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Correspondence

Characterization of microsatellite markers in the genera *Anguis* and *Pseudopus* (Reptilia: Anguidae)

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Microsatellite markers together with mitochondrial DNA (mtDNA) represent a golden standard in most of population-genetic and behavioural studies. Due to their high polymorphism they were successfully used for estimation of population differentiation and gene flow, the rate of hybridization and introgression, and for inferring mating systems and extra-pair paternities. To amplify microsatellite loci by polymerase chain reaction (PCR), primers must be developed from the DNA that flanks the specific microsatellite repeats. These regions of DNA are highly variable, thus primer-binding sites are not well conserved among distantly related taxa and must be designed de novo for each species or a group of closely related species (Primer et al. 1996, Barbará et al. 2007). In this paper we describe a new set of microsatellites for two genera of legless lizards (Squamata: Anguidae), *Anguis* Linnaeus, 1758 and *Pseudopus* Merrem, 1820.

The genus *Anguis* (slow worms) includes five morphologically very similar, but phylogenetically deeply diverged species (Gvoždík et al. 2010, 2013). While *A. fragilis* Linnaeus, 1758 and *A. colchica* (Nordmann, 1840) are widely distributed in Europe and western Palaearctic, *A. graeca* Bedriaga, 1881, *A. cephallonica* Werner, 1894 and *A. veronensis* Pollini, 1818 are endemic to the Balkan and Apennine Peninsula, respectively. The slow-worm species meet and potentially hybridize in the Balkans, the Alpine region and Central Europe (Gvoždík et al. 2013, Szabó & Vörös 2014, Jablonski et al. 2016). The genus *Pseudopus* (glass lizards), phylogenetically closely related to the genus *Anguis* (Macey et al. 1999, Pyron et al. 2013), includes only one extant species, the European glass lizard, *P. apodus* (Pallas, 1775), distributed from the Adriatic part of the Balkans to central Asia (Sindaco & Jeremcenko 2008).

In our previous studies (Gvoždík et al. 2010, 2013, Jablonski et al. 2016, 2017, Jandzik et al. 2018) we investigated phylogeny and genetic diversity of both genera using nucleotide variability in one (*Anguis*; dominating with the ND2 gene) or two (*Pseudopus*; ND2 and cytb) mtDNA fragments, and three nuclear genes (PRLR, RAG1, C-mos). However, mtDNA is maternally inherited molecule susceptible to interspecific introgression, and its application as a marker does not allow inferring the rate of hybridization and indisputable species identification. The three nuclear genes sequenced in the previous studies are relatively conservative and show low intraspecific variation. With the general aim to increase resolution in our future studies we decided to develop and apply more variable microsatellite markers in both mentioned genera. First, we tested nine loci published for *A. fragilis* by Geiser et al. (2013), then we designed an entirely new set of microsatellites. This should provide the necessary tools for the community, which will allow more profound studies of various aspects of slow-worm and European glass lizard biology, such as genetic variation, gene flow, hybridization, and paternity.

The aim of this methodological study was (i) to design a new set of microsatellite markers for *A. fragilis*, (ii) to test if these new markers as well as microsatellites previously published (Geiser et al. 2013) amplify in other *Anguis* species and their closest recent relative *P. apodus*, and (iii) to test whether selected microsatellite markers are suitable for discrimination between closely related *Anguis* species.

Table 1. Repetitive motif and primer sequences of the new microsatellite loci.

Locus	Repetitive motif	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
AnFr08	TG	TACTGGTCAGTCCGGGTTTC	GATGTTGGACCAGGCATTCT
AnFr12	AG	CCTCTGCAAGGAAAAGAAAGAA	CCATCACCCCTGAAACATTC
AnFr15	AC	TGGTTGGGGAGAACTTCAGA	AGCTGTGTTGAGTTCTGGCA
AnFr17	TG	ACGGGATATAGAGCAGGGGT	CAGTGCCACATCTCAGCCTA
AnFr21	CT	TGTTTTACAGGCCCTTCCAC	CCCTCATGTACGATAGTCCCA
AnFr31	AG	TGTGTGGGCATGTGTAGTCA	GAAAATGCTCCCCAATTGAA
AnFr35	AC	CAGCCAAGGAGTCTGTTTTCA	GCTTTGTCAGGGAGATTTGC
AnFr36	AC	CCATTGTCACCAAGCCTTTT	TCAGCATTCTGATTTCAGGAGTTA

