



The limits of mtDNA analysis for determining the provenance of invasive species: a midwife toad example

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Abstract. For most if not all European herpetofauna, range-wide mtDNA phylogeographies have been published. This facilitates establishing the provenance of introduced populations. However, precision is contingent on the spatial genetic structure across the range of the taxon under study and, in particular, from where within that range the introduction was sourced. In the Netherlands, the common midwife toad, *Alytes obstetricans*, only naturally occurs in the extreme southeast and is on the decline there. Yet, introduced populations thrive elsewhere in the country. We use mtDNA analysis to try to determine the origin of two introduced populations along the Dutch coast, in the city of The Hague and the dune area Meijendel. We compiled a database of hundreds of individuals from throughout the distribution range and added over 130 individuals from both native and introduced populations from the Netherlands, Belgium and Germany. The mtDNA haplotypes found in the introduced populations are associated with postglacial expansion. The main haplotype predominates in the natural range in the Netherlands, but also occurs much more widely across western Europe, north of the Pyrenees. A closely related haplotype, newly identified from The Hague, was not found in the native Netherlands range, suggesting an origin from abroad. The combination of low phylogeographic resolution and low sampling density in the postglacially colonized part of the range hampers our ability to determine the provenance of the introduced *A. obstetricans* populations.

Keywords: alien species, *Alytes obstetricans*, exotic species, invasion genetics, phylogeography.

Introduction

Numerous species have shown the ability to thrive outside of their natural distribution range (Simberloff, 2013). Such invasive species pose a major threat to global biodiversity (Bellard et al., 2016; Pyšek et al., 2020; Diagne et

al., 2021). Generally, by the time that invasive species are discovered, they have firmly established themselves (Hulme, 2006). At that point, the details on how introduced populations were founded (typically illegally) may be difficult to recover. One piece of information that can help to retrace the population's introduction history

is from where within the native range it originated. Such knowledge could help to home in on potential routes of anthropogenic dispersal.

MtDNA analysis is regularly used to determine the origin of invasive populations, for example Alpine newts (*Ichthyosaura alpestris*) introduced in New Zealand (Arntzen et al., 2016) and smooth newts (*Lissotriton vulgaris*) in Australia (Tingley et al., 2015). However, precision depends on where from within the natural distribution range of the species under study the introduced population was sourced. This is because the degree of geographical genetic structuring varies in space (Avice, 2000; Hewitt, 2000). For instance, New Zealand *I. alpestris* carries a haplotype that naturally occurs only in Tuscany, Italy (Recuero et al., 2014; Arntzen et al., 2016). Yet, for Australian *L. vulgaris* the origin is less clear-cut: they carry a haplotype that was not previously identified in the wild, but phylogenetic analysis shows it belongs to a lineage that is dispersed across Germany, the Czech Republic, Slovakia, and Hungary (Babik et al., 2005; Tingley et al., 2015).

The common midwife toad (*Alytes obstetricans*) is widely distributed in western Europe (fig. 1). Across its range, the mtDNA phylogeography of *A. obstetricans* (based on the mtDNA gene ND4) has been studied intensively (Gonçalves et al., 2015). There is extensive geographical genetic structure on the Iberian Peninsula, where six distinct mtDNA lineages are found, referred to as haplogroups by Gonçalves et al. (2015). Only one of these haplogroups occurs north of the Pyrenees (fig. 1). Most, but not all, of these lineages correspond to recognized subspecies (Gonçalves et al., 2015; Dufresnes and Hernandez, 2021), and one of these has recently been proposed to represent a closely related sister species (*A. (o.) algomavarii*; Dufresnes and Martínez-Solano, 2020). Mito-nuclear discordance is rampant, with nuclear DNA-defined clades not recovered as monophyletic with mtDNA and extensive geographical mismatch between nuclear DNA and mtDNA (Maia-Carvalho et al., 2014;

Gonçalves et al., 2015; Dufresnes and Martínez-Solano, 2020; Dufresnes and Hernandez, 2021; Lucati et al., 2022; Ambu et al., submitted). Despite the complicated evolutionary and taxonomical history of *A. obstetricans*, the geographical genetic structure of its mtDNA facilitates testing the provenance of introduced populations.

