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Citation

Koster, S., Prins, N., Dufresnes, C., France, J. M., Visser, M. C. de, Struijk, R. P. J. H., & Wielstra, B. M. (2023). The conservation paradox of an introduced population of a threatened species: spadefoot toads in the coastal dunes of the Netherlands. *Amphibia-Reptilia*, *44*(1), 11-18. doi:10.1163/15685381-bja10106

BRILL
Amphibia-Reptilia 44 (2023): 11-18
brill.com/amre

The conservation paradox of an introduced population of a threatened species: spadefoot toads in the coastal dunes of the Netherlands

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Received 26 May 2022; final revision received 3 September 2022; accepted 22 September 2022;
published online 1 December 2022; published in print 27 February 2023
Associate Editor: Sebastian Steinfartz

Abstract. Species that are threatened in their native range may actually prosper as introduced populations. To investigate how such introduced populations were established involves determining from where within the natural range the founder individuals originated. This can be accomplished through mtDNA barcoding. The common spadefoot toad (*Pelobates fuscus*) naturally occurs in the south and east of the Netherlands and has shown a rapid decline. Yet, a flourishing introduced population was recently discovered in the coastal dunes in the west of the country. We use mtDNA barcoding to determine the provenance of the introduced population. We sampled both native and introduced populations from the Netherlands and compared our sequences to haplotypes from across the entire distribution range. The mtDNA haplotypes found in the introduced population are distinct from those naturally occurring in the Netherlands and point towards an origin in the Pannonian Basin, on the boundary between Central and Southeastern Europe. Paradoxically, the thriving *P. fuscus* population in the Dutch coastal dunes should be considered a conservation risk to local biodiversity, even though within the native range in the Netherlands the species is severely threatened. Our study illustrates the complicated conservation questions associated with species that are both native and invasive.

Keywords: alien species, DNA barcoding, exotic species, invasion genetics, *Pelobates fuscus*, phylogeography.

Introduction

Some species can thrive outside of their natural distribution range (Simberloff, 2013). Such invasive species pose a major problem for biodiversity conservation (Bellard et al., 2016; Pyšek et al., 2020; Diagne et al., 2021). What if a species that is threatened in its native range becomes invasive? This has been dubbed the "conservation paradox of endangered and invasive species" (Marchetti and Engstrom, 2016). One might expect that the trade in endangered species is relatively restricted and that, as a consequence, they are less likely to be introduced. Yet, there are multiple cases of endangered species that have established populations outside their native range and are having a negative effect on local biodiversity (Marchetti and Engstrom, 2016). When investigating the inception of such an unexpected introduction, one key question to ask is: from where within the natural distribution range of the species did the founders originate? Answering this question typically requires genetic tools.

DOI:10.1163/15685381-bja10106

DNA barcoding utilizes short DNA markers, usually mtDNA markers, that are conserved enough so they can be compared across a wide range of taxa, but are also variable enough to capture interspecific variation (Hebert et al., 2003; Mir et al., 2021). Due to a fast rate of evolution and single-parental mode of inheritance, mtDNA typically displays strong geographical genetic structuring within species (Avise, 2000). For a given species, newly produced mtDNA barcodes can now directly be compared, through online databases such as GenBank and BOLD, to barcodes from a large number of individuals covering many geographically distinct populations (Mir et al., 2021). This makes mtDNA barcoding a powerful tool for determining the provenance of introduced species.

The common spadefoot toad, *Pelobates fuscus*, occurs in lowland areas across Central and Eastern Europe (Sillero et al., 2014; Dufresnes et al., 2019b). In the Netherlands, at the western fringe of its natural distribution range, *P. fuscus* is restricted to Pleistocene sandy soils in the east and south of the country (Creemers and van Delft, 2009). The species has shown a 74% decline between 1950 and 2007 and is listed as 'threatened' on the national Red List (van Delft et al., 2007). The situation became so severe that in 2012-2016 Amsterdam Royal Zoo ARTIS, Wildlands Adventure Zoo (Emmen) and Reptile, Amphibian and Fish Conservation Netherlands (RAVON) piloted an *ex situ* breeding programme in which egg strings taken from the wild were hatched and raised in captivity, after which tadpoles or juveniles were reintroduced in extinct or depleted populations (Struijk and Bosman, 2015; Struijk et al., 2016).