Table 2. Number of microsatellite alleles (N_A), expected heterozygosity (H_E) and a range of allele size (in base pairs, bp) for five species of the genus *Anguis* and *Pseudopus apodus*. * Microsatellite loci previously published by Geiser et al. (2013), (–) H_E was not calculated for monomorphic loci.

Locus	*A. fragilis* (n=7)			*A. colchica* (n=8)			*A. graeca* (n=9)			*A. cephallonica* (n=8)			*A. veronensis* (n=8)			*P. apodus* (n=8)		
	N_A	H_E	Size (bp)	N_A	H_E	Size (bp)	N_A	H_E	Size (bp)	N_A	H_E	Size (bp)	N_A	H_E	Size (bp)	N_A	H_E	Size (bp)
AnFr08	3	0.385	290–323	6	0.929	294–311	7	0.824	284–314	3	0.492	292–300	5	0.833	302–313	not amplified		
AnFr12	4	0.396	188–206	3	0.242	198–208	2	1.000	200–204	1	–	186	5	0.758	196–204	3	0.692	202–206
AnFr15	2	0.264	154–162	2	0.233	154–156	6	0.791	148–176	2	0.500	144–150	5	0.792	150–168	1	–	154
AnFr17	2	0.264	309–318	2	0.264	316–318	3	0.242	283–318	1	–	318	3	0.530	272–318	2	0.264	299–301
AnFr21	4	0.736	200–221	4	0.712	205–228	5	0.758	210–217	5	0.667	210–220	6	0.835	204–235	2	0.533	202–204
AnFr31	2	0.363	206–210	2	0.485	208–210	3	0.633	204–210	1	–	207	3	0.711	174–212	1	–	193
AnFr35	5	0.788	270–352	6	0.733	268–281	6	0.780	342–355	8	0.825	238–256	5	0.857	282–368	3	0.658	276–280
AnFr36	3	0.385	241–255	5	0.742	245–263	6	0.797	248–257	1	–	245	6	0.846	250–262	1	–	246
AF19*	6	0.802	117–168	5	0.775	135–149	7	0.850	126–149	2	0.533	148–151	7	0.833	162–180	3	0.592	135–142
AF22*	4	0.712	230–260	4	0.700	227–245	3	0.608	223–240	1	–	232	5	0.775	235–256	1	–	231
AF24*	2	0.143	116–133	1	–	113	4	0.542	106–127	2	0.264	113–116	7	0.867	116–148	1	–	117
AF34*	3	0.591	236–240	6	0.817	145–247	7	0.842	234–253	1	–	222	6	0.850	217–244	1	–	220
AF37*	not amplified			not amplified			not amplified			not amplified			not amplified			7	0.792	142–165
AF38*	3	0.733	195–208	1	–	121	8	0.817	179–208	5	0.780	209–227	7	0.775	186–200	4	0.650	212–222
AF44*	4	0.714	151–163	6	0.808	138–163	6	0.627	151–171	8	0.892	146–187	6	0.750	146–176	3	0.692	163–171
AF47*	8	0.901	153–183	5	0.742	172–193	4	0.647	161–187	3	0.714	167–172	3	0.242	151–163	2	0.233	153–177
AF50*	5	0.824	142–151	7	0.817	135–155	11	0.941	131–155	7	0.802	141–153	8	0.900	138–165	3	0.592	129–133