In the Netherlands *A. obstetricans* only occurs naturally in the extreme southeast, in the province of Limburg (fig. 1). The species has been introduced elsewhere in the Netherlands (Creemers and van Delft, 2009). We study two introduced populations from the coast, The Hague and Meijendel, situated c. 165 kilometers from the closest native populations (fig. 1). The earliest reports for The Hague date from 1993, with regular reports since 2005, and for Meijendel from 2006 (National Database Flora & Fauna, NDFF). Because these introductions were illegal, no further information is available on the introduction history. We use mtDNA analysis to try and determine the provenance of these midwife toads.

Materials and methods

Sampling and DNA extraction

Buccal swabs were taken from two adults from Meijendel and nine adults from The Hague and skin swabs were taken from 11 tadpoles from Meijendel (fig. 1, supplementary table S1). We used 4N6FLOQSwabs (Copan) and stored samples at -20°C in 96% ethanol. DNA was extracted using the Wizard[®] Genomic DNA purification kit (Promega). Another 110 DNA extracts were obtained from Lemmers et al. (2020) and covered 31 localities from the southeast Netherlands, Belgium and Germany (fig. 1, supplementary table S1). We obtained 208 *A. obstetricans* sequences from Gonçalves et al. (2015) that mainly cover the Iberian Peninsula (fig. 1, supplementary table S1).

PCR and sequencing

Because of poor initial PCR results using the general primers employed in Gonçalves et al. (2015), we designed specific internal primers based on the *A. obstetricans* sequences from Gonçalves et al. (2015) in Geneious Prime 2021.1.1 (www.geneious.com), to amplify a 578 bp fragment of the mtDNA gene ND4, namely AlytesND4_196F (CCGCTGCACCTTATTCAAACCC) and AlytesND4_814R (CTAAGTGACCCGCGGAATTG). For the 132 newly

