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Slow worm, *Anguis fragilis* (Reptilia: Anguidae) as a species complex: Genetic structure reveals deep divergences

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ABSTRACT

Phylogenetic relationships of the Western Palearctic legless lizard genus *Anguis* were inferred based on a fragment of mitochondrial DNA and two nuclear protein-coding loci, *C-mos* and *PRLR*. *A. cephallonica* from the Peloponnese was confirmed as a valid species. It is the sister taxon to a clade comprising all other evolutionary lineages, which were shown to represent three distinct species: (1) *A. fragilis* sensu stricto occurring in Western and Central Europe, the north-western Balkans, with possibly isolated populations in the eastern Balkans, and presumably also in western Scandinavia and Italy; (2) *A. colchica* distributed from the eastern Czech Republic and the Baltic region eastward to northern Iran, presumably also in eastern Scandinavia, and the north-eastern Balkans; (3) *A. graeca* restricted to the southern Balkans, and partially sympatric with *A. cephallonica*. According to the more variable mitochondrial marker, *A. graeca* appears to be the sister species to *A. colchica*, and these taxa together form a sister clade to *A. fragilis*, whereas the less variable nuclear markers show *A. colchica* to be closer to *A. fragilis*. The C-mos gene has not provided substantial variation within this species complex, while the PRLR gene, which was used for the first time in phylogeographic study in a reptile, distinguished all species successfully. Intra-specific differentiation of *A. colchica* is discussed, and subspecific status of the Caucasian and Caspian populations is proposed. The uncovered genetic differences should be taken into account in all future biogeographical, morphological and ecological studies, as well as in conservation.

1. Introduction

Two species of the legless lizard slow worm, *Anguis* (Reptilia: Anguidae) are currently recognized: *A. cephallonica* Werner, 1894 and *A. fragilis* Linnaeus, 1758 (Arnold, 2002; Völkl and Alfermann, 2007). Whereas the first species is restricted to the Peloponnese and adjacent islands of Zakynthos, Kephallenia and Ithaca, the second one is widespread in the Western Palearctic region. Traditionally, two forms, regarded by some authors as different subspecies (e.g. Arnold, 2002; Musters and in den Bosch, 1982), or alternatively as morphotypes (e.g. Cabela and Grillitsch, 1989; Grillitsch and Cabela, 1990), have been distinguished within *A. fragilis* – western *A. f. fragilis* and eastern *A. f. colchica* (Nordmann, 1840). Morphological differentiation (e.g. prefrontal shield position, ear opening condition, number of scales around the midbody, blue dorsal spotting) and taxonomic status of these forms has been subject to several morphological and biogeographical studies (e.g. Beshkov, 1966; Lác, 1967; Musters and in den Bosch, 1982; Shcherban', 1976; Voipio, 1962; Wermuth, 1950). Also a long contact zone between both forms has been suggested to occur in the north–south direction from the west of Finland and the Baltic Sea coast, through Central Europe (along the border between the Czech Republic and Slovakia) to the north-western Balkans (Dely, 1981; Petzold, 1971; Völkl and Alfermann, 2007). A rather complex pattern of distribution of different morphotypes and their intermediates in the Balkan populations has been explained as sympatric occurrence of both forms (Arnold, 2002; Beshkov, 1966; Cabela and Grillitsch, 1989; Grillitsch and Cabela, 1990; Musters and in den Bosch, 1982; Stojanov, 2001), or as evidence for the existence of an intermediate form (Mayer et al., 1991). However, it is evident that external morphology is not fully concordant with extant intraspecific subdivision, and questions of the taxonomic status as well as interrelationships of the given forms have remained open until the present study.

With the primary aim to elucidate the phylogenetic relationships and taxonomic positions of the populations distributed along the presumptive contact zone of two slow-worm forms in the Czech Republic and Slovakia (Bárta and Tyrner, 1972; Kminiak,

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1992; Lác, 1967; Moravec, 1997; Rozínek et al., 2001), as well as the status of the Balkan populations, we focused our attention on genetic variation of *A. fragilis* using a broad-scale phylogeographic approach based on mitochondrial and nuclear DNA sequence data.

2. Material and methods

2.1. Sampling

Tissue samples of individual *Anguis* specimens (n = 50; 43 localities) were obtained from museum voucher specimens (National Museum, Prague, Czech Rep., NMP; Natural History Museum of Crete, Irakleio, Greece, NHMC) and road-killed individuals. Oral swabs or miniature tail biopsy were occasionally taken from live animals. Three mitochondrial sequences and one nuclear (*C-mos*) sequence of four individuals originating from three different regions were taken from GenBank, together with two sequences of two outgroup species (Table 1 and Fig. 1). We sampled populations of *A. fragilis* along a west – east transect through the Czech Republic and Slovakia. Another sample set covered the area of Greece and adjacent territories of the southern Balkans. Individuals of several populations from distant areas (Iberian Peninsula, British Isles, Baltic and Caucasus regions, and northern Iran) were used for comparison. Closely related *Anguis cephallonica*, as well as more distant *Hyalosaurus koellikeri* Günther, 1873 and *Pseudopus apodus* (Pallas, 1775) (generic affiliation sensu Macey et al., 1999) were employed as outgroup species. Both the Caucasian (*P. a. apodus*) and the Balkan (*P. a. thracius* Obst, 1978) populations of *Pseudopus* were included into the analyses to compare genetic distinctiveness between populations of these distant territories within the outgroup *Pseudopus*, as well as the ingroup *Anguis*.

2.2. Laboratory procedures

Total genomic DNA was extracted from tissue samples using different commercial kits following manufacturers' protocols. A fragment of mitochondrial DNA (mtDNA) and two nuclear genes (nDNA) were targeted for molecular phylogenetic analyses. Mitochondrial DNA comprised the complete NADH dehydrogenase subunit 2 gene (*ND2*), five complete transfer RNA genes – tryptophan, alanine, aspargine, cysteine, tyrosine (*tRNA-Trp*, *tRNA-Ala*, *tRNA-Asn*, *tRNA-Cys*, *tRNA-Tyr*) and the light-strand replication origin which is located between *tRNA-Asn* and *tRNA-Cys*. PCR primers were taken (H5934) or modified (L4437n: 5′-AAGCTATTGGGCCCA TACC-3′) from Macey et al. (1997). Amplification of mtDNA sequences involved an initial cycle of denaturation at 94 °C for 2 min, and 35 subsequent cycles of 94 °C for 35 s, 50 °C for 35 s and 72 °C for 1 min, followed by a final extension step of 72 °C for 10 min. Sequencing was performed using a combination of PCR primer (H5934) and newly designed internal primers AinND2F (5′-CCCAAGACYTAACAACA-3′), AND2inR2 (5′-ATGAAGCCGGATAGA GG-3′) and AND2inRc (5′-ATGAAGCCGGATAGTGG-3′; specific for *A. cephallonica*). In one sample (from Montenegro) only partial *ND2* sequence (744 bp) was obtained due to low quality of source DNA.

Two nuclear protein-coding loci were chosen as independent markers for comparison of their genetic pattern to the mitochondrial signal. Oocyte maturation factor (*C-mos*) gene was sequenced as this gene has been used in several squamate phylogeny studies (e.g. Slowinski and Lawson, 2002). Secondly, prolactin receptor (*PRLR*) gene was used herein for the first time in phylogeny/phylogeography of reptiles, as Townsend et al. (2008) suggested this marker to be the most variable nuclear protein-coding locus in squamate reptiles tested in their comparative study. *C-mos* was amplified using primers S77 and S78 (Lawson et al., 2005) and *PRLR* with primers PRLR_f1 and PRLR_r3 (Townsend et al., 2008). Both nuclear genes were amplified according to the following PCR program: initial cycle of denaturation at 94 °C for 7 min, 40 subsequent cycles of 94 °C for 40 s, 48 °C for 30 s and 72 °C for 1 min, followed by a final extension step of 72 °C for 7 min. Sequencing was performed using the PCR primers. All sequencing was done by Macrogen Inc. (Seoul, S. Korea, http://www.macrogen.com). Sequences of all particular haplotypes have been deposited in GenBank (FJ666554–FJ666589 for mtDNA fragment; GQ285104–GQ285118 for *PRLR*; GQ285119–GQ285123 for *C-mos*).

2.3. Phylogenetic analyses

All alignments were performed in BioEdit 7.0 (Hall, 1999) and tRNAs were aligned with respect to their secondary structures following Macey et al. (1999). The complete mtDNA alignment included a 1428 bp stretch. However, two positions within *tRNA-Trp*, and one within *tRNA-Cys*, respectively, were excluded from phylogenetic analyses because of unique insertions present only within the outgroup samples (the Albanian *P. apodus*, and *H. koellikeri*, respectively). The ND2 gene sequences were examined by translation with the vertebrate mitochondrial genetic code into amino acids using DnaSP 4.50 (Rozas et al., 2003). No stop codons were detected. The same program was used to estimate the average genetic distances between particular taxa or populations. This was done for the entire mitochondrial fragment, the ND2 gene solely, and nuclear *PRLR* and *C-mos* fragments. The whole mitochondrial fragment, as well as the nuclear genes, if appropriate, also served to estimate the average intra-specific and intra-population variation. The computed distances were based on uncorrected p-distances, and only distinct haplotypes were assessed. One mitochondrial haplotype was omitted from the calculations because of its incompleteness (the sample from Montenegro).

Alignment of nuclear genes was prepared by hand as the genes are protein-coding exons with no indels (*C-mos* segment = 555 bp; *PRLR* segment = 544 bp). In the *PRLR* fragment, three individuals showed more than one heterozygous position. For haplotype inference of such cases, a coalescent-based Bayesian method of Phase 2.1 (Stephens and Scheet, 2005; Stephens et al., 2001) as implemented in DnaSP 5.00 (Librado and Rozas, 2009) was employed. The analyses were run multiple times (5×) with different seeds for the random-number generator and checked if gametic phase estimation was consistent through the runs according to goodness-of-fit values. Each run was conducted under the parent-independent mutation model with a burn-in-period of 100 followed by 1000 iterations. No stop codons were detected in nuclear haplotypes as inferred by Phase 2.1 as checked by translation with the universal nuclear genetic code using BioEdit 7.0 (Hall, 1999). However, there were two samples phased with low statistical support (see Section 3), and only one of them (Czech sample from locality No. 3) was used for subsequent analyses and checked for both options of possible haplotype combinations. The other (Slovenian) sample was not directly included into the analyses because of its low probabilities of gametic-phase inference. Haplotype networks for both the *C-mos* and *PRLR* phased data were constructed using the statistical parsimony algorithm implemented in TCS 1.21 (Clement et al., 2000) under the 95% limit of parsimony. For further phylogenetic computations all sequences, mt and phased nDNA, were sorted into distinct haplotype data sets using Collapse1.2 (Posada, 2006).

The best-fit model of sequence evolution was selected using jModelTest 0.1.1 (Posada, 2008). Likelihood scores for each particular model were computed through maximum likelihood (ML) optimized trees using the implemented PhyML algorithm (Guindon and Gascuel, 2003). As Posada and Buckley (2004) argued that the Akaike information criterion (AIC; Akaike, 1974) and the Bayesian information criterion (BIC; Schwarz, 1978) offer important

Table 1
Specimens examined, localities, museum voucher numbers (NMP = National Museum, Prague, Czech Rep.; NHMC = Natural History Museum of Crete, Irakleio, Greece) or references, and haplotype names (and GenBank accession numbers; given only once for each haplotype for sequences obtained within this study) of one mitochondrial (ND2 and tRNAs) and two nuclear (C-mos, PRLR; slash indicates heterozygotes) markers. N = number of individuals sequenced for mtDNA fragment; nDNA was sequenced only in one individual per locality in each case.

