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Hybridisation complicates the conservation of *Natrix* snakes in the Netherlands

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Reptiles worldwide are hit hard by the sixth mass extinction (Böhm et al. 2013, Ceballos et al. 2017). While habitat destruction is the most important factor in biodiversity loss, the introduction of exotic species is a close second (PIMENTEL et al. 2001, SAX & GAINES 2003, KRAUS 2015). When introduced species are related to natives, hybridisation may compromise the genetic integrity of natives, which may lead to unintended loss of population fitness (FITZPATRICK & SHAFFER 2007, ALLENDORF et al. 2010). However, the fitness of local populations may be increased if genes with an adaptive advantage are introduced, for example when local populations have become inbred or are below viable population size (RIESEBERG et al. 1999, ABBOTT et al. 2013, KELLY & PHILLIPS 2018). One well-monitored example of genetic rescue is found in Sweden, where an inbred snake population (*Vipera berus*) was rescued by introducing males from a genetically variable population nearby (MADSEN et al. 1999, 2004). Hence, introduction of non-natives is sometimes conducted to boost native populations, but negative effects such as loss of locally adaptive alleles and outbreeding depression should be carefully considered when performing such genetic rescue (WEEKS et al. 2011, WHITELEY et al. 2015, HAMILTON & MILLER 2016, RUSSO et al. 2018).

We illustrate the conservation complications related to anthropogenic hybridisation, using a case involving grass snakes (*Natrix*) in the Netherlands. Based on mitochondrial DNA (mtDNA) and nuclear DNA, KINDLER et al. (2017) distinguish western *N. helvetica* from eastern *N. natrix* and found that the two species meet in a narrow hybrid zone with very limited gene flow in Western Europe. The spe-

cies occurring in the Netherlands is *N. helvetica* (KINDLER et al. 2017, STUMPEL & JANSSEN 2017). North of the documented hybrid zone, on the boundary between the Netherlands and Germany, the two species appear to be allopatric (STUMPEL & JANSSEN 2017). Sparse *Natrix* records, not identified to the species level, do occur in the region (DE WIJER et al. 2009).

Despite worldwide population declines in reptiles, Dutch reptile populations appear to be stable or growing (JANSSEN & DE ZEEUW 2017). The distribution of *Natrix* in the Netherlands has a long history of human interference, with intentional displacement by naturalists recorded at least as far back as the second half of the 19th century (SCHLEGEL 1862), and may stretch as far back as the Neolithic (LENDERS & JANSSEN 2014). Recent records evidence that several populations in the west of the Netherlands (in the vicinity of populations 7 and 8, Fig. 1) were founded by (unauthorised) translocations of native individuals from neighbouring areas (VAN DER LUGT & SIEBELINK 2003). Illegal release of exotic *N. natrix* derived from Italy and Turkey is thought to have occurred here as well (VAN DER LUGT & SIEBELINK 2003). Another introduction in the south of the Netherlands (in the vicinity of population 9) is thought to have concerned *N. n. persa* (ELZENGA 1974, VAN BUGGENUM & HERMANS 1986, VAN BUGGENUM 1992, BUGTER et al. 2014).

Morphological observations seem to corroborate the history of *Natrix* introduction. Snakes with two white-yellow dorsolateral stripes, a feature normally observed in *N. n. persa* (KREINER 2007), have regularly been reported in the west of the Netherlands (vicinity of populations

7 and 8). The phenotype of *N. helvetica* includes lateral blocked stripes and is easy to distinguish from *N. n. persa*. A recent study from Switzerland reported the occurrence of mtDNA haplotypes of native (*N. helvetica*) and introduced (both *N. helvetica* and *N. natrix persa*) grass snakes, associated with the occurrence of offspring with diverse colour patterns (DUBEY et al. 2017).

Despite the difference in climate, it would not be surprising if exotic species would be able to establish viable populations in the Netherlands (BUGTER et al. 2014). It is known that native grass snakes survive freezing conditions during hibernation in wetlands in both natural hibernacula as well as dry structures built by humans, such as rail embankments (VAN DER LUGT & SIEBELINK 2003). Furthermore, nesting opportunities for snakes are provided by manure heaps (GÜNTHER & VÖLK, 1996, LÖWENBORG et al. 2010, 2012, LENDERS & JANSSEN 2014). Although there is no indication of a population decline of Dutch grass snakes, grass snakes are indicated as vulnerable on the Red List of Reptiles and protected by national law (CBS 2005, VAN DELFT et al. 2007, JANSSEN & DE ZEEUW 2017). Conservation efforts are taken to ensure survival of existing populations and facilitate dispersal among them.

