



Short Note

Saint Patrick, what about legless lizards? Tracing the mitochondrial affiliation and possible origin of *Anguis* (Squamata: Anguidae) population in Ireland

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Abstract. Ireland is home to only one native lizards' species, the viviparous lizard and one of the few areas of the world where there are no native snakes. However, since the end of the 19th century, there have been reports indicating the presence of legless lizards, *Anguis fragilis* Linnaeus, 1758 population whose origin is unknown. As this genus has been intensively studied genetically and taxonomically in the past decade, we have decided to investigate newly obtained mtDNA sequences (ND2 gene) to trace affiliation of the *Anguis* population in Ireland. We genetically examined 12 individuals from the Burren area (western Ireland) and compared them to available data. The Irish population was identified as belonging to *A. fragilis*, specifically to the most common haplotype of the Illyrian-Central European haplogroup. This genetic affiliation is shared by populations across a wide European region, including the western Balkans, Spain, France, western Hungary, Austria, Germany, Latvia, Poland, the Czech Republic, Russia, Sweden, Norway, and Great Britain. Our findings showed that the Irish population does not exhibit unique mitochondrial variability. This haplotype is present across the range of the species, including Great Britain, which is not in contrast with the earlier hypothesis that nearby populations from this area, along with human-mediated introductions, could be the source of *Anguis* populations in Ireland. This is thus potentially the first documented case of *Anguis* introduction out of the native range. However, further research, including broader sampling in Great Britain and genomics, is required to determine and trace its exact origin.

Keywords: *Anguis fragilis*, Europe, human-mediated introduction, mitochondrial diversity, United Kingdom.

Due to the dynamic paleogeography and high habitat diversity, the current biodiversity in Europe is very rich (Blondel et al., 2010). In recent years, development in molecular taxonomy has greatly improved our inference of the diversity of reptiles in Europe (Speybroeck et al., 2020). Evaluating variability in DNA we were able to have better view on the evolution-

ary history and origin of such diversity (e.g., Kaliantzopoulou et al., 2011; Kindler et al., 2017; Marzahn et al., 2016; Jablonski et al., 2019). This also applies to slow worms (genus *Anguis*), which are legless reptiles belonging to lizards distributed in the Western Palearctic (Jablonski et al., 2021). Since the time of Carl Linnaeus, they have been considered as a mono-

typic genus with developed subspecific taxonomy. Later, Grillitsch and Cabela (1990) presented that the Peloponnese populations form endemic species *A. cephalonica* Werner, 1894 and recent studies (Gvoždík et al., 2010, 2013, 2023) revealed that the genus is represented by five species of different levels of their genetic diversity (Thanou, Giokas and Kornilios, 2014; Jablonski et al., 2016, 2017; Dufresnes et al., 2023). Two of these species have wider distribution (*A. colchica* and *A. fragilis*) forming long contact zone throughout Central Europe and the Balkans (Szabó and Vörös, 2014; Gvoždík et al., 2021; Jablonski et al., 2021). Despite their large distribution range, we do not have any direct evidence of their introductions out of its natural range (Jablonski et al., 2021).

Ireland belongs among other areas of a potential biogeographic and/or conservation importance. There is only one known native terrestrial reptile species, the common lizard *Zootoca vivipara* (Lichtenstein, 1823) that appears to have a widespread distribution across the whole island (King et al., 2011). Moreover, Ireland, one of the few regions worldwide, is lacking legless reptiles, particularly snakes. This absence is traditionally attributed to local legends surrounding Saint Patrick but, in fact, it is strongly connected to the historical formation of the island since the Pliocene (Harrison, 2015). However, a population of *Anguis* with unknown origin and species affiliation has been reported from the island since the beginning of the 19th Century (museum specimen NMINH: 2008.84.206; N.T. Monaghan, pers. comm.) and McGuire and Marnell (2000) expect that the introduction from Great Britain is the most likely scenario for their origin. If so, Ireland populations should be affiliated to *A. fragilis* Linnaeus, 1758 which is also distributed in Great Britain. Nevertheless, the recent surge in documented observations of these lizards from various locations across the island does not preclude the potential for a refugial character (natural colonisation) of the Irish population (Parry, 2020; Jablonski et al., 2021). Whereas it has

never been tested, we would like to bring the first evidence from the mitochondrial DNA and identify their species and genetic affiliation with suggestions to possible areas of their origin.

