Phylogeography and genetic structure of the slow worms *Anguis cephallonica* and *Anguis graeca* (Squamata: Anguidae) from the southern Balkan Peninsula

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**Abstract.** Two slow worm species are distributed at the southernmost part of the Balkan Peninsula: *Anguis cephallonica*, an endemic of the Peloponnese and the islands Zakynthos, Ithaki and Kephallonia, and *A. graeca*. Here, we investigate the intraspecific genetic diversity of *A. cephallonica* from the Peloponnese and Kephallonia and analyse *A. graeca*, from the northern Peloponnese, where it is found in sympatry with *A. cephallonica*. MtDNA and nDNA phylogenetic analyses confirm the genetic similarity of Peloponnesian and Kephallonian populations of *A. cephallonica* and reveal significant mtDNA genetic variation within it, probably related to the occurrence of multiple subrefugia in the Peloponnese. Peloponnesian *A. graeca* populations are genetically similar to non-Peloponnesian conspecifics implying recent dispersal to the Peloponnese. In contrast to the genetic markers, morphological characteristics (such as the number of mid-body scale-rows) failed to distinguish between Peloponnesian *A. cephallonica* and *A. graeca*. Although the former species is believed to be well-differentiated from its congeneric taxa, a thorough morphological study is needed.

**Keywords:** *Anguis cephallonica, Anguis graeca*, biogeography, Kephallonia, morphological comparison, Peloponnese, phylogeny, refugia.

*Anguis* is a genus of legless lizards, commonly known as slow worms. Until recently, this genus included two morphologically described species: *A. cephallonica* Werner, 1894 and *A. fragilis* Linnaeus, 1758 (Arnold and Ovenden, 2002). Recent analyses of genetic markers (mitochondrial and nuclear) in combination with morphological data, revealed four cryptic species within *A. fragilis* (Gvoždík et al., 2010, 2013). These species, which seem to present a parapatric distribution with few narrow contact zones and even hybridization zones (Szabo and Vörös, 2014), are *A. colchica* (Nordmann, 1840), *A. fragilis* Linnaeus, 1758, *A. graeca* Bedriaga, 1881 and *A. veronensis* Pollini, 1818 (Gvoždík et al., 2013). In the same studies, beside the recognition of phylogenetic lineages, interrelationships and systematic implications, the phylogeography of the genus was discussed. The diversification history of extant taxa probably dates back to the Late Miocene (approx. 5.7 Mya), relating to the geological and climatic configurations of the Messinian Salinity Crisis (MSC), although the fossil record provides evidence that the genus’ presence in Europe dates back to the Early Eocene (approx. 50 Mya) (Augé, 2003). The most basal phylogenetic split within the genus is the one resulting in *A. cephallonica* or *A. cephalonica/A. veronensis*, since a poorly supported relationship between the two is shown (Gvoždík et al., 2013).

*Anguis cephallonica* is distributed in the Peloponnese and the islands of Zakynthos, Ithaki and Kephallonia. In the past, populations from the islands were considered an endemic taxon, namely *A. fragilis var. cephalonica* Werner, 1894, and Peloponnesian populations were assigned to *A. fragilis peloponnesiacus* Štěpánek, 1937. Currently, morphological and electrophoretic data (Grillitsch and Cabela, 1990; Mayer, Grillitsch and Cabela, 1991) render them a single taxon, i.e. the species *A. cephallonica*.

The morphology of *Anguis* species has not been thoroughly studied, especially under the light of recently discovered molecular diversification. The morphological characters used so...
far have revealed statistically significant differences between some species [e.g. *A. fragilis* and *A. colchica* (Dely, 1981) or *A. veronensis* and *A. fragilis* (Gvoždík et al., 2013)], but their variation broadly overlaps. However, *A. cephallonica* was considered to be clearly and unambiguously different from all other slow worm species (Dely, 1981; Grillitsch and Cabela, 1990; Valakos et al., 2008; Gvoždík et al., 2013). The morphological differentiation of *A. cephallonica* from other *Anguis* (Dely, 1981; Grillitsch and Cabela, 1990) relies on the following criteria: higher NSR (number of scale rows around mid-body), absence of ear opening, prefrontal scales not in contact, discontinued or absent vertebral line, high contrast of ventrolateral coloration and undulated neck-line. Slow worms from the northern parts of the Peloponnese that did not show these features were assigned to the taxa *A. fragilis fragilis* by Grillitsch and Cabela (1990), i.e. current *A. graeca*.