A microsatellite library was produced commercially through the high throughput DNA sequencing developed by the Genoscreen company (Lille, France) and applying 454 GS-FLX genetic analyser (Roche, Basel, Switzerland). Sequencing, based on a sample of a slow-worm individual from Bratislava (Slovakia), resulted in finding 704 microsatellite loci of the best quality and their corresponding primers. Out of these loci we selected 36 based on the following criteria: (1) preferentially loci with dinucleotide repeats were chosen because they were expected to have a higher mutation rate and polymorphism; (2) motifs that contained the same nucleotide in consecutive position (e.g. TTC or AGG) were excluded; (3) only loci with unique priming sites were chosen (i.e. if a primer sequence occurred in the whole dataset twice, such locus was excluded); (4) sequences of repetitive motifs and flanking regions were compared to avoid the same sequence is being amplified by two different primer pairs; (5) sequences with homopolymers of six and more nucleotides (e.g. AAAAAA) situated between a priming site and microsatellite repeats were excluded. Finally, we tested 33 di-, 1 tri- and 2 tetranucleotide loci. Microsatellites were initially amplified using GoTaq Green Master Mix (Promega, Madison, WI, USA) with 1.5 mM $MgCl_2$, 0.2 μM of each primer and 1 μL of DNA. PCR conditions were as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final extension step at 72°C for 10 min. Out of 36 loci, 14 were successfully amplified and yielded clear PCR products on a 2% agarose gel. Because agarose electrophoresis has a low resolving power and does not allow to score polymorphism in microsatellites, the 14 selected loci were subsequently analysed by a capillary electrophoresis using primers fluorescently labelled with 6-FAM, NED, PET, and

VIC. Besides newly designed microsatellite loci, we also tested nine microsatellites previously designed for *A. fragilis* (Geiser et al. 2013). In total, 23 microsatellite markers were amplified using Qiagen multiplex kit (Qiagen, Hilden, Germany). PCR with fluorescently labelled primers was performed in a total volume of 10 µL containing 1× Qiagen Master Mix, 1 µL of DNA and 0.2 µM of each primer. PCR amplification involved an initial cycle of denaturation at 95°C for 15 min and 30 subsequent cycles of 94°C for 30 s, 58°C (for newly designed primers) or annealing temperature according to Geiser et al. (2013) for 90 s, and 72°C for 60 s, followed by a final extension step at 60°C for 30 min. PCR products were run on an ABI 3700 genetic analyser (Applied Biosystems, Foster City, CA, USA) with a LIZ-500 size standard. Peaks were visualized using the software GeneMapper 3.7 (Applied Biosystems) and were scored manually. Seventeen loci [nine previously published by Geiser et al. (2013) and eight dinucleotide repeats designed in this study] generated clear patterns on electropherograms and were analysed statistically (Tables 1–2). The loci yielding clear PCR products on the agarose gel but not on the electropherograms were not further analysed.

In total, we tested 40 *Anguis* individuals, whose mtDNA identity was ascertained using species-specific haplotypes in a 732-bp long mitochondrial fragment ND2 (Jablonski et al. 2016). The samples of each particular species originating from different allopatric localities from throughout the range were chosen to cover polymorphism in microsatellites (Appendix). In addition to the previously unpublished samples, we used slow-worm samples genotyped for the ND2 fragment in our recent study (Jablonski et al. 2016). All sequences are stored in GenBank (National Center for Biotechnology Information, NCBI; https://www.ncbi.nlm.nih.gov/genbank/). After testing the microsatellites in all five *Anguis* species, the same loci were tested in eight individuals of *P. apodus*.

A number of alleles (N_A) and expected heterozygosity (H_E) were calculated using the program GenAlEx 6.5 (Peakall & Smouse 2006). Besides information about polymorphism in particular loci we tested if microsatellite markers are suitable for distinguishing among the five *Anguis* species. Therefore, we conducted the Bayesian clustering as implemented in the program Structure (Pritchard et al. 2000, Falush et al. 2007) and a multivariate Principal Coordinate Analysis (PCoA) implemented in the program GenAlEx 6.5. Using Structure, the admixture and uncorrelated-allele models were applied. The analysis was based on runs of 10^6 iterations, following a burn-in period of 100 000 iterations. A series of twenty independent runs for the fixed K = 5 was made, where K is the number of expected clusters, in our case corresponding to the five *Anguis* species. Admixture coefficients (q) from Structure analyses were visualized using Distruct implemented in Clumpak (Copelman et al. 2015). Bayesian clustering and PCoA were not applied to *P. apodus* samples.

