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An ancient lineage of slow worms, genus *Anguis* (Squamata: Anguidae), survived in the Italian Peninsula

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ABSTRACT

Four species of legless anguid lizard genus *Anguis* have been currently recognized: *A. fragilis* from western and central Europe, *A. colchica* from eastern Europe and western Asia, *A. graeca* from southern Balkans, and *A. cephallonica* from the Peloponnese. Slow worms from the Italian Peninsula have been considered conspecific with *A. fragilis*, despite the fact that the region served as an important speciation center for European flora and fauna, and included some Pleistocene glacial refugia. We used mitochondrial and nuclear DNA sequences to investigate the systematic and phylogenetic position of the Italian slow-worm populations and morphological analyses to test for phenotypic differentiation from *A. fragilis* from other parts of Europe. Our phylogenetic analyses revealed that Italian slow worms form a distinct deeply differentiated mtDNA clade, which presumably diverged during or shortly after the basal radiation within the genus *Anguis*. In addition, the specimens assigned to this clade bear distinct haplotypes in nuclear PRLR gene and show morphological differentiation from *A. fragilis*. Based on the differentiation in all three independent markers, we propose to assign the Italian clade species level under the name *Anguis veronensis* Pollini, 1818. The newly recognized species is distributed throughout the Italian Peninsula to the Southern Alps and south-eastern France. We hypothesize that the Tertiary Alpine orogeny with subsequent vicariance might have played a role in differentiation of this species. The current genetic variability was later presumably shaped in multiple glacial refugia within the Italian Peninsula, with the first splitting event separating populations from the region of the Dolomite Mountains.

1. Introduction

Due to the complex geological history and habitat diversity, the Mediterranean region encompassing the Balkan, Italian, and Iberian peninsulas played a crucial role for the origin of the biodiversity of the European fauna and its subsequent diversification. For many taxa the peninsulas represent the radiation centers, areas with the highest in-group diversity and centers of endemism (Blondel et al., 2010; Hidalgo-Galiana and Ribera, 2011). The origin of many of the oldest extant lineages of terrestrial animal groups dates back to the Oligocene, while the origin of the younger lineages could be related to the Late Oligocene–Early Miocene separation of Tethys and Paratethys with the diversification being mainly driven by subsequent vicariance during the Miocene (Oosterbroek and Arntzen, 1992). Later, in the Quaternary, climate changes had strong effect on European fauna and caused extinctions or repeated range contractions and expansions in many species or their populations. While populations survived glaciations in refugia situated primarily within the three main Mediterranean peninsulas, they expanded to the northern areas during the warmer interglacial periods and particularly after the last glaciation (e.g. Feliner, 2011; Hewitt, 1996, 1999; Taberlet et al., 1998; Weiss and Ferrand, 2007). Within vertebrates, genetic patterns of less vagile taxa, such as amphibians and reptiles, were particularly influenced by population contractions and expansions and their assemblages thus often better reflect location of glacial refugia than contemporary climate (Araújo and Pearson, 2005; Araújo et al., 2006).

Slow worms, legless lizards of the genus *Anguis* Linnaeus, 1758, inhabit a large territory of the Western Palearctic region including

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all three southern European peninsulas (Völkl and Alfermann, 2007). They form a species complex with the highest diversity found in the Balkan Peninsula, where all four currently recognized species occur, *A. cephallonica* Werner, 1894, *A. colchica* (Nordmann, 1840), *A. fragilis* Linnaeus, 1758, and *A. graeca* Bedriaga, 1881 (Gvoždík et al., 2010; Jablonski, 2013). *Anguis fragilis* has been believed to be the only species of the genus distributed in the Iberian and Italian Peninsulas, although only taxonomic identity of the Spanish samples was confirmed with molecular-phylogenetic methods (Gvoždík et al., 2010). So far, no controversy has been raised regarding the taxonomic identity of the slow worms from the Italian Peninsula, although a potentially important role of this peninsula for the diversification of reptiles is suggested by the endemic occurrence of other reptile taxa. Autochthonous populations of the lacertid lizard *Podarcis siculus* and the skink *Chalcides c. chalcides*, revalidation of the snake *Zamenis lineatus* from southern Italy (Lenk and Wüster, 1999), and description of the Sicilian endemic pond turtle *Emys trinacris* (Fritz et al., 2005) could serve as examples.

The basal divergence within the slow-worm lineage pre-dates the Quaternary and was estimated to have occurred during the Late Miocene based on the molecular-evolutionary rate of the studied mitochondrial DNA fragment (Gvoždík et al., 2010). However, relatively rich fossil record indicates that the genus *Anguis* is very old and could be dated further back to the Eocene or Oligocene, with the oldest fossils resembling members of the genus known from the Early Eocene of France (Augé, 2003; Estes, 1983; Hecht and Hoffstetter, 1962).

Taking all this into account, we could formulate several possible hypotheses about the origin and relationships of the slow worms from the Italian Peninsula. Virtually no evidence provided so far about phenotypic differentiation and no doubts raised about taxonomic identity of a relatively common lizard allow us to hypothesize that the peninsula might have been colonized relatively recently after the Quaternary glaciations from their supposed refugia in the north-western Balkans or southern France (Gvoždík et al., 2010). Another possibility, which is not mutually exclusive with our first hypothesis, is that they could have survived glaciations in one or more refugia located within the Italian Peninsula. According to this hypothesis, the Italian populations would represent a unique evolutionary lineage presumably divergent from other slow-worm taxa. In this scenario, their origin could be dated back even to relatively distant history and pre-Quaternary processes such as vicariance related to the Tertiary Alpine orogeny, which might also have played a role in their differentiation and shaping diversity and distribution.

Here, we used phylogenetic and phylogeographic approaches employing mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) sequence data to find whether the slow worms from the Italian Peninsula are genetically differentiated from *A. fragilis* and other slow worms, and if so, to infer their phylogenetic relationships within the genus. Furthermore, we performed a morphological comparison of the Italian slow worms with *A. fragilis* from adjacent parts of Europe to study the pattern of phenotypic differentiation within this group.

2. Material and methods

2.1. Sampling

Tissue samples of slow worms from Italy ($N = 14$) and additional European localities ($N = 18$) were obtained mainly from road-killed individuals and museum voucher specimens (see Table 1). Oral swabs, blood droplets or miniature tail biopsies were taken from a small number of living animals. We also supplemented our data set with sequences used in Gvoždík et al. (2010) corrected in respect to one sample of *A. graeca* previously erroneously allocated to Užice, Serbia, but in fact originating from southern Albania. Mitochondrial DNA sequence of another *A. graeca* specimen from Montenegro was completed by the previously missing fragment. Genetic material is listed in Table 1 and distribution of the sampling localities is depicted in Fig. 1. We used three taxa of two related Western Palearctic anguid genera (sensu Macey et al., 1999) [*Hyalosaurus koellikeri* Günther, 1873 and two subspecies of *Pseudopus apodus* (Pallas, 1775)] as outgroups in phylogenetic analyses, as well as in calculations and comparison of the genetic distances.

For morphological comparison we used 179 preserved vouchers of *A. fragilis* and Italian slow worms from several European museums (see Table 2). Slow worms were *a priori* grouped based on the samples distribution and species' identification obtained by genotyping individuals from the respective areas (Italian Peninsula and south-eastern France $N = 86$ and *A. fragilis* from other parts of Europe $N = 93$; Fig. 2, Table 1). Specimens from the areas of potential hybrid zones (see Section 3.3) were excluded from the analyses.

2.2. Molecular laboratory procedures

We amplified the complete mtDNA gene encoding NADH dehydrogenase subunit 2 (*ND2*) along with five subsequent transfer RNA genes (*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*. Primers L4437n and H5934 were used for both PCR and sequencing (Gvoždík et al., 2010; Macey et al., 1997). Additional internal forward (AinND2F) and reverse (AND2inR2, AND2inRc) primers were employed for sequencing (Gvoždík et al., 2010). We also amplified and sequenced a fragment of the nuclear protein-coding gene for prolactin receptor (*PRLR*) with primers PRLR_f1 and PRLR_r3 (Townsend et al., 2008). As the oocyte maturation factor gene (*C-mos*) is characterized by a low variation within the slow-worm complex (Gvoždík et al., 2010), only two individuals from southern Italy were amplified and sequenced for this gene with primers S77 and S78 (Lawson et al., 2005) to compare them with the other taxa. For further details on laboratory procedures see Gvoždík et al. (2010). All sequencing was performed by Macrogen Inc. (Seoul, S. Korea, http://www.macrogen.com) and sequences of each new unique haplotype have been deposited in GenBank (see Table 1 for all accession numbers).

2.3. Phylogenetic analyses

Sequences were aligned using Clustal W (Thompson et al., 1994) implemented in BioEdit 7.0 (Hall, 1999) and secondary structure of tRNAs were taken into account (sensu Macey et al., 1999). The complete mtDNA alignment included a 1428 bp stretch, although two positions within *tRNA-Trp*, and one within *tRNA-Cys*, respectively, were excluded from phylogenetic analyses because of unique insertions present only within the outgroups (*P. apodus thracius* and *H. koellikeri*). No stop codons in *ND2* were detected when sequences were translated using the vertebrate mtDNA genetic code in the program DnaSP 5.10 (Librado and Rozas, 2009). We calculated uncorrected *p*-distances in PAUP* 4.0b10 (Swofford, 2003) using all individuals and subsequently averaged manually for the taxa. Four individuals from the newly sequenced ones showed more than one heterozygous position in the *PRLR* fragment (544 bp). Haplotype inference of these cases was conducted by a coalescent-based Bayesian algorithm provided by Phase 2.1 (Stephens et al., 2001; Stephens and Scheet, 2005) implemented in DnaSP 5.10 (Librado and Rozas, 2009). Sequences from Gvoždík et al. (2010) complemented the data set and helped to increase probabilities of haplotype estimates. The analyses were run five

Table 1
List of the slow-worm material used for molecular analyses. Asterisk denotes samples used in the previous study. Museum acronyms: BEV – Biogeography and Ecology of Vertebrates, herpetological collection of the UMR 5175 CEFE in Montpellier; NMP – National Museum Prague, Czech Republic; MNVNKNU – The Museum of Nature at V.N. Karazin National University, Kharkov, Ukraine; ZSM – Bavarian State Collection of Zoology Munich, Germany.

Map	Taxon	Locality	Coordinates		Museum no./reference	Haplotype (GenBank acc. nos.)	
			N	E		mtDNA	*PRLR*
	***Anguis veronensis* (Italian clade)**	**France**					
1		Les Mayons	43.32	06.36	BEV.10381	v11 (KC881558)	Pv1/Pv1 (KC881563)
2		Andon – Gréolières	43.79	06.86	BEV.8148	v10 (KC881557)	Pv1/Pv1
3		Mercantour	44.07	07.51	–	v5 (KC881552)	–
4		Villars sur Var	43.94	07.09	BEV.8147	v9 (KC881556)	Pv1/Pv1
		Italy					
5		Manie, Liguria	44.20	08.37	–	v4 (KC881551)	Pv1/Pv1
6		Portofino, Liguria	44.31	09.20	–	v3 (KC881550)	Pv1/Pv1
7		Lorsica, Liguria	44.42	09.28	–	v3	Pv1/Pv1
8		Bianzano, Lombardia	45.75	09.94	–	v6 (KC881553)	Pv1/P0 (see above/KC881566)
9		Trento-Lagorai, Trentino Alto Adige	46.15	11.46	–	v15 (KC88156)	Pv1/Pv3 (see above/KC881565)
10		Cordelle, Forno di Zoldo, Veneto	46.35	12.14	–	v14 (KC881561)	–
11		Roccagnano, Umbria	43.38	12.11	–	v8 (KC881555)	Pv1/Pv1
12		Cantiano, Marche	43.47	12.63	–	v7 (KC881554)	–
13		Suso, Lazio	41.51	13.08	–	v13 (KC881560)	Pv1/Pv1
14		Bassiano, Monte Croce, Lazio	41.56	13.10	–	v13	Pv1/Pv1
15[a]		Monti Pizzi, Abruzzo	41.94	14.20	–	v12 (KC881559)	Pv2/Pv2 (KC881564)
16[b]		Torrente Peglio, Campania	40.31	15.58	–	v1 (KC881548)	Pv1/Pv1
17		Pollino, Basilicata	40.04	16.10	–	v2 (KC881549)	Pv1/Pv1
	Anguis fragilis	**Spain**					
18*		Vilarmiel, Galicia	42.48	−07.12	Albert et al. (2009)	f7 (EU443256)	–
19		Torla, Pyrenees Mts.	42.62	−00.11	–	f14 (KC881545)	Pf1/Pf1 (GQ285105)
		France					
20		Cabrières	43.58	30.37	BEV.9248	f15 (KC881546)	Pf1/Pf1
21		Le Tholy	48.08	60.76	BEV.11018	f14	Pf1/Pf4n (see above/GQ285108.2)
		United Kingdom					
22*		Kent, Kingsferry Bridge	51.25	00.75	Ast (2001)	f1 (FJ66654)	–
		Czech Republic					
23*		Stráž nad Ohří	50.33	13.10	–	f1	Pf3/Pf4n[f] (GQ258107/see above)
24*		Nové Údolí	48.83	13.80	–	f1	–
25*		Malá Skála	50.63	15.18	–	f1	–
26*		Ondřejovice	50.25	17.35	–	f3 (FJ66656)	–
27*		Rantířov	49.41	15.52	–	f2 (FJ66655)	Pf1/Pf1
28*		Nejdek	48.82	16.77	–	f1	–
		Slovakia					
29*		Bratislava	48.15	17.07	–	f1	Pf3/Pf3
		Slovenia					
30		Založče	45.90	13.90	–	f6 (FJ66659)	Pf1/Pf1
31		Kozina	45.60	13.95	–	f9 (KC881540)	–
		Croatia					
32		Prezid, Velebit Mts.	44.25	15.80	–	f1	Pf1/Pf4n
		Bosnia and Herzegovina					
33		Korita	43.03	18.49	BEV T4022	f11 (KC881542)	–
		Serbia					
34[c]		Užice	43.86	19.84	–	f10 (KC881541)	–
		Greece					
35*		Mesoropi	40.89	24.06	–	f4 (FJ66657)	Pf2/Pf3 (GQ285106/see above)