In the current work we revisit and complement the aforementioned studies, focusing on the southern Balkan Peninsula, where *A. cephallonica* and *A. graeca* occur. We use a phylogeographic approach, discussing the historical biogeography of the studied taxa, under the working hypotheses that (1) *A. cephallonica* has an intraspecific genetic structure that corresponds to the geography and palaeogeographical history of the region, (2) *A. graeca* populations from the Peloponnese are the result of secondary dispersal of slow worms from the northern continental parts of Greece, and (3) there should be a consensus of the molecular groupings (mtDNA and nDNA) with the current morphological assignments.

A total of 21 *Anguis* specimens were collected from 19 localities (fig. 1, and online Supplementary table S1). These specimens were assigned to either *A. cephallonica* or *A. graeca*, based on NSR (34-36 and 24-32, respectively), which is considered a valid diagnostic morphological character that distinguishes *A. cephallonica* from other slow worms (Grillitsch and Cabela, 1990; Valakos et al., 2008;
Gvoždík et al., 2013). Additionally, six other morphological characters were examined (ear-opening, type of prefrontal scale position, presence and intensity of vertebral line, presence and intensity of the border between dorsal and lateral coloration, presence of blue spots, and the type of demarcation line in the lateral neck area), following Grillitsch and Cabela (1990).

Sequences of these specimens were combined with previously published ones from Gvoždík et al. (2010, 2013). In all of the specimens collected here, 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 a fragment of the nuclear protein-coding gene of the prolactin receptor (PRLR). Sequencing was conducted by Macrogen Inc. (Seoul, S. Korea, http://www.macrogen.com). For further details on the laboratory procedures (primers, conditions, selected outgroups) see Gvoždík et al. (2010, 2013).

DNA sequences were aligned using ClustalX v.2.0.12 (Larkin et al., 2007). The genetic divergences, as percentage of uncorrected p-distance values, between selected groups were estimated in MEGA5 (Tamura et al., 2011). Phylogenies were built using Bayesian Inference (BI), Maximum Likelihood (ML) and Neighbor-Joining (NJ). For the first time, we used three separate models of evolution for the three codon positions of ND2 and one model for the tRNAs (HKY + G for first and second position, Hasegawa, Kishino and Yano, 1985: GTR + G for third position and tRNAs, Tavaré, 1986). The best partitioning strategy was selected with the use of Bayes factors (for the exact procedure see e.g. Kornilios et al., 2012), and the most suitable models of DNA substitution with jModeltest v.0.1.1 (Posada, 2008), under the Bayesian Information Criterion.

Bayesian analysis (eight incrementally heated Markov chains/2 = 10⁶ generations/four runs) was performed in MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003). Stationarity was confirmed and the number of “burnin” trees (10%) was decided with TRACER v1.5.0 (Rambaut and Drummond, 2007). A majority-rule consensus tree was generated from the remaining trees. Partitioned ML analysis was carried out with RAXML 7.2.7 (Stamatakis, 2006), with each partition having its own GTR|GAMMA model (Stamatakis, 2006), since GTR is the only substitution model for each partition having its own GTRGAMMA model (Stamatakis, 2006), with all other substitution models being encompassed within this model (Felsenstein, 2004). A NJ tree was calculated in MEGA5. In ML and NJ analyses, nodal support was tested via 1000 bootstrap replicates (Felsenstein, 1985).

The genealogical relationships between mtDNA and nDNA haplotypes were assessed with haplotype networks constructed using the statistical parsimony algorithm implemented in the program TCS v.1.21 (Clement, Posada and Crandall, 2000), under the 95% connection limit of parsimony.

