



## Correspondence

### Aliens in the Netherlands: local genetic pollution of barred grass snakes (Squamata: Serpentes: Natricidae)

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Grass snakes are widely distributed across the Palaearctic, ranging from north-western Africa through most of Europe to Central Asia (MERTENS & WERMUTH 1960, KABISCH 1999). Recent studies based on genetic data recognize three full species that show limited hybridization in their geographic contact zones: *Natrix astreptophora*, *N. helvetica*, and *N. natrix* sensu stricto (POKRANT et al. 2016, KINDLER et al. 2017, SCHULTZE et al. 2019, 2020, ASZTALOS et al. 2020). *Natrix astreptophora* (SEOANE, 1884) is distributed from the northern Maghreb region through the Iberian Peninsula to south-eastern France (POKRANT et al. 2016, ASZTALOS et al. 2020, FRITZ & SCHMIDTLER 2020). The barred grass snake, *N. helvetica* (LACEPÈDE, 1789), occurs in France, Great Britain, the Benelux countries, in the Rhine region, Switzerland, and Italy and ranges across the Alps to southernmost Bavaria (KINDLER et al. 2017, GLAW et al. 2019, FRITZ & SCHMIDTLER 2020, SCHULTZE et al. 2020). The common grass snake, *N. natrix* sensu stricto (LINNAEUS, 1758), has the largest distribution range, from the Rhine region eastwards to Lake Baikal in Central Asia (KINDLER et al. 2017, FRITZ & SCHMIDTLER 2020).

Intentionally introduced or accidentally translocated grass snakes are known from several countries (France: ASZTALOS et al. 2020; Germany and Great Britain: KINDLER et al. 2017; Italy: SCHULTZE et al. 2020; Netherlands: VAN RIEMSDIJK et al. 2020; Switzerland: DUBEY et al. 2017), and in these cases it seems likely that alien grass snakes interbreed with local native populations. In the Netherlands, *N. h. helvetica* is the naturally occurring grass snake taxon,

being widely distributed across the country (KABISCH 1999, DE WIJER et al. 2009, KINDLER et al. 2017, STUMPEL & JANSSEN 2017). However, mtDNA data revealed that at least three Dutch populations are compromised by alien grass snakes (VAN RIEMSDIJK et al. 2020, STRUIJK et al. 2020): Alphen aan den Rijn, Krimpenerwaard (both in the province of South Holland), and Brunssummerheide (province of Limburg).

In 1983, grass snakes were illegally released in Alphen aan den Rijn that originated allegedly from Ravenna (north-eastern Italy). In addition, 15 years later, one dice snake (*N. tessellata*) and two adult *N. natrix* were illegally released in the same area. The latter snakes were captive bred from an unstriped spotted male from north-eastern Italy (region of Treviso-Trieste-Udine) and a striped female from Selçuk (Ephesus), Turkey (STRUIJK et al. 2020). Near Krimpenerwaard, grass snakes most likely originating in Austria (Neusiedler See) and Romania started appearing since 1980 (STRUIJK et al. 2020), and for Brunssummerheide the introduction of '*N. n. persa*', i.e., of striped grass snakes, was reported (ELZENGA 1974, VAN BUGGENUM & HERMANS 1986, VAN BUGGENUM 1992, BUGTER et al. 2014). The preliminary results of VAN RIEMSDIJK et al. (2020) suggested interbreeding with native *N. h. helvetica*.

VAN RIEMSDIJK et al. (2020) sequenced two mitochondrial genes (cyt *b* and ND4) of 43 grass snakes and compared these data with basic colour pattern characters (striped, unstriped). Besides mitochondrial haplotypes of the native barred grass snake (*N. h. helvetica*), haplotypes

representing mtDNA lineages 4 and 7 of *N. natrix* were recorded. These haplotypes were frequently, but not exclusively, found in striped grass snakes. This strongly suggests hybridization, even though there is no direct genetic evidence, because mtDNA is exclusively inherited in the maternal line.

Furthermore, the introduction of a dice snake at Alphen aan den Rijn implies that hybridization with this species should be considered as well. One of the snakes from Alphen aan den Rijn (Fig. 1) strikingly resembles photos of supposed hybrids between *N. tessellata* and *N. natrix* sensu lato in MEBERT et al. (2011).

The present study aims at clarifying whether hybridization between alien *N. natrix* and native *N. h. helvetica* occurs in the Netherlands. We also test for potential hybridization with dice snakes. For doing so, we use 162 samples of dice snakes and grass snakes (Supplementary document S1) and combined a nuclear genomic marker system (microsatellite loci) with information from mtDNA sequences.