(*continued on next page*)

Table 1 (*continued*)

Map	Taxon	Locality	Coordinates		Museum no./reference	Haplotype (GenBank acc. nos.)	
			N	E		mtDNA	*PRLR*
36*		Lepida – Megalo Livadi junction	41.37	24.63	NHMC 80.3.92.2	f5 (FJ66658)	Pf2/Pf2
		Germany					
37		Murnauer Moos	47.63	11.15	ZSM 176/2009	f1	P0/P0
38		München	48.17	11.47	ZSM 1922/2008	f13 (KC881544)	P0/P0
		Austria					
39		Hallstatt	47.55	13.66	–	f12 (KC881543)	Pf1/P0
	Hybrid *fragilis–veronensis*	**Italy**					
40		Campiolo, Friuli-Venezia Giulia	46.40	13.17	–	f8 (KC881539)	Pf1/Pv1
		Slovenia					
41*		Bohinj Lake, Stara Fužina	46.29	13.90	NMP6V 72692	f6	Pf1/Pv1[g]
	Anguis colchica	**Czech Republic**					
42*		Hostětín	49.05	17.88	NMP6V 73238	c2 (FJ666577)	–
43*		Štramberk	49.58	18.10	NMP6V 72822	c3 (FJ666578)	Pc1/Pc1 (GQ285112)
		Slovakia					
44*		Rovné	48.92	18.95	–	c1 (FJ666576)	Pc1/Pc1
45*		Šuňava	49.03	20.08	–	c1	–
46*		Chlmecká skalka	48.88	21.93	–	c1	Pc1/Pc1
		Poland					
47		Kamieniec	50.45	21.25	–	c12 (KC881543)	–
48*		Bocki	52.65	23.05	–	c4	Pc1/Pc1
		Lithuania					
49*		Marcinkonys	54.04	24.44	–	c6 (FJ666581)	Pc1/Pc1
50*		Paluše	55.33	26.10	–	c6	Pc1/Pc1
		Ukraine					
51		Sokyryany	48.49	27.42	–	c6	–
52		Sharivka	50.04	35.43	MNVNKNU G1445	c6	–
		Romania					
53*		Finatale Clujuluj	46.83	23.62	–	c5 (FJ666580)	Pc1/Pc1
		Russia					
54*		Babukal, Krasnodarsky Territory	43.67	39.63	Macey et al. (1999)	c11 (AF085622)	–
		Turkey					
55*		Hopa	41.40	41.44	NMP6V 73694	c10 (FJ666585)	Pc2/Pc2 (FJ666584)
		Georgia					
56*		Vardzia – Apnia road	41.37	43.27	–	c9 (FJ666584)	Pc2/Pc3 (see above/GQ285114)
57*		Telavi	41.92	45.49	–	c9	Pc3/Pc5 (see above/GQ285116)
		Iran					
58*		Motalla Sara-ye Lemir	38.20	48.87	NMP6V 72678	c7 (FJ666582)	Pc2/Pc2
59*		Nowshar	36.65	51.50	NMP6V 72680	c8 (FJ666583)	Pc2/Pc4
	Anguis graeca	**Montenegro**					
60*		Ulcinj	41.93	19.21	NMP6V 71272	g14b[e] (FJ666573.2)	–
		Albania					
61*		Diviakë	40.95	19.47	–	g13 (FJ666572)	Pg1/Pg1 (GQ285109)
62*		Dukat	40.21	19.58	–	g15 (FJ666574)	–
63*		Himarë	40.68	19.66	–	g7 (FJ666566)	–
64*[d]		Syri i Kaltër	39.92	20.19	–	g8 (FJ666567)	Pg1/Pg1
65*		Ersekë, Shelegurë Lake	40.32	20.67	–	g4, g5 (FJ666563, FJ666564)	–
66*		Korce	40.61	20.82	NMP6V 73232	g16 (FJ666575)	–

	Greece						
67*	Kerkyra, Korfu	39.59	19.90	NHMC 80.3.92.22	g11 (FJ666570)		Pg1/Pg2 (see above/GQ285110)
68*	Gliki, Acherondas	39.33	20.55	–	g9, g12 (FJ666568, FJ666571)		–
69*	Aoos River, near Konitsa	40.05	20.76	NHMC 80.3.92.17	g6 (FJ666565)		–
70*	Ampelochori	39.53	21.03	NHMC 80.3.92.21	g10 (FJ666569)		Pg1/Pg1
71*	Pertouli	39.54	21.47	NHMC 80.3.92.16	g2 (FJ666561)		–
72*	Fylakti	39.30	21.68	NHMC 80.3.92.4	g2		–
73*	Stomio	39.89	22.62	–	g3 (FJ666562)		Pg1/Pg1
74*	Pefki – Artemision, Evvoias	39.01	23.23	NHMC 80.3.92.18-19	g2		–
75*	Kryoneritis, Evvoia	38.93	23.28	NHMC 80.3.82.5	g2		Pg3/Pg3 (GQ285111)
76*	Mornos River	38.49	22.06	–	g1 (FJ666560)		Pg1/Pg1
	Anguis cephallonica						
	Greece						
77*	Gialova, Peloponnese	36.95	21.70	–	ce1 (FJ666586)		Pce1/Pce1 GQ285104)
78*	Stymfalia Lake, Peloponnese	37.88	22.48	NHMC 80.3.92.1	ce2 (FJ666587)		Pce1/Pce1

[a] Additional nuclear gene (*C-mos*) sequenced – haplotypes Cfc1/Cfc1 (GQ285120).
[b] Additional nuclear gene (*C-mos*) sequenced – haplotypes Cv1/Cv1 (KC881538).
[c] Sample, although listed, not used in the previous study (Gvoždík et al., 2010). The haplotypes before erroneously allocated to Užice, Serbia belong to the sample from Syri i Kaltër, Albania (no. 64).
[d] Sample used in the previous study although erroneously allocated to Užice, Serbia.
[e] Missing fragment from the previous study was completed.
[f] The haplotype estimation was revised. See text for details.
[g] Newly phased sequence.

times 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. All haplotypes were estimated with high probability ($\geqslant$0.94) and all were used in subsequent phylogenetic analyses. The two individuals sequenced for *C-mos* were homozygous in the analyzed 555 bp stretch. No stop codons were detected in nuclear haplotypes as checked by translation with the universal nuclear genetic code using BioEdit 7.0 (Hall, 1999). Haplotype networks for the gametic-phased nuclear data were constructed using the statistical parsimony algorithm implemented in TCS 1.21 (Clement et al., 2000) under the 95% limit of parsimony.

Haplotype data sets prepared by Collapse 1.2 (Posada, 2006) were used for subsequent phylogenetic analyses. We tested four data partitioning strategies reflecting functional categories (protein-coding portion and transfer RNAs) and codon positions (1st, 2nd, 3rd) for the mtDNA marker and three for the nuclear PRLR gene (Table 3). For each data partition the best model of sequence evolution was selected with jModelTest 2.1.2 (Darriba et al., 2012) based on the Bayesian information criterion (BIC). The data were analyzed under the Bayesian approach (BA) with MrBayes 3.2 (Ronquist et al., 2012) for each partitioning strategy. Parameters for each partition were unlinked and rates were allowed to vary independently. Two separate runs, with four chains for each run, of six million generations were conducted simultaneously with sampling every 100th tree. First 20% of trees were discarded as the burn-in after inspection for stationarity of log-likelihood scores of sampled trees and check for convergence of different runs in Tracer 1.5 (Rambaut and Drummond, 2009). Majority-rule consensus trees were drawn from the post-burn-in trees and the posterior probabilities (bpp) were calculated as the frequency of samples recovering any particular clade.

The optimal partitioning strategy was selected based on the performance comparison of the Bayesian analyses of each partition scheme done by pair-wise comparisons of twice the natural logarithm of the Bayes factor, $2\ln(BF_{21})$. Positive values exceeding 10 were used as a threshold for preferring the second strategy over the first strategy (Brandley et al., 2005; Brown and Lemmon, 2007; Kass and Raftery, 1995; see Table 4).

We further performed partitioned maximum likelihood (ML) analyses with the most appropriate partition scheme (Table 4) for the mtDNA and PRLR nuclear markers using RAxML 7.2.6 (Stamatakis, 2006). The general time-reversible model with rate heterogeneity was applied for each partition independently and clade support was assessed by 1000 bootstrap pseudoreplicates. A topologically constrained ML tree (*A. fragilis* and the Italian populations monophyletic) was also calculated and the Shimodaira–Hasegawa test (SH test; Shimodaira and Hasegawa, 1999) as implemented in RAxML applied to evaluate a significance of difference in likelihood scores between the constrained and unconstrained trees.

2.4. Morphological comparison

To compare slow worms from the Italian Peninsula with *A. fragilis* from other parts of Europe we used 10 metric, 11 meristic and 6 categorical characters for univariate and multivariate statistics (for the list of all characters and their definitions see Supplementary data Table S1). The two groups of slow worms were first tested for differences in their body length (snout-vent length, SVL) with *t*-test. Subsequently the SVL was used as a covariate to test for the differences in the tail length (TL) with analysis of covariance (ANCOVA). Differences in all head dimensions (two head length measurements, head height, head width, nostril length, rostrum length, eye length, distance rostrum-eye)

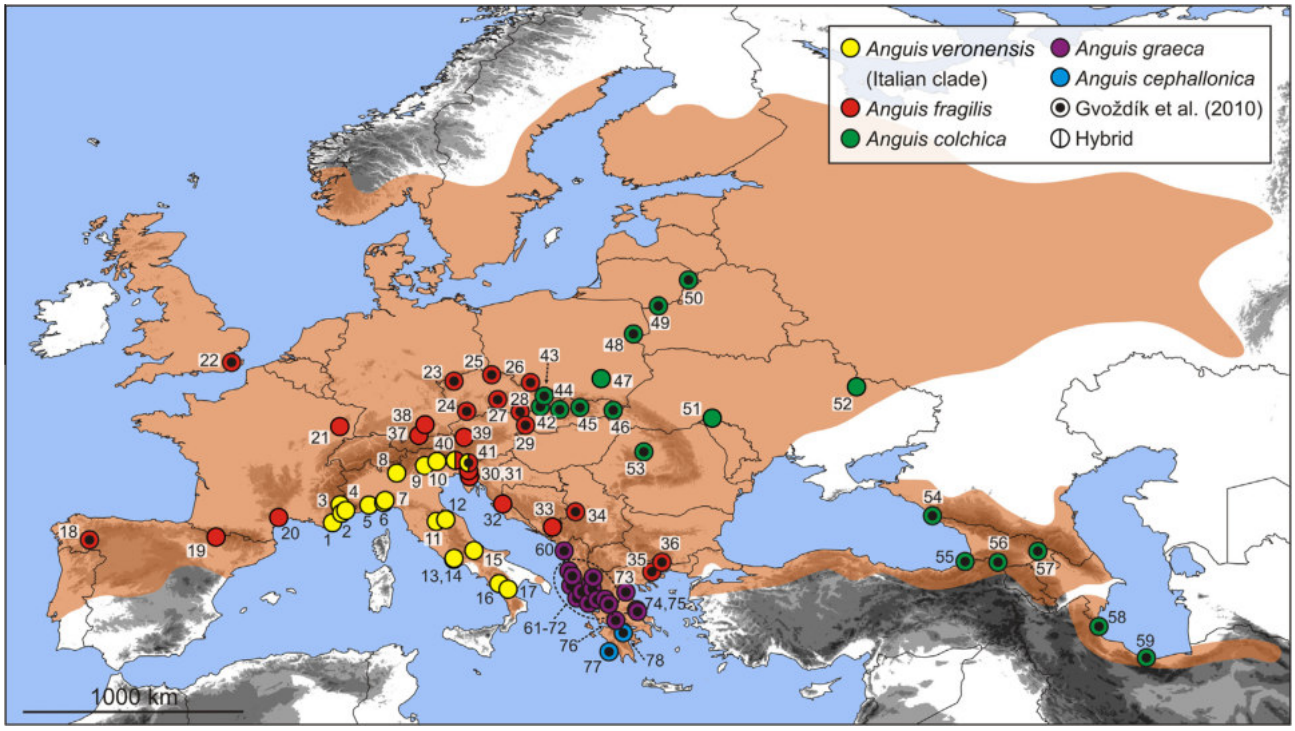

Fig. 1. Map showing the origin of the slow-worm samples used for molecular analyses and the distribution range of the genus (ochre). For the list of localities see Table 1.