Map	Species	Locality	Latitude	Longitude	*N*	Museum No./ Reference	Haplotypes (GenBank Acc. Nos.)		
							mt DNA fragment	*C-mos*	*PRLR*
	Anguis cephallonica	**Greece**							
1		Gialova, Peloponnese	36.95° N	21.70° E	1	–	ce1 (FJ666586)	Cce1 (GQ285119)	Pce1 (GQ285104)
2		Stymfalia Lake, Peloponnese	37.88° N	22.48° E	1	NHMC 80.3.92.1	ce2 (FJ666587)	Cce1	Pce1
	***Anguis fragilis* s.s.**	**Czech Rep.**							
3		Stráž nad Ohří	50.33° N	13.10° E	1	–	f1 (FJ666554)	Cfc1 (GQ285120)	Pf2/4 (GQ285106/ GQ285108)
4		Nové Údolí	48.83° N	13.80° E	1	–	f1	–	–
5		Malá Skála	50.63° N	15.18° E	1	–	f1	–	–
6		Rantířov	49.41° N	15.52° E	1	–	f2 (FJ666555)	Cfc1	Pf1 (GQ285105)
7		Nejdek	48.82° N	16.77° E	1	–	f1	–	–
8		Ondřejovice	50.25° N	17.35° E	1	–	f3 (FJ666556)	–	–
		Greece							
9		Mesoropi	40.89° N	24.06° E	1	–	f4 (FJ666557)	Cfc1	Pf2/3 (see above/ GQ285107)
10		Lepida–Megalo Livadi junction	41.37° N	24.63° E	1	NHMC 80.3.92.2	f5 (FJ666558)	Cfc1	Pf2
		Slovakia							
11		Bratislava	48.15° N	17.07° E	1	–	f1	Cfc1	Pf3
		Slovenia							
12		Bohinj Lake, Stara Fužina	46.29° N	13.90° E	1	NMP6V 72692	f6 (FJ666559)	Cfc1	(GQ285118)[b]
		Spain							
13		Vilarmiel, Galicia	42.48° N	07.12° W	1	Albert et al. (2009)	f7 (EU443256)	–	–
		UK							
14a		Kent, Kingsferry Bridge, England	51.25° N	00.75° E	1	Ast (2001)	f1 (AF407536)		–
14b		Kent, Isle of Sheppey, England	51.25° N	00.75° E	1	Slowinski and Lawson (2002)	–	Cfc1 (AY099972)	–
	Anguis graeca	**Albania**							
15		Diviakë	40.95° N	19.47° E	1	–	g13 (FJ666572)	Cg1/2 (GQ285122/ GQ285123)	Pg1 (GQ285109)
16		Himarë	40.68° N	19.66° E	1	–	g7 (FJ666566)	–	–
17		Dukat	40.21° N	19.58° E	1	–	g15 (FJ666574)	–	–
18		Korce	40.61° N	20.82° E	1	NMP6V 73232	g16 (FJ666575)	–	–
19		Ersekë, Shelegurë Lake	40.32° N	20.67° E	2	–	g4, g5 (FJ666563, FJ666564)	–	–
		Greece							
20		Kerkyra, Korfu	39.59° N	19.90° E	1	NHMC 80.3.92.22	g11 (FJ666570)	Cg1	Pg1/2 (see above/ GQ285110)
21		Gliki, Acherondas	39.33° N	20.55° E	2	–	g9, g12 (FJ666568, FJ666571)	–	–
22		Aoos River, near Konitsa	40.05° N	20.76° E	1	NHMC 80.3.92.17	g6 (FJ666565)	–	–

23		Ampelochori	39.53° N	21.03° E	1	NHMC 80.3.92.21	g10 (FJ666569)	Cg1	Pg1
24		Pertouli	39.54° N	21.47° E	1	NHMC 80.3.92.16	g2 (FJ666561)	–	–
25		Fylakti	39.30° N	21.68° E	1	NHMC 80.3.92.4	g2	–	–
26	Region of type locality of *A. fragilis* var. *graeca*	Mornos River	38.49° N	22.06° E	3	–	g1 (FJ666560)	Cg1	Pg1
27		Stomio	39.89° N	22.62° E	1	–	g3 (FJ666562)	Cg1	Pg1
28		Pefki–Artemision, Evvoias	39.01° N	23.23° E	2	NHMC 80.3.92.18-19	g2	–	–
29		Kryoneritis, Evvoia	38.93° N	23.28° E	1	NHMC 80.3.92.5	g2	Cg1	Pg3 (GQ285111)
		Montenegro							
30		Ulcinj	41.93° N	19.21° E	1	NMP6V 71272	g14[a] (FJ666573)	–	–
		Serbia							
31		Užice	43.86° N	19.84° E	1	–	g8 (FJ666567)	Cg1	Pg1
	Anguis colchica incerta	**Czech Rep.**							
32		Hostětín	49.05° N	17.88° E	1	NMP6V 73238	c2 (FJ666577)	–	–
33		Štramberk	49.58° N	18.10° E	1	NMP6V 72822	c3 (FJ666578)	Cfc1	Pc1 (GQ285112)
		Lithuania							
34	Region of type locality of *A. incerta*	Paluše	55.33° N	26.10° E	2	–	c6 (FJ666581)	Cfc1	Pc1
35	Region of type locality of *A. incerta*	Marcinkonys	54.04° N	24.44° E	2	–	c6	Cfc1	Pc1
		Poland							
36		Bocki	52.65° N	23.05° E	1	–	c4 (FJ666579)	Cfc1	Pc1
		Romania							
37		Finatale Clujuluj	46.83° N	23.62° E	1	–	c5 (FJ666580)	Cfc1	Pc1
		Slovakia							
38		Rovné	48.92° N	18.95° E	1	–	c1 (FJ666576)	Cfc1	Pc1
39		Šuňava	49.03° N	20.08° E	1	–	c1	–	–
40		Chlmecká skalka	48.88° N	21.93° E	1	–	c1	Cfc1	Pc1
	Anguis colchica colchica	**Georgia**							
41		Vardzia–Apnia road	41.37° N	43.27° E	1	–	c9 (FJ666584)	Cfc1	Pc2/3 (FJ666584/ GQ285114)
42		Telavi	41.92° N	45.49° E	1	–	c9	Cfc1	Pc3/5 (see above/ GQ285116)
		Russia							
43		Babukal, Krasnodarsky Territory	43.67° N	39.63° E	1	Macey et al. (1999)	c11 (AF085622)	–	–
		Turkey							
44		Hopa	41.40° N	41.44° E	1	NMP6 V 73694	c10 (FJ666585)	Cfc1	Pc2
	Anguis colchica orientalis	**Iran**							
45		Motalla Sara-ye Lemir	38.20° N	48.87° E	1	NMP6 V 72678	c7 (FJ666582)	Cfc2 (GQ285121)	Pc2
46		Nowshar	36.65° N	51.50° E	1	NMP6 V 72680	c8 (FJ666583)	Cfc1	Pc2/4 (see above/ GQ285115)
	Pseudopus apodus apodus	Dedop'lis Tskaro, **Georgia**	41.43° N	46.10° E	1	–	Paa1 (FJ666588)	–	PPaa1 (GQ285117)
		Voskresenskaya, Chechenia, **Russia**	43.35° N	46.10° E	1	Macey et al. (1999)	Paa1 (AF085623)	–	–
	Pseudopus apodus thracius	Diviakë, **Albania**	40.95° N	19.47° E	1	–	Pat1 (FJ666589)	–	–
	Hyalosaurus koellikeri	Kenitra, 10 km S, **Morocco**	34.27° N	06.60° W	1	Macey et al. (1999)	AF085621	–	–

[a] Only a fragment of the ND2 gene.
[b] Unphased heterozygous sequence – not directly used in phylogenetic analyses.

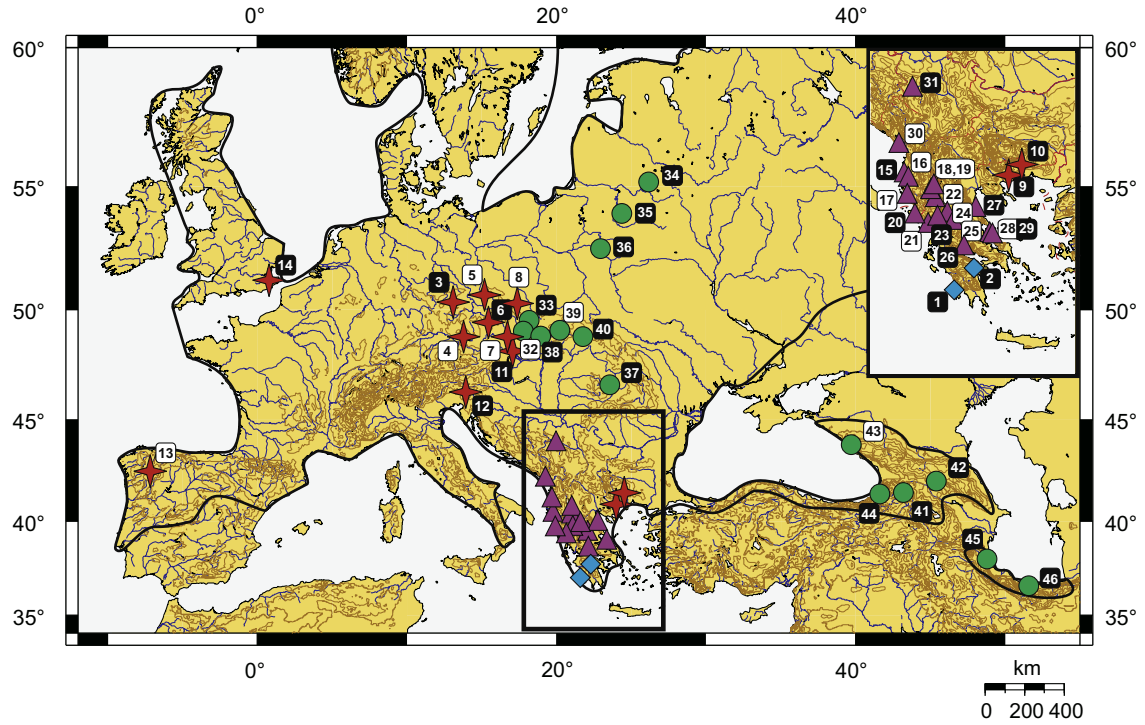

Fig. 1. Map showing localities of specimens used for the molecular analyses. Numbers correspond to those in Table 1; numbers in black squares indicate samples which were sequenced for both mt and nDNA. Black line delimits the distribution of *Anguis* according to Völkl and Alfermann (2007). Rhomboids = *A. cephallonica*; stars = *A. fragilis*; triangles = *A. graeca*; circles = *A. colchica*.

advantages over the hierarchical likelihood-ratio tests, we selected the BIC. In the protein-coding genes (*ND2*, *PRLR*, *C-mos*), we tested the best-fit model selection for both the whole fragments and separately for their codon positions, while the tRNA genes were tested only as a whole fragment. Finally, using the implemented Consense program from the PHYLIP package (Felsenstein, 2005), we obtained for mtDNA fragment the model-averaged phylogenetic tree as inferred from the 50% majority-rule consensus tree of 88 ML tree topologies, one for each model, weighted according to the BIC weights. This allowed us to estimate phylogenetic uncertainty due to model selection (Posada, 2008).

