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Amphibia-Reptilia (2024) DOI:10.1163/15685381-bja10181

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Short Note

Extreme genetic depletion upon postglacial colonization hampers determining the provenance of introduced palmate newt populations

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Received 15 February 2024; final revision received 11 April 2024; accepted 3 May 2024;
published online 24 May 2024
Associate Editor: Guillermo Velo-Antón

Published with license by Koninklijke Brill BV | DOI: 10.1163/15685381-bja10181

| ISSN: 0173-5373 (print) 1568-5381 (online)

Abstract. MtDNA barcoding is regularly applied to determine the provenance of invasive species. Variation in spatial genetic structuring across a species' range, typically high within glacial refugia and low in postglacially colonized areas, influences the precision of this approach. The palmate newt (*Lissotriton helveticus*) has been introduced north of its native range inside the Netherlands. We conduct mtDNA barcoding to try and retrace the origin of the introduced localities. A large increase in sample size, particularly focusing on temperate Europe, emphasizes that the palmate newt shows practically no genetic variation outside the Iberian Peninsula glacial refugium. While we find a haplotype previously only known from the Iberian Peninsula inside the native range in Belgium, the haplotype present in the introduced Dutch populations occurs widely throughout the native range north of the Iberian Peninsula. Although mtDNA barcoding can be a powerful tool in invasion biology, the palmate newt case exposes its limitations.

Keywords: amphibians, invasive species, *Lissotriton helveticus*, mtDNA barcoding, phylogeography.

Invasive species – defined here following Simberloff (2013) as species that have established a reproductive population outside of their native range – are one of the main drivers of the current sixth mass extinction (Bellard et al., 2016; Nunes et al., 2019; Pyšek et al., 2020; Bellard et al., 2021; Dueñas et al., 2021). Documenting introduced populations and tracing their origins are key steps when considering conservation actions (Allendorf et al., 2010; Vliegenthart et al., 2023). For this purpose, mtDNA barcoding is commonly employed, given its cost effectiveness (Hebert et al., 2003; Mir et al., 2021; Gostel and Kress, 2022). Many taxa show extensive geographical genetic structure across their natural range (Avise, 2000), and for most European amphibians, mtDNA phylogeography datasets have been published (e.g., Babik et al., 2005; Martínez-Solano et al., 2006; Fijarczyk et al., 2011; Recuero et al., 2012; Vences et al., 2013; Wielstra et al., 2013; Dufresnes et al., 2019b; Ambu et al., 2023; Dufresnes et al., 2024). Such detailed insights into geographic mtDNA structure facilitate the identification of introduced populations and enable these populations to be linked to their region of origin within the natural range.

However, the degree of geographical genetic structuring within taxa varies considerably in space (Avise, 2000). Typically, genetic variation is concentrated in glacial refugia, whereas it is generally low or absent in postglacially colonized terrain (Hewitt, 2000). Furthermore, phylogeographic sampling is typically biased towards glacial refugia, thereby reducing the likelihood to capture genetic variation present in postglacially colonized areas (Vliegenthart et al., 2023). Therefore, the accuracy with which mtDNA barcoding can pinpoint the provenance of invasive species strongly depends on the source locality of the introduced population. As a consequence, their origin can be determined with high spatial resolution in some cases (Dubey et al., 2019; Dufresnes et al., 2019a; Koster et al., 2023; Kuijt et al., 2023; Robbemont et al., 2023), but remains imprecise in others (de Brouwer et al., 2023; Vliegenthart et al., 2023).

The palmate newt (*Lissotriton helveticus*) occurs in the Iberian Peninsula and in western and (a small part of) central Europe (Sillero et al., 2014). In the Netherlands, the species is native to the south, in the provinces Limburg and Noord-Brabant, but has been introduced north of the Rhine river, with around a dozen introduced localities currently known (Creemers and van Delft, 2009; https://www.ravon.nl/Soorten/Soortinformatie/vinpootsalamander; fig. 1). An mtDNA-based phylogeographical study for the palmate newt demonstrates that most of its genetic diversity is present within the Iberian Peninsula (Recuero and García-París, 2011). Genetic depletion further north is also strongly suggested, albeit based on limited sampling. In this study, we aim to understand the introduction history of palmate newts by (1) mtDNA barcoding localities from the introduced range within the Netherlands and (2) conducting a dense phylogeographical survey of the postglacially colonized natural range.

