three haplotypes (AF01, AF04 and AC01) were identical (at least regarding the overlapping 765 bp) with haplotypes previously published by Gvoždík et al. (2010) (see fig. 1 and table 1). When restricted only to the Carpathian basin samples, *A. fragilis* population was more diverse (five haplotypes, haplotype diversity = 0.477 (0.115), nucleotide diversity π = 0.00223 (0.00083)) than *A. colchica* (two haplotypes, haplotype diversity = 0.286 (0.1), nucleotide diversity π = 0.00075 (0.0005)).

Eigth variable positions were detected in the analyzed 1043 bp segment of the Rag1 gene, defining altogether 10 genotypes, from which five (AfR04, AfR05, AfR06, AfR07 and AcR02) had heterozygous SNPs (fig. 1). Seven genotypes (AfR01-AfR07) were found in samples that belonged to the *A. fragilis* ND2 clade, and another three genotypes (AcR01-AcR03) in

samples belonging to the *A. colchica* mitochondrial lineage.

In seven animals, all collected in the putative contact zone (six in the Budai Mountains, one in the Pilis Mountains; fig. 1), and belonging to the *A. fragilis* mitochondrial clade, two Rag1 SNPs (on position 72 and 237, defining genotypes AfR06 and AfR07) contained alleles that were found in *A. colchica* but not in *A. fragilis* lineages outside of the hybrid zone (fig. 1).

The Danube River proved to have an important role in biogeography of Central European herpetofauna, either as a pathway or as a barrier for dispersal (e.g. Vörös et al., 2006). Our molecular data confirmed the theory of Dely (1972) based on morphological traits that within the Carpathian Basin *A. fragilis* is distributed west, while *A. colchica* east of the Danube. The hybrid zone of the two taxa seems to be located at the Pilis and Budai Mountains, again confirming the previous findings of Dely (1972). This hybrid zone lies at the meeting point of the two taxa with long independent evolutionary history recolonizing Europe from different glacial refugia. *Anguis fragilis* might have expanded to western Europe from a glacial refugium located in the north-western Balkans (Gvoždík et al., 2010) similarly to *Natrix natrix* lineage 3 (Kindler et al., 2013), while *A. colchica* populations most probably survived the last glaciations in southern Caspian, Caucasian, and presumably Carpathian refugia, recolonizing Eastern and Northern Europe (Gvoždík et al., 2010). This pattern is similar to expansion of *Emys orbicularis* lineage II presented by Fritz et al. (2007).

The fact that different *Anguis* taxa are able to hybridize to some extent where their ranges come into contact, e.g. *A. fragilis* and *A. colchica* in the Carpathian Basin (present study) and *A. fragilis* and *A. veronensis* in the Italian Alps (Gvoždík et al., 2013) could call their taxonomic status into question. However, detailed molecular studies on *Anguis* contact zone coupled with crossing experiments would help to understand these hybrid systems and clarify their species status.

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