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Short Note

MtDNA barcoding illuminates native diversity and introduction pathways of slow worms in The Netherlands

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Abstract. MtDNA barcoding is a powerful tool for determining the identity and provenance of introduced (cryptic) species. We mtDNA barcode *Anguis* slow worms from the Netherlands. We show that a non-native population (Zuid-Kennemerland National Park) that was established over a century ago concerns the same species that occurs natively: *Anguis fragilis*. It carries a haplotype that naturally occurs from The Netherlands to the Balkans. While a lineage from the Balkans up to Western Europe occurs throughout The Netherlands, we detect a lineage from France and Spain in one locality in the southwest of the country. We associate this record with the import of hay bales from France, used for horse feed. In the central Netherlands we find a haplotype previously only reported from Poland, potentially reflecting an unknown introduction or an underestimation of the haplotype's distribution in the poorly sampled postglacially colonized part of the range.

Keywords: *Anguis fragilis*, biogeography, introduced species, phylogeography, translocation.

Introduced species pose a major conservation concern due to their potential to prey on, compete with, spread disease to and hybridize with native species (Simberloff, 2013; Bellard et al., 2016; Pyšek et al., 2020; Theodoropoulos et al., 2025a). Many reptile species have been introduced by human activities beyond their native range (Kraus, 2015). However, it is not always straightforward to identify an introduced species as it might belong to a cryptic species complex (Bickford et al., 2007; Hending, 2025). MtDNA barcoding (Hebert et al., 2003; Mir et al., 2021) is a powerful tool for identifying to which (cryptic) species a suspect individual belongs and determining from which part of the native range it derives.

The slow worm genus *Anguis* is an example of a cryptic species complex that has only recently been resolved through genetic analyses (Gvoždik et al., 2010, 2013, 2023; Jablonski et al., 2016). As a result, five species are now recognized: *A. fragilis* in western and northern Europe, *A. colchica* in (south)eastern Europe and western Asia, *A. graeca* in the southern Balkan Peninsula, *A. cephallonica* in the Peloponnese, and *A. veronensis* in the Italian Peninsula. In The Netherlands, *A. fragilis* occurs natively in the south and east of the country (Creemers and van Delft, 2009). However, slow worms have also been introduced into the Dutch coastal dunes, presumably around 1900, and a self-sustaining population is now present in Zuid-Kennemerland National Park (Creemers and van Delft, 2009; fig. 1A). We mtDNA barcode Dutch slow worms from the native and introduced range to address two questions: (1) Which slow worm species is involved in the introduced population?; and (2) From where do these introduced slow worms originate?

We obtained 148 slow worm samples from 46 localities (a mixture of oral swabs, skin sheds and roadkills; supplementary table S1; Fig. 1A). DNA was extracted using the Wizard® Genomic DNA purification kit (Promega). We amplified the entire ND2 (NADH dehydrogenase 2) mtDNA gene using the primers H5934 (5′-AGRGTGCCAATGTCTTTGTGRTT-3′) from Macey et al. (1997) and L4437n (5′-AAGCTA TTGGGCCCATACC-3′) from (Gvoždik et al., 2010). PCRs were performed in 12 μl reactions, containing 0.06 μl of both forward and reverse primer (0.05 μM end concentration of each primer), 6 μl Qiagen multiplex PCR master mix, 4.88 μl purified water and 1 μl of DNA extract. PCR conditions were: a hot start for 15 min at 95°C, followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 1 min at 55°C and extension for 1 min at 72°C, and extension at 72°C of 10 min. Sanger sequencing was outsourced to Macrogen and forward and reverse sequences were compiled, edited and trimmed in Geneious Prime 2025.0.2 (https://www.geneious.com). We limited our downstream analyses to 729–732 bp of ND2 (variable due to a 3 bp deletion in *A. fragilis* compared to the other slow worm species) because this fragment has been widely studied in previous studies (e.g. Jablonski et al., 2016, 2017; Oskyrko et al., 2023), allowing for direct comparison.