were tested with multivariate analysis of covariance (MANCOVA) with SVL used as a covariate and also with univariate ANCOVAs. Meristic characters (number of dorsal, ventral, anal, subcaudal, supraocular, submaxillar, supralabial scales, and number of the scale rows around body in four body segments) were compared with multivariate analysis of variance (MANOVA) and *t*-test. The categorical characters were compared for the frequency of their occurrence in both species with chi-square test (type of prefrontal scales position; presence of the ear opening was not statistically compared as we found no variation in this trait) and log-linear regression (coloration and pattern characters; the amount of dorsal spots, presence and intensity of vertebral line, presence and intensity of the border between dorsal and lateral coloration, amount of the dark pigment in the ventral side of the body). Correct groupings of the individuals were tested by discriminant function analysis (DFA) using leave-one-out cross-validation (Lance et al., 2000). The sexes were analyzed separately to avoid misinterpretation of the differences caused by sexual dimorphism and in all statistical tests only adult specimens with known sex were used. All statistical analyses were performed in SPSS 17.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Mitochondrial DNA

The resulting mtDNA dataset (1425 bp), excluding the outgroups, contained 324 polymorphic sites out of which 278 were parsimony informative, and yielded 60 haplotypes. Thirty-two newly sequenced individuals produced 27 haplotypes, including 24 new ones. The ML (Fig. 3) and BA consensus trees were essentially identical with respect to the topology of the main *Anguis* lineages and recovered the same, previously detected (Gvoždík et al., 2010), clades/species and their relationships: (*A. cephallonica*, (*A. fragilis*, (*A. colchica*, *A. graeca*))). However, from the 24 new haplotypes only nine belonged to the previously recognized clades, one to the *A. colchica* clade (c12 from Poland) and eight to the *A. fragilis* clade (f8–f15; one codon deletion in *ND2* was detected in all new *fragilis* haplotypes). The remaining 15 haplotypes (v1–v15), originating from Italy and south-eastern France, formed a new distinct lineage which we tentatively name "the Italian clade". In both phylogenetic approaches (ML, BA) the Italian clade was recovered as the sister lineage to *A. cephallonica*, however with virtually no support. Therefore the deep relationships within the tree remain unresolved, polytomic, with unclear position of the Italian clade relatively to *A. cephallonica* and the clade containing *A. fragilis*, *A. colchica* and *A. graeca* (showing relatively high support 87/0.97 in ML/BA).

The three newly sequenced individuals of *A. colchica* had two haplotypes, c6 (Ukraine) and c12 (Poland). Both haplotypes belong to the eastern European *A. colchica* subclade, *A. colchica incerta* Krynicki, 1837 (Gvoždík et al., 2010). The newly analyzed individuals with *A. fragilis* haplotypes filled some geographic gaps in the knowledge on distribution of the haplotypes, such as in eastern Spain, southern and eastern France, southern Germany, Austria, extreme north-eastern Italy, Slovenia, Croatia, Bosnia and Herzegovina, and Serbia. No distinctly divergent subclade was detected within the *A. fragilis* clade, which is characterized by relatively low genetic variation in respect to its wide distribution range. On the contrary, the Italian clade possesses relatively high genetic variation with conspicuous, highly supported basal split separating samples from the Dolomite Mountains (Eastern Alps, NE Italy) from all others. The result of the SH test clearly rejected the constrained tree (*A. fragilis* and the Italian clade monophyletic) over the best tree where the two species were not in a sister relationship ($p < 0.01$). Genetic distances between all taxa are shown in Table 5.

3.2. Nuclear DNA

The number of polymorphic sites in the nuclear *PRLR* fragment (544 bp), excluding the outgroup genera, was 20 with nine

Table 2
List of the material of *A. veronensis* and *A. fragilis* used for morphological analyses. Museum acronyms: HNHM – Hungarian Natural History Museum, Budapest, Hungary; MZUF – La Specola – Zoological Museum of the University of Florence, Italy; NMW – Natural History Museum, Vienna, Austria; NMP – National Museum Prague, Czech Republic; MUZAC – Museum of the Department of Biology, Aldo Moro University, Bari, Italy; SNMB – Slovak National Museum Bratislava, Slovakia; ZFMK – Zoological Research Museum Alexander Koenig Bonn, Germany.

Map	Taxon	Locality	Coordinates		*N*	Museum no.
			N	E		
	***Anguis veronensis* (Italian clade)**	**France**				
m1		Mercantour	44.07	07.51	1	–
		Italy				
m2		Fenestrelle, Piemonte	45.04	07.05	1	NMW 25068/6
m3		Camposilvano-Passo della Fugazzo, Veneto	45.76	11.15	1	NMW 37294
m4		Levico, Trentino	46.01	11.30	2	NMW 25059/1-2
m5		Badalucco, Liguria	43.92	07.81	6	NMW 27434–27439
m6		Borgomaro, Liguria	43.98	07.95	3	MZUF 27401, 27404, 27405
m7		Lucinasco, Liguria	43.97	07.96	9	27408, 27410, 27413, 27415, 27416, 27418, 27420, 27421, 27423
m8		San Lazzaro Reale, Liguria	43.88	08.02	1	MZUF 6970
m9		Altare, Liguria	44.34	08.34	1	MZUF 12807
m10		Torre del Mare, Liguria	44.24	08.44	2	MZUF 27286, 27287
m11		Genova, Liguria	44.40	08.93	1	HNHM 58.772.1
m12		Cavi di Lavagna, Liguria	44.29	09.38	2	NMW 25077/1-2
m13		Pulica, Toscana	44.15	10.05	2	MZUF 7595, 7597
m14		Campagrina, Toscana	44.06	10.26	1	MZUF 7584
m15		Lucca 1, Toscana	43.82	10.50	1	ZFMK 761618
m16		Lucca 2, Toscana	43.84	10.57	4	MZUF 12836, 12838, 12839, 12840
m17		Cutigliano, Toscana	44.10	10.76	6	MZUF 12877–12880, 12882, 12883
m18		Scarlino, Toscana	42.91	10.85	1	MZUF 37293
m19		Gambassi Terme, Toscana	43.56	10.98	1	ZFMK 59086
m20		Monticiano, Toscana	43.14	11.18	1	MZUF 37271
m21		Giogoli, Toscana	43.73	11.20	3	MZUF 12906, 12907, 12910
m22		Pratolino, Toscana	43.86	11.30	1	MZUF 26584
m23		San Jacopo, Toscana	43.86	11.31	1	MZUF 26524
m24		Monteloro, Toscana	43.82	11.37	1	MZUF 26523
m25		Borselli, Toscana	43.80	11.54	1	MZUF 26521
m26		Upacchi, Toscana	43.51	12.00	1	ZFMK 64938
m27		Bevagna, Umbria	42.93	12.61	1	MZUF 12843
m28		Riofreddo, Lazio	42.04	13.01	1	–
m29		Fiumata, Lazio	42.25	13.10	1	MZUF 31963
m30		Roma, Lazio	41.29	13.25	1	ZFMK 53034
m31		Opi, Abruzzo	41.78	13.85	1	MZUF 17991
m32		Val Fondillo, Abruzzo	41.75	13.87	1	MZUF 31962
m33		Caramanico, Abruzzo	42.16	14.00	4	MZUF 12853–12856
m34		Lagoon of Lesina, Puglia	41.90	15.36	1	MUZAC 1696
m35		Foresta Umbra, Puglia	41.82	16.03	1	MZUF 31964
m36		Roccarainola, Campania	40.97	14.56	1	MZUF 25128
m37		Paestum, Campania	40.42	15.00	2	NMW 25051/1-2
m38		Agro di Lagonegro, Basilicata	40.13	15.77	2	MZUF 18731, 18737
m39		Bosco di Monticchio, Basilicata	40.64	15.81	1	MZUF 12816
m40		Spinazzola, Puglia	40.97	16.07	1	MUZAC 1005
m41		Valloncino di Ruggio, Basilicata	39.97	16.09	1	MZUF 34696
m42		Pollino, Basilicata	40.05	16.13	1	MUZAC 1253
m43		Mongiana-Serra San Bruno, Calabria	38.55	16.33	5	MZUF 12899–12903
m44		Lago di Cecita, Calabria	39.37	16.50	1	MZUF 22772
m45		La Corsonara, Calabria	39.37	16.55	1	MZUF 22773
m46		Veglia, Calabria	40.34	17.95	3	NMW 25025/5-7
	Anguis fragilis	**Spain**				
m47		Aragon	42.23	−00.58	1	ZFMK 72046
m48		Lerida	42.25	00.90	1	ZFMK 58056
m49		Mont Sant	41.13	01.25	1	NMW 25062/3
m50		Coll de Nargo	42.17	01.32	1	ZFMK 62678
m51		Barcelona surroundings	41.42	02.13	1	ZFMK 35707
		Andorra				
m52		Andorra	42.55	01.60	1	ZFMK 16556
m53		Soldeu	42.58	01.67	1	ZFMK 62677
		France				
m54		Ariége 1	42.94	01.06	1	ZFMK 87343
m55		Ariége 2	42.99	01.16	1	ZFMK 86800
m56		Gabas	42.60	02.89	1	ZFMK 51749
		Luxembourg				
m57		Junglinster	49.70	06.24	2	ZFMK 41129, 41130
		Germany				
m58		Wesel	51.72	07.68	1	ZFMK 88087

(*continued on next page*)

Table 2 (*continued*)