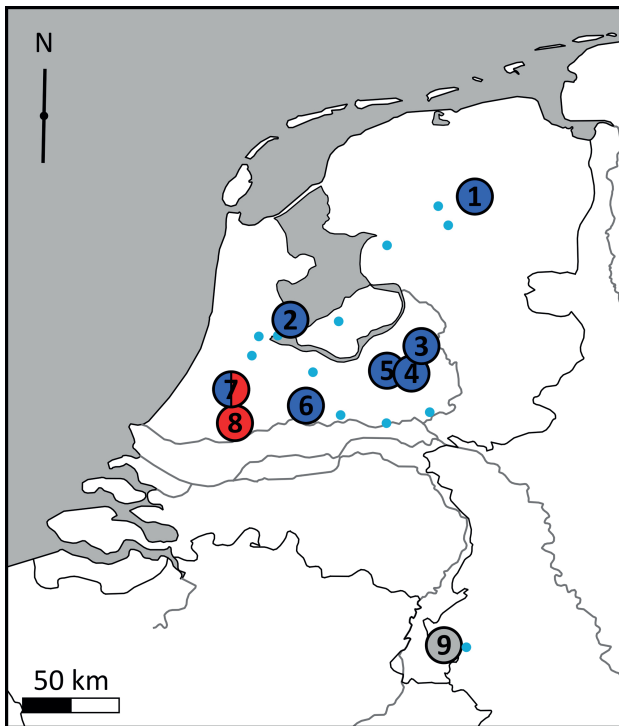


Figure 1. Sampling of grass snakes (*Natrix*) in the Netherlands. Large numbered dots are new localities and small dots are from KINDLER et al. (2017). Blue reflects *N. helvetica* mtDNA haplotypes (clade E in Fig. 2), red (clade 4, for which the closest occurrence is an introduced population in eastern Germany) and grey (clade 7, Cyprus and the west coast of Turkey, *N. c. cyprica*) reflect *N. natrix* mtDNA haplotypes.

Table 1. Sampling of nine Dutch *Natrix* populations. * Populations where the presence of dorsolateral stripes was previously recorded.

Pop. Location	n	Dorsolateral stripes (n)	mtDNA <i>N. helvetica</i> / <i>N. natrix</i>
1 Fochteloërveen	1	0	1/0
2 Marken	3	0	3/0
3 Vaassen	1	0	1/0
4 Asselsche Heide	1	0	1/0
5 Kootwijkerveen	1	0	1/0
6 Houten	1	0	1/0
7 Alphen aan den Rijn*	7	6	2/5
8 Krimpenerwaard*	27	10	0/27
9 Brunssummerheide*	1	Unknown	0/1

Previous records of exotic *Natrix* individuals in the Netherlands consist of anecdotal and indirect evidence. To monitor the current genetic make-up of *Natrix* populations in the Netherlands, genetic data may provide insight into where introductions have led to populations with exotic genotypes. The two grass snake species *N. helvetica* and *N. natrix* are represented by distinct mtDNA clades (KINDLER et al. 2013, 2017, 2018). Therefore, mtDNA can be used to verify if multiple *Natrix* taxa co-exist in the Netherlands, if these snakes are introduced or native, and if conservation efforts should consider the presence of multiple taxa and their potential hybridisation. Based on an mtDNA phylogeography for Dutch *Natrix*, in combination with phenotypical information, we discuss natural and anthropogenic hybridization in the context of conservation.

Tissue samples or saliva swabs from 43 Dutch snakes from 9 populations (Fig. 1) were collected with permission from the conservation organisations Stichting Het Zuid-Hollands Landschap and Natuurmonumenten. The emphasis of sampling was on populations 7 and 8, which are suspected to include introduced snakes (Table 1). For each individual the presence of dorsolateral stripes was recorded. DNA was extracted with the Qiagen DNeasy Blood & Tissue Kit. Fragments of two mitochondrial markers (ND4, 565 bp and cyt *b*, 899 bp) were amplified following KINDLER et al. (2013). Sanger sequencing was done commercially at BaseClear B.V., Leiden, the Netherlands. Sequences were edited in Sequencher 4.10.1 (Gene Codes Corporation, MI USA) and collapsed into haplotypes with FaBox (VILLESEN 2007). Haplotypes were aligned against the NCBI website using BLAST (ALTSCHUL et al. 1990). Previously published haplotypes were identified and recorded using haplotype numbers (KINDLER et al. 2013, 2017). As the sequences generated here are ~200 bp shorter, all possible matches with haplotypes (100% identity) are reported. One cyt *b* haplotype was entirely new, and was added to NCBI (MN585707). The new haplotype was combined with the datasets of KINDLER et al. (2013, 2017) in Mesquite (MADDISON & MADDISON 2015). To determine to

which clade the new haplotype belongs, a maximum likelihood phylogenetic tree was built with RAXML 8.2.4, with 1,000 rapid bootstrap replicates and a search for the best scoring maximum likelihood tree, treating each mtDNA marker as a separate partition and using the nucleotide substitution model GTR+G (STAMATAKIS 2014).

