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Phylogeography and Contact Zones of the Common Grass Snake in Poland (Squamata: Natricidae: *Natrix natrix*)

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ABSTRACT

Until now, the genetic identity of common grass snakes (*Natrix natrix*) in Poland remained poorly understood. This study presents the first comprehensive phylogeographic analysis for Poland using mitochondrial DNA sequences (*cyt b* and *ND4+tRNAs*) and 13 nuclear microsatellite loci. A total of 982 individuals from Poland and adjacent countries were analysed. Three mitochondrial lineages were identified, with partially overlapping distributions. A Bayesian cluster analysis of microsatellite data revealed three genetic clusters corresponding to distinct mitochondrial lineages and the subspecies *N. n. natrix*, *N. n. vulgaris*, and *N. n. scutata*, respectively, albeit with extensive admixture. The mitochondrial lineage of the nominotypical subspecies occurs in the centre and west of the country, the lineage of *N. n. vulgaris* mainly in the south and southeast, and the lineage of *N. n. scutata* mainly in the very east. However, Poland represents a complex hybrid zone, with widespread admixture and many individuals with mitonuclear discordance, indicating extensive gene flow. Nuclear genomic signatures of *N. n. scutata* were found to be widely distributed, also far beyond the occurrence of the respective mitochondrial lineage in eastern Poland. This provides evidence for a dynamic hybrid zone moving from the east to the west and for a westward expansion of *N. n. scutata*. The present phylogeographic pattern reflects complex postglacial range dynamics, namely the admixture of *N. n. natrix*, a Central European survivor during the last glaciation, with the postglacial invaders *N. n. vulgaris* from the southeast and *N. n. scutata* from the east.

1 | Introduction

Grass snakes of the genus *Natrix* Laurenti, 1768 are widely distributed across the Palearctic region, with most species confined to the Western Palearctic. The genus comprises five species. Two are well-defined water snake species: the phylogenetically earliest diverging species, the viperine snake *Natrix maura* (Linnaeus, 1758), and the dice snake *Natrix tessellata*

(Laurenti, 1768). *Natrix maura* inhabits Northwestern Africa, the Iberian Peninsula, and parts of Western Europe, while *N. tessellata* ranges from Italy and Central Europe to the Balkans and Western and Central Asia (Sindaco et al. 2013). *Natrix tessellata* is the sister species to the remaining three *Natrix* species (Guicking et al. 2006; Schöneberg et al. 2023), the so-called grass snakes. This clade contains three semiaquatic species (Pokrant et al. 2016; Kindler et al. 2017; Speybroeck et al. 2020): the red-eyed grass snake *Natrix astreptophora* (Seoane, 1884), found in the Iberian Peninsula, adjacent France and Northwestern Africa; the barred grass snake *Natrix helvetica* (Lacepède, 1789), inhabiting Western Europe including Italy and Great Britain; and the common grass snake *Natrix natrix* (Linnaeus, 1758) sensu stricto, which is the most widely distributed species and sister to a clade containing the two other grass snake species. The range of *N. natrix* spans from Fennoscandia and Central Europe across northeastern Italy and the Balkans and extends eastward into Central Asia to the Lake Baikal region in Siberia (Russia) and Mongolia, a distribution exceeding 6300 km in west–east direction (Fritz and Schmidtler 2020; Asztalos, Ayaz, et al. 2021; Schöneberg et al. 2023). *Natrix natrix* is composed of four subspecies that differ in genetics and morphology (Asztalos, Ayaz, et al. 2021; Fritz and Ihlow 2022). These subspecies hybridise rampantly in wide secondary contact zones (Kindler et al. 2017; Asztalos, Ayaz, et al. 2021). Three of the four subspecies occur in Central and Eastern Europe: *Natrix natrix natrix* (Linnaeus, 1758), distributed in the northern part of Central Europe and western Fennoscandia; *Natrix natrix vulgaris* (Laurenti, 1768) from southern and southeastern Central Europe; and *Natrix natrix scutata* (Pallas, 1771) occurring from easternmost Central Europe to Lake Baikal. With respect to biogeography, it has been inferred that the nominotypical subspecies is a glacial survivor, whereas the two other subspecies reached Central Europe during postglacial range expansions from the Balkans and the east (Kindler et al. 2017, 2018; Simonov et al. 2024).