Phylogenies were constructed using maximum likelihood (ML), Bayesian inference (BI), maximum parsimony (MP), and neighbor-joining (NJ). For ML analyses, PhyML 3.0 (Guindon and Gascuel, 2003) was employed using the best-fit models [TrN + I + G, Tamura and Nei (1993) for mtDNA; HKY, Hasegawa et al. (1985) for PRLR; TPM3uf, Kimura (1981) for the combined nuclear-gene tree]. We set an option of 10 random starting BioNJ trees. The best of the nearest neighbor interchange (NNI), and the "new" subtree pruning and regrafting algorithm (SPR; Hordijk and Gascuel, 2005) of branch swapping was used as a tree topology search, with options to optimize the topology and branch lengths. We computed bootstrap values based on 1000 resampled data sets (Felsenstein, 1985), as well as the approximate likelihood-ratio test for branches (aLRT; Anisimova and Gascuel, 2006) as branch supports. Bayesian analyses were performed with MrBayes 3.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Partitioned models were applied according to genes and codon positions (see Table 2). In the tRNAs fragment of mtDNA, the likelihood settings corresponded to the general time-reversible model with a proportion of invariant sites and rate heterogeneity (GTR + I + G; Tavaré, 1986), which is the closest approximation of the TrN + I + G model (best model) available in MrBayes. All MrBayes analyses were performed with two runs and four chains for each run for six million generations, and sampling every 100th tree. First 300 trees (burn-in value) were discarded, as log-likelihood scores of sampled trees plotted against the generation time showed that stationarity was fully achieved after the first 30,000 generations in all data sets. A majority-rule consensus tree was then produced from the remaining trees after discarding the burn-in trees, and the posterior probabilities calculated as the frequency of samples recovering any particular clade (Huelsenbeck and Ronquist, 2001). The BI analysis was run three more times in all data sets with random starting trees, and the results were compared to check for local optima. Within MP analyses, all characters were equally weighted, gaps (in mtDNA) were treated as a fifth state, and a heuristic search was conducted with 100 random taxon stepwise-addition replicates using tree bisection and reconnection (TBR) branch swapping. The topology was reconstructed using 50% majority-rule consensus of most-parsimonious trees, and support values were

Table 2
Results of the substitution-model selections as inferred by jModelTest (Posada, 2008) under the Bayesian information criterion (BIC).

Fragment/Partition	Model selected (BIC)	References
mtDNA fragment	TrN + I + G	Tamura and Nei (1993)
ND2 – 1st codon position	HKY + G	Hasegawa et al. (1985)
ND2 – 2nd codon position	HKY + G	Hasegawa et al. (1985)
ND2 – 3rd codon position	HKY + G	Hasegawa et al. (1985)
tRNAs fragment	TrN + I + G	Tamura and Nei (1993)
PRLR	HKY	Hasegawa et al. (1985)
PRLR – 1st codon position	JC	Jukes and Cantor (1969)
PRLR – 2nd codon position	K80	Kimura (1980)
PRLR – 3rd codon position	F81	Felsenstein (1981)
C-mos	K80	Kimura (1980)
C-mos – 1st codon position	JC	Jukes and Cantor (1969)
C-mos – 2nd codon position	JC	Jukes and Cantor (1969)
C-mos – 3rd codon position	K80	Kimura (1980)
PRLR – *C-mos* combined	TPM3uf	Kimura (1981)

assessed using 1000 bootstrap pseudoreplicates (Felsenstein, 1985). MP and NJ analyses were performed with PAUP* 4.0b10 (Swofford, 2003). The NJ analyses were executed twice: first time with uncorrected *p*-distances, and second time with distances based on the best model. The branch support was evaluated by bootstrap analysis (Felsenstein, 1985) with 10,000 pseudoreplicates. MP and NJ were employed also on the ND2-translated and PRLR-translated amino acid data sets. The settings were the same as for the DNA analyses, gaps were treated as a 21st amino acid in MP, and mean character difference was used as a distance measure in NJ. All distinct haplotypes were used in the amino acid analyses as well, and checked for synonymous and non-synonymous mutations.

3. Results

3.1. Mitochondrial DNA sequence diversity

Among the 1425 base pair positions examined, 409 were variable, 296 of which were parsimony informative. Several indels have occurred in all tRNA genes, except for *tRNA-Ala* and *tRNA-Asn*, and in the light-strand replication origin. One codon deletion in the ND2 gene was detected even in one ingroup clade (see below). For phylogenetic analyses, a data set of 39 distinct haplotypes, including outgroups, was used. Within the procedure of substitution-model selection, the BIC selected the TrN + I + G (Tamura and Nei, 1993) model, which was used in ML analysis. It identified the most likely tree with log likelihood (ln*L*) = −5696.45 (Fig. 2). Model-averaged phylogeny (Posada and Buckley, 2004; Posada, 2008) showed exactly the same topology of the main clades as the most likely tree (not shown). Moreover, all main clades were supported by all most important models as weighted by the BIC suggesting that the different models support the same topology of the main lineages in our data set. All independent BI runs identified essentially identical topologies and likelihood estimates (mean ln*L* = −5428.80). MP analysis produced six most-parsimonious trees with a length of 747 steps (consistency index, CI = 0.656; retention index, RI = 0.869). All trees had identical topologies with respect to the main clades. Also both NJ trees, generated with uncorrected *p*-distances and the TrN + I + G (Tamura and Nei, 1993) distances, were consistent in their general topologies, and similar in the bootstrap support values.

The ND2-translated amino acid data set consisted of 345 characters, 78 were variable, of which 54 were parsimony informative. MP produced 357 most-parsimonious trees with a length of 137 steps (CI = 0.715; RI = 0.889). All MP trees were congruent in the topologies of the main clades, although the bootstrap support for branching patterns was low, yielding a polytomy of most of the main clades in the bootstrap majority-rule consensus tree. A similar polytomy was obtained by the NJ algorithm after bootstrap analysis. The NJ tree with branch lengths is depicted in Fig. 3.

In all mtDNA nucleotide analyses, *A. cephallonica* from Peloponnese was the sister lineage to a clade comprising all other lineages within the radiation (Fig. 2). However, deep divergences were

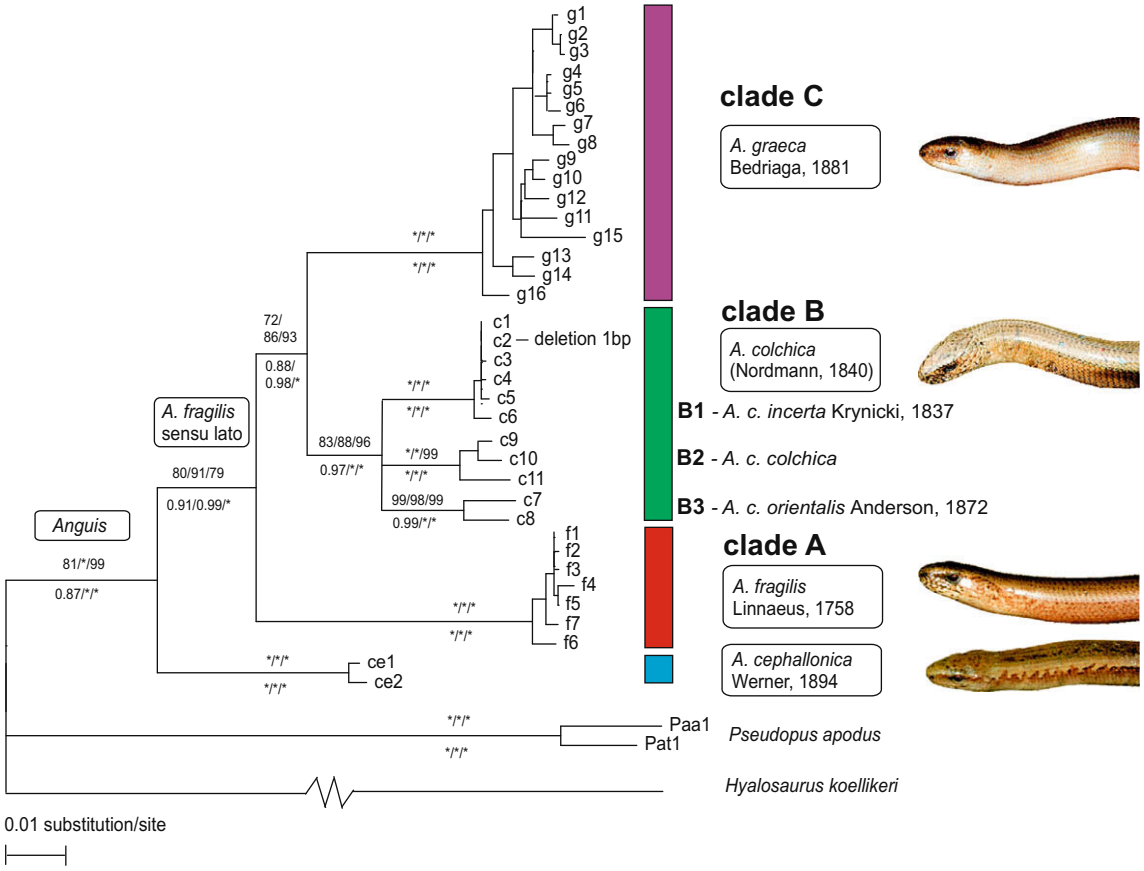

Fig. 2. Maximum likelihood haplotype tree showing the *Anguis* phylogeny as inferred from the ND2 and five tRNAs mtDNA sequences. Substitution model TrN + I + G with following values was used: substitution rate matrix AC = AT = CG = GT = 1.00, AG = 26.43, CT = 9.17; proportion of invariable sites P_{inv} = 0.091; gamma shape rate variation among sites α = 0.201; base frequencies *A* = 0.33, *C* = 0.32, *G* = 0.13, *T* = 0.22. Numbers above branches indicate bootstrap support values for maximum likelihood/maximum parsimony/neighbor-joining analyses. Numbers below branches indicate the PhyML (Guindon and Gascuel, 2003) approximate likelihood-ratio test for branches values/Bayesian posterior probability values/uncertainty due to model selection. Asterisk indicates full support (100 or 1.00) for particular clade. Haplotype names as presented in Table 1.