We first collapsed Dutch mtDNA sequences into haplotypes using the DNA to haplotype collapser function in FaBox (Villesen, 2007). We then took one sequence each of the five cryptic *Anguis* species (Mikulíček et al., 2018; Dufresnes et al., 2023; Altmanová et al., 2024; Skórzewski et al., 2025), namely *A cephallonica* (GenBank Accession MG797453), *A. colchica* (PP549459), *A. graeca* (OR352107), *A. fragilis* (PP549518) and *A. veronensis* (OP255969), aligned the Dutch mtDNA haplotypes and constructed a maximum likelihood phylogeny using the IQ-TREE webserver (Trifinopoulos et al., 2016), to determine with which species the Dutch *Anguis* haplotypes clustered. We used *Pseudopus apodus* as an outgroup (GenBank Accession MW400934; taken from Jablonski et al., 2021a). We used ModelFinder (Kalyaanamoorthy et al., 2017) to determine the most appropriate partitioning scheme and model of sequence evolution (with HKY+F+I for codon 1, HKY+F+I for codon 2 and TN+F for codon 3) and 1000 ultrafast bootstrap replicates (Hoang et al., 2017) to determine branch support.

Because all Dutch *Anguis* mtDNA barcodes clustered with *Anguis fragilis*, we then collected ND2 sequences collected from 248 individuals from 185 localities across the native *A. fragilis* range from previous studies (Ast, 2001; Albert et al., 2009; Gvoždik et al., 2010, 2013, 2021, 2023; Szabó and Vörös, 2014; Jablonski et al., 2016, 2017, 2021b; Mikulíček et al., 2018; Margaryan et al., 2021; Dufresnes et al., 2023; Altmanová et al., 2024; Skórzewski et al., 2025) and again collapsed these (including the Dutch haplotypes) into haplotypes with FaBox (supplementary table S2).

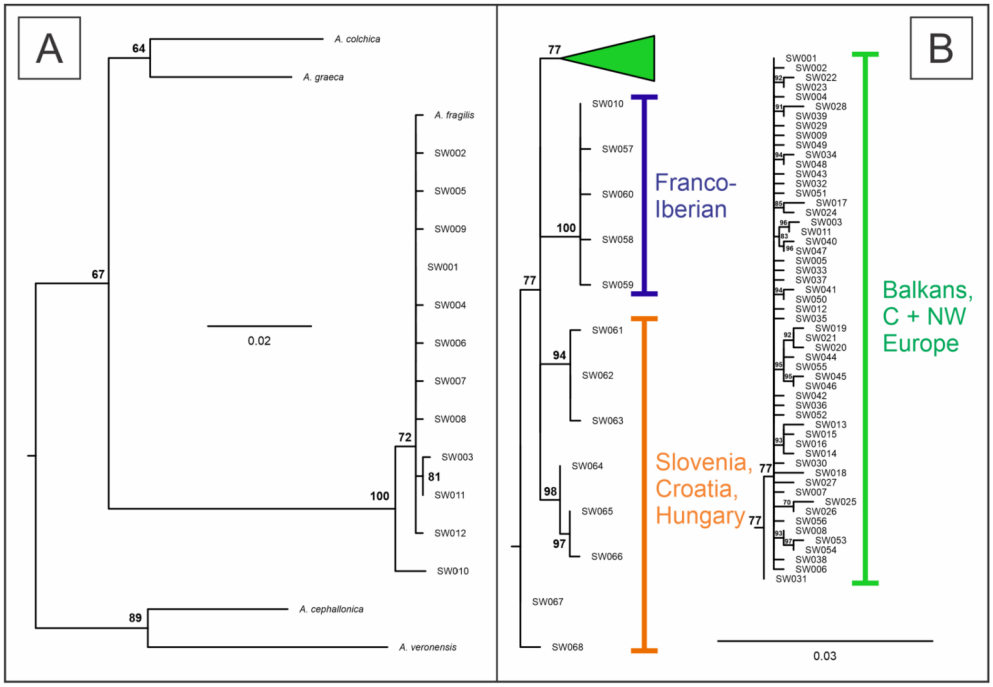

Figure 1. Maximum likelihood phylogeny of mtDNA (ND2) phylogeographical lineages of slow worms (*Anguis*). The outgroup is not shown. The scale bar represents the number of substitutions per site. Support values are ultrafast bootstrap support values (only values >60 are displayed). (A) A phylogeny including representatives of all five *Anguis* species as well as all haplotypes found in The Netherlands. (B) A phylogeny of all *Anguis fragilis* haplotypes. Colours used to denote groups of haplotypes match fig. 2. Haplotype codes correspond to supplementary tables 1 and 2.

