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Discovery of an Italian slow worm (*Anguis veronensis* Pollini, 1818) population on a Western Mediterranean Island confirmed by genetic analysis

Julien Renet[1,*], Daniela Lucente[5], Michel Delaugerre[2], Olivier Gerriet[3], Grégory Deso[4], Chiara Abbattista[5], Roberta Cimmaruta[5]

[1] *Conservatoire d'espaces naturels de Provence-Alpes-Côte d'Azur, Pôle Biodiversité régionale, 96, rue Droite, 04200, Sisteron, France. *Corresponding author.* E-mail: julien.renet@cen-paca.org
[2] *Conservatoire du littoral, Résidence St Marc, 2, rue Juge Falcone, 20200, Bastia, France*
[3] *Muséum d'Histoire naturelle de Nice, 60, boulevard Risso, 06300, Nice, France*
[4] *Association herpétologique de Provence Alpes Méditerranée, Hameau du Nivernais, 84100, Orange, France*
[5] *Department of Ecological and Biological Sciences, Tuscia University, Largo dell'Università snc, 01100, Viterbo, Italy*

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Abstract. The genus *Anguis* is known to be mainly continental in the Mediterranean area, and accordingly it has never been recorded in Western Mediterranean islands. Here we report for the first time the presence of the slow worm in a Western Mediterranean island, the Ile Sainte-Marguerite from Lérins archipelago (southeastern France). The molecular analysis of ND2 and PRLR genes assigned the specimens to *A. veronensis* Pollini, 1818 and showed that they are genetically related to the mainland population from Les Mayons, in mainland France.

Keywords. *Anguis veronensis*, Lérins archipelago, haplotype network.

Phylogenetic classification of the genus *Anguis* (Reptilia: Anguidae) has undergone deep changes over the last years. Although morphologically identical, four widespread species were genetically identified from the western Palearctic: *A. fragilis* Linnaeus, 1758, *A. graeca* Bedriaga, 1881, *A. colchica* (Nordmann, 1840) and *A. cephallonica* Werner, 1894 (Gvoždík et al., 2010). Such pattern of genetic differentiation is likely the result of geographic barriers (mainly mountain systems) leading to the isolation of allopatric populations in the area, according to the "refugia within refugia" model (Jablonski et al., 2016).

More recently, slow worms from the Italian peninsula have been recognized as a fifth separate species (former *A. fragilis* becoming *A. veronensis* Pollini, 1818) according to strong genetic divergence and morphological differentiation of the sampled specimens (Gvoždík et al., 2013). While contact zones have been discovered between *A. fragilis* and *A. veronensis* in eastern Italy and Slovenia (Gvoždík et al., 2013), the distribution limits of *A. veronensis*, as well as sympatric areas with *A. fragilis*, remain poorly known in the western part of the range, despite the relatively high number of samples genetically studied (Fig. 1).

In the Mediterranean area, the genus *Anguis* is known to be mainly continental with some insular populations (Table 1) and accordingly it was never recorded in western Mediterranean insular environments (including all Tyrrhenian, Baléaric and Galite islands, Delaugerre and Cheylan, 1992; Cabela, 1997; Turrisi and Vaccaro, 1998; Galan, 2002; Vanni and Nistri, 2006; Zanghellini, 2006; Sindaco and Jeremčenko, 2008; Geniez and Cheylan, 2012; Ineich,

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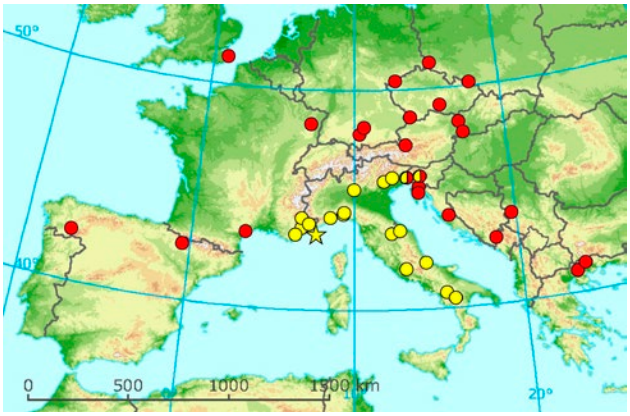

Fig. 1. Localization of Ile Sainte-Marguerite (yellow star) on a distribution map reporting the localities where *A. veronensis* (in yellow) and *A. fragilis* (in red) have been genetically studied so far. Redrawn from Gvoždík et al. (2013).