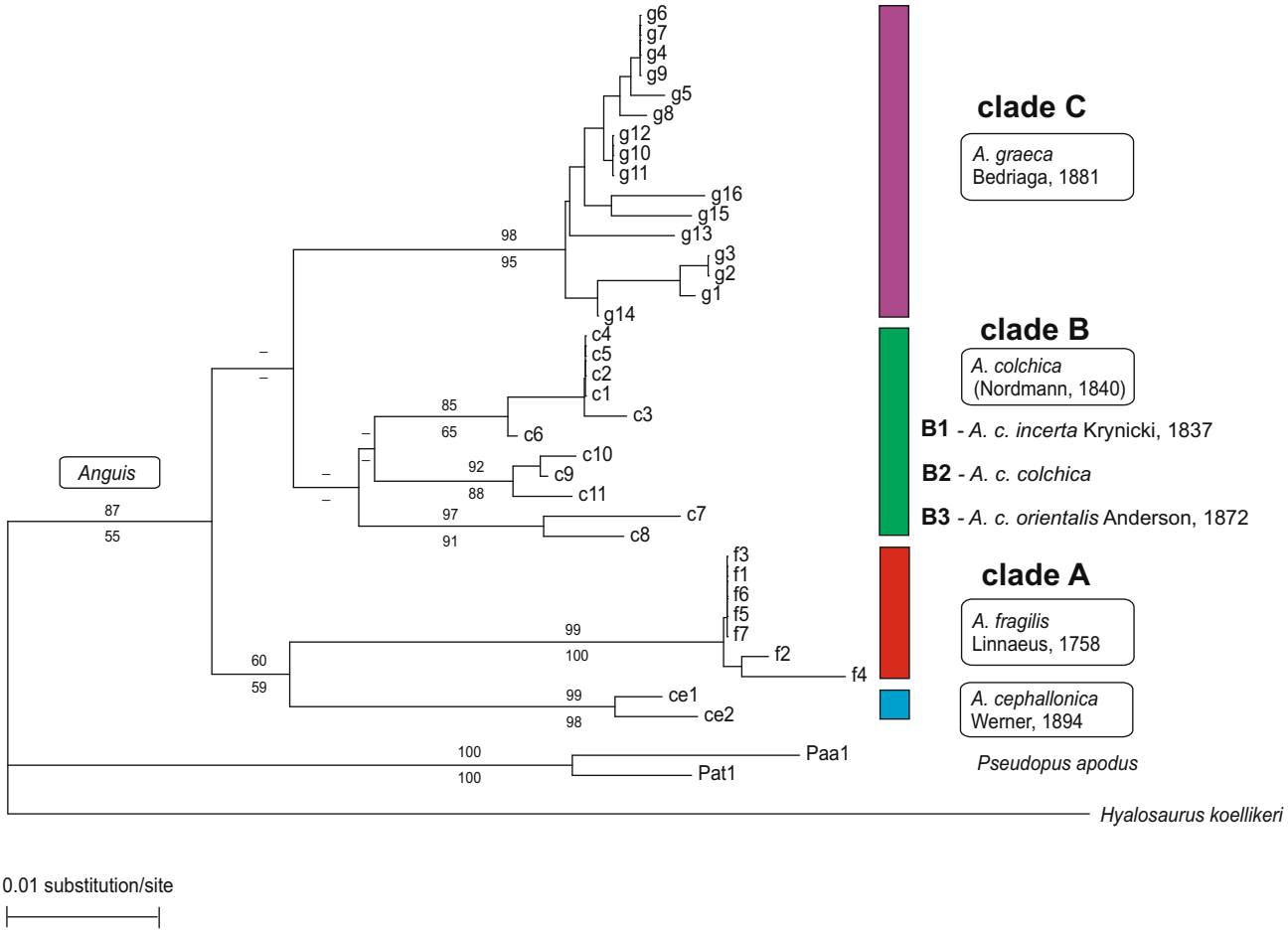

Fig. 3. Neighbor-joining tree based on amino acid sequences of the translated ND2 gene. Numbers above and below branches indicate bootstrap support values for neighbor-joining and maximum parsimony analyses, respectively. Sample names correspond to the haplotype names as in Fig. 2 and Table 1.

uncovered within the cluster of *A. fragilis* sensu lato (s.l.). This cluster (80/91/79/0.90 = ML/MP/NJ bootstrap values/BI posterior probability) is divided into three main clades. Clade A (100/100/100/1.00) consists of haplotypes from the Iberian Peninsula, the British Isles, Central Europe, Slovenia, and two isolated samples in northeastern Greece. Clade B (83/88/96/1.00) was found in four regions: (a) the Carpathians, (b) the Baltic region, (c) Caucasus, and (d) the Caspian region. These four regions clustered into three distinct subclades: B1 (100/100/100/1.00) consisting of haplotypes from the Carpathians and the Baltic region, B2 (100/100/99/1.00) corresponding to the Caucasus area and B3 (99/98/99/1.00) with two samples from the southern Caspian region. However, the mutual relationships between these subclades remain unresolved. Clade C (100/100/100/1.00) was found geographically located in the southern Balkan Peninsula (Greece, Albania, southern Montenegro, western Serbia), and is genetically very diverse, although without deeply divergent subclades. Clades B and C are likely sister clades (72/86/93/0.97).

Analyses of the translated *ND2* data set also showed several well supported distinct lineages within *Anguis*, congruent with the DNA lineages (Fig. 3), i.e. a high number of nucleotide substitutions were non-synonymous. However, topologies of the lineages within the ND2-translated trees are rather unsupported, forming a polytomy, including particular subclades of the mitochondrial clade B, which are considered distinct within the bootstrap analyses, but without clear relationships to each other and to the other clades (see the bootstrap support, or rather non-support in Fig. 3). Contrary to the nucleotide-based topology, *A. cephallonica* seems to be the sister lineage to the clade A, although with only low support (59/60 = MP/NJ bootstrap values). In contrast to the deep divergences between the clades in the ND2-translated data set, the geographically distant individuals from Spain, Slovenia, some Czech individuals, a Slovak sample, and even one individual from Greece (clade A) had only synonymous mutations.

From the mitochondrial analyses two main results we may highlight: (1) Topologies of all mt phylograms and the fact that the mitochondrial divergences between *A. cephallonica* and any main mt clade of *A. fragilis* s.l. (7.0–7.8%) are similar to those among the main clades within *A. fragilis* s.l. (5.8–8.1%) argue for species-level status of these main lineages (Table 3, Figs. 2 and 3). (2) Presence of the unique deletion of one codon within the ND2 gene in the clade A is an unusual type of mutation, even between different genera within the family Anguidae (Macey et al., 1999).

3.2. Nuclear DNA sequence diversity

Only a parsimony haplotype network was applied to the *C-mos* data set, as little variation was present within *Anguis* (5 distinct haplotypes; Fig. 4). The only heterozygous site in the only heterozygous sample (locality No. 15, Albania) was uncovered. Thus there was no problem with the inference of gametic phases within this diploid marker. Haplotype of *A. cephallonica* was the most distant within the genus, seven mutational steps away from the most common haplotype (Cfc1), which was shared by the mitochondrial clades A and B with the exception of one sample from Iran

Table 3
Genetic distances in percentage between the taxa (populations) based on uncorrected *p*-distances below and above diagonals. Average intraspecific (-population) variation at diagonals. In mtDNA, distances based on the whole mtDNA fragment below and at the diagonal (in bold), on the ND2 gene solely above the diagonal. In nDNA, the PRLR gene (in bold) below the diagonal and in front of the slash, and the C-mos gene above the diagonal and behind the slash.

Fragment	Uncorrected *p*-distances (%)	*H. koellikeri*	*P. apodus*	*P. a. apodus*	*P. a. thracius*	*A. cephallonica*	*A. fragilis*	*A. graeca*	*A. colchica*	*A. c. incerta* (Europe)	*A. c. colchica* (Caucasus)	*A. c. orientalis* (Caspian)
mtDNA	*H. koellikeri*	–	17.9	–	–	15.8	17.4	16.4	15.4	–	–	–
	P. apodus	**16.0**	**2.9**	–	–	13.8	14.6	15.0	14.1	–	–	–
	P. a. apodus	–	–	–	3.1	–	–	–	–	–	–	–
	P. a. thracius	–	–	**2.9**	–	–	–	–	–	–	–	–
	A. cephallonica	**14.1**	**12.1**	–	–	**0.5**	9.0	9.2	8.6	–	–	–
	A. fragilis	**15.6**	**13.2**	–	–	**7.8**	**0.5**	9.2	8.1	–	–	–
	A. graeca	**15.0**	**13.1**	–	–	**7.6**	**8.1**	**1.3**	7.0	–	6.9	–
	A. colchica	**14.0**	**12.4**	–	–	**7.0**	**7.1**	**5.8**	**2.4**	–	–	–
	A.c. incerta (Europe)	–	–	–	–	–	–	–	–	**0.2**	4.0	3.8
	A.c. colchica (Caucasus)	–	–	–	–	–	–	**5.9**	–	**3.5**	**1.2**	4.4
	A. c. orientalis (Caspian)	–	–	–	–	–	–	–	–	**3.2**	**3.6**	**1.6**
PRLR/*C-mos*	*H. koellikeri*	–	–	–	–	–	–	–	–	–	–	–
	P. apodus	–	–	–	–	–	–	–	–	–	–	–
	P. a. apodus	–	–	–	–	–	–	–	–	–	–	–
	P. a. thracius	–	–	–	–	–	–	–	–	–	–	–
	A. cephallonica	–	**1.5**	–	–	–	1.4	1.5	1.4	–	–	–
	A. fragilis	–	**2.0**	–	–	**1.5**	**0.3**/–	0.4	–	–	–	–
	A. graeca	–	**1.8**	–	–	**0.9**	**1.3**	**0.5**/0.2	0.4	–	–	–
	A. colchica	–	**1.9**	–	–	**1.4**	**0.7**	**1.2**	**0.3**/0.2	–	–	–
	A. c. incerta (Europe)	–	–	–	–	–	–	–	–	–	–	–
	A. c. colchica (Caucasus)	–	–	–	–	–	–	–	–	**0.5**	**0.2**/–	–
	A. c. orientalis (Caspian)	–	–	–	–	–	–	–	–	**0.3**	**0.4**	**0.2**/–

(mt clade B, locality No. 45). Six of the seven mutational steps were, however, synonymous. Samples from the mt clade C were distinguished from the most common haplotype Cfc1 by one (or two) unique mutation step(s).

The *PRLR* data set contained higher variation than the *C-mos*, although still not very high (compare genetic distances in Table 3). Seven slow-worm individuals were heterozygous, three of which in more than one site (2, 3, 4 sites). One of the three individuals (sample from the locality No. 9, Greece) was phased with high probabilities above 0.95, the second one with low probability in one from three heterozygous positions (probability 0.51; locality No. 3, Czech Rep.), and the third one with low probabilities in three from four heterozygous sites (probabilities around 0.70; locality No. 12, Slovenia). The latter was not further used in the analyses, while the second one was employed and both haplotype possibilities as inferred from the ambiguous site were checked. Parsimony haplotype network was applied for 13 distinct haplotypes within *Anguis* (Fig. 5B). The Czech sample with one ambiguous position did not change the network substantially when the other haplotype combination was applied (not shown). One haplotype stayed common with one homozygous sample (Pf3, instead of previous Pf2), while the other one stayed unique on the tip of the network. Among 544 base pairs in total within the *PRLR* fragment, 23 base pair positions were variable, ten of which were parsimony informative, including the outgroup genus *Pseudopus* (18 variable, 9 parsimony informative in *Anguis*). The HKY substitution model (Hasegawa et al., 1985) was used in ML analysis and different codon position-partitioned models (as in Table 2) in BI analyses. ML analysis identified the most likely tree with ln*L* = −927.53 (Fig. 5A). All independent BI runs yielded essentially identical topologies and likelihood estimates (mean ln*L* = −932.73). MP analysis produced four most-parsimonious trees with a length of 25 steps (CI = 0.960; RI = 0.955). Majority-rule consensus tree resulted in the same topology as ML and BI phylograms. NJ trees computed based on uncorrected *p*-distances and the HKY distances showed also the same topologies and were similar in the bootstrap support values. The PRLR-translated amino acid sequence data set consisted of 181 characters, 18 of which were variable, and eight of these variable characters were parsimony informative. MP produced four most-parsimonious trees with a length of 21 steps (CI = 0.952; RI = 0.938). MP majority-rule consensus tree and NJ tree (both not shown) were very similar to those of the nucleotide sequence data set (Fig. 5A). The most important difference was that nucleotide haplotypes Pf1 and Pf2, differing only in one synonymous mutation, formed a single haplotype in the amino acid data set.