Ironically, in 2020 it became clear that a, for Dutch standards, thriving population of spadefoot toads has become established in the northwest of the Netherlands, in the dune area Callantsoog, over 90 kilometres from the closest natural populations (Struijk, 2020; Jansen and Putters, 2021). To determine which *Pelobates* species is concerned (several closely related *Pelobates* species can only be reliably distinguished with genetic data; Dufresnes et al., 2019a) and to establish what the provenance of the Callantsoog population is, we conduct mtDNA barcoding of both native and introduced spadefoot toads from the Netherlands, and compare our sequences to an extensive database of haplotypes sampled across the entire distribution range of the genus *Pelobates* (Crottini et al., 2007; Dufresnes et al., 2019a).

Materials and methods

Sampling, DNA extraction and PCR

We included 42 samples from 11 localities in the Netherlands (fig. 1, supplementary table S1). Buccal swabs were taken from 18 adult spadefoot toads from the introduced Callantsoog population, using 4N6FLOQSwabs (Copan), and samples were stored at −20°C in 96% ethanol. Tissues for another 24 samples were available from native populations (roadkills and casualties from the *ex situ* breeding programme). DNA was extracted using the Wizard® Genomic DNA purification kit (Promega). We amplified a 657 bp fragment of the mitochondrial cytochrome b gene using the primer pair PB-cytB-F1 / H15915-short2 (Dufresnes et al., 2019a). PCRs were performed in 12 μ l reactions containing 0.06 μ l forward and 0.06 μ l reverse primer (0.05 μM end concentration of each primer), 7.2 μ l QIAGEN multiplex PCR master mix, 3.68 μ l purified water and 1μ 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. Bidirectional Sanger sequencing was performed commercially by BaseClear B.V. Sequences were edited using Geneious Prime 2021.1.1 (https://www.geneious.com).

Genetic analyses

Consensus sequences were manually aligned in Geneious Prime 2021.1.1 to a database of *Pelobates* haplotypes built from previous studies (Eggert et al., 2006, PopSet: 84374649 on GenBank; Crottini et al., 2007, Popset: 119393800; Dufresnes et al., 2019a, Popset: 1721807813). The alignment was 657 bp long. Our haplotype naming convention followed Dufresnes et al. (2019a; see supplementary table S1 for details). We considered all 31 *P. fuscus* and 12 *P. vespertinus* haplotypes known and a single haplotype for the other species in the genus (*P. balcanicus*, *P. syriacus P. cultripes*, and *P. varaldii*). The Haplotype Collapser function in FaBox (Villesen, 2007) was used to check which previously identified haplotypes our 42 newly sequenced individuals possessed. To visualize the relative genetic divergence among haplotypes, Bayesian phylogenetic inference was performed using MrBayes 3.2.7 (Ronquist et al., 2012).