The sequences resulting from the mtDNA analysis were deposited in GenBank (Acc. Nos. KJ634782-KJ634801, online Supplementary table S1). The overall topology of the phylogenetic tree is in agreement with that of Gvoždík et al. (2010, 2013), distinguishing the same lineages (species) and respective interrelationships. A noticeable difference is that our dataset strongly supports the monophyly of A. cephalonica/A. veronensis group (BI pp values = 0.98; ML bootstrap values = 83; NJ bootstrap values = 99) (fig. 2A). Therefore, the scenario proposed by Gvoždík et al. (2013) for the early diversification of Anguis seems quite plausible. According to that, slow worms probably inhabited Europe before the Quaternary, and began to diversify in two groups approximately 5.7 Mya, due to geographic separation and vicariance events south of the Alps. During the MSC in the Late Miocene (5.96-5.33 Mya), the southern region was probably inhabited by the A. cephalonica/A. veronensis ancestor. The mean p-distance value between these taxa, estimated here for the mtDNA marker, is 6.3%. Based on the molecular evolution rate of the studied mtDNA region (Macey et al., 1999; Gvoždík et al., 2013), the time of divergence between A. cephalonica and A. veronensis was approximately 4.8 Mya, in the Early Pliocene. Similarly, the estimated divergence-time between A. graeca and A. colchica was 4.6 Mya (p-distance 6.0%), with both events probably related to that period’s climatic oscillations.

Regarding the nuclear marker PRLR, two of our specimens failed to amplify (Supplementary table S1). For the remaining samples, the nuclear-marker phylogenetic-assignments agreed with the ones from the mitochondrial marker, i.e. no incongruence between the markers was observed that would imply possible introgression. All “mitochondrial” A. graeca were assigned to the Pg1 haplotype (Acc. No. GQ285109, Gvoždík et al., 2010). Despite the high levels of mtDNA diversity of A. cephalonica, all individuals shared the same PRLR haplotype, namely Pce1 (GQ285104, Gvoždík et al., 2010). Thus, the haplotype network (not shown) of the nuclear marker PRLR is identical to the one from Gvoždík et al. (2010, 2013).
MtDNA and nDNA analyses from the present study confirm the genetic connectivity between the former taxa *A. fragilis var. cephalonica* described from the islands and *A. fragilis peloponnesiacus* from Peloponnese. They are situated within the same phylogenetic group, with no apparent genetic differentiation. There is significant mtDNA genetic variation within *A. cephalonica*, comparable to other *Anguis* species with much larger geographic distributions (*A. fragilis*, *A. graeca*), and it shows some geographic structure. Specifically, its intraspecific divergence begins with the clear separation of a population from the southernmost parts of the Peloponnese (specimen 296 from Mani Peninsula; fig. 1). The *p*-distance between this specimen and all other *A. cephalonica* populations, including Kephallonia’s, is high (2.4%), corresponding to a phylogenetic split dated some 1.8 Mya. The population from Mani Peninsula forms a separate haplotype network under the 95% connection limit in statistical parsimony, indicating that it represents an Evolutionary Significant Unit (Fraser and Bernatchez, 2001). All other populations form four subgroups, both in the phylogenetic tree and the network (fig. 2A, B). One of them corresponds to Kephallonian individuals, while the other three are roughly situated in the southern, northern and east-central parts of the Peloponnese, respectively (fig. 1). All genetic groupings most probably relate to the occurrence of multiple subrefugia in the Peloponnese corresponding to mountain massifs, which played a key-role in sustaining populations during the Pleistocene periods of unfavorable conditions. A similar pattern is observed in *A. veronensis*, with populations from the Dolomite Mts exhibiting a *p*-distance value of 2.3% from all other conspecific populations, while the occurrence of multiple subrefugia in the southwestern Balkan Peninsula is also inferred for *A. graeca* (Gvoždík et al., 2013). As for the occurrence of *A. cephalonica* on the islands (Kephallonia, Zakynthos, Ithaki), it seems that this is the result of a relatively recent dispersal from the Peloponnese. During the Last Glacial Maximum when the sea level was at −120 m, these islands were connected as one large island, separated from the mainland by narrow straits (Ferentinos et al., 2012).