Combined nuclear data set (*PRLR*, *C-mos*) was analysed by ML and BI, which yielded very similar trees as when based on the PRLR fragment solely. The main difference was that all samples of the mt clade C formed a clade with high statistical support (Fig. 6).

A. cephallonica had the most distant haplotype within *Anguis* haplotypes in the nDNA analyses. Samples from the mt clade C from the southern Balkans were shown consistently distinctive in both nuclear genes from the other nuclear haplotypes of *A. fragilis* s.l. However, variation of the *C-mos* segment was very low within *Anguis* and not practical for a phylogeographic approach. The *PRLR* phylogram showed samples from the mt clade A (Western Europe, haplotypes Pf1–Pf4) monophyletic. Samples from the mt clades B2 and B3 (Caucasus – Caspian region, haplotypes Pc2–Pc5) also formed a monophyletic group. Samples from the mt clade B1 (Eastern Europe, haplotype Pc1) appeared as basal in respect to the two monophyletic groups. Statistical supports of all clades were rather low, although it was clearly caused by general low number of variable sites.

3.3. Estimation of divergence times

Rate of molecular evolution for the mitochondrial region we used was estimated to 0.6–0.7% change per lineage per million

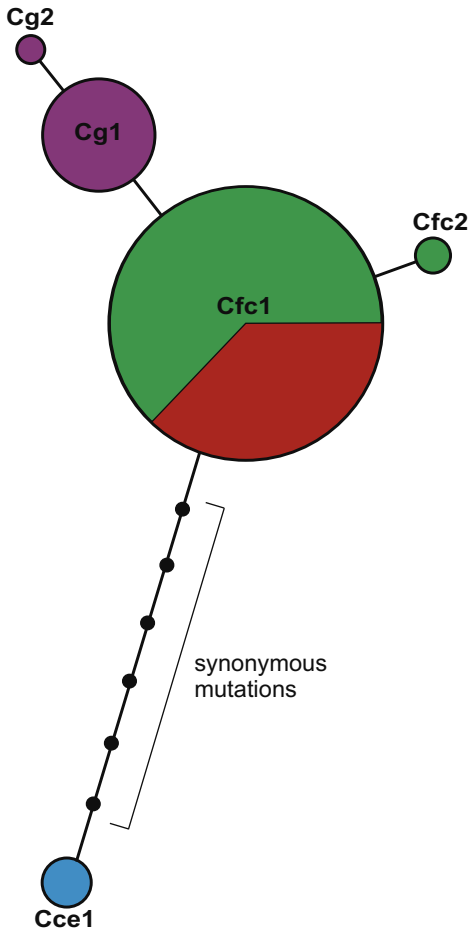

Fig. 4. Haplotype network of the C-mos gene based on the statistical parsimony algorithm. Circle sizes correlate to haplotype frequencies and color to the proper mt clade (species). *A. fragilis* and *A. colchica* share common main haplotype. Six from seven mutational steps (black dots) between *A. cephallonica* and the common *fragilis–colchica* haplotype are synonymous. Haplotype names as listed in Table 1.

years in ectotherm vertebrates based on uncorrected distances (Bermingham et al., 1997; Macey et al., 1998a,b). We found 12.7% of average uncorrected genetic distance between *Anguis* and *Pseudopus*. Applying mean rate of 0.65%, the calculated divergence date 9.8 Mya coincides with the result of Macey et al. (1999). Based on this rate, the basal radiation within *Anguis* could have occurred approx. 5.7 Mya in the late Miocene, followed by further rapid diversification. The lastly diverged lineages according to the mitochondrial gene tree, clade B and C, could have separated approx. 4.5 Mya in the early Pliocene. The three lineages of the clade B could then have started their own evolutionary history during the Pliocene/Pleistocene boundary approx. 2.5–2.8 Mya. At that time they apparently segregated into three different refugia – the southern Caspian, Caucasian, and presumably the Carpathian. However, one has to have in mind that all these calculations are rough, based on uncorrected genetic distances, and thus might be underestimated in the case some substitutional saturation has occurred.

4. Discussion

4.1. Genetic structure and relationships within Anguis

The results of this study reveal that there are not two (*A. cephallonica*, *A. fragilis*), but rather four comparable divergent evolutionary lineages representing separate species in *Anguis*. The observed levels of the mitochondrial sequence divergences within *A. fragilis* s.l. are comparable to those observed between *A. cephallonica* and any of the main mt clades of *A. fragilis* s.l. The mean genetic distance between *Anguis* and its sister genus, *Pseudopus* (Macey et al., 1999), is only 1.8 times greater (12.7%) than average "intra-specific" variation within *A. fragilis* s.l. (7.0%). Furthermore, if we compare the genetic distance between *P. a. apodus* from the Caucasus region and *P. a. thracius* from the Balkans (2.9%) with the respective Caucasian and Balkan populations of *Anguis*, the distance is more than double in the latter (5.9%). Also nuclear protein-coding locus *PRLR* shows genetic structure, which is concordant with the mitochondrial genetic structure in the sense of main mt clades A, B, C, and thus, concordant also with geography. Only mt clade A (Western Europe) forms a monophyletic group in the *PRLR* phylogram, although all samples from the mt clade B (Eastern Europe) form a compact cluster as well, in which each haplotype differs from the neighboring one just in one mutation step. Haplotype Pc1 corresponds to the mt subclade B1 and seems to be the ancestral haplotype from which the haplotypes found in specimens corresponding to the mt (sub)clades A, B2 and B3 derived. This pattern may be caused by incomplete lineage sorting as autosomal loci are known to have fourfold slower rate of lineage sorting (Avise, 2000). On the other hand, samples from the mt clade C are clearly distinguished from the other *A. fragilis* s.l. and *A. cephallonica* haplotypes. Moreover, the results of Mayer et al. (1991), who examined several proteins, i.e. exonic nuclear markers on a set of slow-worm individuals, are in good agreement with ours regarding the general patterns recovered. The study, focused on the question of taxonomic position of *A. cephallonica* (*A. fragilis peloponnesiacus* Štěpánek, 1937 at that time), also distinguished three lineages within the remaining populations of *A. fragilis*. *A. f. fragilis* specimens of Mayer et al. (1991) originated from Austria, i.e. from the region of our *A. fragilis* mt clade A distributed along the southern border of the Czech Republic eastwards to the south-western Slovakia, and into Slovenia. Similarly, *A. f. colchica* sensu Mayer et al. (1991) from Hopa (Turkey) is identical with our mt clade B (or B2, respectively), which includes samples from the same locality (map No. 44 in Fig. 1). Also their intermediate "*fragilis*/*colchica*" form from Feneos (Greece) corresponds well to our mt clade C. Thus, bringing the data of Mayer et al. (1991) into context with our results, it is evident that the "three species concept" within *A. fragilis* s.l. is supported by mitochondrial and nuclear DNA sequence data as well as with protein data.

4.2. Taxonomic implications and nomenclature

In the light of the obtained results and in accordance with the Baker and Bradley's (2006) definition of the genetic species concept (i.e. genetic species is a group of genetically compatible interbreeding natural populations that is genetically isolated from other such groups), we propose full species ranks for the main clades of *A. fragilis* s.l. and following nomenclature:

Clade A = *Anguis fragilis* Linnaeus, 1758, restricted type locality (Mertens and Müller, 1928): "Schweden" [=Sweden]; proposed common name: Common European Slow Worm.
Clade B = *Anguis colchica* (Nordmann, 1840), (new status), type locality: "Abasien" [=Kuban' region, southern Russia] and "Mingrelien" [=region in western Georgia]); proposed common name: Eastern Slow Worm.
Clade C = *Anguis graeca* Bedriaga, 1881 (new status), type locality: "Parnaß-Gebirge, Griechenland" [=Parnas Mts., Greece]; proposed common name: Greek Slow Worm.

The trinomen *A. fragilis colchica* [or more commonly, although incorrectly as masculine *colchicus*; Linnaeus (1758) used the