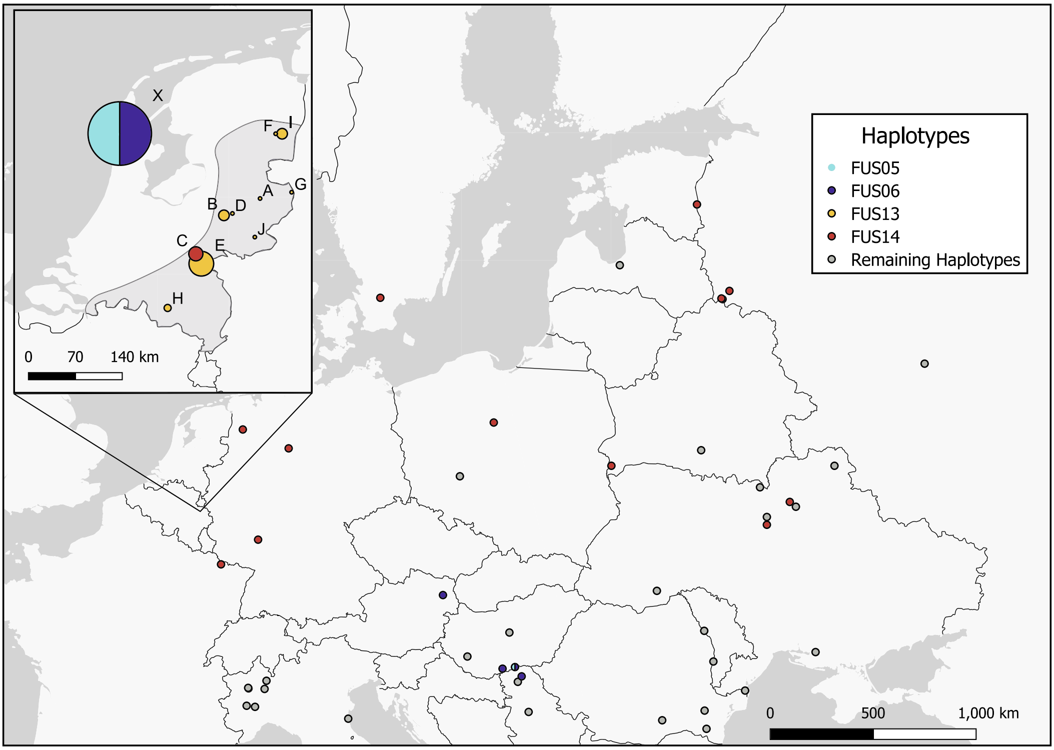

Figure 1. Sampling sites of the common spadefoot toad (*Pelobates fuscus*). The main map shows localities sampled outside and the inset localities sampled inside the Netherlands (see main text for details). A rough outline of the natural distribution range in the Netherlands is shaded grey. Localities that contain haplotypes found in the Netherlands are colour coded; otherwise they are left grey (FUS stands for *P. fuscus*). Sampling details are in supplementary table S1.

The appropriate model of sequence evolution for each codon position (GTR+I, K80+I and HKY+I for codon positions 1, 2 and 3) was determined with jModelTest 2 (Darriba et al., 2012). We ran two, four-chain, one-million-generation runs, with a sampling frequency of 0.001 and a heating parameter of 0.2 in MrBayes and used a 25% burnin. We confirmed that runs converged and ESS values were over 200 in Tracer v1.7 (Rambaut et al., 2018). *Spea bombifrons* (GenBank accession JX564896; Zhang et al., 2013) was included as an outgroup. Furthermore, for the 31 *P. fuscus* haplotypes a TCS haplotype network (Clement et al., 2000) was created in PopART (Leigh and Bryant, 2015).

Results

All haplotypes recovered in the Netherlands were previously identified (Crottini et al., 2007; Dufresnes et al., 2019a; supplementary table S1) and belong to *P. fuscus* (figs. 1-3). In the native Dutch populations two haplotypes (FUS13 and FUS14, differing by one substitution) are present. The former is currently only known from the Netherlands, while the latter has a wider distribution in Europe and has also been reported from Belarus, Estonia, France, Germany, Poland, Russia and the Ukraine (fig. 1, supplementary table S1). In the introduced population at Callantsoog, two haplotypes (FUS05 and FUS06, differing by five substitutions) were recovered that are genetically distinct from the native Dutch ones (together carrying three fixed substitutions compared to FUS13 and FUS14; fig. 3). Neither of these occurs naturally in the Netherlands. Haplotype FUS05 has previously been reported in Serbia and FUS06 in Austria, Hungary and Serbia (fig. 1, supplementary table S1).