2012; Silva-Rocha et al., 2018). However, the presence of an island population was signaled in May, 2011 (Renet and Martinerie, unpublished data) on Ile Sainte-Marguerite (ISM, 43°31’25”N, 7°02’43”E), the largest (210 ha) and most forested island in the Lérins archipelago, located along the southeastern French coasts (Fig. 1).

This discovery raises several questions concerning not only species assignment but also the origin of this population. To answer these questions, a field mission was conducted in May 2016 on Ile Sainte-Marguerite. Six individuals (3 adult females, 1 adult male and 2 subadults) were collected (under tree stumps) inside a high forest composed of Aleppo pine *Pinus halepensis* Miller, 1768 and Holm oak *Quercus ilex* Linnaeus, 1753 in the moat shaded at the “Fort Sainte-Marguerite” (a historical military construction) and in small fresh adjacent valleys. The presence of numerous decaying woods and a thick layer of litter provide optimal ecological condition for this *Anguis* population (Fig. 2). Tissue fragments of 1 mm in length were sampled by tail clipping and stored in Eppendorf tubes filled with ethanol (96%).

Total genomic DNA was extracted using a CTAB protocol slightly modified from Doyle and Doyle (1987). The two genes targeted for the molecular analysis were the mitochondrial NADH dehydrogenase subunit 2 (ND2) and the nuclear prolactine receptor (PRLR), to obtain results comparable with those reported in recent literature (Gvoždík et al., 2013; Jablonski et al., 2016). In literature, ND2 gene was sequenced within a 1428 bp long fragment comprising also transfer RNA genes (Gvoždík et al., 2010, 2013). To target the ND2 gene only, we specifically designed two primer pairs using the software PRIMER3 v. 4.1.0:
AnF1 (5’ACAAAATACTTCCTCACACAAGCA-3’) and AnR1 (5’GAGTATGAAAGTCGYAGGTAGAAG-3’); AnF2 (5’TGAGCCCYATAATTACCTCAATCT-3’) and AnR2 (5’GGGCCATRTTTTGTGYTAGTAGYT-3’).

For the PRLR gene we used the primers PRLR_f1 and PRLR_r3 (Townsend et al., 2008), as reported by Gvoždík et al. (2013). PCR conditions were those reported in Cimmaruta et al. (2015) and Lucente et al. (2016). Purification and sequencing reactions were outsourced to Macrogen Inc. (www.macrogen.com). The sequences obtained were deposited in GenBank (MH316862-5). To assign the obtained sequences to a species of *Anguis*, the recovered ND2 and PRLR haplotypes were compared to the homologous sequences of *A. fragilis*, *A. veronensis*, *A. colchica*, *A. graeca* and *A. cephallonica* recovered in GenBank through a megaBlast search (Clark et al., 2016).

Alignments were performed using the software Mega v. 6.0 (Tamura et al., 2013).

Table 1. Location of the currently known island populations of slow worm in the Mediterranean basin.

Species	Island	Sea area	Country	References
Anguis cephallonica	Kephallonia	Ionian	Greece	Grillitsch and Cabela, 1990; Mayer et al., 1991; Jablonski et al., 2016
	Ithaki	Ionian	Greece	
	Lefkas	Ionian	Greece	
	Zakynthos	Ionian	Greece	
Anguis graeca	Corfu	Ionian	Greece	Tóth et al., 2002; Valakos et al., 2008; Jablonski et al., 2016
	Thasos	Aegean	Greece	
	Euboea	Aegean	Greece	
Anguis fragilis/veronensis (hybrid zone)	Cres	Adriatic	Croatia	Tóth et al., 2006; Kryštufek and Kletečki, 2007; Gvozdík et al., 2010
	KrK	Adriatic	Croatia	
	Košljun	Adriatic	Croatia	
Anguis veronensis	Sainte-Marguerite	Ligurian	France	This study

Fig. 2. Habitat of *Anguis veronensis* from Sainte-Marguerite Island, southeastern France.