Out of 17 microsatellite loci, AF37 and AnFro8 were not amplified in the genus *Anguis* and *P. apodus*, respectively

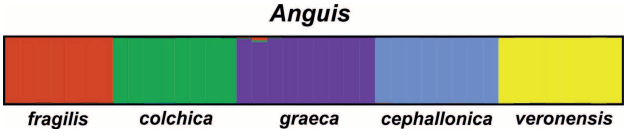

Figure 1. Assignment of slow-worm individuals to the five clusters corresponding to the species *Anguis fragilis*, *A. colchica*, *A. graeca*, *A. cephallonica* and *A. veronensis* based on Bayesian clustering implemented in the program Structure.

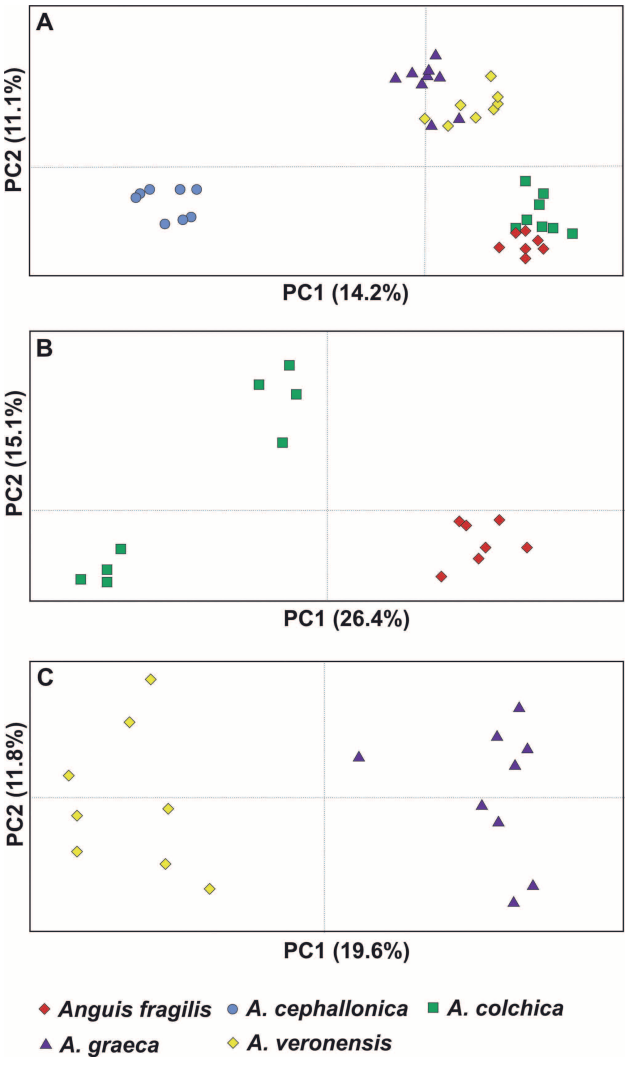

Figure 2. Clustering of *Anguis* individuals as revealed by Principal Coordinate Analysis (PCoA). The plot of two principal coordinates shows a clear separation between three groups of samples: *A. cephallonica*, *A. fragilis*/*A. colchica* and *A. graeca*/*A. veronensis* (A). Samples assigned to *A. fragilis* and *A. graeca* are only partially separated from those assigned to *A. colchica* and *A. veronensis*, respectively. Subsequent analyses clearly separated species pairs *A. fragilis*/*A. colchica* (B) and *A. graeca*/*A. veronensis* (C). Clustering of *A. colchica* samples in two groups (B) might be attributed to the hidden genetic structure and the limited number of analysed individuals.

(Tab. 2), despite AF37 was successfully amplified in *A. fragilis* in a previous study (Geiser et al. 2013). Six loci were monomorphic in *P. apodus* and *A. cephallonica*, two in *A. colchica*; the remaining species showed polymorphism in all amplified loci (Tab. 2). Polymorphic loci possessed 2 to 11 alleles per locus with expected heterozygosity (H_E) values ranged from 0.141 to 1.000.