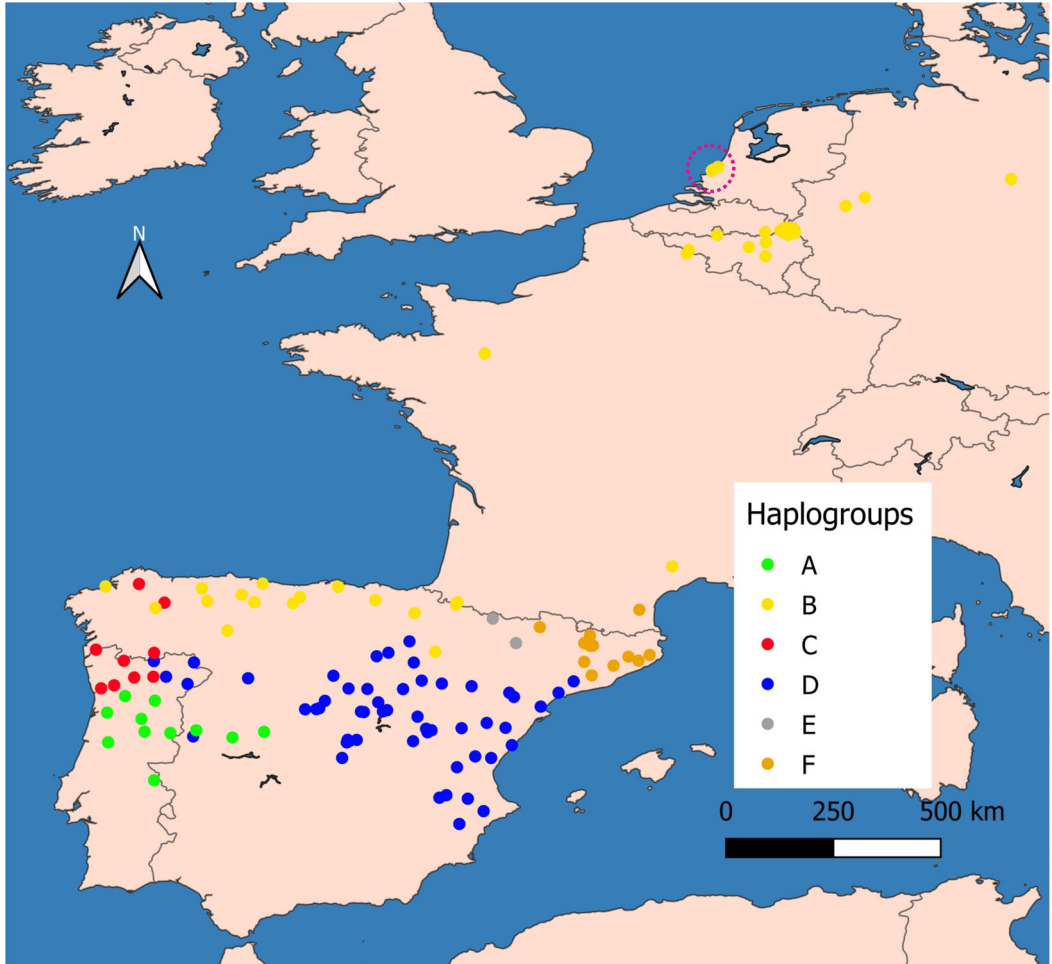


Figure 1. Sampling of the common midwife toad (*Alytes obstetricans*). Samples from the Netherlands, Belgium and Germany were newly studied. The localities on the coast of the Netherlands (Meijendel and The Hague from top to bottom, enclosed by the pink dotted line) are introductions outside of the native range. Localities are color-coded according to the six major ND4 mtDNA clades, referred to as haplogroups by Gonçalves et al. (2015), to which they belong. Sampling details can be found in supplementary table S1.

studied individuals, PCRs were performed in 12 μ l reactions containing 0.06 μ l of both forward and reverse primer (0.05 μ M end concentration of each primer), 7.2 μ l QIAGEN multiplex PCR master mix, 3.68 μ l purified water and 1 μ l 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 a final 30 minutes extension at 60°C. Sequencing was outsourced to BaseClear B.V. and sequences were edited in Geneious Prime 2021.1.1.

Genetic analyses

Consensus sequences for the new individuals were aligned with the *A. obstetricans* database of Gonçalves et al. (2015) and the total alignment was trimmed to equal length. The

Haplotype Collapser function in FaBox (Villesen, 2007) was used to check which previously identified haplotypes (if any) newly sequenced individuals belonged. To place newly identified haplotypes we conducted Bayesian phylogenetic inference with MrBayes v.3.2.6. (Ronquist et al., 2012). jModeltest2 (Darriba et al., 2012) was used to determine the most appropriate model of sequence evolution for each codon position (HKY + I, F81 and GTR + G for positions 1, 2 and 3). A sequence of *A. muletensis* taken from Gonçalves et al. (2015) was used as outgroup (GenBank Accession KT363309). We ran two runs of four-chains for five million generations with a sampling frequency of 0.001 and a heating parameter of 0.2 in MrBayes and used a 25% burnin. Tracer v1.7 (Rambaut et al., 2018) was used to confirm that runs converged and that ESS was above the standard threshold of 200. A median joining haplotype network

was constructed in PopArt under default settings (Leigh and Bryant, 2015).

Results

The 132 new sequences were added to the 208 individuals from Gonçalves et al. (2015), resulting in a database consisting of 340 individuals that were divided into 74 unique haplotypes (supplementary table S1). All new sequences from Belgium and Germany, as well as most samples from the Netherlands, including all Meijndel and the majority of The Hague individuals, possessed a single haplotype: Ao_H1. This haplotype was reported by Gonçalves et al. (2015) in the Spanish and French Pyrenees (Irati Forest) as well as their two non-Pyrenean French sites and their only German locality. Five new haplotypes were identified. Haplotypes Ao_H70, Ao_H71, Ao_H72 and Ao_H73 were found in the southeast of the Netherlands and Ao_74 was found in the introduced population of The Hague (fig. 1, supplementary table S1). All newly identified haplotypes are closely related to Ao_H1 and are part of a clade that received a posterior probability of 0.9 and is

nested inside ‘haplogroup B’ (*sensu* Gonçalves et al., 2015; fig. 2, supplementary fig. S1). This clade, which also includes haplotype Ao_H52 from the French Pyrenees (Irati Forest), contains all *A. obstetricans* sampled north of the Pyrenees so far (supplementary table S1). Our new haplotypes (as well as Ao_H52) all show a single substitution difference from haplotype Ao_H1 (fig. 3).