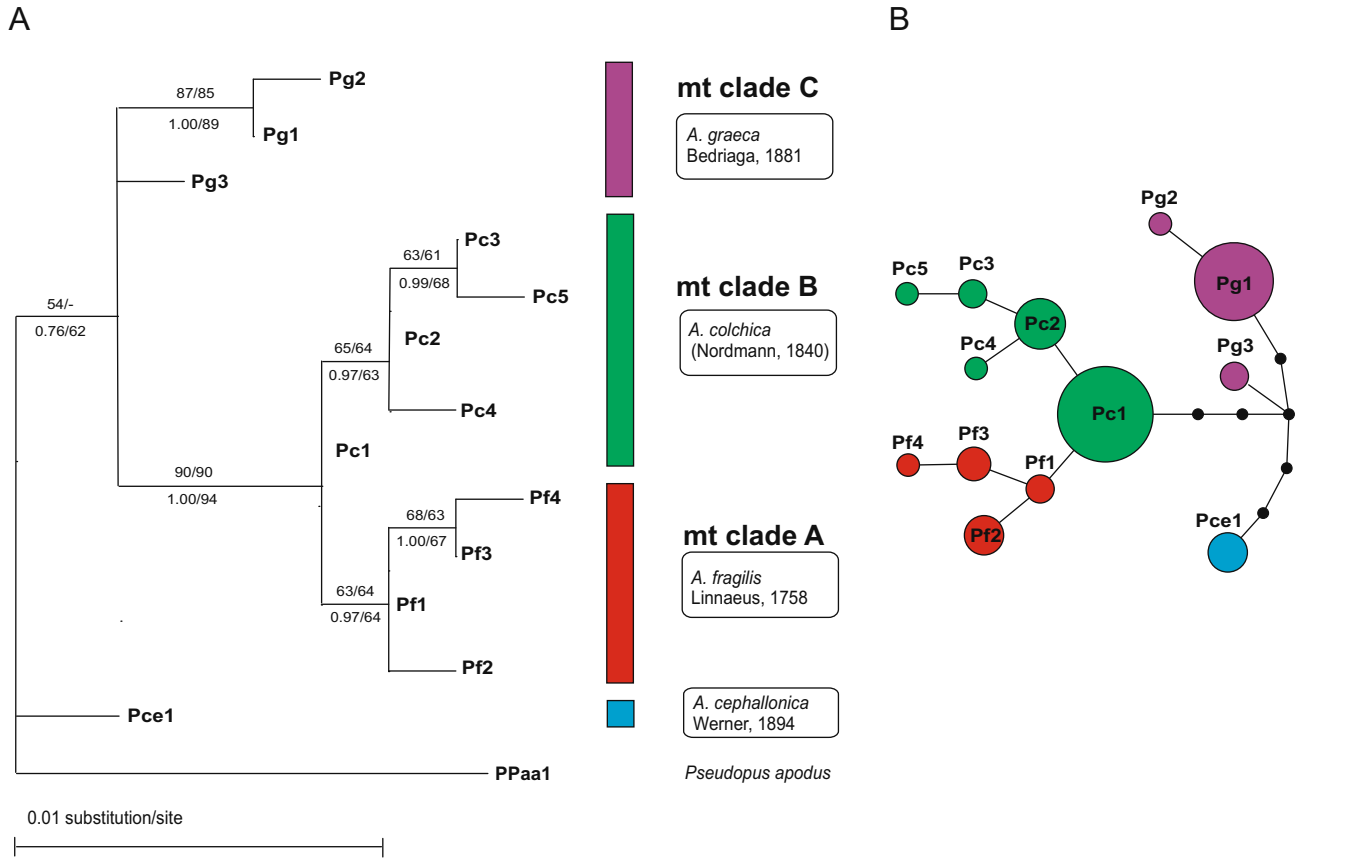

Fig. 5. (A) Maximum likelihood phylogram based on phased haplotypes of nuclear PRLR gene. Parameters for the HKY substitution model were as follows: transitions/transversions ratio = 2.118; base frequencies $A = 0.34$, $C = 0.21$, $G = 0.23$, $T = 0.22$. Numbers above branches are bootstrap support values for maximum likelihood/maximum parsimony, and numbers below branches Bayesian posterior probability values/neighbor-joining bootstrap support. Haplotypes sampled from specimens of mitochondrial clades A–C do not necessarily form corresponding clades in the *PRLR* pattern, but correspond to the mitochondrial clades A–C as shown in Fig. 2 and Fig. 3. (B) Statistical parsimony haplotype network of the same data set, *PRLR*, with circle sizes proportional to haplotype frequencies; small black dots = missing haplotypes. Haplotype names as listed in Table 1.

name *Anguis* in feminine gender as obvious from his originally established names like e.g. *A. maculata*, *A. reticulata*, etc.; see Article 30.1.4.2 of ICZN (1999)] had for a long time been applied for the populations from south-eastern Europe and the Caucasus region (Mertens and Wermuth, 1960). Later the name *colchica* has often been applied to the eastern slow worm populations including the northern ones (Arnold, 2002; Dely, 1981). Our findings indeed confirmed that clade B includes also the Baltic populations from Lithuania, where the type locality (Vilnius) of *Anguis incerta* Krynicki, 1837 is located. This name, previously erroneously considered a junior synonym of *A. f. fragilis* (Mertens and Wermuth, 1960), is older than the name *Otophis eryx* var. *colchica* Nordmann, 1840 (=*A. f. colchica*). However, according to our knowledge, few authors (e.g. Juszczyk, 1974; Štěpánek, 1949) used this name after 1899, in contrast to the name *A. f. colchica* (Nordmann, 1840) which has widely been used in zoological literature throughout the last century (for references see Supplementary data). As all authors known to us, who used the name *incerta* after 1899, explicitly used this name as infra-subspecific, and thus invalid according to ICZN (1999) [see articles 45.5 and 45.6], we believe that both conditions of the Article 23.9.1 of ICZN (1999) [see Supplementary data to discuss the conditions of the Article 23.9.1.2] have been met to promote the nomenclatural stability and consider the younger though prevailingly used name *A. colchica* (Nordmann, 1840) valid as *nomen protectum*. Consequently, we propose to treat *A. incerta* Krynicki, 1837 as *nomen oblitum*.

Regarding the finding that the eastern European populations north of the Caucasus are geographically separated from the Caucasian populations (Völkl and Alfermann, 2007) and show distinct genetic differentiation (Figs. 2 and 3), we propose to treat the east European populations (subclade B1) as distinct subspecies *Anguis colchica incerta* Krynicki, 1837, restricted type locality (Mertens and Wermuth, 1960): "Wilna, Litauen" [=Vilnius, Lithuania] (new status). The same approach we apply for the similarly divergent and presumably geographically isolated Caspian populations from northern Iran and probably from south-eastern Azerbaijan (subclade B3), for which the subspecific name *Anguis colchica orientalis* Anderson, 1872 (new status) (type locality: "Rehst, on the Caspian Sea" [=Rasht, Iran]) is available.

The species rank of the southern Balkan populations requires resurrection of the name *Anguis fragilis* var. *graeca* Bedriaga, 1881 (type locality: Parnas Mts., Greece) from the synonymy of *A. f. fragilis*.

4.3. Distribution and biogeography of the species

The exact distribution pattern of all three species is still little known. The Common European Slow Worm, *A. fragilis* sensu stricto (s.s.), is distributed from the Iberian Peninsula (confirmed on the mitochondrial basis) eastwards to Central Europe (Czech Republic, south-western Slovakia; confirmed by mt and nDNA). The range of this species presumably continues to Hungary west of the Danube River (Musters and in den Bosch, 1982), northwards to western

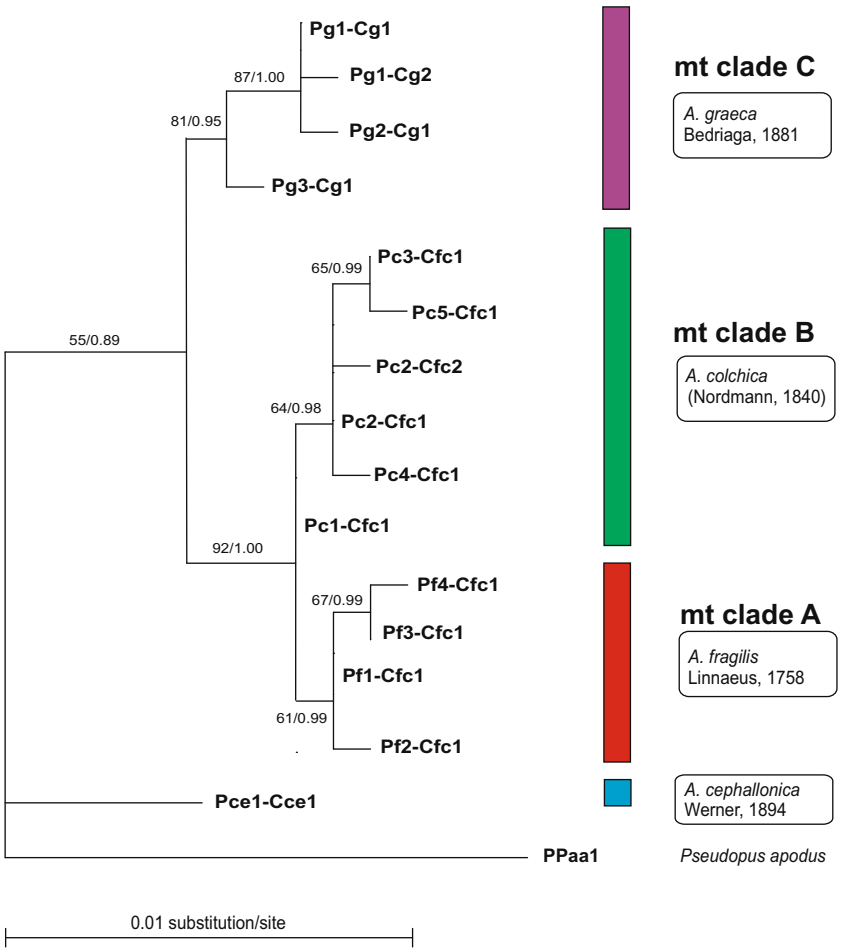

Fig. 6. Maximum likelihood tree based on combined nuclear data sets (*PRLR*, *C-mos*). Parameters for the TPM3 substitution model were: substitution rate matrix AC = CG = 0.32, AG = CT = 3.90, AT = GT = 1.00; base frequencies *A* = 0.32, *C* = 0.21, *G* = 0.23, *T* = 0.24. Numbers by branches are bootstrap support values for maximum likelihood and Bayesian posterior probability values. Haplotype names are combinations of names of the *PRLR* and *C-mos* haplotypes as presented in Table 1.

Scandinavia (Norway, Sweden = type locality), and south-eastwards to the Apennine Peninsula and the north-western Balkans (according to the data from Völkl and Alfermann, 2007). Isolated refugia have also been suggested to occur in the north-eastern Balkans (Lác, 1967; Petzold, 1971), in Romania (Musters and in den Bosch, 1982), and in Bulgaria (Beshkov, 1966; Musters and in den Bosch, 1982). The only Romanian sample we analyzed (from the Transylvanian region) fell within *A. colchica* according to both markers mtDNA and nDNA, respectively. However, based on both independent molecular markers we identified two *A. fragilis* s.s. individuals in north-eastern Greece, which is consistent with the assumption of an isolated refugium in Bulgaria (e.g. in the Rhodope Mts.). The situation in the north-western Balkans is not known at the moment. According to the distribution map in Musters and in den Bosch (1982), it is possible that the Greek (and Bulgarian) *A. fragilis* s.s. populations are not isolated, but rather the species may be continuously distributed west- and southwards the Danube River. However, it is probable that all three species (*A. fragilis* s.s., *A. colchica* and *A. graeca*) meet somewhere in the northern Balkans. Pozzi (1966) mentioned the *fragilis* form to be present in Slovenia and Croatia, and the *colchica* form in Bosnia and Herzegovina, Serbia, Montenegro and F.Y.R.O. Macedonia. This pattern could correspond to *A. fragilis* s.s. (the "*fragilis*" form), whereas the Balkan "*colchica*" morphotype presumably comprised two species, *A. graeca* and *A. colchica*. However, most of the *A. colchica* distribution probably corresponds to the range of the former "*colchica*" form as suggested by Arnold (2002), Dely (1981), Petzold (1971), and Völkl and Alfermann (2007), with the exception of the southern Balkans (Greece, Albania, southern Montenegro, western Serbia), where *A. graeca* is present. For the moment, we documented nominotypic subspecies *A. c. colchica* in Georgia, the Caucasian part of Russia, north-eastern Turkey; further lineages assigned to the subspecies *A. c. incerta* in Lithuania, north-eastern Poland, eastern Czech Republic, Slovakia, Romania, and another subspecies *A. c. orientalis* in northern Iran.

All species are probably mutually parapatric, although partial sympatry is feasible as has already been shown in *A. cephallonica* and *A. graeca* (*A. fragilis* at that time) in northern Peloponnese (Grillitsch and Cabela, 1990; Mayer et al., 1991). Ecological vicariance can be anticipated in the contact zones, as was shown by Beshkov (1966) in Bulgaria. However, there is no information about hybridization of the species at the moment. There are four potential zones of contact, or possibly hybrid zones (*A. cephallonica*/*A. graeca*, *A. graeca*/*A. fragilis*, *A. fragilis*/*A. colchica*, and *A. colchica*/*A. graeca*). No vertebrate species complexes with the same distribution pattern are known to us, however some parallels might be found in anurans, e.g. in *Bombina* (Hofman et al., 2007; Zheng et al., 2009) or *Pelobates* (see maps in Arnold, 2002), with western, eastern and southern taxa.