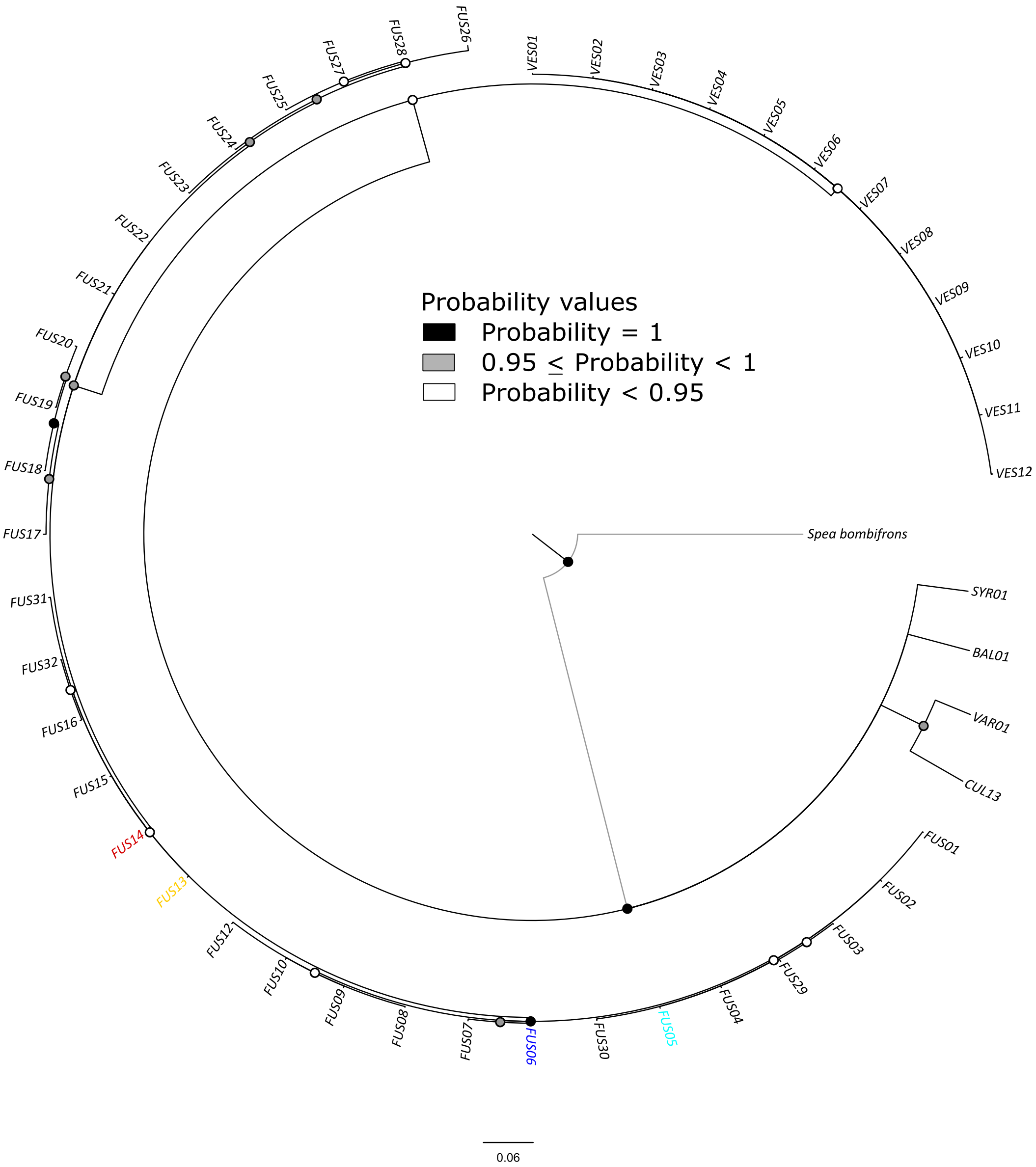

Figure 2. Phylogenetic tree for common spadefoot toad (*Pelobates fuscus*) and Pallas's spadefoot toad (*P. vespertinus*). Haplotypes relevant to the current study are colour coded; the remainder is left grey (details in Table S1). Haplotype abbreviations are: FUS = *P. fuscus*, VES = *P. vespertinus*, BAL = *P. balcanicus*, SYR = *P. syriacus*, CUL = *P. cultripes*, and VAR = *P. varaldii*.