FIGURE 1 | (A) Grass snake from northeastern Poland (Białowieża; photo: H. Bringsøe) in comparison with (B) a typical *Natrix natrix scutata* from Ukraine (Haidary, Kharkiv Oblast; photo: O. Zinenko). Note the pale versus orange occipital crescents and the general body coloration.

Although the general phylogeography of *N. natrix* is well studied, the postglacial range dynamics in eastern Central Europe remain largely unknown due to a lack of samples from Poland. The mitochondrial lineages of the few previously studied samples from that country corresponded to all three subspecies (Kindler et al. 2013, 2017; Asztalos, Ayaz, et al. 2021), suggesting that these taxa could meet somewhere in Poland. Also, it remained a possibility that the Central European population of the nominotypical subspecies is connected along the Carpathian mountain chain to a relict occurrence of the same mitochondrial lineage in the southern Balkans. If so, this would contradict the hypothesis that *N. n. natrix* is a glacial survivor in Central Europe (Kindler et al. 2018). Furthermore, another unexplored possibility is that *N. n. vulgaris* moved from the southern Balkans along this route northwards into Poland. The few previous genetic records for Poland suggested that the easternmost subspecies *N. n. scutata* reaches the eastern border of the country (Kindler et al. 2013, 2017; Asztalos, Ayaz, et al. 2021), but it remained unclear how far west the subspecies or its genetic signatures reach. Morphologically, most *N. n. natrix* and *N. n. vulgaris* are difficult to tell apart, whereas *N. n. scutata* is characterised by its unique orange-coloured occipital crescents and dark body coloration (Fritz and Ihlow 2022; see also Figure 1). In a recent investigation using iNaturalist records, the latter authors found a few grass snakes resembling *N. n. scutata* along the eastern border of Poland and a single similar individual in the centre of the country, whereas the vast majority of snakes morphologically matched the other two subspecies.

The present study aims at resolving the intricate situation in Poland. In doing so, we apply the same mitonuclear approach as previous studies allowing us to merge our new data with the previously assembled dataset covering neighbouring regions (Kindler et al. 2013, 2014; Glaw et al. 2019; Schultze et al. 2019, 2020; Asztalos, Ayaz, et al. 2021; Asztalos, Glaw, et al. 2021; Neumann et al. 2024). We use two mitochondrial markers, the *cyt b* gene and the *ND4* gene plus adjacent DNA coding for *tRNAs*. In addition, we use 13 microsatellite loci for detecting population structure, gene flow, and hybridisation.

The objectives of this study are: (1) to map the distribution of the three subspecies across Poland, (2) to identify and characterise their secondary contact zones, (3) to assess the occurrence and extent of hybridisation among the subspecies, and based on these objectives, (4) to place the genetic pattern in Poland into the previously inferred framework for the postglacial colonisation history of *N. natrix* in eastern Central Europe, and (5) to corroborate or reject the previous hypothesis

(Kindler et al. 2018) that *N. n. natrix* is a glacial survivor in Central Europe.

2 | Materials and Methods

2.1 | Study Area

This study analyzes the phylogeography of the common grass snake in eastern Central Europe, specifically Poland. Covering an area of approximately 312,000 km^2, the country is bordered by Germany to the west, the Czech Republic and Slovakia to the south, Ukraine and Belarus to the east, and Lithuania along with the Russian exclave of Kaliningrad to the northeast; the northern boundary is formed by the Baltic Sea coast. Most of Poland consists of lowlands and only the southern border region lies within mountain chains—the Sudetes in the west and the Carpathians in the east, where the Tatra Mountains rise to approximately 2500 m above sea level (m a.s.l.). The Sudetes and Carpathians are separated by a depression, the Moravian Gate with an average altitude of only 270 m a.s.l. *Natrix natrix* is broadly distributed across Poland, occupying a wide range of both natural and anthropogenically influenced habitats, from lowland floodplains to montane zones up to 1150 m a.s.l. (Juszczyk 1987; Sura 2018).