Forty-three of our samples originated in the Netherlands and were already studied by VAN RIEMSDIJK et al. (2020) for mtDNA and morphology; four additional Dutch samples supplemented our data set (Fig. 2; Table 1). All of these samples were genotyped at 13 microsatellite loci used in previous studies on grass snakes (POKRANT et al. 2016, KINDLER et al. 2017, SCHULTZE et al. 2019, 2020, ASZTALOS et al. 2020), and for the four new samples two mitochondrial genes (cyt *b*, ND4+tRNAs) were sequenced as described in KINDLER et al. (2013) and POKRANT et al. (2016).

The mtDNA sequences were aligned with all previously identified haplotypes of grass snakes (KINDLER et al. 2013, 2017, 2018, POKRANT et al. 2016, SCHULTZE et al. 2019, 2020, ASZTALOS et al. 2020) using BIOEDIT 7.0.5.2 (HALL 1999), resulting in a 1,117-bp-long alignment of 283 cyt *b* sequences and an 866-bp-long alignment of 196 ND4+tRNAs sequences. Using TCS 1.21 (CLEMENT et al. 2000), explora-



Figure 1. Hybrid between *Natrix h. helvetica* and *N. natrix vulgaris* from Alphen aan den Rijn, morphologically resembling putative hybrids between *N. natrix* sensu lato and *N. tessellata*. Photo: R. P. J. H. STRUIJK.

tory networks were drawn for haplotype determination (gaps coded as fifth character state, connection limit of 100 steps; networks not shown). This resulted in the identification of one new haplotype for each mtDNA fragment (mtDNA lineage 7; Supplementary document S1), whereas the remaining sequences matched known haplotypes (see below). The new haplotype gy12 (ENA accession number LR963483) for DNA coding for ND4 and tRNAs differs by one mutation step from the previously known haplotype gy11. The new cyt *b* haplotype gy14 (ENA accession number LR963484) differs by three mutation steps from the previously known haplotype gy3.

For microsatellite analyses, data of our samples from the Netherlands were merged with those from KINDLER et al. (2017) for Dutch *N. h. helvetica* ( $n = 35$ ). Furthermore, data for 20 genotypically pure *N. n. natrix* (mtDNA lineage 3), 20 genotypically pure *N. n. vulgaris* (mtDNA lineage 4), 20 representatives of mtDNA lineage 7 of *N. natrix* of unclear subspecific identity (see FRITZ & SCHMIDTLER 2020), and 20 newly genotyped *N. tessellata* from Turkey were added (Supplementary document S1). The resulting data set was analysed using Principal Component Analyses (PCAs) as implemented in the R package ADEGENET 2.1.1 (JOMBART 2008). This approach uses exclusively genetic information, without population genetic presumptions, and avoids any population-specific bias that might occur due to the overrepresented Dutch snakes.

A PCA for the whole data set ( $n = 162$ ) revealed three clusters corresponding to *N. h. helvetica*, *N. natrix*, and *N. tessellata*, respectively (Fig. 3A). The 27 samples from Krimpenerwaard clustered with *N. natrix* (all samples had haplotypes of mtDNA lineage 4 of *N. natrix*). The remaining snakes from Alphen aan den Rijn and Brunssummerheide had an intermediate position between *N. h. helvetica* and *N. natrix* (nine individuals) or clustered within *N. natrix* (one sample from Brunssummerheide). In addition, one out of five grass snakes from another Dutch locality (Houten) had an intermediate position between *N. h. helvetica* and *N. natrix*. The 10 intermediate snakes harboured mtDNA haplotypes of mtDNA lineage E (*N. h. helvetica*) and mtDNA lineages 4 and 7 of *N. natrix* (Supplementary document S1), supporting hybrid status. The cluster of *N. tessellata* was highly distinct and remote from the two others.

For a second PCA round, the data for *N. tessellata* were removed to obtain a better resolution for the remaining data. The processed sample of 142 genotypes resulted in two clusters, one for *N. h. helvetica* and another one for *N. natrix* (Fig. 3B, top). The results of the first PCA were confirmed: The snakes from Krimpenerwaard (mtDNA lineage 4) clustered completely within *N. natrix* as did one sample (mtDNA lineage 7) from Brunssummerheide. The 10 putative hybrid grass snakes from Alphen aan den Rijn, Brunssummerheide, and Houten were now placed largely outside the 95% confidence intervals of both *N. helvetica* and *N. natrix*, again in an intermediate position. Within *N. natrix*, samples corresponding to mtDNA lineage 7 were quite distinct from the others.