Map	Taxon	Locality	Coordinates		*N*	Museum no.
			N	E		
m59		Bonn 1	50.72	07.11	2	ZFMK 89092, 89410
m60		Bonn 2	50.70	07.13	1	ZFMK 88856
m61		Wuppertal	51.25	07.17	2	ZFMK 88085, 88086
m62		Wahner Heide	50.85	07.17	1	ZFMK 75085
m63		Mayern-Maria Laach	50.38	07.24	2	ZFMK 88079, 88080
m64		Neunkirchen-Seelscheid	50.85	07.34	1	ZFMK 89409
m65		Spay	50.26	07.64	1	ZFMK 83958
m66		Seck Westerburg	50.57	08.05	1	ZFMK 76386
m67		Herborn	50.68	08.31	1	ZFMK 89408
m68		Kronberg-Taunus	50.18	08.50	1	ZFMK 71617
m69		Dreieich-Sprendlingen	50.03	08.69	2	ZFMK 88077, 88078
m70		Frankfurt am Main	50.15	08.78	4	ZFMK 88069, 90549–90551
m71		Spessart-Bad Orb	50.23	09.36	1	ZFMK 57711
m72		Probsteierhagen	54.36	10.28	2	ZFMK 85181, 85182
m73		Probstei-Hessenstein	54.39	10.38	1	ZFMK 85183
m74		Rügen	54.31	13.35	3	HNHM 62.547.1, 2010.48.1-2
m75		Koblentzer See	53.53	14.13	1	ZFMK 67202
m76		Thale	51.75	11.02	1	HNHM 62.552.1
m77		Quarmbach	51.77	11.13	1	ZFMK 71614
m78		Schwarza-Tal	50.63	11.17	1	ZFMK 57810
m79		Eltmann	49.96	10.65	1	ZFMK 64736
m80		Eisenberg-Pfalz	49.56	08.07	1	ZFMK71021
m81		Hirschhorn-Neckar	49.44	08.90	1	ZFMK 87328
m82		Rutesheim	48.80	08.95	6	ZFMK 39621, 40577–40580
m83		Oberweissach	48.92	09.50	1	ZFMK 55935
m84		Fischbach, Schluchsee	47.84	08.16	1	ZFMK 55935
m85		Blumberg	47.85	08.53	1	ZFMK 64737
m86		Oberpfalz	49.02	12.09	1	ZFMK 84808
m87		Niederneuching	48.25	11.82	1	ZFMK 71168
		Austria				
m88		Admont	47.57	14.46	1	NMW 22861
m89		Hauenstein	47.19	15.08	1	ZFMK 54787
m90		Schneeberg	47.79	15.91	2	ZFMK 60779, 65112
m91		Wien 2	48.24	16.29	1	HNHM 62.550.1
m92		Wien 1	48.20	16.38	1	NMW 13977
m93		Hipples	48.50	16.41	1	–
m94		Eisenstadt	47.86	16.52	2	ZFMK 65100, 86678
m95		Zurndorf	47.98	17.04	2	ZFMK 68657, 71996
		Croatia				
m96		Pula	44.88	13.87	1	ZFMK 63024
m97		Učka	45.25	14.20	1	HNHM 82.65.1
m98		Beli, Cres	45.11	14.35	1	ZFMK 68472
m99		Malinska, Krk	45.12	14.53	2	ZFMK 41467, NMP 6V 35063
m100		Nivice, Krk	45.16	14.54	1	ZFMK 41468
m101		Fort Opus	43.01	17.56	2	NMW 25025/1-2
m102		Ragusa	42.67	18.12	1	NMW 25025/3
		Bosnia and Herzegovina				
m103		Oštrelj	44.48	16.40	3	NMP6V 74385/13-
m104		Kozara	45.04	16.91	1	NMP6V 74388
m105		Ost	43.97	17.46	1	NMP6V 74382
m106		Travnik	44.22	17.67	1	NMW 8162/1
m107		Jablanica	43.65	17.80	1	NMW 25025/4
m108		Konjič	43.65	17.97	1	SNMB 157
m109		Kukovice	43.57	18.06	1	HNHM 2009.35.1
m110		Nišiči	44.05	18.46	1	–
m111		Sutjeska	43.31	18.66	1	NMP6V 74389
m112		Tjenjište	43.36	18.71	1	NMP6V 74386
m113		Maglič	43.29	18.72	2	NMP6V 74384/1-2
m114		Požarnica	44.53	18.78	2	NMP6V 74387, –

parsimony informative sites. Altogether 17 gametic-phased haplotypes were found, including four new ones (haplotypes Pv1–Pv3 and P0) forming a compact cluster in the parsimony haplotype network positioned in between the *A. colchica–A. fragilis*, *A. graeca* and *A. cephallonica* clusters (Fig. 4). Two new haplotypes (Pv1, P0) filled the positions of hypothetical haplotypes from the previously published network (Gvoždík et al., 2010), while the other two haplotypes (Pv2, Pv3) were derived from the most common haplotype (Pv1).

One *A. fragilis PRLR* haplotype (Pf4) from Gvoždík et al. (2010) was shown erroneous with a high probability. Its previous coalescent-based Bayesian estimation from a heterozygous individual (Stráž nad Ohří, Czech Republic) was inferred with low probability (0.51) in one of three heterozygous positions (Gvoždík et al., 2010). Estimation within this study with additional data produced the opposite combination of possible haplotype inference with high average probability (0.99). We named the newly inferred haplotype Pf4n and replaced Pf4 in GenBank (GQ285108.2). The individ-

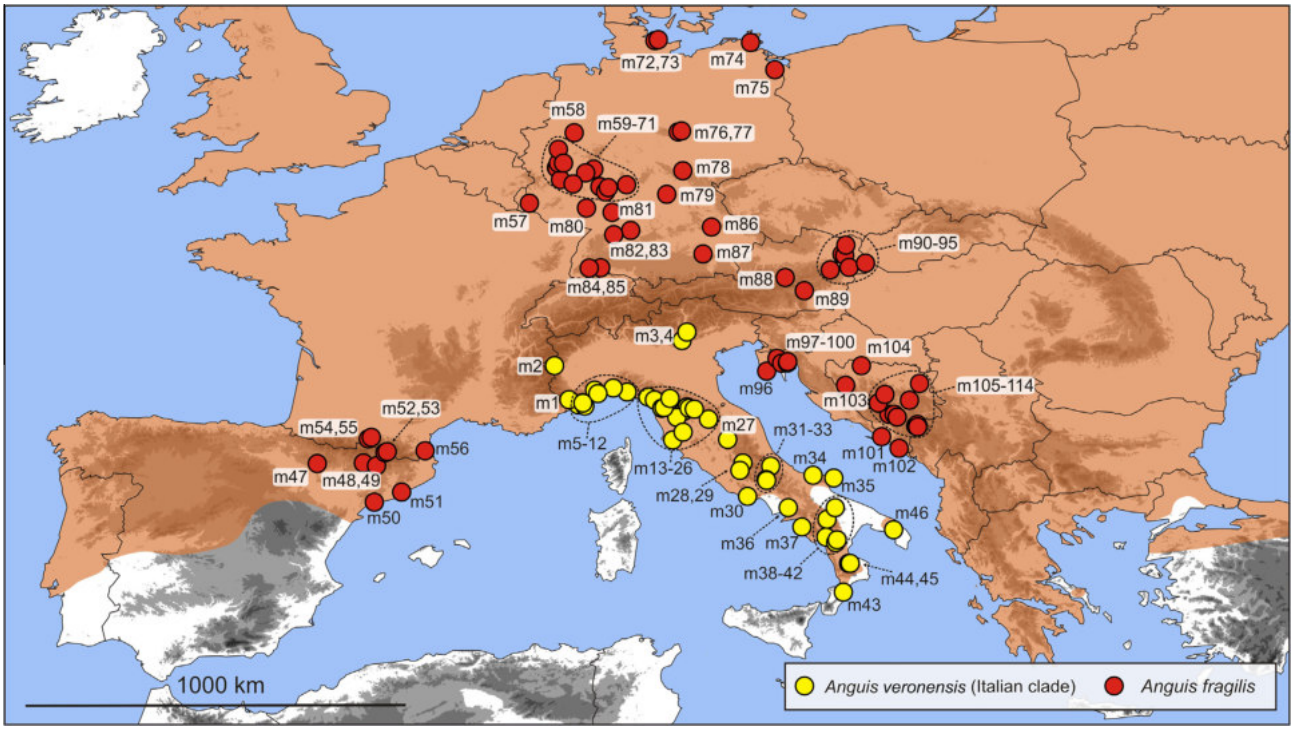

Fig. 2. Map showing the origin of the slow-worm samples used for morphological analyses. For the list of localities see Table 2.

Table 3
Partitioning strategies for the mtDNA fragment and nuclear PRLR gene. Partitions reflect functional categories (protein-coding portion and transfer RNAs) and codon positions (1st, 2nd, 3rd). The table lists the number of partitions (n_{part}), number of parameters (K), partition identities, models selected under the BIC criterion, and natural logarithms of the harmonic means of the likelihood values of Bayesian analyses ($\ln L_{harm}$) for each partitioning strategy. The best partitioning strategies are indicated in bold.

Partition strategy	n_{part}	K	Partition identity	Model	$\ln L_{harm}$
mtP1	1	133	*ND2* + *tRNAs* (unpartitioned)	GTR + G	−6781.2
mtP2	2	262	*ND2*/*tRNAs*	GTR + G/HKY + G	−6781.4
mtP3	3	392	*ND2*-1st + 2nd/*ND2*-3rd/*tRNAs*	GTR + I + G/HKY + G/HKY + G	−6596.9
mtP4	**4**	**516**	***ND2*-1st/*ND2*-2nd/*ND2*-3rd/*tRNAs***	**HKY + G/HKY + I/HKY + G/HKY + G**	**−6547.6**
nP1	1	38	*PRLR* (unpartitioned)	HKY	−999.7
nP2	**2**	**75**	***PRLR*-1st + 2nd/*PRLR*-3rd**	**HKY/F81**	**−986.8**
nP3	3	106	*PRLR*-1st/*PRLR*-2nd/*PRLR*-3rd	JC/K80/F81	−994.4

Table 4
Evaluation of the optimal partitioning scheme for mtDNA and PRLR nuclear markers separately assessed by pair-wise comparisons of twice the natural logarithm of the Bayes factor, $2\ln(BF_{21})$. Positive values exceeding 10 were used as a threshold for preferring the second strategy over the first strategy (Brandley et al., 2005; Brown and Lemmon, 2007; Kass and Raftery, 1995). The best partitioning strategies are in bold. For partition identities see Table 3.

$2\ln(BF_{21})$	mtP1	mtP2	mtP3
mtP1	–		
mtP2	−0.4	–	
mtP3	368.6	369.0	–
mtP4	**467.2**	**467.6**	**98.6**
$2\ln(BF_{21})$	nP1	**nP2**	nP3
nP1	–		
nP2	**25.8**	–	
nP3	10.6	**−15.2**	–

ual from Stráž nad Ohří thus possesses the haplotype combination Pf3/Pf4n (see also Table 1).

The nuclear *C-mos* fragment (555 bp) possesses much lower genetic variation in slow worms as was already pointed out before (Gvoždík et al., 2010). Eleven sites were polymorphic within *Anguis*, with nine being parsimony informative. However, most of the nucleotide variation contributed to the differentiation of *A. cephallonica*, with all mutations being synonymous. One of the two southern Italian samples sequenced for this marker had the most common haplotype Cfc1 (shared with almost all analyzed *A. fragilis* and *A. colchica*), while the other sample had a new haplotype (Cv1) derived by one mutational step from the most common haplotype (Fig. 5).

3.3. Comparison of mtDNA and nDNA and signs of hybridization

Comparing the overall diversity of the mtDNA and nuclear *PRLR* fragments with respect to the Italian populations (including the populations from south-eastern France), it is evident that they form a unique lineage in mtDNA and a compact cluster of closely related haplotypes in *PRLR*, distinct from other populations. There was congruence in this pattern in all Italian and south-eastern French samples with the exception of two individuals from extreme north-eastern Italy (Campiolo) and Slovenia (Stara Fužina): both specimens were characterized by a combination of the most common *A. fragilis* and Italian *PRLR* haplotypes (Pf1/Pv1) and *A. fragilis* mtDNA (haplotypes f8 and f6, respectively). These two samples clearly show hybrid origin with mixed genotypes of *A.*

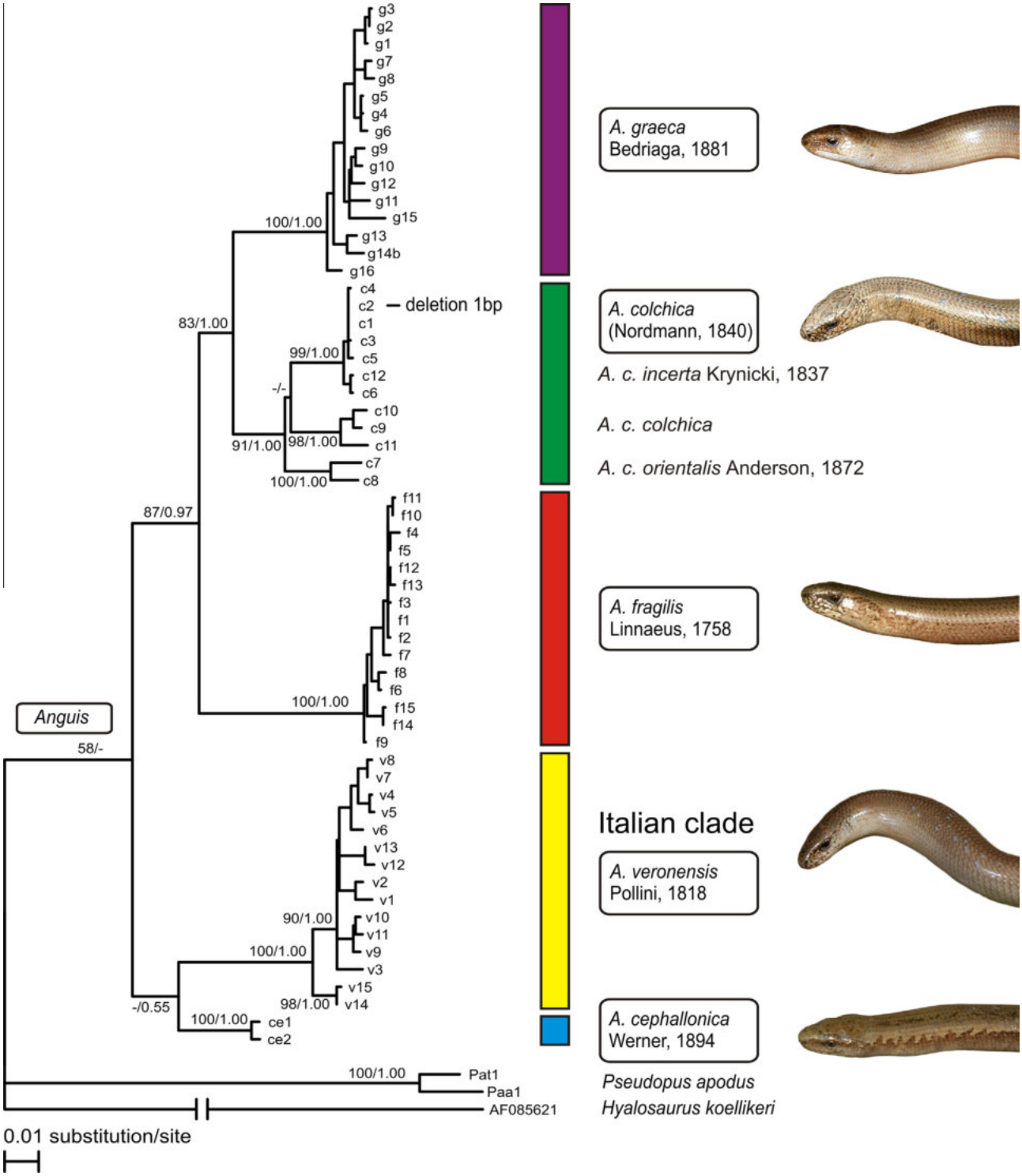

Fig. 3. Maximum-likelihood phylogeny of the slow worms, based on sequences of the 1428 bp fragment of *ND2* and *tRNAs* (*tRNA-Trp*, *tRNA-Ala*, *tRNA-Asn*, *tRNA-Cys*, *tRNA-Tyr*). Statistical support for the major clades is expressed as the percentage bootstrap values and Bayesian posterior probabilities (bpp). Branch support values <50/0.50 bootstrap/bpp are not indicated.