We obtained new mtDNA data for 93 individuals from 49 localities – including 20 individuals from six introduced

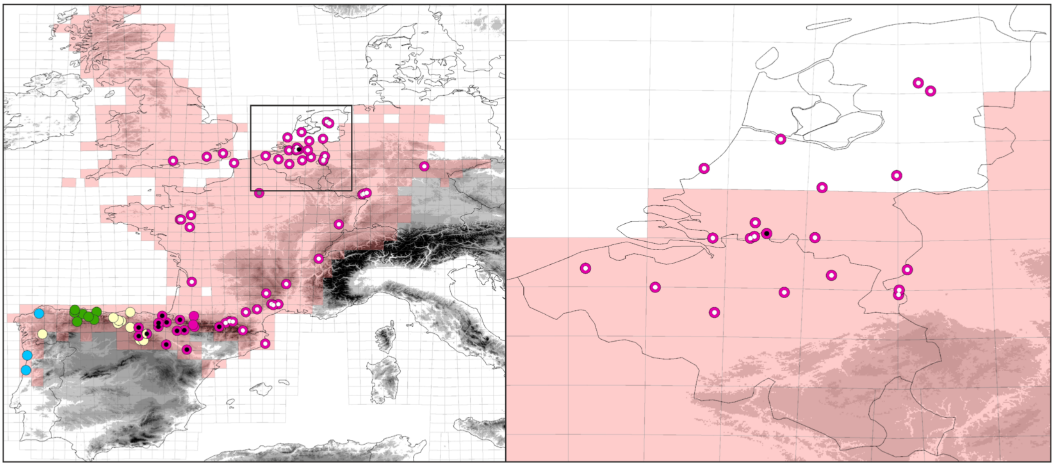

Figure 1. Distribution map and sampling scheme for the palmate newt (*Lissotriton helveticus*). Circles represent sampled localities and the colors reflect the geographic groups sensu Recuero and García-París (2011). Dots inside these circles represent haplotypes discussed further in the main text (Lhel006 = black; Lhel016 = white; Lhel027/Lhel028 = grey). The pink grid squares show the palmate newt distribution range at 50 × 50 km resolution based on Sillero et al. (2014).

populations (fig. 1; supplementary table S1). For 54 of these individuals samples consisted of skin swabs, of which many were collected by citizen scientists. The outer surface of the newt was swabbed (40 times in total, 10 times on the dorsal, ventral and each lateral side) with Copan 155C Rayon swabs. Swabs were stored dry and frozen at −20°C as soon as feasible. We also included 39 tissue samples (taken from earlier studies (Johanet et al., 2011; Palomar et al., 2021), the tissue collection of the CEFE CNRS – EPHE collection of reptiles and amphibians of the Biogeography and Ecology of the Vertebrates team in Montpellier (BEV collection), or newly collected). DNA was extracted using the Wizard® Genomic DNA purification kit (Promega).

We amplified a 644 base pair fragment of the COI (Cytochrome Oxidase I) mtDNA gene. We used the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′; Folmer et al., 1994) and COI-H (5′-TCAGGGTGACCAAAAAATCA-3′; Machordom et al., 2003) that were previously applied in *L. helveticus* by Recuero and García-París (2011). 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), 7.2 μl QIAGEN multiplex PCR master mix, 3.68 μl purified water and 1 μl of DNA extract. PCR conditions were: a hot start for 15 minutes at 95°C, followed by 35 cycles of denaturation for 30 seconds at 95°C, annealing for 1 minute at 55°C and extension for 1 minute at 72°C, and extension at 72°C for ten minutes. Sanger sequencing was outsourced to BaseClear B.V. and sequences were edited and trimmed in Geneious Prime 2023.1.1.