Out of 40 slow-worm individuals analysed for 16 amplified loci (Tab. 2), seven were assigned to *A. fragilis*, nine to *A. graeca*, and eight to *A. colchica*, *A. cephallonica*, and *A. veronensis* based on the Bayesian clustering in Structure. One individual originally assigned to *A. fragilis* based on its mitochondrial ND2 fragment was unequivocally (with the posterior probability 0.981) assigned to *A. graeca* based on microsatellite markers. We considered this individual to be *A. graeca* with introgressed mtDNA of *A. fragilis*. The specimen originated from the locality Spuž in Montenegro (Appendix), which is situated approximately 60 km north of the expected contact zone of both species (Jablonski et al. 2016). Other slow-worm samples were assigned to a particular Structure cluster (species) with the posterior probabilities 0.935–0.995 (Fig. 1). Also PCoA analysis clearly separated different slow-worm species (Fig. 2A). A plot of the first two principal coordinates showed a clear separation between three groups of samples: *A. cephallonica*, *A. fragilis*/*A. colchica* and *A. graeca*/*A. veronensis*. Samples assigned to *A. fragilis* and *A. graeca* were only partially separated from those assigned to *A. colchica* and *A. veronensis*, respectively. However, subsequent analyses using only genotypes of *A. fragilis*/*A. colchica* (Fig. 2B) and *A. graeca*/*A. veronensis* (Fig. 2C) clearly separated the two species pairs. Samples of *A. colchica* clustered in two groups (Fig. 2B). This pattern might be attributed to the hidden genetic structure and the limited number of analysed samples.

Newly designed microsatellite loci as well as those previously published (Geiser et al. 2013) seem to be suitable markers for identification of slow-worm species and represent a new tool for population-genetic studies of the genera *Anguis* and *Pseudopus*.

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Appendix

List of samples of the five species of the genus *Anguis* and *Pseudopus apodus* genotyped for microsatellite markers. The individual 4457 possessed mtDNA of *A. fragilis*, but was assigned to *A. graeca* based on microsatellite markers. *Available from: https://www.ncbi.nlm.nih.gov/genbank/