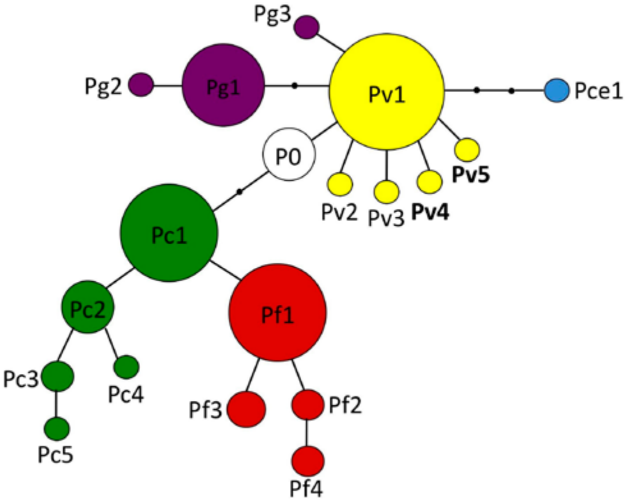

Fig. 3. Statistical Parsimony Network based on PRLR sequences and including the sequences both from Gvoždík et al. (2013) and from this study (Isle Sainte-Marguerite). Haplotypes are named as in Gvoždík et al. (2013); the newly found haplotypes Pv4 and Pv5 are in bold.

A statistical parsimony network was constructed for nuclear PRLR haplotypes using TCS v. 1.21 with 95% as disconnection limit (Clement et al., 2000), by including the haplotypes found in this study and those reported by Gvoždík et al. (2013).

The gene fragments obtained from the six individuals from Ile Sainte-Marguerite (ISM) were 662 bp long for ND2 and 544 bp for PRLR. The sequences recovered for ND2 did not show stop codons and provided a single haplotype for all the six sampled specimens (MH316865). A megaBlast search in GenBank showed that these six sequences were identical to KC881558, which belongs to *A. veronensis* from Les Mayons (mainland municipality in the nearby department of Var) as reported in Gvoždík et al. (2013).

We found three different haplotypes for the PRLR fragment (MH316862-3-4): four specimens showed the same haplotype recovered by Gvoždík et al. (2013) as the most frequent in *A. veronensis* (Pv1) in homozygosity, while the other two specimens resulted to be heterozygous. They showed two haplotypes both recorded for the first time, being Pv1/Pv4 and Pv1/Pv5 their genotypes. The haplotype network showed that the two new haplotypes are derivative from the most widespread Pv1 and differ by a single mutation each (Fig. 3).

To our knowledge, this finding attests for the first time the presence of the slow worm on a Western Mediterranean island, Ile Sainte-Marguerite from Lérins archipelago (southeastern France). The molecular analysis carried out has allowed identifying the specimens as the Italian species *A. veronensis*. This is not an unexpected result, since *A. veronensis* ranges not only in the Italian peninsula but also in southeastern France (Gvoždík et al., 2013). However, the origin of this island population was not straightforward, since possible anthropogenic dispersal could not be ruled out. The habits of *A. veronensis* are indeed continental and the crossing of geographic barriers as a sea channel, even if 1.3 km wide and of a few meters of depth, may be challenging for this species. For example, a recent study correlated the genetic heterogeneity of the Balkan species (in particular *A. graeca* and *A. cephallonica*) to the terrain ruggedness (Jablonski et al., 2016). Although in the cited case the geographic barriers were represented specifically by altitudinal differences and steep slopes, slow worms are small legless lizards with semi-fossorial habits and consequently low dispersal ability (Haley, 2014). Despite this, the data showed that the slow worm population from Ile Saint-Marguerite is naturally derived from the one living on the nearby mainland. Indeed, the gene pool of this population is characterized by the presence of the same mitochondrial ND2 haplotype observed at Les Mayons (v11) and of the most common nuclear PRLR haplotype (Pv1). The population of Ile Saint-Marguerite also showed two newly found PRLR haplotypes, derived from Pv1, so suggesting that this is likely a natural population that has been inhabiting Lérins archipelago for a long period of time. It might be the remnant of a former continental population that has been severed by sea level rise that occurred 5000 to 7000 years ago (Lambeck and Bard, 2000).