Dorsolateral striping was present in 47% of individuals from populations 7 and 8. No stripes were reported in any of the other populations studied, although dorsolateral striping was reported previously in population 9 as well (JANSSEN 2009). The 43 Dutch snakes possess 3 ND4 and 4 cyt *b* haplotypes. Ten individuals possess a *N. helvetica* haplotype (Fig. 2, belonging to clade E in KINDLER et al. 2013, 2017) and 33 individuals a *N. natrix* haplotype (Fig. 2, clade 4 and 7, Supplementary Table S1). All haplotypes of *N. helvetica* were previously reported in western Europe (KINDLER et al. 2013, 2017). When combining the information on the geographical origin of ND4 and cyt *b* haplotypes, the *N. natrix* haplotypes found in the Netherlands occur naturally in southern and eastern Europe, up to central Germany; haplotypes previously found further west (in western Germany and Great Britain) are introductions (KINDLER et al. 2013, 2017). One individual from population 9 possesses a haplotype of *N. n. cypriaca*, which is endemic to Cyprus and the west coast of Turkey (Fig. 2, clade 7, KINDLER et al. 2013). There is no correlation between mtDNA type and the presence of dorsolateral stripes (Supplementary Table S1).

We confirm the presence of three *Natrix* mtDNA clades in the Netherlands, typical of two recently recognized grass snake species *N. natrix* (clades 4 and 7; represented by the

colours red and grey in Fig. 1) and *N. helvetica* (clade E; blue). Based on sampling throughout the Dutch *Natrix* range, only *N. helvetica* is thought to occur naturally in the Netherlands (KINDLER et al. 2017). The origin of the *N. natrix* introduction could not be connected to the previously suggested source in Italy. It appears that individuals from population 7 possess both native mtDNA from *N. helvetica* and exotic mtDNA from *N. natrix*, whilst individuals of population 8 only possess exotic mtDNA (Fig. 1). KINDLER et al. (2017) previously reported geographically restricted genetic admixture in the hybrid zone between *N. natrix* into *N. helvetica*, but noted the natural occurrence of asymmetric mtDNA introgression from *N. natrix* into *N. helvetica*. This pattern of asymmetric introgression could reflect eastward hybrid zone movement, which would be in line with *N. natrix* 'enclaves' nested within *N. helvetica* territory (CURRAT et al. 2008, TOEWS & BRELSFORD 2012, WIELSTRA et al. 2017a,b). However, the two *N. natrix* mtDNA clades found in the Netherlands occur far eastwards from the natural hybrid zone and hence their presence in the Netherlands cannot be explained by natural processes.

No direct relation between exotic mtDNA and deviating phenotypes was found. However, the appearance of both striped and unstriped snakes in those populations possessing native and non-native mtDNA haplotypes suggests hybridisation occurs between native and non-native *Natrix* individuals. These findings are in line with previous findings in populations where both *Natrix* species are present, and offspring were observed to possess a wide range of phenotypes (DUBEY et al. 2017). Unmonitored or unformed hybridisation between distinct species is not new and is especially problematic if species status is still under debate (e.g.; VAN RIEMSDIJK et al. 2017a, b). The complicated issue of 'genetic pollution', the replacement of native alleles by non-native counterparts, should thus be taken into account in the conservation of Dutch *Natrix* (MEILINK et al. 2015). 'Genetic pollution' should also be considered in the context of improving connectivity among Dutch *Natrix* populations: without species identification based on nuclear DNA, the spread of *N. natrix* alleles into the range of *N. helvetica* might inadvertently be promoted. To assess the impact of 'genetic pollution' on Dutch *Natrix* we urge that a follow-up study incorporating nuclear DNA is conducted, focussing on the southwestern Dutch populations.

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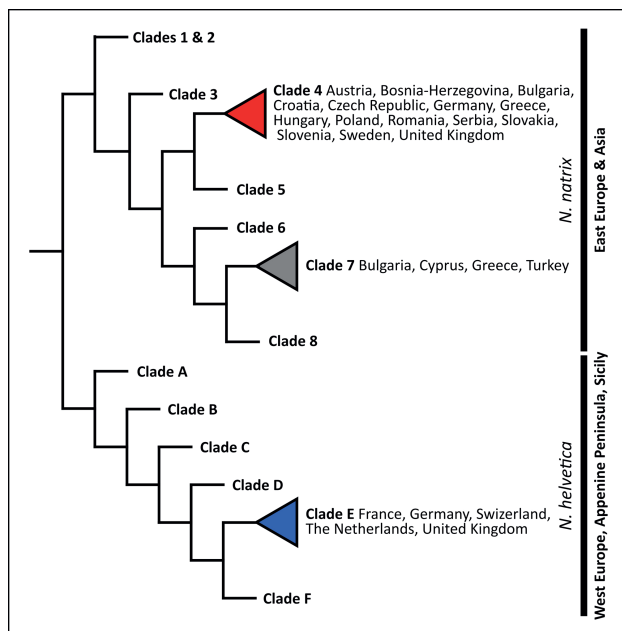


Figure 2. Placement of Dutch *Natrix* haplotypes in the range-wide phylogeography of grass snakes by Kindler et al. (2013, 2017). Dutch haplotypes were allocated to clade E (*N. helvetica*) and clades 4 and 7 (*N. natrix*).

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Supplementary data

The following data are available online:

Supplementary document 1. Sample overview including population numbers (corresponding to Fig. 1 of the article), sample ID, information on morphology, sex, life stage, location coordinates, haplotypes from the Kindler publications, clade, and countries where the haplotypes were previously found.