4.4. Intra-specific genetic variation

Uncovered intra-specific genetic variation had a very different pattern within each particular species studied. The most diverse mtDNA variation was found within *A. graeca*. It is probably tied to the fact that we sampled most of the species' range, which is lo-

cated in the zone of an important glacial refugium (Joger et al., 2007; Taberlet et al., 1998). Different sublineages in several microrefugia are thus likely to have persisted. On the other hand, the genetic uniformity of *A. fragilis* s.s. is surprising. Although our sampling of the species range is not comprehensive, we included geographically very distant populations such as the Iberian versus Slovak, or even the Greek, to prevent a possible bias caused by under-sampling. Nevertheless, the average intra-specific mtDNA variation is very low (0.5%), and most of the mutations within the ND2 gene were found to be synonymous. It is possible that the Spanish as well as the Greek populations resulted from a recent colonization, and the refugium of *A. fragilis* s.s. was located elsewhere. Here, Apennine Peninsula, southern France, and/or the north-western Balkans, which we did not sample (with the exception of one Slovenian sample, which is phylogenetically outside the group formed by other mt haplotypes of *A. fragilis* s.s. examined) come into account. The second possible explanation, however far less likely, is that there is a low substitution rate within this species. It would also mean that the origin of this species dated to the late Miocene based on mean mt genetic distance comparison is underestimated. As discussed above, *A. colchica* shows the most distinct intra-specific differentiation, which is probably related to the existence of several separate Pleistocene refugia. In comparison to the Caucasian (B2) and Caspian (B3) subclades, the European subclade (B1) shows relatively lower genetic variation. We could hypothesize that it is due to incomplete sampling, as we have virtually no individuals from prospective refugia of the subclade B1, which could be located in the Carpathian Basin, or in the north-eastern Balkans.

4.5. Current knowledge of morphological differentiation

The morphology of the newly recognized species is not known sufficiently, as many available morphological studies (Lác, 1967; Shcherban', 1976; Voipio, 1962; Wermuth, 1950) dealt with samples containing mixtures of different species. Nonetheless, some morphological traits historically distinguishing two morphotypes (e.g. Dely, 1981), i.e. "*fragilis*" and "*colchica*", could be roughly applied to *A. fragilis* s.s. (prefrontal shields in broad contact; ear opening indistinct; 24–26 scales around the midbody; blue dorsal spotting infrequent, present only in males) and *A. colchica* (prefrontal shields usually separated, sometimes in point contact and only rarely in broad contact; ear opening usually distinct, visible; 26–30 scales around the midbody; blue dorsal spotting frequent in males and occasionally present also in females), respectively. *A. graeca* remains morphologically the most enigmatic, as its populations are known to display intermediate or mosaic characters of the "*fragilis*" and "*colchica*" morphotypes (Cabela and Grillitsch, 1989; Grillitsch and Cabela, 1990). Detailed morphological study of the species complex is under preparation by the authors.

4.6. Conservation

The slow worm, *A. fragilis* s.l., has been suggested as Least Concern under the IUCN criteria (Cox et al., 2006). It is believed to be a widely distributed and quite common species. However, its cryptic ecology complicates a proper evaluation of populations' densities and possible threat *in situ*. Beside these general complications for evaluation of conservation status, the genetic structure has totally been omitted so far. This should be changed now, considering the slow worm, *A. fragilis* s.l., to be composed of three species, *A. fragilis* s.s., *A. colchica* and *A. graeca*. The first two species are seemingly widespread across Western and Eastern Europe, respectively, while *A. graeca* seems to be a more geographically limited species, which calls for further attention to the Mediterranean Basin as a global biodiversity hotspot (Myers et al., 2000) and to the importance of the Balkan Peninsula in particular.

The evolutionary differentiation within the genus *Anguis* presented in our study should be taken into account in all future conservation efforts, as well as in all biogeographical, morphological, ecological, and/or ethological studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.01.007.

References

Akaike, H., 1974. A new look at the statistical model identification. IEEE Trans. Automatic Control 19, 716–723.

Albert, E.M., San Mauro, D., García-París, M., Rüber, L., Zardoya, R., 2009. Effect of taxon sampling on recovering the phylogeny of squamate reptiles based on complete mitochondrial genome and nuclear gene sequence data. Gene 441, 12–21.

Anisimova, M., Gascuel, O., 2006. Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. Syst. Biol. 55, 539–552.

Arnold, E.N., 2002. Reptiles and amphibians of Europe, second ed. Princeton Univ. Press, Princeton and Oxford.

Ast, J.C., 2001. Mitochondrial DNA evidence and evolution in Varanoidea (Squamata). Cladistics 17, 211–226.

Avise, J.C., 2000. Phylogeography: The History and Formation of Species. Harvard University Press, Cambridge.

Baker, R.J., Bradley, R.D., 2006. Speciation in mammals and the genetic species concept. J. Mammal. 87, 643–662.

Bárta, Z., Tyrner, P., 1972. Finding of *Anguis fragilis colchicus* in Moravia, and notes to the subspecies affiliation of slow worms from the Ore Mts. Ochrana fauny (Bratislava) 6, 84 (in Czech).

Bermingham, E., McCafferty, S.S., Martin, A.P., 1997. Fish biogeography and molecular clocks: perspectives from the panamanian isthmus. In: Kocher, T.D., Stepien, C.A. (Eds.), Molecular Systematics of Fishes. Academic Press, San Diego, pp. 113–128.

Beshkov, V., 1966. Investigations on the systematics and distribution of the slow worm (*Anguis fragilis* L.) in Bulgaria. Bull. Inst. Zool. Mus. 21, 185–201 (in Bulgarian with Russian and German Abstracts).

Cabela, A., Grillitsch, H., 1989. Zum systematischen Status der Blindschleiche (*Anguis fragilis* Linnaeus, 1758) von Nordgriechenland und Albanien (Squamata: Anguidae). Herpetozoa 2, 51–69.

Clement, M., Posada, D., Crandall, K.A., 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9, 1657–1659.

Cox, N., Chanson, J., Stuart, S. (Eds.), 2006. The Status and Distribution of Reptiles and Amphibians of the Mediterranean Basin. IUCN, Gland and Cambridge.

Dely, O.G., 1981. *Anguis fragilis* Linnaeus 1758 – Blindschleiche. In: Böhme, W. (Ed.), Handbuch der Reptilien und Amphibien Europas. Band 1. Echsen (Sauria) 1. AULA-Verlag, Wiesbaden, pp. 241–258.

Felsenstein, J., 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. 17, 368–376.
Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.
Felsenstein, J., 2005. PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle (USA). http://evolution.genetics.washington.edu/phylip.html.
Grillitsch, H., Cabela, A., 1990. Zum systematischen Status der Blindschleichen (Squamata: Anguidae) der Peloponnes und der südlichen Ionischen Inseln (Griechenland). Herpetozoa 2, 131–153.
Guindon, S., Gascuel, O., 2003. A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. Syst. Biol. 52, 696–704.
Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.
Hasegawa, M., Kishino, K., Yano, T., 1985. Dating the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174.
Hofman, S., Spolsky, C., Uzzell, T., Cogălniceanu, D., Babik, W., Szymura, J.M., 2007. Phylogeography of the fire-bellied toads *Bombina*: independent Pleistocene histories inferred from mitochondrial genomes. Mol. Ecol. 16, 2301–2316.
Hordijk, W., Gascuel, O., 2005. Improving the efficiency of SPR moves in phylogenetic tree search methods based on maximum likelihood. Bioinformatics 21, 4338–4347.
Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17, 754–755.
ICZN, 1999. International Code of Zoological Nomenclature, fourth ed. The International Trust for Zoological Nomenclature, London.
Joger, U., Fritz, U., Guicking, D., Kalyabina-Hauf, S., Nagy, Z.T., Wink, M., 2007. Phylogeography of western Palaearctic reptiles – Spatial and temporal speciation patterns. Zool. Anz. 246, 293–313.
Jukes, T.H., Cantor, C.R., 1969. Evolution of protein molecules. In: Munro, H.M. (Ed.), Mammalian Protein Metabolism. Academic Press, New York, pp. 21–132.
Juszczyk, W., 1974. Amphibians and reptiles in our country, Państwowe Wydawnictwo Naukowe, Warszawa (in Polish).
Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.
Kimura, M., 1981. Estimation of evolutionary distances between homologous nucleotide sequences. Proc. Natl. Acad. Sci. USA 78, 454–458.
Kminiak, M., 1992. *Anguis fragilis* Linnaeus, 1758. In: Baruš, V., Oliva, O. (Eds.), Fauna of Czechoslovakia, vol. 26, Reptiles – Reptilia. Academia, Praha, pp. 101–106 (in Czech with English Abstract).
Lác, J., 1967. To the systematics of the slow worm (*Anguis fragilis* L.) and its distribution in Slovakia. Biológia (Bratislava) 22, 908–921 (in Slovak with German and Russian Abstracts).
Lawson, R., Slowinski, J.B., Crother, B.I., Burbrink, F.T., 2005. Phylogeny of the Colubroidea (Serpentes): new evidence from mitochondrial and nuclear genes. Mol. Phylogenet. Evol. 37, 581–601.
Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452.
Linnaeus, C., 1758. Systema naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. vol. 1, 10th ed.. Laurentii Salvii, Holmiae.
Macey, J.R., Larson, A., Ananjeva, N.B., Fang, Z., Papenfuss, T.J., 1997. Two novel gene orders and the role of light-strand replication in rearrangement of the vertebrate mitochondrial genome. Mol. Biol. Evol. 14, 91–104.
Macey, J.R., Schulte II, J.A., Ananjeva, N.B., Larson, A., Rastegar-Pouyani, N., Shammakov, S.M., Papenfuss, T.J., 1998a. Phylogenetic relationships among agamid lizards of the *Laudakia caucasia* species group: testing hypotheses of biogeographic fragmentation and an area cladogram for the Iranian Plateau. Mol. Phylogenet. Evol. 10, 118–131.
Macey, J.R., Schulte II, J.A., Larson, A., Fang, Z., Wang, Y., Tuniyev, B.S., Papenfuss, T.J., 1998b. Phylogenetic relationships of toads in the *Bufo bufo* species group from the eastern escarpment of the Tibetan Plateau: a case of vicariance and dispersal. Mol. Phylogenet. Evol. 9, 80–87.
Macey, R.J., Schulte II, J.A., Larson, A., Tuniyev, B.S., Orlov, N., Papenfuss, T.J., 1999. Molecular phylogenetics, tRNA evolution, and historical biogeography in anguid lizards and related taxonomic families. Mol. Phylogenet. Evol. 12, 250–272.
Mayer, W., Grillitsch, H., Cabela, A., 1991. Proteinelektrophoretische Untersuchungen zur Systematik der südgriechischen Blindschleiche (Squamata: Anguidae). Herpetozoa 4, 157–165.
Mertens, R., Müller, L., 1928. Liste der Amphibien und Reptilien Europas. Abhandlungen der Senckenbergischen Naturforschenden Gesellschaft 41, 1–62.
Mertens, R., Wermuth, H., 1960. Die Amphibien und Reptilien Europas. W. Kramer, Frankfurt am Main.
Moravec, J., 1997. *Anguis fragilis* Linnaeus, 1758. In: Nečas, P., Modrý, D., Zavadil, V. (Eds.), Czech recent and fossil amphibians and reptiles. An atlas and field guide. Edition Chimaira, Frankfurt am Main, pp. 78–79.
Musters, C.J.M., in den Bosch, H.J.A., 1982. Einige Bemerkungen zu den Unterarten von *Anguis fragilis* L., mit Berücksichtigung niederländischer Exemplare (Reptilia: Sauria: Anguidae). Salamandra 18, 196–204.
Myers, N., Mittermeier, R.A., Mittermeier, C.G., da Fonseca, G.A.B., Kent, J., 2000. Biodiversity hotspots for conservation priorities. Nature 403, 853–858.
Petzold, H.-G., 1971. Blindschleiche und Scheltopusik. Die Familie Anguidae. Die Neue Brehm-Bücherei 448. A. Ziemsen, Wittenberg-Lutherstadt.
Posada, D., 2006. Collapse: Describing haplotypes from sequence alignments. Downloadable from http://darwin.uvigo.es.
Posada, D., 2008. JModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256.
Posada, D., Buckley, T.R., 2004. Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Syst. Biol. 53, 793–808.
Pozzi, A., 1966. Geonemia e catalogo ragionato degli Anfibi e dei Rettili della Jugoslavia. Natura (Milano) 57, 5–55.
Ronquist, F., Huelsenbeck, J.P., 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
Rozas, J., Sánchez-Delbarrio, J.C., Messeguer, X., Rozas, R., 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19, 2496–2497.
Rozínek, K., Jr., Zavadil, V., Mikátová, B., 2001. Slow Worm – *Anguis fragilis* Linnaeus, 1758. In: Mikátová, B., Vlašín, M., Zavadil, V. (Eds.), Atlas of the distribution of reptiles in the Czech Republic. AOPK ČR, Brno-Praha, pp. 82–101 [207–213] (bilingual in Czech and English).
Schwarz, G., 1978. Estimating the dimension of a model. Annals of Statistics 6, 461–464.
Shcherban', M.I., 1976. To the intraspecific systematics of the slow worm (Reptilia, Sauria, *Anguis fragilis* L.). Sborn. Trud. Zool. Mus. (Kiev) 36, 81–83 (in Russian).
Slowinski, J.B., Lawson, R., 2002. Snake phylogeny: evidence from nuclear and mitochondrial genes. Mol. Phylogenet. Evol. 24, 194–202.
Štěpánek, O., 1949. Amphibians and reptiles of the Czech countries with respect to the fauna of Central Europe. Arch. Přír. Výzk. Čech. 1, 1–125 (in Czech with French Abstract).
Stephens, M., Scheet, P., 2005. Accounting for decay of linkage disequilibrium in haplotype inference and missing data imputation. Am. J. Hum. Genet. 76, 449–462.
Stephens, M., Smith, N.J., Donnelly, P., 2001. A new statistical method for haplotype reconstruction from population data. Am. J. Hum. Genet. 68, 978–989.
Stojanov, A., 2001. Beobachtung einer Kopulation zwischen den beiden Unterarten der Blindschleiche (*Anguis fragilis* L.). Historia Naturalis Bulgarica 13, 155–157.
Swofford, D.L., 2003. PAUP . Phylogenetic Analysis Using Parsimony (and other methods). Version 4. Sinauer Associates, Sunderland.
Taberlet, P., Fumagalli, L., Wust-Saucy, A.-G., Cosson, J.-F., 1998. Comparative phylogeography and postglacial colonization routes in Europe. Mol. Ecol. 7, 453–464.
Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512–526.
Tavaré, S., 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. In: Miura, R.M. (Ed.), Some mathematical questions in biology – DNA sequence analysis. American Mathematical Society, Providence, pp. 57–86.
Townsend, T.M., Alegre, R.E., Kelley, S.T., Wiens, J.J., Reeder, T.W., 2008. Rapid development of multiple nuclear loci for phylogenetic analysis using genomic resources: an example from squamate reptiles. Mol. Phylogenet. Evol. 47, 129–142.
Voipio, P., 1962. Multiple phaneromorphism in the European slow-worm (*Anguis fragilis*) and the distributional and evolutionary history of the species. Ann. Zool. Soc. 'Vanamo', 23, 1–20.
Völkl, W., Alfermann, D., 2007. Die Blindschleiche–die vergessene Echse. Z. Feldherpetologie, Beiheft 11. Laurenti-Verlag, Bielefeld.
Wermuth, H., 1950. Variationsstatistische Untersuchung der Rassen- und Geschlechtsmerkmale bei der Blindschleiche (*Anguis fragilis* Linné). Dtsch. Zool. Z. 1, 81–121.
Zheng, Y., Fu, J., Li, S., 2009. Toward understanding the distribution of Laurasian frogs: a test of Savage's biogeographical hypothesis using the genus *Bombina*. Mol. Phylogenet. Evol. 52, 70–83.