COI sequences were available for 100 individuals from 35 populations from Recuero and García-París (2011) and our newly produced COI sequences were added to this list. The Haplotype Collapser function in FaBox (Villesen, 2007) was used to collapse sequences into haplotypes. We followed Recuero and García-París (2011) and partitioned the haplotypes into four geographic mtDNA groups. The resulting COI haplotype database provides a comprehensive overview of genetic variation across the natural range and we introduce a new naming system (supplementary table S1). Our COI haplotype database also allows individuals from introduced populations to be linked to the part of the natural range that they originate from, based on their COI haplotype.

We conducted a maximum likelihood inference tree search using the IQ-TREE web server (Trifinopoulos et al., 2016). For this analysis, ModelFinder (Kalyaanamoorthy et al., 2017) was used to automatically determine the most appropriate model of sequence evolution for each codon position (TNe, K2P + G4 and TIM2e + I for positions 1, 2 and 3, respectively). We used default settings, which included 1000 ultrafast bootstraps (Hoang et al., 2017) to determine branch support. Samples of *Triturus cristatus* (Genbank Code: EU880336, taken from Zhang et al., 2008) and *Lissotriton vulgaris* (EU880339, taken from Zhang et al., 2008) were used as outgroups. A median spanning haplotype network was constructed in PopArt 1.7 under default settings (Leigh and Bryant, 2015). This analysis allowed us to determine which geographic mtDNA group the newly identified COI haplotypes belonged to.

Our database of 193 COI sequences represents 93 newly sequenced and 100 previously published individuals (from 49 and 35 localities) and encompasses 28 haplotypes (figs 1-3; supplementary table S1). All but three of the newly sequenced individuals possess one of the 26 previously identified haplotypes. Two new

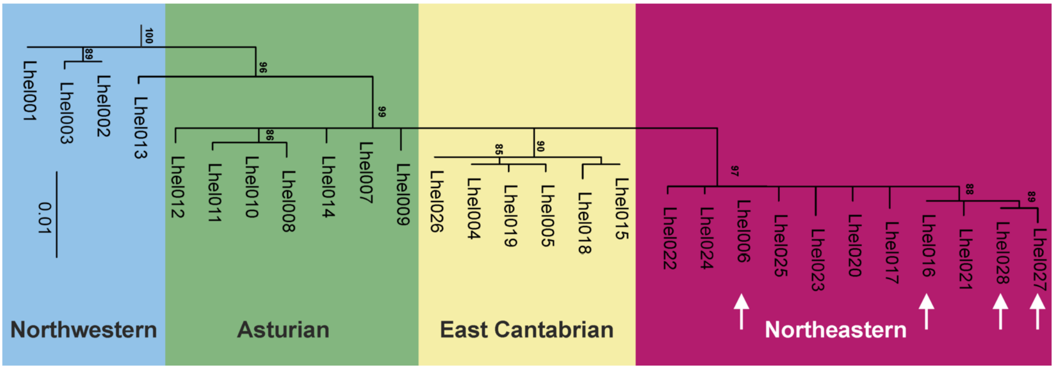

Figure 2. Maximum likelihood phylogeny for the palmate newt (*Lissotriton helveticus*) based on a 644 base pair fragment of COI. Haplotypes are partitioned into geographic groups according to Recuero and García-París (2011). Arrows denote haplotypes that are further discussed in the main text. The outgroup is not shown.