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Table 1. Dorsal colour pattern and genetic identity of grass snakes from nine Dutch populations. Column mtDNA: Lineage E (*Natrix h. helvetica*), lineages 4 and 7 (*N. natrix*). Most mtDNA data are from VAN RIEMSDIJK et al. (2020). No mtDNA data are available for one sample from Houten\*. \* mtDNA and microsatellite data for three samples from KINDLER et al. (2017).

No.	Locality	n	Dorsolateral stripes (n)	mtDNA lineage		Microsatellite identity
				E/4/7	<i>helvetica/natrix/admixed</i>	
1	Fochteloërveen	1	0	1/0/0		1/0/0
2	Marken	3	0	3/0/0		3/0/0
3	Vaassen	1	0	1/0/0		1/0/0
4	Asselsche Heide	1	0	1/0/0		1/0/0
5	Kootwijkerveen	1	0	1/0/0		1/0/0
6	Houten*	5	0	4/0/0		4/0/1
7	Alphen aan den Rijn	7	6	2/5/0		0/0/7
8	Krimpenerwaard	27	10	0/27/0		0/27/0
9	Brunssummerheide	3	1	0/0/3		0/1/2

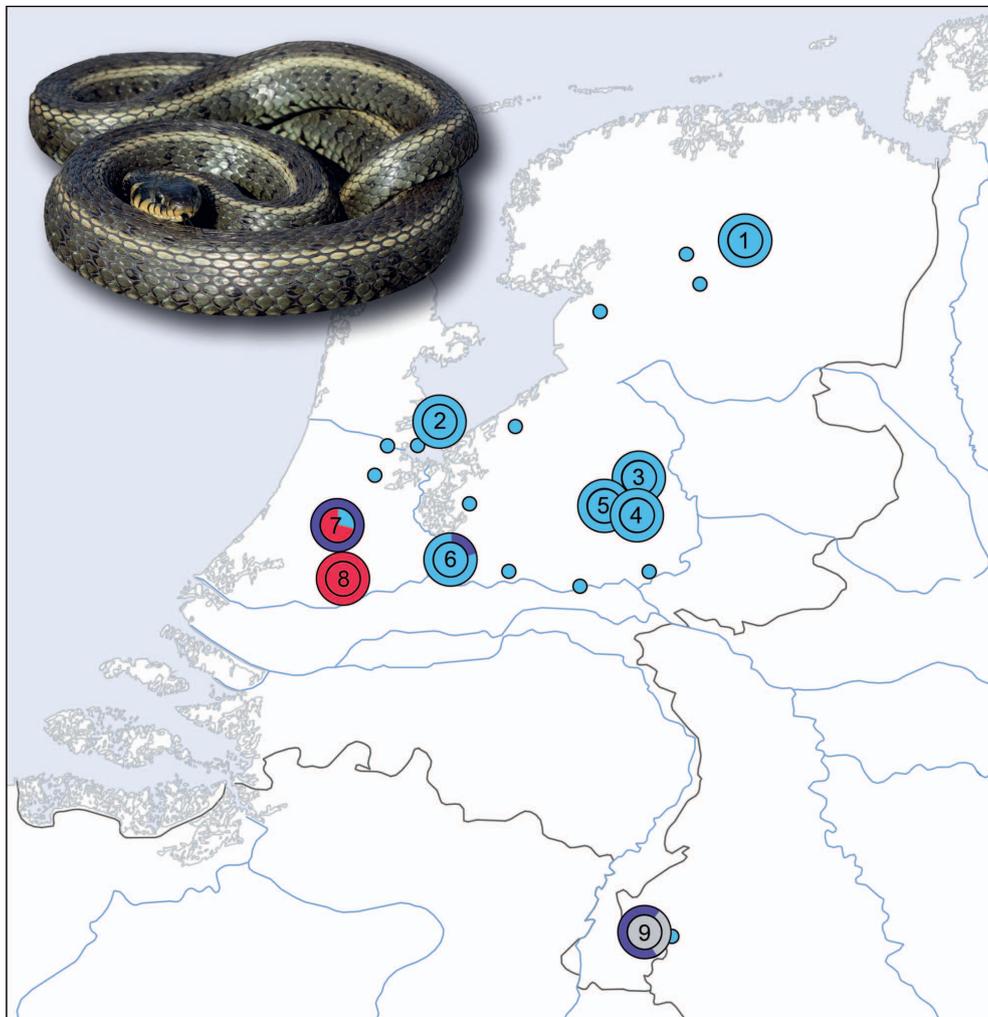


Figure 2. Studied samples of grass snakes (*Natrix h. helvetica*, *N. natrix* and their hybrids) from the Netherlands. Numbers for large symbols refer to Table 1. For large symbols, the colour of the core corresponds to mtDNA lineage (blue = lineage E of *N. helvetica*; red = lineage 4 and grey = lineage 7 of *N. natrix*) and the colour of the edge indicates genotypic identity according to microsatellite data (blue = *N. h. helvetica*; red = *N. n. vulgaris*; purple = admixed; grey = *N. natrix* subsp.). Small symbols are pure *N. h. helvetica* based on both mtDNA and microsatellites (data from KINDLER et al. 2017). Inset: Striped *Natrix n. vulgaris*, Krimpenerwaard. Photo: S. GULDEMOND.

Our nuclear genetic evidence allows the firm conclusion that in the Netherlands an introduced population of *N. n. vulgaris* is established at the Krimpenerwaard site. However, in the PCA (Fig. 3B, top) the samples from Krimpenerwaard appeared somewhat distinct from other *N. natrix* samples, including our reference samples of *N. n. vulgaris*.

This putative distinctness could reflect inbreeding caused by the low number of founder individuals, as supported by the statistically highly significant inbreeding coefficient for Krimpenerwaard snakes ( $F_{is} = 0.137$ ,  $p < 0.001$ ) and a heterozygote deficit ( $H_o = 0.44376$  vs.  $H_e = 0.51284$ , calculated in ARLEQUIN 3.5.2.2; EXCOFFIER & LIESCHER 2010).