Discussion

While mtDNA can potentially provide precise information on the provenance of introduced populations, the mtDNA data obtained from the Dutch introduced coastal *A. obstetricans* populations provide little resolution. Most individuals of the introduced populations from The Hague, and all individuals from Meijndel, carry the same mtDNA haplotype that predominates across the Dutch native range of the species (Ao_H1). This same haplotype is distributed over a vast area that stretches from the Pyrenees northwards across France and is also found in Belgium and Germany. This illustrates a first drawback of phylogeography from

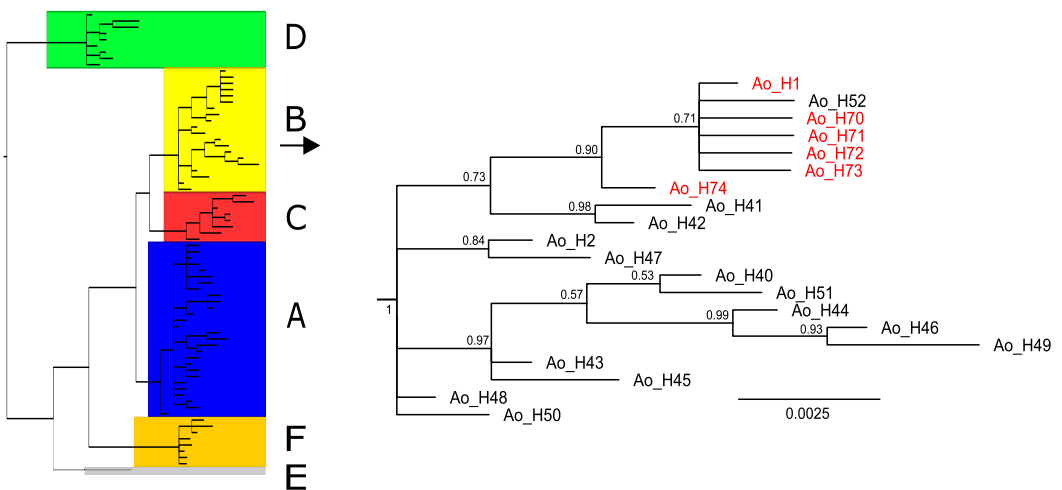


Figure 2. The 50% majority-rule consensus tree from the Bayesian phylogenetic analysis of 74 ND4 mtDNA haplotypes of the common midwife toad (*Alytes obstetricans*). The inset shows the six major mtDNA clades referred to as haplogroups by Gonçalves et al. (2015) and details can be consulted in supplementary fig. S1. The main figure zooms in on haplogroup B and the haplotypes identified in the newly studied localities are in red. Haplotype details can be found in supplementary table S1.