In 2021, the second author (NP) collected 12 DNA tissue samples of *Anguis* from five localities (supplementary table S1) from the Burren area (in western part of the Republic of Ireland). The genus *Anguis* is not protected in Ireland (Nelson et al., 2019) and all samples were collected from private lands (oral permissions) or non-protected areas. The Burren area is a karst landscape centered in County Clare and Galway, on the west coast of Ireland (around 530 km²), within the circle made by the villages of Lisdoonvarna, Corofin, Gort, Kinvara and the Burren National Park (Feehan, 1991). The outer tip (~1 cm) of the tails was removed by gently squeezing with a pair of tweezers and stored in 96% ethanol. Total genomic DNA was extracted from the tissue samples using the E.Z.N.A.[®] Tissue DNA Kit (Omega Biotek, Inc., USA). We amplified a portion of mitochondrial DNA (mtDNA) comprising the NADH dehydrogenase 2 (ND2) gene using primers (L4437n, H5934) and PCR protocol following (Gvoždík et al., 2010). The PCR products were purified using the EPPiC Fast (A&A Biotechnology, Poland). Following the methodology of Jablonski et al. (2016), the sequencing was performed by MacroGen Europe Inc. (The Netherlands; <http://www.macrogen-europe.com>) and new sequences have been deposited in GenBank under accession numbers OQ341636-OQ341647 (supplementary table S1). For species identification the Irish sequences were checked via BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences were edited using Geneious Prime v.2020.1 (<https://www.geneious.com>) and final alignment contains 732 bp-long fragments. The alignment was performed with MAFFT v.7 (Katoh and Standley, 2013) and overall included 206 sequences of *A. fragilis* from Ireland and published studies (see supplementary table S1). Using DnaSP 5.10 (Librado and Rozas, 2009), stop codons

were not detected. The same program was used to calculate p -distances among the main haplogroups. P -distances were not corrected for multiple substitutions, substitution rate biases, or differences in evolutionary rates. Nevertheless, such estimation appears appropriate for the recent evolutionary times here involved. To assess the diversity of ND2 haplotypes, a 95% maximum parsimony haplotype network was constructed and visualized using the TCS algorithm in the software programs TCS 1.21 (Clement, Posada, and Crandall, 2000) and tcsBU (Santos et al., 2016). In order to provide a comprehensive overview of *Anguis* distribution in Ireland, we compiled a dataset of distribution information and generated a map based on four sources: (1) our own data collected between 2015 and 2021 (February to November); (2) published data from Allingham (1913), Baring (1915), McGuire and Marnell (2000), and Parry (2020); (3) surveys conducted among local residents; and (4) data obtained from local databases, i.e., the National Biodiversity Data Centre (<https://biodiversityireland.ie/>). The software QGIS 3.10.8 (QGIS Development Team, 2022) was used for map creation and range calculation. All primary data can be found in supplementary table S2.

The genetic analysis using BLAST comparison confirmed the affiliation of the Irish (the Republic of Ireland) population to *A. fragilis*, specifically to the Illyrian-Central European haplogroup (ICE) as described by Jablonski et al. (2016). This is the most common haplogroup observed, which is shared by individuals from various countries including Bosnia and Herzegovina, Croatia, Montenegro, Serbia, Slovenia, Spain, France, western Hungary, Austria, Germany, Latvia, Poland, the Czech Republic, Russia (Kaliningrad Oblast), Slovakia, Sweden, Norway, and the United Kingdom (Great Britain; fig. 1B, C). In our study, a total of 47 ND2 haplotypes were identified within the species, indicating relatively low genetic diversification (p -distance $\leq 1.1\%$). These findings confirm the geographically divided presence

of haplotype diversity in *A. fragilis*, consistent with the five haplogroups previously identified by Jablonski et al. (2016). Additionally, we established a haplogroup termed here “Western Europe”, which was detected in France and Spain and demonstrated a close genetic affinity to haplogroups found in the northwest Balkans (Alpine-Pannonian, Carniolan, and North Adriatic).

Based on our comprehensive review of distribution data, records of *Anguis* in Ireland date back to the first notice in “November 1893, Fitzwilliam Street, Dublin” documented in the collections of the National Museum of Ireland – Natural History (voucher number NMINH: 2008.84.206; N.T. Monaghan, pers. comm.). In total, our data include 109 records, comprising 63 newly obtained data, 16 published records, and 20 records from the National Biodiversity Data Centre (fig. 1A, supplementary table S2). The estimated distribution range of the species in Ireland covers approximately 273 km², which accounts for approximately 0.3% of the island’s total territory. During our fieldwork, we encountered the species in open and well-vegetated habitats, such as calcareous grassland and scrub areas with limestone pavement outcrops (fig. 2B–C). The soil composition in the Burren area predominantly consists of horizontal layers of bare limestone, interspersed with some brown soil in the valleys. Average air temperatures ranged from 4–6°C in January to 15°C in July, while soil temperatures typically did not drop below 6°C. Thanks to our comprehensive yet geographically constrained field studies carried out from 2015 to 2021, most records (103) originate from the Burren area. Notably, only two records dating back to 1998 were documented outside this timeframe. In 2018, a single find was reported on Inishmaan Island by a local tourist, approximately 35 km away from the Burren area. Two additional records were documented in 2020, one from Mullingar (located in the central part of Ireland) and the other from Minane Bridge in County Cork (southern part of Ireland). Three