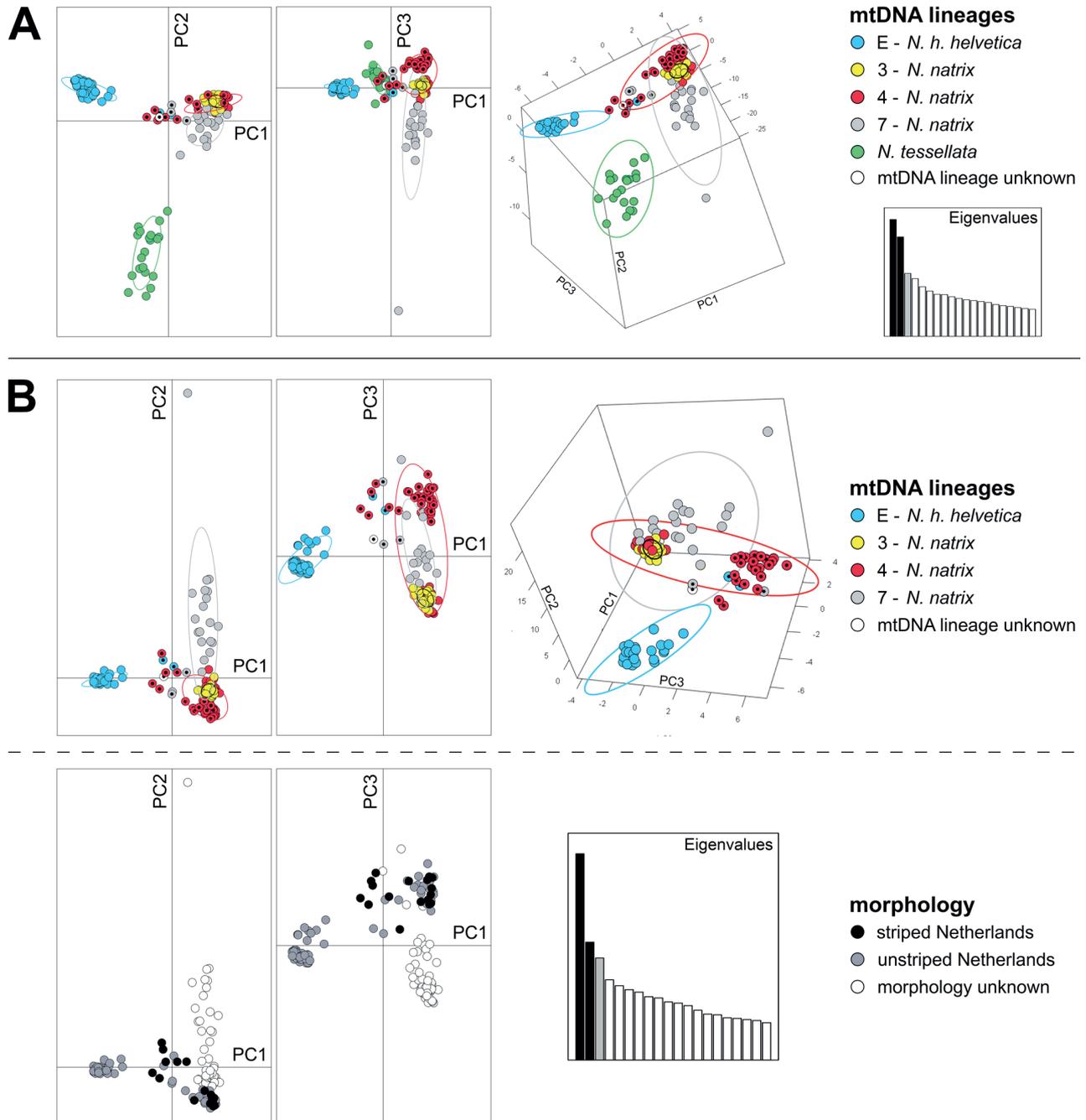


Figure 3. Principal Component Analyses for grass snakes and dice snakes (*Natrix* spp.) based on microsatellite data of A) the entire data set ( $n = 162$ ) and B) the data set without *N. tessellata* ( $n = 142$ ). Samples are coloured according to their mitochondrial identity (A, B top) or back pattern (B bottom). In A and B (top), symbols of putative hybrids and non-native *N. natrix* from the Netherlands are marked by a black dot. Oval outlines correspond to 95% confidence intervals; the confidence interval for mtDNA lineage 3 is shown in black. For A) PC1 explains 6.1% of variance, PC2 5.2%, and PC3 3.3%; for B) PC1 explains 7.7% of variance, PC2 4.4%, and PC3 3.8%.

According to our results, the genotypically intermediate samples from Alphen aan den Rijn, Brunssummerheide, and Houten, bearing mtDNA haplotypes of *N. h. helvetica* (mtDNA lineage E) and *N. natrix* (mtDNA lineages 4 and 7), represent interspecific hybrids. The record of a pure *N. natrix* (mtDNA lineage 7) at Brunssummerheide suggests that at least some pure individuals of this species still occur there. Our findings are also supported by morphology (Fig. 3B, bottom), because some of these snakes are striped, a morphological trait that never occurs in *N. helvetica* but is frequently found in the south-eastern distribution range of *N. natrix* (MERTENS 1946, KABISCH 1999, FRITZ & SCHMIDTLER 2020). According to our results, hybrids may bear back stripes as well (Supplementary document S1).

Despite the observation of a snake with an odd phenotype pointing towards potential hybridization with *N. tessellata* at Alphen aan den Rijn, this hypothesis can be rejected based on our genetic data; rather it seems that hybridization between *N. helvetica* and *N. natrix* can result in aberrant phenotypes (see also DUBEY et al. 2017).