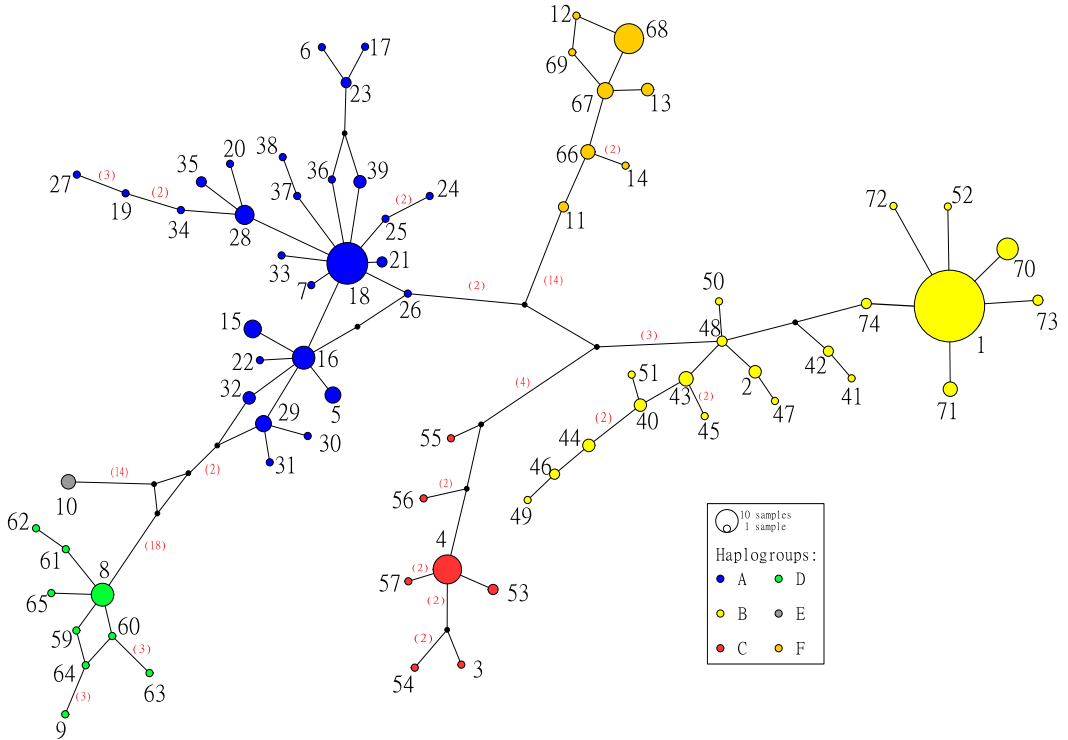


Figure 3. Median Joining Network of 74 ND4 mtDNA haplotypes of the common midwife toad (*Alytes obstetricans*). Pie size reflects haplotype frequency and red numbers between parentheses reflect the number of substitutions (if greater than 1). The prefix ‘Ao_H’ is omitted from haplotype labels. Haplotype details can be found in supplementary table S1.

the perspective of mtDNA analysis of introduced populations. As a consequence of the Quaternary Ice Age’s oscillation between cold (glacial) and warm (interglacial) cycles, the duration that habitable conditions were available for a particular species varies in space (Hewitt, 2000). For many of Europe’s temperate species the Iberian, Italian and Balkan Peninsulas act as so-called glacial refugia that remain suitable throughout glacial-interglacial cycles, while central and northern Europe are only habitable during the relatively short interglacial periods. Most genetic diversity is typically present in glacial refugia (Hewitt (2000) called this ‘southern richness’, from a northern hemisphere point of view). On the other hand, the region that was colonized postglacially is genetically homogenic (Hewitt (2000) referred to this as ‘northern purity’).

Another, closely related, haplotype (Ao_74) was newly identified from The Hague. Despite our dense sampling across the native *A. obstetricans* range in the Netherlands, this haplotype was not found here. While it could have potentially evolved *in loco*, this is suggestive of a foreign origin for the population from The Hague, but outside of the Netherlands this haplotype has not been reported either. Sampling in the most comprehensive mtDNA phylogeography to date meticulously covers Spain, Portugal and southern France, but north of the Pyrenees sampling resolution is low (Gonçalves et al., 2015). This illustrates a second drawback of phylogeography from the perspective of mtDNA analysis of introduced populations (directly related to the first): typically, sampling focusses on the genetically diverse glacial refugia, whereas postglacially colonized regions are poorly sampled. Additional sampling in the

postglacial range outside of the Netherlands could reveal a more precise geographical source for the The Hague population.

We suggest that, to address the provenance of the introduced *A. obstetricans* populations in the Netherlands, we need to look beyond mtDNA. RAD-sequencing has already been used in *A. obstetricans* to support the status of a newly described subspecies (Dufresnes and Hernandez, 2021), to propose elevating a former subspecies to the level of species (Dufresnes and Martínez-Solano, 2020), and to determine genetic differentiation within and between population in the southeast of the Netherlands (Lemmers et al., 2020), and could provide the required spatial resolution. Our mtDNA data does make clear that such a study should particularly focus on the postglacially colonized section of the *A. obstetricans* range.

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Supplementary material. Supplementary material is available online at:
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