We made the total 729 bp haplotype dataset (supplementary table S3) available via GenBank (Accessions PV862286-PV862353). We constructed another maximum likelihood phylogeny using the same approach in IQ-TREE (with K3Pu+F+G4 for codon 1, HKY+F for codon 2 and TN+F for codon 3) and *P. apodus* as outgroup.

We identify 12 *Anguis* mtDNA haplotypes in The Netherlands, of which three have been reported in previous studies (i.e., already present in our database) and eight were newly identified (supplementary tables s1 and s2). All haplotypes belong to *A. fragilis* (figs. 1a). Within *A. fragilis* we see some substructuring (fig. 1b). Most Dutch mtDNA haplotypes belong to a clade distributed in the Balkan Peninsula and central and northwestern Europe, while one belongs to a clade otherwise found in France and Spain (figs. 1 and 2).

We detect only *A. fragilis* mtDNA haplotypes in The Netherlands. The most frequent haplotype, SW001, is widespread in the native *A. fragilis* range, from The Netherlands to the Balkan Peninsula (supplementary table s2). This haplotype is also found in the introduced population in Zuid-Kennemerland National Park. In contrast to some herpetofauna introductions in the Dutch coastal dunes that involve non-native populations (*Pelobates* spadefoot toads, Koster et al., 2023; *Pelophylax* waterfrogs, Theodoropoulos et al., 2025b) or even non-native species (*Hyla* tree frogs, Kuijt et al., 2023), other introduced populations are genetically indistinguishable from native ones based on mtDNA (*Triturus* newts, de Brouwer et al., 2023; *Mesotriton* newts, Robbemont et al., 2023; *Alytes* midwife toads, Vliegenthart et al., 2023). Similarly, for *A. fragilis* in Zuid-Kennemerland National Park, the mtDNA data do not allow us to determine whether they