The network analysis confirmed that the PRLR haplotypes of *A. veronensis* are not shared with other species, and occupy an internal position within the network, further supporting the idea that *A. veronensis* lineage is ancestral within the genus, as already reported based on previous molecular and karyological data (Gvoždík et al., 2013; Mezzasalma et al., 2013).

The data presented show that small islands may host genetically variable slow worm populations, despite *Anguis* was considered mainly a mainland distributed genus. The south-eastern France is confirmed as inhabited by *A. veronensis*, but a more detailed sample of the southern French coasts would allow identifying western boundaries with *A. fragilis*.

Given the age of this lineage of *Anguis*, the species were likely already present on Balkan, Italian and SE Provence continental areas at the time islands were severed by sea level rise, after the Last Glacial Maximum. The reason why the species is nowadays so uncommon in insular context is more likely related to climate and ecological constraints such as scarceness of fresh forested habitats and summer drought, prevailing on Western Mediterranean islands.

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REFERENCES

Cabela, A. (1997): *Anguis fragilis*. In: Atlas of Amphibians and Reptiles in Europe, pp. 196-197. Gasc, J.P., Cabela, A., Crnobrnja-Isailovic, J., Dolmen, D., Grossenbacher, K., Haffner, P., Lescure, J., Martens, H., Martínez Rica, J.P., Maurin, H., Oliveira, M.E., Sofianidou, T.S., Veith, M., Zuiderwijk A., Eds, Societas Europaea Herpetologica & Muséum National d'Histoire Naturelle, Paris, France.

Cimmaruta, R., Lucente, D., Nascetti, G. (2015): Persistence, isolation and diversification of a naturally fragmented species in local refugia: the case of *Hydromantes strinatii*. PLoS One **10**: e0131298.

Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Sayers, E.W. (2016): GenBank. Nucleic Acids Res. **44**(D1): D67-72.

Clement, M., Posada, D., Crandall, K.A. (2000): TCS: A computer program to estimate gene genealogies. Mol. Ecol. **9**: 1657-1659.

Delaugerre, M., Cheylan, M. (1992): Atlas de répartition des Batraciens et Reptiles de Corse. P.N.R.C./E.P.H.E., Ajaccio.

Doyle, J.J., Doyle, J.L. (1987): A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. **19**: 11-15.

Galán, P. (2002): *Anguis fragilis*. In: Atlas y Libro Rojo de los Anfibios y Reptiles de España, pp. 240-242. Pleguezuelos, J.M., Marquez, R., Lizana, M., Eds, Dirección General de Conservación de la Naturaleza-Asociación Herpetológica Española (2ª impresión), Madrid.

Geniez, P., Cheylan, M. (2012): Les Amphibiens et les Reptiles du Languedoc-Roussillon et régions limitrophes. Atlas biogéographique. Biotope, Muséum national d'Histoire naturelle, Mèze, Paris.

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**: 460-72.

Gvoždík, V., Benkovský, N., Crottini, A., Bellati, A., Moravec, J., Romano, A., Sacchi, R., Jandzik, D. (2013): An ancient lineage of slow worms, genus *Anguis* (Squamata: Anguidae), survived in the Italian Peninsula. Mol. Phylogenet. Evol. **69**: 1077-92.