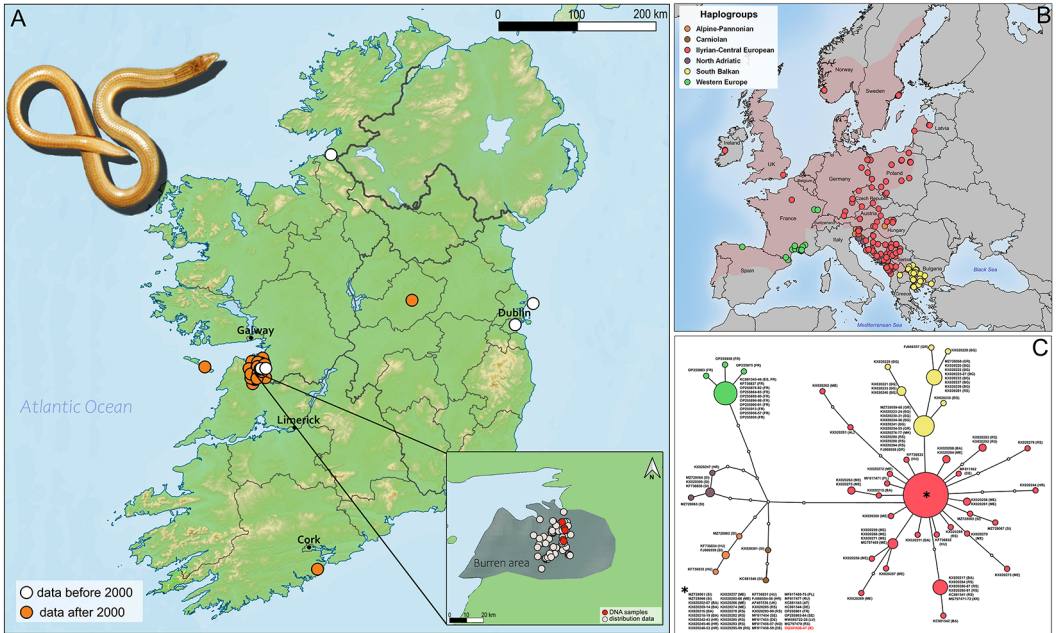


Figure 1. Map and haplotype network of the native and introduced distribution of *Anguis fragilis* based on mitochondrial data. (A) Distribution of *Anguis fragilis* in Ireland, based on localities (white circles – data before 2000, orange circles – data after 2000). Detail view on records of *A. fragilis* in the Burren area are given in the inset map. The Burren area is marked with gray, red circles refer to localities where DNA samples were collected, white circles are distribution data. (B) The distribution of mitochondrial haplogroups (ND2) of *A. fragilis*. The species distribution range (red shading) is re-drawn according to Jablonski et al. (2021). (C) Parsimony haplotype network of analyzed data: circle size is proportional to the number of samples under the same haplotype. Open circles represent missing haplotypes. The colors of haplotypes follow the colors on the map (B). GenBank numbers of samples from Ireland are highlighted in red. Two-letter abbreviation country codes follow ISO 3166-1 alpha-2. Inset photography: an adult male from the Burren area, Ireland.

records were also identified prior to 2000. During our fieldwork, we collected specimens of both male and female individuals, as well as juveniles (fig. 2A), suggesting active reproduction of *A. fragilis* populations in Ireland.

Due to the relatively challenging interpretation of morphology alone, identifying *Anguis* species can be difficult without knowledge of their geographic origin (Gvoždík et al., 2010, 2013; Benkovský et al., 2021). Therefore, tracing the origin of Irish populations (currently known only from the Republic of Ireland) was nearly impossible without genetic evidence, which is why we employed this approach using the rich mitochondrial dataset of previous studies (supplementary table S1). Although our data solely rely on mitochondrial DNA, the overall findings align with previous research conducted in Europe, where *A. fragilis* has been

detected (Gvoždík et al., 2010, 2013, 2023; Jablonski et al., 2017). This species is distributed from Portugal to the Baltics (Latvia), as well as from Scandinavia (Norway, Sweden) to Greece and Bulgaria (Gvoždík et al., 2010, 2023; Jablonski et al., 2016, 2017, 2021). The phylogeographic signal observed in Irish slow worms is not exclusive; it aligns with closely located populations in Great Britain and the majority of Central Europe, a pattern analogous to that observed in the alpine newt population through mtDNA barcoding (Robbemont et al., 2023). The star-like structure observed in the ICE haplogroup of *A. fragilis*, where Irish populations belong, implies recent post glacial re-colonization events of this haplogroup (Jablonski et al., 2016, 2017). This potentially involves rapid spatial expansion into more northerly regions of Europe, including