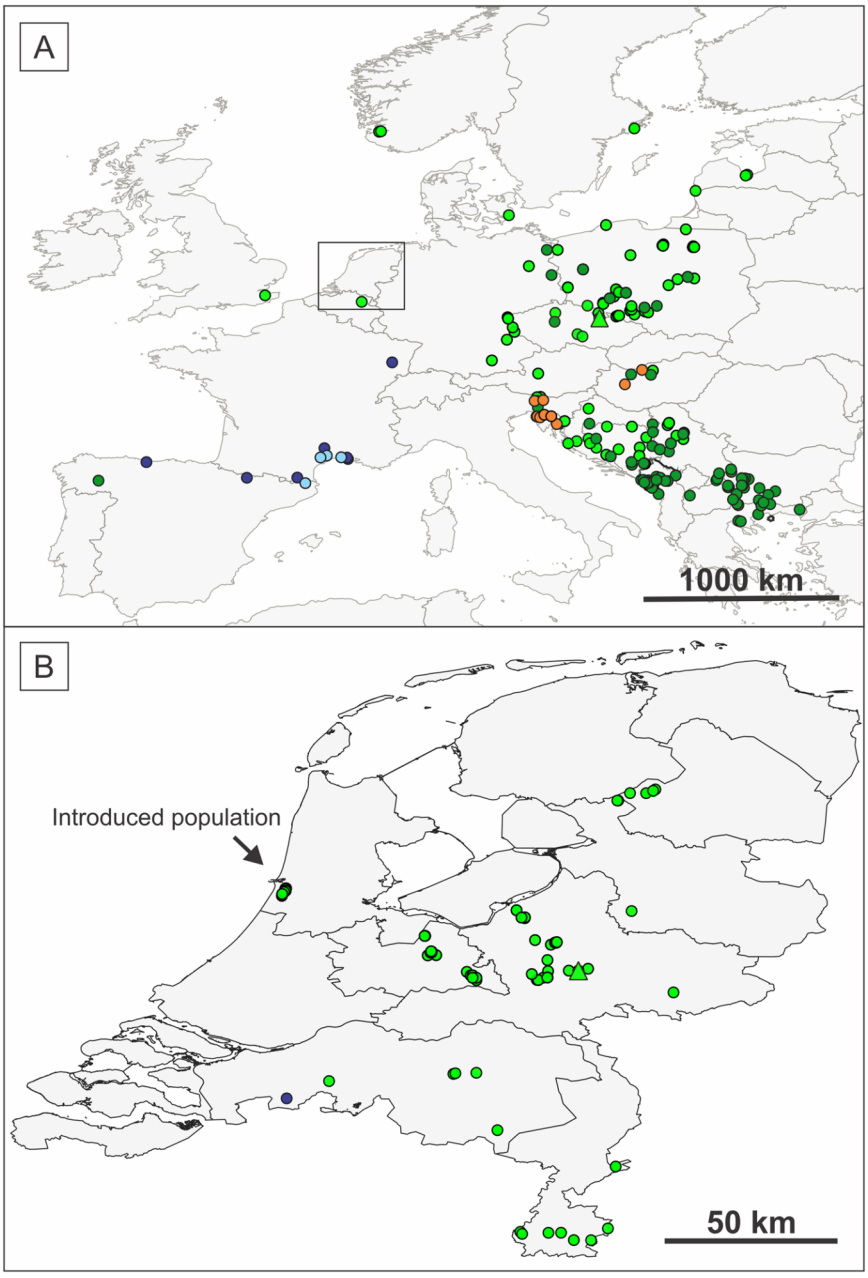

Figure 2. Distribution map of mtDNA (ND2) phylogeographical lineages of slow worms (*Anguis fragilis*). Distribution of groups of haplotypes in Europe (A) and in The Netherlands (B). Colours correspond to fig. 1. The lighter shades of green and the darker shades of blue on the map for Europe reflect populations in which haplotypes are found that also occur in The Netherlands. The green triangle concerns haplotype SW009, discussed in the main text.

originate from within The Netherlands or from abroad; a study employing nuclear DNA data could provide the required geographical genetic resolution.

A noteworthy finding is the presence of a haplotype, SW010, that belongs to a clade otherwise found in France and Spain. Haplotypes from the rest of The Netherlands belong to a

clade otherwise found in the Balkan Peninsula and central and northwestern Europe. At first glance, this finding appears to support a scenario of postglacial recolonization of western Europe from distinct glacial refugia, with secondary contact occurring in The Netherlands, as observed in the great crested newt, *Triturus cristatus* (de Brouwer et al., 2023). However, the locality is a horse farm that uses hay bales imported from France as horse feed. Given that multiple dead and flattened slow worms were found on the terrain, while subsequent surveys did not reveal a resident population, we accept the explanation that the corpses turned up via imported hay. Mowing casualties are known to occasionally turn up in commercial hay feed (Bergmans and Zuiderwijk, 1986).

Within the native Dutch range we also identified one haplotype, SW009, that was previously only reported from Poland (Skórzewski et al., 2025). This might indicate an introduction, but, given the limited sampling outside of presumed glacial refugia, we cannot rule out the possibility that this mtDNA haplotype is more widely distributed. It is a common limitation of phylogeographic studies that postglacially colonized areas remain undersampled, which hampers our ability to detect introductions (Vliegenthart et al., 2023; Elfering et al., 2024). We emphasize the value of increased sampling outside of glacial refugia, both to refine phylogeographic patterns and to provide reference data for studies on species introductions.

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Supplementary materials. Data is available on https://doi.org/10.1163/15685381-bja10243 under Supplementary Materials.

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