Haley, T. (2014): A metapopulation of the lizard *Anguis fragilis* (Squamata: Anguidae) on a local scale in Dorset, Great Britain, as indicated by spatial distribution and movement. Phyllomedusa **13**: 91-98.

Ineich, I. (2012): *Anguis fragilis* Linnaeus, 1758. In: Atlas des Amphibiens et Reptiles de France, pp. 200-201. Lescure, J., de Massary, J.C., Eds, Biotope, Mèze, Muséum national d'Histoire naturelle, Paris.

Jablonski, D., Jandzik, D., Mikulíček, P., Džukić, G., Ljubisavljević, K., Tzankov, N., Jelić, D., Thanou, E., Moravec, J., Gvoždík, V. (2016): Contrasting evolutionary histories of the legless lizards slow worms (*Anguis*) shaped by the topography of the Balkan Peninsula. BMC Evol. Biol. **16**: 99.

Kryštufek, B., Kletečki, E. (2007): Biogeography of small terrestrial vertebrates on the Adriatic landbridge islands. Folia Zool. **56**: 225-234.

Lambeck, K., Bard, E. (2000): Sea-level change along the French Mediterranean coast for the past 30 000 years. Earth Planet. Sci. Lett. **175**: 203-222.

Lucente, D., Renet, J., Gailledrat, M., Tillet J., Nascetti, G., Cimmaruta, R. (2016): A new population of European cave salamanders (genus *Hydromantes*) from west-central France: relict or introduction? Herpetol. Bull. **138**: 21-33.

Mayer, W., Grillitsch, H., Cabela, A. (1991): Proteinelektrophoretische Untersuchungen zur Systematik der südgriechischen Blindschleiche (Squamata: Anguidae). Herpetozoa **4**: 157-165.

Mezzasalma, M., Guarino, F.M., Aprea, G., Petraccioli, A., Crottini, A., Odierna, G. (2013): Karyological evidence for diversification of Italian slow worm populations (Squamata, Anguidae). Comp. Cytogenet. **7**: 217.

Silva-Rocha, I., Montes, E., Salvi, D., Sillero, N., Mateo, J.A., Ayllón, E., Pleguezuelos, J.M., Carretero, M.A. (2018): Herpetological history of the Balearic Islands: When aliens conquered these islands and what to do next. In: Histories of Bioinvasions in the Mediterranean, pp. 105-131. Queiroz, A.I., Pooley, S., Eds, Springer, Dordrecht.

Sindaco, R., Jeremčenko, V.K. (2008): The Reptiles of the Western Palearctic. 1. Annotated Checklist and Distributional Atlas of the Turtles, Crocodiles, Amphisbaenians and Lizards of Europe, North Africa, Middle East and Central Asia. Belvedere, Latina, Italia.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013): MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. **30**: 2725-2729.

Tóth, T., Krecsák, L., Madsen, T., Újvári, B. (2002): Herpetofaunal locality records on the Greek Island of Corfu. Herpetozoa **15**: 149-169.

Tóth, T., Grillitsch, H., Farkas, B., Gál, J., Susic, G. (2006): Herpetofaunal data from Cres Island, Croatia. Herpetozoa **19**: 27-58.

Townsend, T.M., Alegre, E.R., 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.

Turrisi, G.F., Vaccaro, A. (1998): Contributo alla conoscenza degli Anfibi e dei Rettili di Sicilia. Boll. Accad. Gioenia Sci. Nat. **30**: 5-88.

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.

Vanni, S., Nistri, A. (2006): Atlante degli Anfibi e dei Rettili della Toscana. Università di Firenze, Museo di Storia Naturale, Sezione di Zoologia La Specola e Regione Toscana, Firenze.

Zanghellini, S. (2006): *Anguis fragilis* Linnaeus, 1758. In: Atlante degli anfibi e dei Rettili d'Italia / Atlas of Italian Amphibians and Reptiles, pp. 426-429. Sindaco, R., Doria, G., Razzetti, E., Bernini, F., Eds, Societas Herpetologica Italica, Edizioni Polistampa, Firenze.