fragilis and the Italian slow worms. Further, three samples from southern Germany and central Austria had *PRLR* haplotype (P0) derived by one mutational step from the most common Italian haplotype in combination with *A. fragilis* mtDNA haplotypes (f1, f12, f13). This might indicate either some level of gene flow between the Italian slow worms and *A. fragilis* or, as we suppose, an ancestral state of P0 haplotype within *Anguis*, i.e. the result of incomplete lineage sorting (see Table 1, Fig. 1).

3.4. Morphological comparison

Our analyses show that adult *A. fragilis* and the Italian slow worms differ in the relative length of the intact tail [ANCOVA, SVL as a covariate; males: $F(1,20) = 9.641$, $p = 0.006$; females: $F(1,36) = 25.332$, $p < 0.001$] (Fig. 6a), with Italian slow worms having longer tails than *A. fragilis* in both males (mean ± SE; Italian slow worms: 206.3 ± 5.4 mm, $N = 12$; *A. fragilis*: 186.6 ± 9.0 mm,

Table 5
Average genetic distances in percentage between all taxa of the genus *Anguis* and outgroup genera *Pseudopus* and *Hyalosaurus* based on uncorrected *p*-distances of the investigated mtDNA fragment (*ND2* and *tRNAs*). In bold, distances related to *A. veronensis*, in italics within-group distances are given. *Anguis veronensis* 2 represents the clade from the Dolomite Mountains (haplotypes v14 and v15), while *A. veronensis* 1 all remaining *A. veronensis* populations.

	A. graeca	*A. colchica*	*A. fragilis*	***A. veronensis***	***A. veronensis* 1**	***A. veronensis* 2** (Dolomites)	*A. cephallonica*	*P. apodus*	*P. a. apodus*
A. graeca	*1.2*								
A. colchica	5.8	*1.8*							
A. fragilis	8.0	7.0	*0.6*						
A. veronensis	**8.8**	**9.2**	**9.2**	***1.4***					
***A. veronensis* 1**	–	–	–	–	***1.4***				
***A. veronensis* 2** (Dolomites)	–	–	–	–	**2.3**	***0.1***			
A. cephallonica	7.6	7.1	7.8	**6.1**	–	–	*0.5*		
P. apodus	13.1	12.4	13.2	**12.9**	–	–	12.2	*1.9*	
P. a. apodus	–	–	–	–	–	–	–	–	–
P. a. thracius	–	–	–	–	–	–	–	–	2.9
H. koellikeri	14.9	14.0	15.6	**15.1**	–	–	14.1	16.0	–

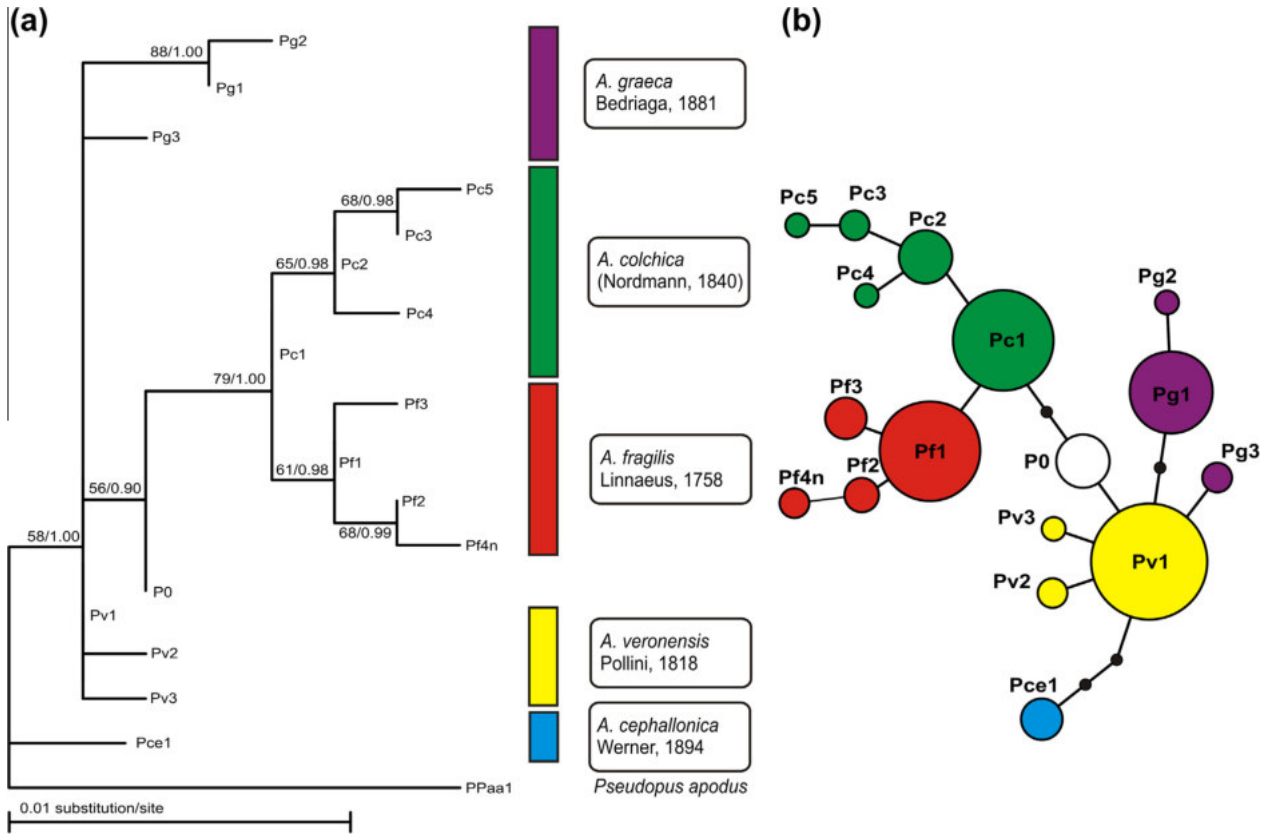

Fig. 4. (a) Maximum likelihood phylogram based on phased haplotypes of the nuclear PRLR gene. Numbers above branches are bootstrap support values for maximum likelihood and Bayesian posterior probability values. (b) Statistical parsimony haplotype network of *PRLR*, with circle sizes proportional to haplotype frequencies and colors matching the main clades (species) in the mtDNA phylogenetic tree. Small black dots = extinct or unsampled haplotypes. Haplotype P0 is presumably an ancestral haplotype found in *A. fragilis* and *A. veronensis* from the Alpine region. Haplotype names as listed in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$N = 11$) and females (Italian slow worms: 196.4 ± 5.8 mm, $N = 22$; *A. fragilis*: 183.9 ± 5.1 mm, $N = 18$). Consequently, both species also differ in the number of subcaudal scales [*t*-test; males: $t(21) = -3.002$, $p = 0.007$; females: $t(37) = -3.695$, $p = 0.001$] (Fig. 6b) with Italian slow worms having more subcaudal scales than *A. fragilis* in both males (Italian slow worms: 150.5 ± 2.4, $N = 12$; *A. fragilis*: 140.7 ± 2.1, $N = 11$) and females (Italian slow worms: 149.1 ± 1.5, $N = 22$; *A. fragilis*: 141.2 ± 1.6, $N = 17$). In females this result was confirmed by post hoc test after significant MANOVA of body scales [$F(11,21) = 2.671$, $p = 0.025$] where none of the remaining characters differed between the two groups. In males, the same analysis was non-significant, presumably due to the small number of specimens with intact tail (results not shown). Both sexes of Italian slow worms have larger heads than *A. fragilis* (MANCOVA; males: $F(8,32) = 2.937$, $p = 0.014$; females $F(8,60) = 2.274$, $p = 0.034$), but only in males the post hoc tests showed differences in head width, head height and anteorbital length (see Supplementary data, Tables S1 and S2).

Both groups also differ in the frequency of the types of the prefrontal scales position (Pearson chi-square; $\chi^2(3) = 12.096$, $p = 0.006$) with significantly higher frequency of type C prefrontal scales position in Italian slow worms (28% in Italian slow worms vs. 6% in *A. fragilis* on average; Fig. 7). We have not found sufficient variation in the external ear opening presence in Italian slow

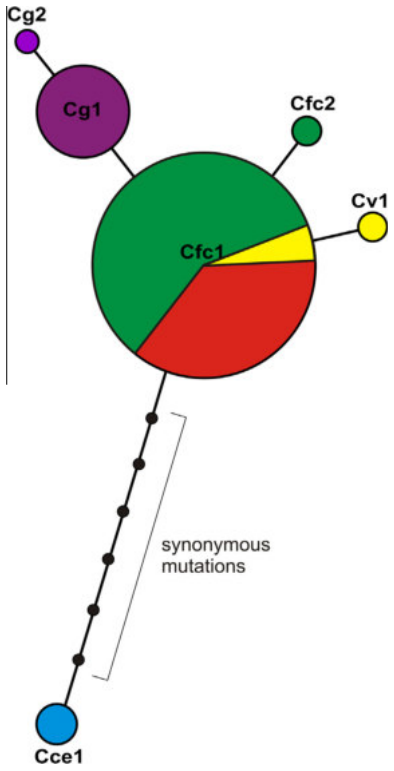

Fig. 5. Haplotype network of the C-mos gene based on the statistical parsimony algorithm (95% limit of parismony). Circle sizes correspond to haplotype frequencies and colors match the main mtDNA clades (species). *Anguis fragilis* and *A. colchica* share the common main haplotype (Cfc1) and the same haplotype was found in one of the two analyzed specimens of *A. veronensis*. Haplotype names as listed in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

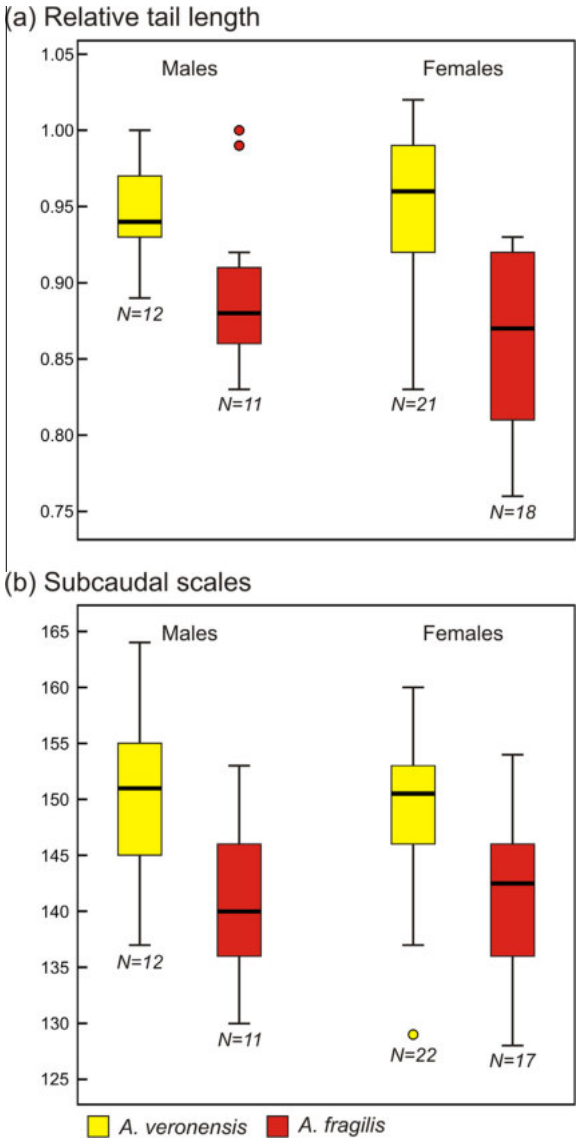

Fig. 6. Box-plots of the relative tail lengths (a) and subcaudal scale numbers (b) in adult *A. veronensis* and *A. fragilis* (only specimens with intact tails were considered). The tail lengths were standardized to the length of the male slow worm with the longest SVL and subsequently normalized to the ratio of the obtained standardized tail length to the longest male standardized tail length (male $TL_{max} = 1.00$). Upper and lower limit of the box represent upper and lower quartile, respectively, the bar inside the box represents median, the ends of whiskers show extreme values, and the individual dots are outliers.

worms and *A. fragilis*, and therefore the character could not have been compared between the groups. The ear opening is not visible in Italian slow worms, which is a typical state in *A. fragilis* as well.