Discussion

We confirmed that the introduced population from Callantsoog corresponds to the species *P. fuscus*, rather than the morphologically similar but genetically distinct *P. vespertinus* (fig. 2; Crottini et al., 2007; Litvinchuk et al., 2013; Dufresnes et al., 2019a). Based on the two haplotypes detected, the source of the population is clearly located outside the Netherlands, in the Pannonian Basin or surrounding regions (which have been poorly sampled, see fig. 1). One haplotype (FUS06) has been documented from a wide area in Central Europe, encompassing

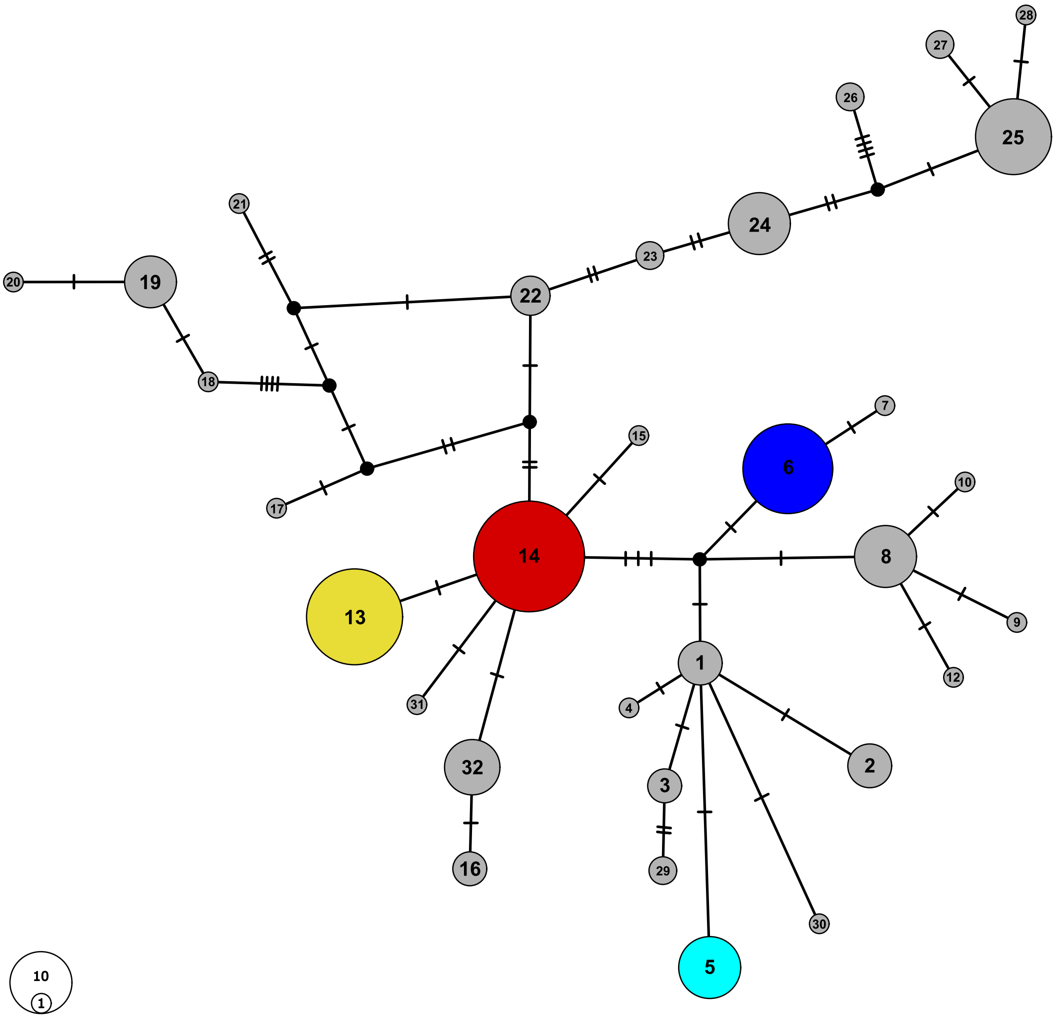

Figure 3. Haplotype network for the common spadefoot toad (*Pelobates fuscus*) Haplotypes relevant to the current study are colour coded; the remainder is left grey (details in supplementary table S1). The prefix 'FUS' is not shown for the haplotype codes.

Austria, Hungary and Serbia, while the other (FUS05) has been reported from Serbia only. Given the low sampling resolution in the Pannonian Basin, we consider it likely that the latter haplotype is more widespread, and we refrain from restricting our estimation on the origin of the Callantsoog population to Serbia (Eggert et al., 2006; Crottini et al., 2007).