The Peloponnese has served as an important agent for producing and sustaining reptile diversity. Several endemic taxa, beside *A. cephalonica*, occur in this region: the endemic genus *Hellenolacerta* (Arnold, Arribas and Carranza, 2007), *Podarcis peloponnesiacus* (Bibron and Bory, 1833), *Algyroides moreoticus* Bibron and Bory, 1833, which also occurs on the three islands of Kephallonia, Zakynthos and Ithaki, and *Ophiomorus punctatissimus* (Bibron and Bory, 1833). For other reptiles, Peloponnesian populations are genetically distinct from those occurring in continental Greece, e.g. *Podarcis tauroicus* (Pallas, 1814) or *Vipera ammodytes* (Linnaeus 1758) (Poulakakis et al., 2005; Ursenbacher et al., 2008). Finally, several species have never managed to disperse to the Peloponnese by overcoming the sea-barrier, or have done so recently and are only distributed in its northern parts. The role of the Peloponnese is related to its position at the southernmost edge of the Balkan Peninsula and its complicated palaeogeographical history. Palaeogeographical maps show that the Peloponnesian region was connected to the Greek mainland until the end of the Miocene (5.3 Mya), while at the beginning of the Pliocene (from 5 to 3 Mya) it was disconnected with a wide sea-barrier (Dermitzakis, 1990). From that point on, climatic oscillations and resulting sea-level fluctuations led to repeated connection/disconnection cycles (see Perissoratis and Conispoliatis, 2003), during which animals could disperse to the Peloponnese.

An example of secondary dispersal to the Peloponnese is the one of the slow worms. Specifically, *A. graeca* has managed to disperse to the Peloponnese where it is found in sympatry with the endemic *A. cephalonica*. Our results show that this event has happened recently, probably during one of the glacial peri-
Figure 2. (A) Phylogenetic relationships (BI tree), among the Anguis specimens of the present study, combined with sequences from GenBank. Numbers in terminal nodes refer to specimen codes (fig. 1, Supplementary table S1) and haplotypes from Gvoždík et al. (2010, 2013). Numbers near the nodes are BI posterior probabilities (≥0.50), ML bootstrap values and NJ bootstrap values (≥50) (∗ = 1.00 pp and 100 bootstraps). (B) Anguis cephallonica mtDNA parsimony network. Lines represent a mutational step, black circles missing haplotypes and open circles known haplotypes. The circle area is proportional to the number of individuals. Probable ancestral haplotypes are given as rectangles. Specimen No. 296 from Mani Peninsula forms a separate haplotype network (not shown). (C) Respective network for A. graeca, including samples of the present study and their connection to haplotypes g1 to g12 (Gvoždík et al. 2010). Haplotypes g13-g14b, g15 and g16 (Gvoždík et al. 2013) form three separate networks, respectively (not shown).

ods of the Late Pleistocene, or even more recently (Holocene), since the phylogenetic tree and network (fig. 2A, C) show genetic affinities of Peloponnesian A. graeca to east Balkan ones, i.e. populations inhabiting the areas east of the Pindos Mountain Range. This could imply colonization through the current landbridge connecting the Peloponnes to the remaining continental Greece in the east (fig. 1).

Our molecular data refute the morphological discrimination between these two Anguis species, at least according to the characters of Dely (1981) and Grillitsch and Cabella (1990). Although all Peloponnesian specimens that are grouped in the graeca mitochondrial and nuclear lineages showed low NSR (24–26), four Peloponnesian specimens (3, 91, 194, 272) clustering in the cephallonica lineages showed equally low NSR (Supplementary table S1). Moreover, coloration, undulation of the neck-line, contact of the prefrontal scales and ear opening diverged from the expected patterns in many specimens of the cephallonica lineage. Despite the small size of our sample which cannot suffice for statistical analyses, our results corroborate with previous stud-
ies and suggest that currently used morphological characters, and specifically the NSR, do not allow morphological discrimination between *Anguis* species. Morphological features could have resulted from convergent phenotype evolution or represent an ancestral condition in this genus (Gvoždík et al., 2010, 2013). Alternatively, Peloponnesian specimens morphologically similar to *A. graeca* but genetically assigned to *A. cephallonica*, could be hybrids between the two sympatric species. However, these individuals were found in southern Peloponnese, where only *A. cephallonica* is presumably distributed (Grillitsch and Cabela, 1990).

Finally, more detailed sampling and analysis of microsatellite markers is needed in order to examine the occurrence of possible hybridization between *A. cephallonica* and *A. graeca* in the northern Peloponnese and to investigate the intraspecific genetic variation of the former species in the southern Peloponnese. Future work should also focus on a detailed morphological analysis, for the re-evaluation of shared phenotypic patterns apparent in both species and the detection of those morphological characters that may be of diagnostic value.

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**References**


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