We show that the worry concerning ‘genetic pollution’ of native grass snake populations through introduced alien individuals, recently expressed in several papers (SCHULTZE et al. 2019, VAN RIEMSDIJK et al. 2020, STRUIJK et al. 2020), has already become a reality in the Netherlands. There is extensive hybridisation between the native and invasive grass snake species in two Dutch populations, previously highlighted as containing *N. natrix* mtDNA (VAN RIEMSDIJK et al. 2020). These snakes show different degrees of genetic admixture, suggesting backcrossing to parents and/or crosses between hybrids; F1 hybrids are evidently not sterile.

Furthermore, grass snakes are known to be highly mobile animals (MADSEN 1984, WISLER et al. 2008), with home ranges of up to 40 ha. This mobility poses a serious threat beyond the immediate introduction sites. This is evinced by the record of a hybrid snake at Houten (Table 1, Supplementary document S1), which is approximately 30 km east of the introduced *N. natrix* population in the Krimpenerwaard site. While the origin of the relatively isolated ‘native’ *N. helvetica* populations in South Holland could be debated (STRUIJK et al. 2020), Houten decisively concerns the core native range of *N. helvetica* in the Netherlands.

We urge dedicated genetic screening of additional grass snake populations that might be affected by genetic pollution to delineate how far foreign alleles have spread. Microsatellite analyses, combined with mtDNA sequencing, offer here a straightforward and cost-efficient approach that delivers reliable and fast results. The results of such investigations could serve as a sound basis for conservation decisions on how genetically compromised populations should be managed.

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## Supplementary data

The following data are available online:

Supplementary document S1. *Natrix* samples used in the present study.