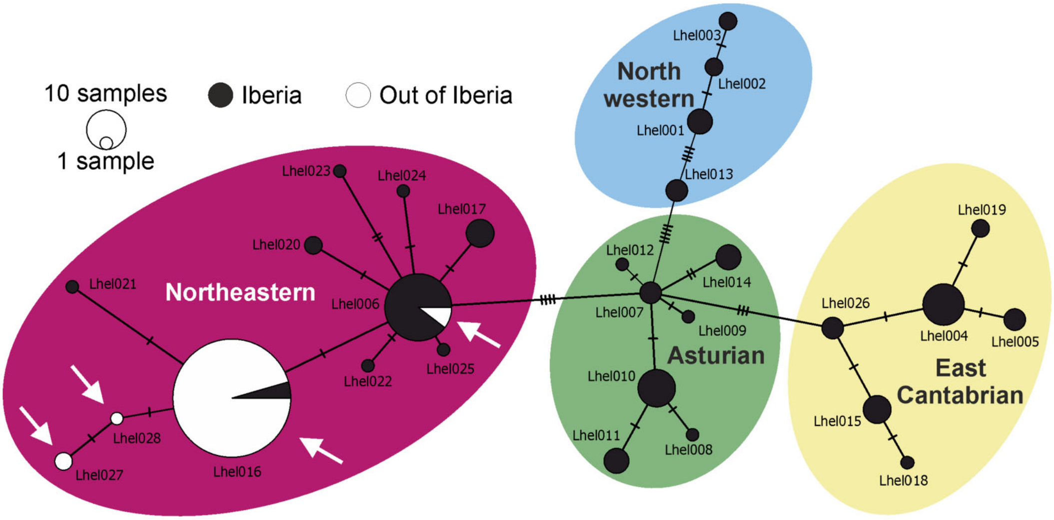

Figure 3. Haplotype network for the palmate newt (*Lissotriton helveticus*) based on a 644 base pair fragment of COI. Haplotypes are partitioned into geographic groups according to Recuero and García-París (2011). Arrows denote haplotypes that are further discussed in the main text.

haplotypes were found, one present in a single individual and one present in two individuals.

The topology of our phylogeny is identical when including both *T. cristatus* and *L. vulgaris* as outgroup (fig. 2) or only *T. cristatus* or *L. vulgaris* (data not shown). We highlight the “geographic groups” recognized by Recuero and García-París (2011). We recover the Northwestern geographic group as paraphyletic and basal in relation to the three remaining geographic groups. The Asturian geographic group is paraphyletic as well and we recover both the Northeastern and East Cantabrian geographic groups as clades that are nested inside it. We show that the East Cantabrian geographic group extends to the northwest of Spain (fig. 1).

Most mitochondrial DNA diversity is found on the Iberian Peninsula and the extreme south of France (26 haplotypes are present in the region denoted as “Iberia” in fig. 3). North of the Iberian Peninsula and the extreme south of France, where we sampled extensively, a single haplotype, Lhel016, occurs almost exclusively (the region denoted as “out of Iberia” in fig. 3), with two exceptions. One case concerns the presence of two newly identified haplotypes in northern France (figs. 1-2): Lhel027 and Lhel028. The other case concerns the locality Meerle from Belgium, where hap Lhel006 is found. Otherwise, haplotype Lhel006 only occurs in and south of the Pyrenees (fig. 1). All the introduced localities in the Netherlands contain haplotype Lhel016 (supplementary table S1).

The phylogenetic structure we recover in the palmate newt slightly deviates from Recuero and García-París (2011). This likely reflects the fact that we base our analysis on COI only, whereas Recuero and García-París (2011) also included the D-loop (which we excluded because this marker is relatively problematic to align). The main topological difference is that our phylogeny suggests that the deepest genetic divergence is positioned in the west of the Iberian part of the range (where the Northwestern geographic group is distributed; figs. 1-2), rather than in the center (where the Asturian geographic group is distributed). Another conflict, which reflects our increased sample size, is that the East Cantabrian geographic group actually extends almost 300 kilometers further west than previously assumed (well outside of Cantabria; fig. 1). A denser sampling, particularly in the northwest of the Iberian Peninsula, would help clarify the distribution and extent of comingling of geographical groups.

Despite almost doubling the sample size of Recuero and García-París (2011), we only identified two new COI haplotypes in the palmate newt. This is likely due to our explicit focus on the postglacially colonized part of the range. In this respect, our findings provide strong support that only a single haplotype (Lhel016) is widely distributed outside the Iberian Peninsula (fig. 1). This phylogeographic pattern underlines that the palmate newt represents an extreme case of genetic depletion associated with postglacial range expansion. However, the two new haplotypes identified in northern France underscore the premise that sparse sampling outside glacial refugia leads to an underestimation of the haplotype diversity in the postglacially colonized part of the range (fig. 1; with one notable exception from Belgium, but see below).