Figure 2. The representative of *Anguis fragilis* population from Ireland and the natural habitat. (A) An adult male and four juveniles of *A. fragilis*. (B-C) Habitat of *A. fragilis* in the Burren area, Ireland. Photographs by N. Parry.

Great Britain. Although the fossil evidence of the genus *Anguis* from Great Britain dates to the Pleistocene (Benton and Spencer, 1995), there is no evidence of natural ancient colonization to Ireland. The island's connection to and isolation from Great Britain by the Irish Sea (Groote, Lewis, and Stringer, 2018), coupled with its glaciation during past glacial maxima, have played a significant role in shaping the present-day low diversity of reptiles on the island (Harrison, 2015). Similarly, Great Britain experienced waves of colonization and subsequent extinctions of reptile populations depending on the connection to mainland and climatic conditions since Pleistocene (e.g., Sommer et al., 2009; Allentoft, Rasmussen and Kristensen, 2018) and currently only six species of native reptiles are present in the Great Britain. Our data thus are in line with the most common presumption that the local populations of *Anguis* in Ireland originated from the Great Britain through human-mediated introductions dating

back at least 130 years ago. Such interpretation is consistent with previous research and historical records (see Allingham, 1913; Baring, 1915; McCarthy, 1977; McGuire and Marnell, 2000).

The British Isles are known for their history of non-native reptile introductions (e.g., Langton et al., 2011), making it unsurprising that *Anguis* may have been introduced from Great Britain to Ireland in the past (see below). In this context, the presence of Irish population of the genus *Anguis* potentially represents the first documented occurrence of a reproductively viable population introduced outside the native range of these legless lizards. The first find of this species dates from 1893 (NMINH: 2008.84.206 from the National Museum of Ireland – Natural History). Unfortunately, no further information is available regarding this finding, and it is therefore possible that the individual may have originated from captivity (see Allingham, 1913). Another record of the slow worm was published on Lambay Island, near

Dublin in 1906 (Baring, 1915). Interestingly, during the same period, reports emerged regarding the introduction of other reptile species to the island, including *Testudo graeca*, *Mauremys leprosa*, *Pseudopus apodus*, and *Lacerta viridis* (Baring, 1915). It is worth noting that such introductions were also commonplace in various regions of Europe at that time (Adolph, 1922; Pax, 1925; Kehlmaier, Zinenko, and Fritz, 2020; Kolenda et al., 2020). In this context, an intriguing record of *A. fragilis* was documented by Allingham (1913) in Ballyshannon, County Donegal, located in the northern part of Ireland. The journal editor, upon publication, commented that while the slow worm is not considered native to Ireland, it was frequently kept in vivariums and therefore most likely introduced (Allingham, 1913). As there are no further findings in this location, it would confirm that these observations were only anecdotal. The first finding of *A. fragilis* in the Burren area was dated 1972 (McCarthy, 1977) but it also includes notes that slow worms were present there already during the 60s. At that time, there was also an introduction of *L. viridis* in Ireland (Cabot, 1965). Considering the historical context, the presence of identical haplotype diversity, and the increasing number of records from recent observations, it strongly suggests that the population growth of *A. fragilis* in Ireland is a relatively recent phenomenon.

In recent years, there has been a growing interest in the potential introduction of herpetofauna to new regions within Europe (Asztalos et al., 2021; Doorn et al., 2021; de Brouwer et al., 2022; Kuijt et al., 2022; Vliegenthart et al., 2022; Robbemont et al., 2023). Consequently, the possibility of introduced species and their spreading in Ireland is on the rise, while active measures are being taken to prevent such introductions in the neighboring Great Britain (e.g., *Ichthyosaura alpestris*; Lever, 2003; Wells, 2007; Morris, 2018). Based on our findings, it appears that the distribution range of the slow worm in Ireland may be considerably larger than previously assumed. However,

further field studies are required to validate this presumption. Additionally, collecting additional samples from the British Isles and employing a wider array of genetic markers would enhance our understanding of the potential sources of introductions. Consequently, it is essential to dedicate more attention to mapping, further distribution modelling, and conservation efforts, as well as investigating the ecological and niche adaptations of slow worms in Ireland.

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