The log-linear analysis of the coloration, which also takes into account correlation among the individual characters, was not significant, while the simple chi-square test showed higher frequency of the occurrence of black abdominal coloration in Italian slow worms in comparison to *A. fragilis* (Pearson; $\chi^2(3) = 12.507$, $p = 0.006$).

Discriminant function analyses (DFA) showed high rate of successful posterior assignment to the respective groups (sexes treated separately): 90.5% (males) and 94.4% (females) in DFAs of metric characters, 86.4% and 90.9% in scale characters, and 100% in combined datasets of both metric and scale characters. However after cross-validation procedure the rates considerably decreased to 52.4% and 66.7% in DFAs of metric characters, 54.5% and 60.6% in scale characters, and 52.4% and 84.4% in combined datasets of both metric and scale characters, in males and females, respectively.

For the summary descriptive statistics see Supplementary data (Table S2).

4. Discussion

4.1. Genetic structure and relationships in Anguis

The results of this study reveal a new evolutionary lineage within the genus *Anguis*. This lineage was detected within the Italian Peninsula and is distributed up to the southern Alps and to south-eastern France (Figs. 1 and 2). All specimens of the Italian mtDNA clade (Fig. 3) are also characterized by distinct *PRLR* haplotypes that are not shared by other slow-worm lineages (Fig. 4b). According to the mtDNA genealogy, the new evolutionary lineage forms part of a basal polytomy within *Anguis* not yet fully resolved. Additionally, the structure in the nuclear PRLR gene reveals that the new *Anguis* lineage occupies an internal position inside the network, supporting an ancestral position within the identified lineages of the genus. Genetic distances between the new *Anguis* evolutionary lineage and all other currently recognized species are larger (8.3% on average) than the interspecific distances among the other lineages within the genus (7.2%) and represent about two thirds of the genetic distance between the genera *Anguis* and *Pseudopus* (12.8% in uncorrected *p*-distances; Table 5). Therefore the genetic distances in the mtDNA markers indicate rather interspecific than intraspecific differentiation. Multilocus inference of phylogenetic relationships within *Anguis* is currently ongoing

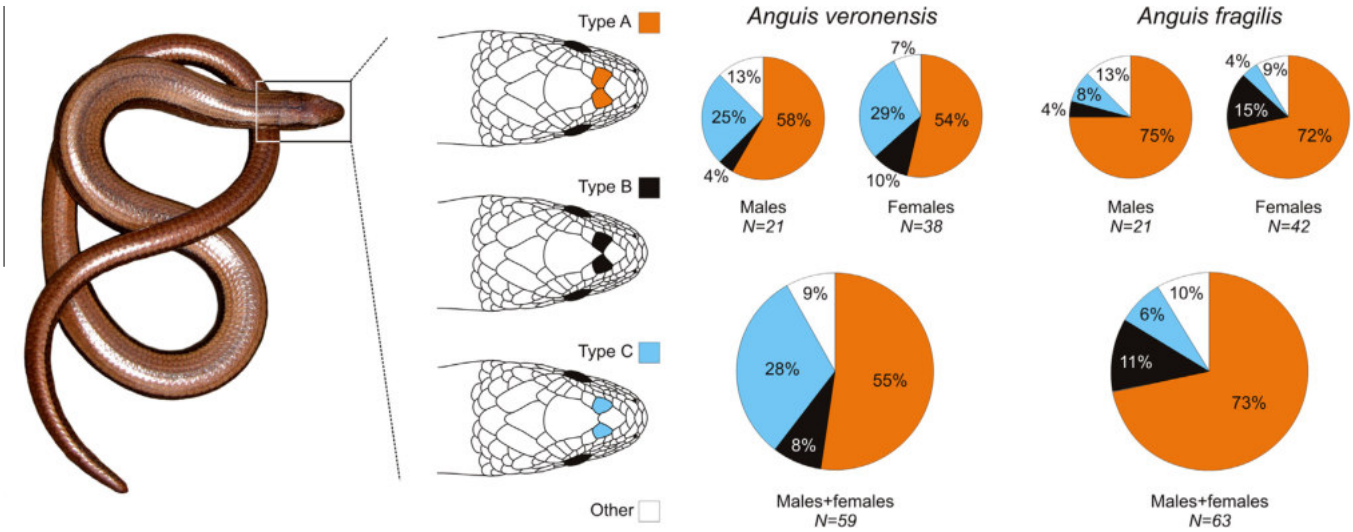

Fig. 7. Prefrontal scales position types in *A. veronensis* and *A. fragilis*. The pie plots show frequencies of the occurrence of each position type.

(Bellati et al., in preparation) and should ascertain more precisely the phylogenetic position of all slow worms.

4.2. Taxonomy and nomenclature

We propose to resurrect the name *Anguis veronensis* Pollini, 1818 for the populations representing the evolutionary lineage of slow worms from the Italian Peninsula and south-eastern France (type locality "Caldiero e la Ruota", Verona Province, Italy; Pollini, 1818).

The recent changes in the taxonomy of the genus *Anguis* proposed by part of our team (Gvoždík et al., 2010) were originally justified on the basis of the genetic species concept (Baker and Bradley, 2006), according to which the genetic species represents a group of genetically compatible interbreeding natural populations that is genetically isolated from other such groups. The newly recognized species *A. veronensis* conforms to this definition, however, we also showed that the lineage has a long independent evolutionary history, represents a lineage of ancestral descendent populations, and maintains its identity from other lineages both on genetic (mtDNA and nDNA) and morphological level. Based on these criteria, *A. veronensis* also represents an evolutionary species as defined by Simpson (1951) and Wiley (1978). With present knowledge we can diagnose *A. veronensis* on the basis of unique nucleotide composition of the ND2 and PRLR genes. Proper morphological diagnosis is presently only possible with respect to parapatric species *A. fragilis*, in comparison to which *A. veronensis* has relatively longer tail (Fig. 7), more subcaudal scales, relatively more robust head and higher rate of the type C of prefrontal scales position (prefrontal scales not in contact; Fig. 6). Both mentioned species were considered conspecific for a long time and based on current evidence they share the largest number of phenotypic characters from among all species of the genus. A detailed morphological analysis of the entire genus is under preparation (Benkovský et al., in preparation).

Several junior synonyms of *A. veronensis* with the type localities within the range of this taxon can be found in Supplementary data (S3). The common name we propose for *A. veronensis* is "Italian slow worm".

4.3. Historical biogeography of Anguis veronensis

Italian slow worms have not been studied using molecular markers prior to this study and no concerns about their morphological similarity to other *A. fragilis* populations have arisen so far. Consequently, the Italian populations have been taxonomically assigned to *A. fragilis*, a species which is distributed throughout western Europe. However, our analyses have not confirmed close phylogenetic relationships of the Italian slow worms with *A. fragilis*, rather it seems that the lineage that we designated *A. veronensis* evolved shortly after the basal split within the genus, independently from the lineage comprising the common ancestor of *A. fragilis*, *A. colchica* and *A. graeca* (as suggested by high support for the clade formed by these three species).

The estimation of divergence times based on the molecular evolution rate of the studied mtDNA region placed the basal radiation of extant forms of *Anguis* to the Late Miocene approx. 5.7 Mya (Gvoždík et al., 2010). However, relatively rich and well preserved fossil record of anguines provides evidence that the genus *Anguis* is very old. There are several Eocene, Oligocene and Early Miocene records from various regions of Europe, mainly from the western part of the genus range (e.g. Klembara, 1979, 1981; Estes, 1983; Augé and Smith, 2009), with the oldest known fossils of the genus resembling the recent species from the Early Eocene locality Prémontré in France, approx. 50 Mya (Augé, 2003). Although fossil records only confirm the presence of slow worms from the Middle Pleistocene in the Italian Peninsula (Delfino, 2004), the relative abundance in other parts of western Europe offers a possibility that the slow worms might have been present in the region for a long time before the Quaternary. It is thus possible that slow worms colonized the peninsula in the Early or Middle Miocene, after the land bridge between the Apennines and the rest of the European continent formed (ca. 20 Mya, Early Miocene; Rögl, 1999), and later gene flow between these newly established populations and more northerly populations has become significantly restricted due to processes connected to the Tertiary Alpine orogeny (Blondel et al., 2010). Geographic separation and vicariance south of the Alps might have subsequently resulted in divergence from the remaining European slow-worm populations. During the Messinian Salinity Crisis in the Late Miocene (ca. 5.96–5.33 Mya), when the large parts of the Mediterranean Sea bottom were exposed connecting the Apennines with Balkans, this southern region might have been inhabited by a population which not only gave rise to *A. veronensis*, but also to another ancient lineage, *A. cephallonica* from the present Peloponnese. Although our mtDNA tree clusters these two taxa together, the support for this group, and thus the phylogenetic evidence for this scenario, is very low.

Although diversification of many European reptile taxa happened during the Late Pliocene or in Pleistocene as a consequence of climate oscillations and range changes, there is growing evidence that many reptile lineages diverged earlier during the Miocene, with snakes being usually older than lizards (Joger et al., 2007; Blondel et al., 2010; Dubey and Shine, 2011).

Considering the data presented herein, the origin of the Italian slow worms via Pleistocene colonization of the Italian Peninsula by *A. fragilis* from the Balkan and/or southern French refugia seems implausible and the Pleistocene climate oscillations rather played a role in shaping its more recent genetic diversity and distribution. The newly recognized species is sub-structured into several subclades showing relatively high intraspecific variation. Such diversity would be expected if the genetic variation had been shaped in multiple microrefugia dislocated in the Italian Peninsula and/or in south-eastern France. This is in concordance with the 'refugia-within-refugia' hypothesis (Gomez and Lunt, 2007), originally proposed for the Iberian Peninsula, and the observed pattern of genetic variation could have been shaped during the Pleistocene climatic oscillations. Several other reptiles and vertebrates had multiple Pleistocene refugia in the Italian Peninsula, but besides higher overall diversity in the southern part of the peninsula, no other general pattern can be observed (see e.g. Böhme et al., 2007; Bellati et al., 2011; Canestrelli et al., 2011; Podnar et al., 2005; Stefani et al., 2012; Ursenbacher et al., 2006). In *A. veronensis*, haplotypes v14 and v15 (Table 1; Fig 1, localities 9 and 10) form a sister lineage to all other Italian slow worms with a relatively deep intraspecific divergence (2.3% uncorrected *p*-distance; Table 5), suggesting that the area of their distribution in the Dolomite Mts. might be connected to the firstly separated microrefugium. Here in deep warmer southern valleys, the slow worms could have survived the cold glacial periods and later expanded to the surrounding areas. The region of the Dolomite Mts. has already been suggested as a microrefugium for several species of mountain plants (Ronikier et al., 2008; Schönswetter et al., 2005), while the butterfly *Parnassius mnemosine* and the snail *Charpenteria itala* have been reported to have refugia in the Southern Alps (Gratton et al., 2008; Scheel and Hausdorf, 2012). Also the salamander *Salamandra atra aurorae* is endemic to the Dolomite Mts., and according to the available data, it seems that this form is genetically differentiated from the neighboring conspecific populations (Riberon et al., 2001, 2004). In another amphibian, *Rana temporaria*, the Italian population in the eastern Alps presents private haplotypes, which did not contribute to the colonization of other European areas after the glaciations (Stefani et al., 2012). This suggests that both these amphibian species presumably had a glacial refugium in this region.