The same haplotype that naturally occurs in almost the entire range north of the Iberian Peninsula, including the native range in the Netherlands (Lhel016), is also the one that is found in all introduced Dutch localities (fig. 1). This means that the provenance of these introduced localities cannot be determined with a high degree of accuracy. A similar lack of phylogeographic resolution has hampered the ability to determine the provenance of other amphibian populations introduced in the Netherlands, namely the alpine newt *Ichthyosaura alpestris*, the great crested newt *Triturus cristatus*, the common tree frog *Hyla arborea* and the common midwife toad *Alytes obstetricans* (de Brouwer et al., 2023; Kuijt et al., 2023; Robbemont et al., 2023; Vliegenthart et al., 2023).

In Belgium, in one locality (Meerle) that is located within the natural range of the palmate newt, we unexpectedly found haplotype Lhel006, which is otherwise only present on the Iberian Peninsula (fig. 1). There is a similar finding for the great crested newt, where a haplotype otherwise only known from northwestern Romania was recovered in Belgium as well (in the locality Temse, located c. 50 kilometers away from Meerle) (de Brouwer et al., 2023). Except for a laboratory error (which we consider unlikely because the relevant samples were not processed together with Iberian samples), there are three scenarios that could explain an outlier palmate newt haplotype in Belgium: (1) the haplotype occurs naturally north of the Iberian Peninsula, albeit at low frequency; (2) the same haplotype has independently evolved locally, given that it is only one substitution different from the widespread haplotype Lhel016; or (3) newts carrying this haplotype have been introduced in Belgium. Scenario 1 and 2 seem unlikely because we densely sampled the area north of the Iberian Peninsula and the haplotype has an extremely local distribution in Belgium. Therefore, we consider scenario 3, introduction, to best explain our unexpected observation.

The introduction of non-native palmate newts within the native range introduces the risk of ‘genetic pollution’, where local alleles are replaced by invasive ones via introgressive hybridization (Meilink et al., 2015). Nuclear DNA should be studied to determine the degree and extent of genetic admixture between native and introduced palmate newts. The introduced palmate newt populations (but also the native ones) also overlap with the congeneric smooth newt *L. vulgaris* (Sillero et al., 2014; Wielstra et al., 2018). The risk of genetic pollution between these species seems negligible, given that hybridization where their natural ranges overlap is extremely rare and only F1 hybrids have ever been recorded (Arntzen et al., 1998; Dubey et al., 2019; Miralles et al., 2023).

Although mtDNA barcoding has proven itself to be a powerful tool in revealing the provenance of many invasive populations, the limitations of the technique are exposed in cases such as the palmate newt. The Dutch introduced populations contain the mtDNA haplotype that is dominant in the vast postglacially recolonized part of the range. Given that the species occurs naturally in the southern part of the Netherlands, a Dutch origin may appear a reasonable assumption. However, the unexpected finding of an Iberian palmate newt haplotype inside the native range in Belgium suggests that such an interpretation might be overly simplistic. In fact, for some introduced populations anecdotal evidence points towards a northwestern French origin (G. Timmermans, pers. comm.). Here the same haplotype that is found in the native and introduced populations is also present, meaning our mtDNA cannot reject (or confirm) this possibility. Nuclear DNA should be consulted to increase the precision with which the provenance of Dutch palmate newt localities can be determined.

Acknowledgements. Permits for fieldwork are FF/75A/2016/015 and FF/75A/2016/022, provided via Reptile, Amphibian and Fish Conservation Netherlands (RAVON), for the Netherlands, ANB/BL-FF/V19-00209-212 for Belgium, n°04/2012 for France and PA/356/2021/2023 for Spain. People who helped with sampling are listed in Table S2. Dutch land owner organizations that provided field permits are Natuurmonumenten (Society for Preservation of Nature Monuments in the Netherlands), het Drentse Landschap and Staatsbosbeheer (State Forestry).

Supplementary material. Supplementary material is available online at:
https://doi.org/10.6084/m9.figshare.25755690

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