SUPPLEMENTARY DATA to

Slow worm, *Anguis fragilis* (Reptilia: Anguidae) as a species complex: Genetic structure reveals deep divergences. *Molecular Phylogenetics and Evolution.*

by

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According to the Article 23.9.1.2 of ICZN (1999) the application of the Principle of Priority is moderated and prevailing usage must be maintained when "the junior synonym or homonym has been used for a particular taxon, as its presumed valid name, in at least 25 works, published by at least 10 authors in the immediately preceding 50 years and encompassing a span of not less than 10 years". To fulfill the Article 23.9.2 requirements and give evidence that conditions of the Article 23.9.1.2 are met, we present the following list of publications where the name "*colchica*" was used as a valid name:

Ananjeva, N.B., Orlov, N.L., Khalikov, R.G., Darevsky, I.S., Ryabov, S.A., Barabanov, A.V., 2006. The reptiles of Northern Eurasia. Taxonomic diversity, distribution, conservation status. Pensoft, Sofia.

Anderson, S.C., 1999. The lizards of Iran. Contributions to Herpetology, Vol. 1. SSAR, Ithaca, New York.

Arnold, E.N., 2002. Reptiles and amphibians of Europe. 2nd ed. Princeton Univ. Press, Princeton and Oxford.

Bárta, Z., Tyrner, P., 1972. [Finding of *Anguis fragilis colchicus* in Moravia, and notes to the subspecies affiliation of slow worms from the Ore Mts.]. Ochrana fauny (Bratislava) 6, 84 (in Czech).

Beshkov, V., 1966. [Investigations on the systematics and distribution of the Slow Worm (*Anguis fragilis* L.) in Bulgaria]. Bull. Inst. Zool. Mus. 21, 185–201 (in Bulgarian with Russian and German abstracts).

Beshkov, V., Nanev, K. 2006. Amphibians and reptiles in Bulgaria. Pensoft, Sofia, Moscow.

Cabela, A., 1997. "*Anguis fragilis* Linnaeus, 1758". In: Gasc, J.-P. et al. (Eds.), Atlas of amphibians and reptiles in Europe. SEH, Mus. Nat. Hist. Naturelle, Paris, pp. 196–197.

Cabela, A., Grillitsch, H., Tiedemann, F., 2001. Atlas zur Verbreitung und Ökologie der Amphibien und Reptilien in Österreich: Auswertung der Herpetofaunischen Datenbank

der Herpetologischen Sammlung des Naturhistorischen Museums in Wien. Umweltbundesamt, Wien.

Dely, O.G., 1981. *Anguis fragilis* Linnaeus 1758 – Blindschleiche. In: Böhme, W. (Ed.), Handbuch der Reptilien und Amphibien Europas. Band 1. Echsen (Sauria) 1. AULA-Verlag, Wiesbaden, pp. 241–258.

Engelman, W.-E., Fritzsche, J., Günther, R., Obst, F. J., 1993. Lurche und Kriechtiere Europas. Neuman Verlag, Radebeul.

Günther, R., Völkl, W., 1996. Blindschleiche – *Anguis fragilis*. In: Günther, R. (Ed.), Die Amphibien und Reptilien Deutschlands. Gustav Fischer Verlag, Jena.

Kminiak, M., 1992. *Anguis fragilis* Linnaeus, 1758. In: Baruš, V., Oliva, O. (Eds.), [Fauna of Czechoslovakia, Vol. 26, Reptiles – Reptilia]. Academia, Praha, pp. 101–106 (in Czech with English abstract).

Lác, J., 1967. [To the systematics of the Slow Worm (*Anguis fragilis* L.) and its distribution in Slovakia]. Biológia (Bratislava) 22, 908–921 (in Slovak with German and Russian abstracts).

Lác, J., 1968. [Reptiles – Reptilia]. In: Oliva, O., Hrabě, S., Lác, J. (Eds.), [Vertebrates of Slovakia I, Fish, amphibians and reptiles], Vyd. Slov. Akad. Vied, Bratislava (in Slovak).

Mertens, R., Wermuth, H., 1960. Die Amphibien und Reptilien Europas. W. Kramer, Frankfurt am Main.

Moravec, J., 1997. *Anguis fragilis* Linnaeus, 1758. In: Nečas, P., Modrý, D., Zavadil, V. (Eds.), Czech recent and fossil amphibians and reptiles. An atlas and field guide. Edition Chimaira, Frankfurt am Main, pp. 78–79.

Musters, C.J.M., in den Bosch, H.J.A., 1982. Einige Bemerkungen zu den Unterarten von *Anguis fragilis* L., mit Berücksichtigung niederländischer Exemplare (Reptilia: Sauria: Anguidae). Salamandra 18, 196–204.

Petzold, H.-G., 1971. Blindschleiche und Scheltopusik. Die Familie Anguidae. Die Neue Brehm-Bücherei 448. A. Ziemsen, Wittenberg-Lutherstadt.

Rozínek, K. jr., Zavadil, V., Mikátová, B., 2001. [Slow Worm – *Anguis fragilis* Linnaeus, 1758]. In: Mikátová, B., Vlašín, M., Zavadil, V. (Eds.), [Atlas of the distribution of reptiles in the Czech Republic]. AOPK ČR, Brno-Praha, pp. 82–101 [207–213] (bilingual in Czech and English).

Shcherban', M. I., 1976. [To the intraspecific systematics of the Slow Worm (Reptilia, Sauria, *Anguis fragilis* L.)]. Sborn. Trud. Zool. Mus. (Kiev) 36, 81–83 (in Russian).

Sindaco, R., Jeremchenko, V.K., 2008. The reptiles of the Western Palearctic. 1. Annotated checklist and distributional atlas of the turtles, crocodiles, amphisbaenians and lizards of Europe, North Africa, Middle East and Central Asia. Edizioni Belvedere, Latina.

Stojanov, A., 2001. Beobachtung einer Kopulation zwischen den beiden Unterarten der Blindschleiche (*Anguis fragilis* L.). Historia Naturalis Bulgarica 13, 155–157.

Trutnau, L., 1975. Europäische Amphibien und Reptilien. Belser Verlag, Stuttgart.

Valakos, E.D., Pafilis, P., Sotiropoulos, K., Lymberakis, P., Maragou, P., Foufopoulos, J., 2008. The amphibians and reptiles of Greece. Edition Chimaira, Frankfurt am Main.

Voipio, P., 1962. Multiple phaneromorphism in the European Slow-Worm (*Anguis fragilis*) and the distributional and evolutionary history of the species. Ann. Zool. Soc. 'Vanamo' 23, 1–20.

Völkl, W., Alfermann, D., 2007. Die Blindschleiche – die vergessene Echse. Z. Feldherpetologie, Beiheft 11. Laurenti-Verlag, Bielefeld.