4.4. Distribution and ecology of Anguis veronensis

The species is currently known only from the territories of Italy and France, but its range presumably extends to the extreme of southern Switzerland as well. It inhabits the major part of the Italian Peninsula except for the south-east (Apulia region; Luiselli et al., 2011; Zanghellini, 2006), although its occurrence seems to be much rarer in southern than in northern Italy (Luiselli et al., 2011; A. Romano, pers. obs., Figs. 1 and 2). Another species, *A. fragilis*, seemingly occurs in the extreme north and north-east of Italy in the Alps where both species meet and to some extent also hybridize. In France, *A. veronensis* only occurs in the south-eastern region Provence-Alpes-Côte d'Azur.

The slow worms in Italy are distributed from sea level up to altitudes above 2.000 m above sea level (a.s.l.) in the Alps. However, both taxa occur in the southern slopes of the Alps, where details of their ranges are not precisely known and therefore it is currently difficult to assess the upper altitudinal limit of *A. veronensis*. The Italian slow worms, genetically identified in this study, originated from localities distributed from sea level (Portofino, Italy) up to 1.520 m a.s.l. (Mercantour, France). In the northern limits of the range the Italian slow worm is relatively common, inhabiting a wide variety of different forest-steppe, steppe and ruderal habitats and not avoiding urban areas. On the contrary, in central and southern Italy the species inhabits a narrower spectrum of habitats, mainly the margins of oak and beech forests and occasionally could also be found in dry habitats such as sand dunes in the Mediterranean macchia (Luiselli et al., 2011).

In the Mediterranean region, the Italian slow worms tend to be active at dusk with occasional nocturnal activity, while other slow-worm populations are rather diurnal and occasionally crepuscular (Luiselli et al., 2011). There is almost no further data on ecology of *A. veronensis* as most ecological studies from the Italian territory were carried out in the areas where *A. fragilis* or possibly also hybrid populations occur (e.g. Capula and Luiselli, 1993; Luiselli et al., 1994).

4.5. Morphological differentiation of Anguis veronensis

Morphological comparisons showed that *A. veronensis* is differentiated from *A. fragilis*. The species differ in the relative tail length and number of subcaudal scales (in intact tail; Fig. 6, Supplementary data Table S2) and in overall robustness of the head. We also found a difference in the frequency of occurrence of the type C of the prefrontal scales position (no contact of the scales; Fig. 7). Another difference we identified, i.e. the frequency of the black abdominal coloration is rather tentative and will need more thorough investigation.

Despite being differentiated with statistical significance, the ranges of all morphological characters broadly overlap between the species. As a consequence, the species cannot be identified unambiguously based on the set of analyzed characters (indeed, successful classification rates of DFA were quite low, particularly for males). A similar pattern was previously found in the differences between *A. fragilis* and *A. colchica*, which could be only discriminated by a combination of several characters (presence of ear opening, prefrontal scales position, number of scales around body, head size; while they do not differ in the relative tail length, see Dely, 1981), but neither of them is strictly diagnostic. On the other hand, *A. cephallonica*, another ancient species with putative sister relationship to *A. veronensis* in the mtDNA genealogy, clearly and unambiguously differs from all other slow-worm species in having higher number of scale rows around body (34–36 vs. 23–32) and by the presence of a characteristic lateral color pattern formed by chocolate-brown undulated line in the anterior part of the body (e.g. Dely, 1981, under the name *A. fragilis peloponnesiacus* Štěpánek, 1937; Grillitsch and Cabela, 1990; Valakos et al., 2008). Finally, *A. graeca* shows intermediate characteristics between *A. fragilis* and *A. colchica* (Cabela and Grillitsch, 1989), and is thus possible to presume that it is morphologically differentiated from *A. veronensis* as well.

Close morphological similarity of *A. veronensis* and *A. fragilis*, which are genetically differentiated, could either have resulted from convergent evolution, or more likely the shared morphological characters could be plesiomorphic and represent an ancestral condition in the genus *Anguis*. In such case, the diagnostic characters of *A. cephallonica* would represent its autapomorphies and the species could be considered morphologically most derived and most divergent from all other slow-worm species. This might presumably be explained as an adaptation to specific environmental conditions of a relatively small area in the Peloponnese. To resolve the evolution of the slow-worm phenotypes, a detailed morphological analysis and multilocus species tree of the entire complex is highly desired.

4.6. Conservation

Due to the relative rarity of the Italian slow worm in southern and central Italy, the species needs to be monitored and its conservation status needs to be properly assessed. As the populations in the marginal parts of the range are particularly vulnerable, conservation measures should be taken to protect the slow-worm populations in southern Italy as well as in south-eastern France. A combined faunistic, ecological and genetic research is needed to confirm the occurrence of the species in southern Switzerland. On the other hand, *A. fragilis* in Italy becomes a species with limited distribution in the northern border areas and, as such, deserves protection at national level.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.05.004.

References

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.

Araújo, M.B., Pearson, R.G., 2005. Equilibrium of species' distributions with climate. Ecography 28, 693–695.

Araújo, M.B., Thuiller, W., Pearson, R.G., 2006. Climate warming and decline of amphibians and reptiles in Europe. J. Biogeogr. 33, 1712–1728.

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

Augé, M., 2003. La faune de Lacertilia (Reptilia, Squamata) de l'Éocène inférieur de Prémontré (Bassin de Paris, France). Geodiversitas 25, 539–574.

Augé, M., Smith, R., 2009. An assemblage of early Oligocene lizards (Squamata) from the locality of Boutersem (Belgium), with comments on the Eocene–Oligocene transition. Zool. J. Linn. Soc. 155, 148–170.

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

Bellati, A., Pellitteri-Rosa, D., Sacchi, R., Nistri, A., Galimberti, A., Casiraghi, M., Fasola, M., Galeotti, P., 2011. Molecular survey of morphological susbpecies reveals new mitochondrial lineages in *Podarcis muralis* (Squamata: Lacertidae) from Tuscan Archipelago (Italy). J. Zool. Syst. Evol. Res. 49, 240–250.

Blondel, J., Aronson, J., Bodiou, J.-Y., Boeuf, G., 2010. The Mediterranean Region: Biological Diversity through Time and Space, second ed. Oxford University Press.

Böhme, M.U., Fritz, U., Kotenko, T., Džukić, G., Ljubisavljević, K., Tzankov, N., Berendokn, T.U., 2007. Phylogeography and cryptic variation within the *Lacerta viridis* complex (Lacertidae, Reptilia). Zool. Scr. 36, 119–131.

Brandley, M.C., Schmitz, A., Reeder, T.W., 2005. Partitioned Bayesian analyses, partition choice, and the phylogenetic relationships of scincid lizards. Syst. Biol. 54, 373–390.

Brown, J.M., Lemmon, A.R., 2007. The importance of data partitioning and the utility of Bayes factors in Bayesian phylogenetics. Syst. Biol. 56, 643–655.

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

Canestrelli, D., Sacco, F., Nascetti, G., 2011. On glacial refugia, genetic diversity, and microevolutionary processes: deep phylogenetical structure in the endemic newt *Lissotriton italicus*. Biol. J. Linn. Soc. 105, 42–55.

Capula, M., Luiselli, L., 1993. Ecology of an alpine population of the slow worm, *Anguis fragilis* Linnaeus, 1758. Thermal biology of reproduction (Squamata, Sauria, Anguidae). Herpetozoa 6, 57–63.

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

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. JModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9, 772.

Delfino, M., 2004. The Middle Pleistocene herpetofauna of Valdemino cave (Liguria, North-Western Italy). Herpetol. J. 14, 113–128.

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.

Dubey, S., Shine, R., 2011. Are reptile and amphibian species younger in the Northern Hemisphere than in the Southern Hemisphere? J. Evol. Biol. 25, 220–226.

Estes, R., 1983. Sauria terrestria, Amphisbaenia. Handbuch der Paläoherpetologie, vol. 10A. Gustav Fischer Verlag, Stuttgart.

Feliner, G.N., 2011. Southern European glacial refugia: a tale of tales. Taxon 60, 365–372.

Fritz, U., Fattizzo, T., Guicking, D., Tripepi, S., Pennisi, M.G., Lenk, P., Joger, U., Wink, M., 2005. A new cryptic species of pond turtle from southern Italy, the hottest spot in the range of the genus *Emys* (Reptilia, Testudines, Emydidae). Zool. Scr. 34, 351–371.

Gomez, A., Lunt, D.H., 2007. Refugia within refugia: the patterns of phylogeographic concordance in the Iberian Peninsula. In: Weiss, S., Ferrand, N. (Eds.), Phylogeography of Southern European Refugia. Springer, pp. 155–188.

Gratton, P., Konopiński, M.K., Sbordoni, V., 2008. Pleistocene evolutionary history of the Clouded Appollo (*Parnassius mnemosyne*): genetic signatures of climate cycles and a 'time-dependent' mitochondrial substitution rate. Mol. Ecol. 17, 4248–4262.

Grillitsch, H., Cabela, A., 1990. Zum systematischen Status der Blindschleichen (Squamata: Anguidae) der Peloponnes und der südlichen Ionischen Inseln (Griechenland). Herpetozoa 2, 131–153.

Gvoždík, V., Jandzik, D., Lymberakis, P., Jablonski, D., Moravec, J., 2010. Slow worm, *Anguis fragilis* (Reptilia: Anguidae) as a species complex: genetic structure reveals deep divergences. Mol. Phylogenet. Evol. 55, 460–472.

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.

Hecht, M.K., Hoffstetter, R., 1962. Note préliminaire sur les Amphibiens et les Squamates du Landénien supérieur et du Tongrien de Belgique. Bull. Inst. R. Sci. Nat. Belg. 38, 1–30.

Hewitt, G.M., 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. Biol. J. Linn. Soc. 58, 247–276.

Hewitt, G.M., 1999. Post-glacial re-colonization of European biota. Biol. J. Linn. Soc. 68, 87–112.

Hidalgo-Galiana, A., Ribera, I., 2011. Late Miocene diversification of the genus *Hydrochus* (Coleoptera, Hydrochidae) in the west Mediterranean area. Mol. Phylogenet. Evol. 59, 377–385.

Jablonski, D., 2013. Mitochondriální diverzita a haplotypová distribuce slepýšů rodu *Anguis* (Reptilia, Anguidae) na Balkánském poloostrově [Mitochondrial diversity and haplotype distribution of the slow worms of the genus *Anguis* (Reptilia, Anguidae) in the Balkan Peninsula]. Unpublished MSc Thesis, Comenius University in Bratislava, Bratislava.

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.

Kass, R.E., Raftery, A.E., 1995. Bayes factors. J. Am. Stat. Assoc. 90, 773–795.

Klembara, J., 1979. Neue Funde der Gattungen *Ophisaurus* und *Anguis* (Squamata, Reptilia) aus dem Untermiozän Westböhmens (ČSSR). Věst. Ústř. Úst. Geol. 54, 163–169.

Klembara, J., 1981. Beitrag zur Kenntnis der Subfamilie Anguinae (Reptilia, Anguidae). Acta Univ. Carol. Geol. 2, 121–168.

Lance, R.F., Kennedy, M.L., Leberg, P.L., 2000. Classification bias in discriminant function analyses used to evaluate putatively different taxa. J. Mammal. 81, 245–249.

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.

Lenk, P., Wüster, W., 1999. A multivariate approach to the systematics of Italian ratsnakes of the *Elaphe longissima* complex (Reptilia: Colubridae): revalidation of Camerano's *Callopeltis longissimus* var. *lineata*. Herpetol. J. 9, 153–162.

Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452.

Luiselli, L., Anibaldi, C., Capela, M., 2011. *Anguis fragilis*. In: Corti, C., Capula, M., Luiselli, L., Sindaco, R., Razzetti, E. (Eds.), Fauna d'Italia: Reptilia, vol. XLV. Calderini, Bologna, pp. 463–467.

Luiselli, L., Capula, M., Anibaldi, C., 1994. Food habits of the slow worm, *Anguis fragilis* (L.), in two contrasting alpine environments. Bull. Soc. Herpetol. France 71/72, 45–48.

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, 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.

Oosterbroek, P., Arntzen, J.W., 1992. Area-cladograms of circum-Mediterranean taxa in relation to Mediterranean palaeogeography. J. Biogeogr. 19, 3–20.

Podnar, M., Mayer, W., Tvrtković, N., 2005. Phylogeography of the Italian wall lizard, *Podarcis sicula*, as revealed by mitochondrial DNA sequences. Mol. Ecol. 14, 575–588.