ID number	mtDNA cluster	GenBank accession number*	Microsatellite cluster	Country	Locality	Coordinates N	Coordinates E
1032	–	–	*Pseudopus apodus*	Albania	Koder	39.824	20.114
1416	–	–	*Pseudopus apodus*	Bulgaria	Alepu	42.360	27.713
1539	–	–	*Pseudopus apodus*	Rep. Macedonia	Nikolich	41.242	22.705
2540	–	–	*Pseudopus apodus*	Kyrgyzstan	Jalal-Abad	40.932	73.028
2900	–	–	*Pseudopus apodus*	Armenia	Garni	40.110	44.730
2996	–	–	*Pseudopus apodus*	Armenia	Noravank	39.683	45.235
4064	–	–	*Pseudopus apodus*	Kyrgyzstan	Tyolyok	43.141	74.042
1796	–	–	*Pseudopus apodus*	Albania	Luadh	40.300	20.110
2023	*Anguis cephallonica*	MG797449	*Anguis cephallonica*	Greece	Poros, Kefalonia	38.150	20.774
2028	*Anguis cephallonica*	MG797450	*Anguis cephallonica*	Greece	Ithaca	38.363	20.721
2034	*Anguis cephallonica*	MG797451	*Anguis cephallonica*	Greece	Ano Kastritsi	38.274	21.829
2247	*Anguis cephallonica*	MG797452	*Anguis cephallonica*	Greece	Chimara	36.646	22.469
2269	*Anguis cephallonica*	KU052866	*Anguis cephallonica*	Greece	Oitylo	36.721	22.395
2294	*Anguis cephallonica*	MG797453	*Anguis cephallonica*	Greece	Chora Getson	36.948	22.254
2313	*Anguis cephallonica*	MG797454	*Anguis cephallonica*	Greece	Ag. Vasilikos	37.076	22.271
2323	*Anguis cephallonica*	MG797455	*Anguis cephallonica*	Greece	Doxas	37.931	22.283
443	*Anguis colchica*	MF817478	*Anguis colchica*	Poland	NP Pieniny, Orlica	49.418	20.457
553	*Anguis colchica*	MG797456	*Anguis colchica*	Slovakia	Poprad - Kvetnica	49.021	20.277
806	*Anguis colchica*	KX020152	*Anguis colchica*	Bulgaria	Izgrev	42.120	27.769
1980	*Anguis colchica*	MG797457	*Anguis colchica*	Slovakia	Trenčianské Teplice	48.904	18.165
4307	*Anguis colchica*	MG797458	*Anguis colchica*	Romania	Dâmbovicioara	45.447	25.220
4308	*Anguis colchica*	MG797459	*Anguis colchica*	Romania	Dâmbovicioara	45.447	25.220
4309	*Anguis colchica*	MG797460	*Anguis colchica*	Romania	Dâmbovicioara	45.447	25.220
4312	*Anguis colchica*	MG797461	*Anguis colchica*	Romania	Drumul Carului	45.476	25.303
563	*Anguis fragilis*	KX020279	*Anguis fragilis*	Serbia	Beograd - Avala	44.689	20.514
629	*Anguis fragilis*	KX020251	*Anguis fragilis*	Croatia	Paklenica, Ramići	44.345	15.482
2624	*Anguis fragilis*	MG797470	*Anguis fragilis*	Serbia	Belo Polje	44.616	20.194
2783	*Anguis fragilis*	MG797471	*Anguis fragilis*	Kosovo	Rekë e Allagës	42.727	20.162
3305	*Anguis fragilis*	MG797472	*Anguis fragilis*	Albania	Runice, Prokletije Mts.	42.467	19.780
4457	***Anguis fragilis***	**MG797469**	***Anguis graeca***	**Montenegro**	**Spuž**	**42.528**	**19.198**
Aba01	*Anguis fragilis*	KX020202	*Anguis fragilis*	Bosnia and Herzegovina	Gornji Podgradci, Kozara Mts.	45.040	16.910
Aba15	*Anguis fragilis*	KX020214	*Anguis fragilis*	Bosnia and Herzegovina	Požarnica	44.530	18.770
1000	*Anguis graeca*	KX020306	*Anguis graeca*	Albania	Milot	41.698	19.747
1590	*Anguis graeca*	MG797462	*Anguis graeca*	Rep. Macedonia	Baba Mts.	41.034	21.221
1596	*Anguis graeca*	MG797463	*Anguis graeca*	Rep. Macedonia	Baba Mts.	41.034	21.221
1797	*Anguis graeca*	MG797466	*Anguis graeca*	Albania	Trebeshine Mts.	40.426	20.068
3762	*Anguis graeca*	MG797467	*Anguis graeca*	Albania	Orikum	40.318	19.431
3763	*Anguis graeca*	MG797464	*Anguis graeca*	Albania	Orikum	40.318	19.431
3764	*Anguis graeca*	MG797465	*Anguis graeca*	Albania	Orikum	40.318	19.431
3813	*Anguis graeca*	MG797468	*Anguis graeca*	Albania	Syri i Kaltër	39.924	20.191
Ait01	*Anguis veronensis*	KC881548	*Anguis veronensis*	Italy	Torrente Peglio	40.310	15.580
Ait02	*Anguis veronensis*	KC881549	*Anguis veronensis*	Italy	Pollino	40.040	16.100
Ait03	*Anguis veronensis*	KC881550	*Anguis veronensis*	Italy	Portofino	44.310	9.200
Ait04	*Anguis veronensis*	KC881551	*Anguis veronensis*	Italy	Manie	44.200	8.370
Ait05	*Anguis veronensis*	KC881552	*Anguis veronensis*	France	Mercantour	44.070	7.510
Ait06	*Anguis veronensis*	KC881553	*Anguis veronensis*	Italy	Bianzano	45.750	9.940
Ait07	*Anguis veronensis*	KC881554	*Anguis veronensis*	Italy	Cantiano	43.470	12.630
Ait08	*Anguis veronensis*	KC881555	*Anguis veronensis*	Italy	Roccagnano	43.380	12.110