While mtDNA barcoding has well-understood limitations (Moritz and Cicero, 2004; Balloux, 2010), our case study illustrates the potential of mtDNA barcoding in invasion science. Clearly, the introduced Callantsoog *P. fuscus* population has non-Dutch ancestry. Yet, future work including nuclear DNA markers could help home in on the geographical region of origin (although this would require finer resolution sampling in the native range as well). Another issue that could be explored with nuclear DNA is whether the introduced population is potentially of mixed ancestry. For example, in the introduced banded newt population in Spain mtDNA of the species *Ommatotriton ophryticus* is fixed, but based on nuclear DNA these newts are genetically admixed between *O.*

ophryticus and *O. nesterovi* (van Riemsdijk et al., 2018).

It is unknown how *P. fuscus* was introduced in Callantsoog, but the most likely scenario is that animals were released by hobbyists who keep and breed amphibians. The amphibian trade is extensive (Schlaepfer et al., 2005) and responsible for the majority of amphibian invasions (Mohanty and Measey, 2019). It used to be illegal to keep native species in the Netherlands. However, this changed in 2017, when a new law, the 'Wet Natuurbescherming', entered into force (https://wetten.overheid.nl/BWBR0037552/2021-07-01). Although native species can since be kept in the Netherlands, these have to be of demonstrably legal origin. As a consequence, native species had to be acquired from breeders abroad (where these species were already allowed to be kept). Considering these developments (in combination with the huge potential reproductive output and low commercial value of amphibians), it is, unfortunately, unsurprising that new introduced populations of alien origin arise.

The terrain where the introduced spadefoot toads occur has a Natura2000 status ('Zwanenwater & Pettemerduinen'). It covers 770 hectares and likely provides the *P. fuscus* population the opportunity to expand southwards. Amongst the native amphibian species occurring in the same waterbodies is the natterjack toad, *Epidalea calamita*, a European and national strictly protected species. The spadefoot toads are likely to compete for resources with other amphibians and will predate on native species (Simberloff et al., 2013). Furthermore, cross-species infection with pathogens deriving from introduced species is particularly common in amphibians (Scheele et al., 2019). The possibility of 'genetic pollution' via anthropogenic hybridization should be taken into account as well, if people were to transplant Callantsoog spadefoot toads to native populations (Meilink et al., 2015). Unfortunately, this is not without precedent; grass snakes *Natrix natrix* from an introduced Dutch population that were caught in a residential area were subsequently 'set free' inside the range of the native barred grass snake *N. helvetica*, with which the species is known to hybridize (Struijk et al., 2020; van Riemsdijk et al., 2020; Asztalos et al., 2021).

We show that the *P. fuscus* population in Callantsoog derives from a geographical population that is genetically diverged from the native Dutch one (Eggert et al., 2006; Crottini et al., 2007) and is obviously introduced. We would argue that, because *P. fuscus* is not globally threatened (Agasyan et al., 2009), the Callantsoog population has no conservation value (Marchetti and Engstrom, 2016; Shaffer, 2018). Paradoxically, the thriving Callantsoog spadefoot toads potentially poses a conservation threat to native biodiversity. Because *P. fuscus* is native to the Netherlands, where it is considered (severely) threatened, and is protected by both national and European law, it is not straightforward to determine what conservation action could potentially be taken for the Callantsoog population (Kuijt et al., 2023). We urge stakeholders to design a plan of action and for now suggest the impact of the Callantsoog spadefoot toads on native biodiversity is closely monitored.

Acknowledgements. Natuurmonumenten allowed us to conduct research on the *P. fuscus* population in Callantsoog. Amsterdam Royal Zoo ARTIS provided DNA samples from their *ex situ* breeding programme. The permit (no. FF/75A/2016/015) to conduct our fieldwork was provided via Reptile, Amphibian and Fish Conservation Netherlands (RAVON). Rolf van Leeningen provided the rough distribution outline in the Netherlands.

Supplementary material. Supplementary material is available online at:
https://doi.org/10.6084/m9.figshare.21213596

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