Pollini, C., 1818. Lettera del sig. dott. Ciro Pollini al Direttore della Biblioteca Italiana intorno ad alcune malattie degli ulivi e ad alcuni serpenti del Veronese, per servire di appendice alla sua Memoria su lo stesso argomento inserita nel T. VIII, p. 63 di questa Biblioteca, ed a quella del sig. Bernardino Angelini intorno al Marasso da noi pure inserita nel T. VII, pag. 451. Bibl. Ital. giorn. lett., sci. art. 9: 236–240.

Posada, D., 2006. Collapse: Describing Haplotypes from Sequence Alignments. <http://darwin.uvigo.es>.

Rambaut, A., Drummond, A.J., 2009. Tracer. MCMC Trace Analysis Tool. BEAST Website. <http://beast.bio.ed.ac.uk>.

Riberon, A., Miaud, C., Grossenbacher, K., Taberlet, P., 2001. Phylogeography of the Alpine salamander, *Salamandra atra* (Salamandridae), and the influence of the Pleistocene climatic oscillations on population divergence. Mol. Ecol. 10, 2555–2560.

Riberon, A., Miaud, C., Guyetant, R., Taberlet, P., 2004. Genetic variation in an endemic salamander, *Salamandra atra*, using amplified fragment length polymorphism. Mol. Phylogenet. Evol. 31, 910–914.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542.

Ronikier, M., Costa, A., Aguilar, J.F., Feliner, G.N., Küpfer, P., Mirek, Z., 2008. Phylogeography of *Pulsatilla vernalis* (L.) Mill. (Ranunculacae): chloroplast DNA reveals two evolutionary lineages across Europe and Scandinavia. J. Biogeogr. 35, 1650–1664.

Rögl, F., 1999. Mediterranean and Pratethys. Facts and hypotheses of an Oligocene to Miocene paleogeography (short overview). Geol. Carpath. 50, 339–349.

Scheel, B.M., Hausdorf, B., 2012. Survival and differentiation of subspecies of the land snail *Charpentieria itala* in mountain refuges in the Southern Alps. Mol. Ecol. 21, 3794–3808.

Schönswetter, P., Stehlik, I., Holderegger, R., Tribsch, A., 2005. Molecular evidence for glacial refugia of mountain plants in the European Alps. Mol. Ecol. 14, 3547–3555.

Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16, 1114–1116.

Simpson, G.G., 1951. The species concept. Evolution 5, 285–298.

Stamatakis, A., 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22, 2688–2690.

Stefani, F., Gentilli, A., Sacchi, R., Razzetti, E., Pellitteri-Rosa, D., Pupin, F., Galli, P., 2012. Refugia within refugia as a key to disentangle the genetic pattern of a highly variable species: the case of *Rana temporaria* Linnaeus, 1758 (Anura, Ranidae). Mol. Phylogenet. Evol. 65, 718–726.

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.

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.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673–4680.

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.

Ursenbacher, S., Conelli, A., Gloay, P., Monney, J.-C., Zuffi, M.A.L., Thiery, G., Durand, T., Fumagalli, L., 2006. Phylogeography of the asp viper (*Vipera aspis*) inferred from mitochondrial DNA sequence data: evidence for multiple Mediterranean refugial areas. Mol. Phylogenet. Evol. 38, 546–552.

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.

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

Weiss, S., Ferrand, N. (Eds.), 2007. Phylogeography of Southern European Refugia. Springer, Dordrecht.

Wiley, E.O., 1978. Evolutionary species concept reconsidered. Syst. Zool. 27, 17–26.

Zanghellini, S., 2006. *Anguis fragilis*. In: Sindaco, R., Doria, G., Razzetti, E., Bernini, F. (Eds.), Atlante degli Anfibi e dei Rettili d'Italia/Atlas of Italian Amphibians and Reptiles. Societas Herpetologica Italica, Edizioni Polistampa, Firenze, pp. 426–429.

SUPPLEMENTARY DATA to

Basal lineage of slow worms, genus *Anguis* (Squamata: Anguidae), survived in the Italian Peninsula

by

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Table S1 List of the analyzed morphological characters with their definitions.

Character analyzed	Definition
Snout-vent length (SVL)	longitudinal length from the rostrum to the posterior margin of the anal scales
Tail length (TL)	longitudinal length from the posterior margin of cloaca to the tail tip; only in specimens with complete tail
Head dimensions	
Head length 1 (HL1)	longitudinal length from the rostrum to the posterior margin of the occipital shield
Head length 2 (HL2)	longitudinal length from the rostrum to the posterior margin of the mandible
Head width (HW)	head width in the level of the largest width
Head height (HH)	head height in the level of the largest height
Nasal opening length (NL)	horizontal length of the nasal opening
Rostrum length (NRL)	longitudinal length between the rostrum and the anterior margin of the nasal opening
Eye length (EYL)	horizontal length of the eye
Anteorbital length (EYRL)	longitudinal length between the rostrum and the anterior eye corner
Scale counts	
Dorsal scales (D)	longitudinal scale count on the dorsal side of the body
Ventral scales (V)	longitudinal scale count on the ventral side of the body
Subcaudal scales (SCD)	longitudinal scale count on the ventral side of the tail
Scales around body 1 (SCR1)	number of the scales around the body in the level of 20th D
Scales around body 2 (SCR2)	number of the scales around the body in the level of the half of D
Scales around body 3 (SCR3)	number of the scales around the body in the level of 5th D anterior to the anal scale
Scales around tail (SCR4)	number of the scales around the body in the level of 20th SCD
Anal scales (A)	number of anal scales
Supraocular scales (SO)	number of supraocular scales
Supralabial scales (SLAB)	number of supralabial scales
Submaxillary scales (SUM)	number of submaxillary scales
Prefrontal shields position (PRF)	A-PRF in broad contact; B-PRF in point contact; C-PRF separated, X-other pattern
Ear opening	ear openings indistinct, ear openings on the form of a shallow depression, ear openings distinct – all types on both sides of the head or on one side only
Pattern and coloration	
Dorsal spots	blue or grey spots absent or present on the dorsal side of the body
Vertebral line	gradient from vertebral line absent to vertebral line on the dorsal side of the body present and prominent
Dorso/lateral border coloration	gradient from prominent border to no border between the dorsal and lateral coloration
Abdominal coloration	gradient from black abdomen to lack of black coloration on the ventral side of the body

Table S2 Descriptive statistics of the metric and meristic (scale counts) morphological data of *A. veronensis* and *A. fragilis*. Data are presented in the form: arithmetic mean ± standard error (minimum - maximum). *N* – number of specimens analyzed, * paired scale counts were taken on the right side of the head.

Morphological character	*Anguis veronensis*				*Anguis fragilis*			
	N	**Males**	***N***	**Females**	***N***	**Males**	***N***	**Females**
Snout-vent length	24	169.67±3.51 (132-210)	40	159.65±3.80 (108-233)	24	159.25±6.09 (100-226)	47	160.38±3.48 (106-227)
Tail length	12	206.25±5.36 (168-234)	22	196.36±5.79 (146-246)	11	186.64±9.04 (135-233)	18	184.72±5.29 (154-251)
Head dimensions								
Head length 1	24	14.14±0.33 (10.50-18.20)	38	12.02±0.23 (9.40-15.20)	24	12.83±0.35 (9.30-16.40)	45	12.17±0.22 (9.20-16.50)
Head length 2	24	15.33±0.42 (12.20-21.10)	38	13.11±0.26 (10.10-16.50)	23	14.16±0.49 (9.70-19.50)	43	13.18±0.27 (9.50-17.90)
Head width	24	10.09±0.25 (7.70-12.60)	36	8.04±0.18 (5.70-10.20)	21	8.37±0.31 (5.80-10.60)	39	8.06±0.17 (6.40-11.20)
Head height	23	7.43±0.20 (5.40-9.80)	36	6.18±0.14 (4.30-7.60)	22	6.26±0.25 (4.10-8.30)	37	6.15±0.17 (4.10-9.60)
Nasal opening length	24	0.64±0.02 (0.40-0.80)	37	0.52±0.02 (0.30-0.90)	20	0.57±0.03 (0.30-0.80)	38	0.55±0.02 (0.30-0.70)
Rostrum length	24	1.40±0.04 (1.00-2.00)	37	1.10±0.03 (0.70-1.50)	20	1.17±0.05 (0.70-1.50)	38	1.15±0.03 (0.80-1.50)
Eye length	24	2.85±0.05 (2.40-3.40)	36	2.52±0.05 (1.80-3.20)	20	2.70±0.09 (2.00-3.50)	39	2.72±0.07 (1.70-3.70)
Anteorbital length	24	5.34±0.14 (3.80-7.00)	36	4.54±0.11 (3.60-5.80)	20	4.56±0.15 (3.20-5.40)	39	4.48±0.10 (3.00-5.80)
Scale counts								
Dorsal scales	24	135.13±0.71 (127-139)	40	135.38±0.49 (129-139)	23	132.91±0.78 (126-141)	41	133.49±0.54 (127-142)
Ventral scales	24	139.33±0.58 (134-144)	38	139.16±0.45 (134-145)	23	137.39±0.63 (132-144)	40	138.63±0.55 (130-145)
Subcaudal scales	12	150.50±2.42 (137-164)	22	149.09±1.46 (129-160)	11	140.73±2.14 (130-153)	17	140.88±1.68 (128-154)
Scales around body 1	24	26.71±0.23 (24-29)	39	26.41±0.13 (26-28)	23	26.65±0.25 (24-29)	42	26.67±0.18 24-30)
Scales around body 2	24	25.50±0.17 (24-26)	40	25.40±0.16 (24-28)	24	25.46±0.26 (23-28)	47	25.34±0.17 (24-28)
Scales around body 3	24	22.08±0.18 (20-24)	41	21.98±0.11 (20-24)	23	21.96±0.12 (20-23)	42	21.98±0.18 (20-24)
Scales around tail	24	13.33±0.20 (12-14)	39	12.97±0.16 (12-14)	20	13.20±0.21 (12-14)	41	12.93±0.15 (12-14)
Anal scales	24	8.29±0.14 (8-10)	40	8.15±0.08 (7-10)	22	8.23±0.13 (8-10)	38	8.18±0.08 (8-10)
Supraocular scales*	24	3.04±0.04 (3-4)	41	3.05±0.05 (2-4)	23	3.09±0.06 (3-4)	43	3.07±0.05 (2-4)
Supralabial scales*	24	8.5±0.10 (8-9)	36	8.5±0.09 (8-10)	21	8.67±0.13 (8-10)	37	8.76±0.10 (8-10)
Submaxillary scales*	24	3.21±0.09 (3-4)	41	3.22±0.08 (2-4)	22	3.36±0.11 (3-4)	42	3.33±0.07 (3-4)

S3 Synonyms of *Anguis veronensis* Pollini, 1818

Junior synonyms

Anguis cinerea Risso, 1826, Hist. nat. Eur. mérid., Nice, Alp. Marit. 3, 88. Type locality: "Nizza" [= Nice], France, restricted by Mertens and Wermuth (1960).

Anguis bicolor Risso, 1826, Hist. nat. Eur. mérid., Nice, Alp. Marit. 3, 89. Type locality: "Nizza" [= Nice], France, restricted by Mertens and Wermuth (1960).

Anguis fragilis var. *albiventris* Bonaparte, 1837, Iconogr. Faun. ital. 2 20, -, Tab., Fig. 2b. Type locality: Italy.

Anguis fragilis var. *nigriventris* Bonaparte, 1837, Iconogr. Faun. ital. 2 20, -, Tab., Fig. 2a. Type locality: Italy.

Anguis fragilis var. *fusca* De Betta, 1857, Atti Accad. Agricolt. Verona 35, 164. Type locality: Non Valley.

Anguis fragilis var. *grisea* De Betta, 1857, Atti Accad. Agricolt. Verona 35, 164. Type locality: Venice and South Tyrol.

Anguis fragilis var. *lineata* De Betta, 1857, Atti Accad. Agricolt. Verona 35, 164. Type locality: Venice and South Tyrol.

Anguis fragilis var. *vulgaris* De Betta, 1857, Atti Accad. Agricolt. Verona 35, 164. Type locality: Venice and South Tyrol.

Anguis fragilis var. *ocellata* Dürigen, 1897, Deutschl. Amph. Rept., 223. Type locality: South Tyrol.

Anguis fragilis var. *vittata* Dürigen, 1897, partim (substitute name for *A. bicolor* Risso, 1826, *A. besseri* Andrzejowski, 1832 and *Anguis fragilis* var. *lineata* De Betta, 1857, unjustified), Deutschl. Amph. Rept., 223.

Anguis fragilis var. *lineomaculata* Dürigen, 1897 (substitute name for *A. cinerea* Risso, 1826, unjustified), Deutschl. Amph. Rept., 224.

Anguis fragilis var. *ventrimaculata* Dürigen, 1897 (substitute name for *A. cinerea* Risso, 1826, unjustified), Deutschl. Amph. Rept., 224.