2.2 | Sampling, Genetic Markers, and Laboratory Procedures

The present study is based on a dataset of 982 *Natrix natrix* that also includes previously processed samples; 243 samples from Poland are new (Table S1), corresponding to tissue samples, buccal swabs, scales, and pieces of shed skin. Invasive sampling was only conducted using roadkills. The new samples were sequenced and genotyped following the protocols of Kindler et al. (2013) and Pokrant et al. (2016). The samples from previous studies (Kindler et al. 2013, 2014; Glaw et al. 2019; Schultze et al. 2019, 2020; Asztalos, Ayaz, et al. 2021; Asztalos, Glaw, et al. 2021; Neumann et al. 2024) included grass snakes from Austria (70), the Czech Republic (38), Denmark (30), Finland (9), Germany (380), Hungary (62), Kazakhstan (2), Lithuania (5), Norway (8), Poland (26), Romania (9), Russia (4), Slovakia (80), Sweden (14), and Ukraine (2).

The same two molecular marker systems were used as in the above-mentioned previous studies: mitochondrial DNA (mtDNA) and nuclear microsatellites. Two mtDNA fragments were sequenced: the cytochrome *b* gene (*cyt b*, 1117 bp) and a fragment comprising the second half of the NADH dehydrogenase subunit 4 (*ND4*) gene with adjacent DNA coding for *tRNAs* (His, Ser, Leu; 867 bp). Nuclear genomic variation was assessed using 13 polymorphic microsatellite loci, grouped into four multiplex sets for genotyping. Detailed information on primers, loci, and PCR conditions is provided in Tables S2 and S3.

2.3 | Mitochondrial DNA Sequence Alignment and Analyses

Mitochondrial DNA sequences were aligned using GENEIOUS R7.0 (https://www.geneious.com) and the MUSCLE algorithm. Sequences from 263 grass snakes from Poland were examined. Among these, 237 were newly sampled and data from 26 individuals were from previous studies (Table S1). For the obtained 193 *cyt b* sequences (176 full-length and 17 partial) and 245 *ND4 + tRNAs* sequences (164 full-length and 81 partial), exploratory Neighbour-Joining trees (NJ) were calculated in MEGA X (Kumar et al. 2018) to determine the mitochondrial lineage (clade) of each sample. Sequences from *Natrix tessellata* were used as outgroup (*cyt b*: OU862672 and *ND4 + tRNAs*: OU862630). For sequences of full length, the individual haplotypes were identified using statistical parsimony networks as implemented in Hapsolutely (Vences et al. 2024). For this purpose, one representative of each previously characterised haplotype of the lineages of *N. n. natrix* (clade 3 or 'yellow lineage' of Kindler et al. 2013, 2017), *N. n. vulgaris* (clade 4 or 'red lineage' of Kindler et al. 2013, 2017) and *N. n. scutata* (clade 8 or 'green lineage' of Kindler et al. 2013, 2017) were included as a reference. Accession numbers of all haplotypes are listed in Table S4.

For mapping the geographic distribution of mitochondrial lineages using QGIS 3.4 (QGIS Development Team 2023), previously published data from Poland and neighbouring countries was included, bringing the tally of individuals with mtDNA data to 976 (Table S1, see there also for sources).

2.4 | Microsatellite-Based Cluster Analyses

The processed dataset contained 960 genotypes corresponding to 247 grass snakes from Poland (221 new) and data for neighbouring countries (Table S1). All microsatellite loci were tested for Hardy–Weinberg equilibrium (HWE) and pairwise linkage disequilibrium (LD) using Arlequin 3.5 (Excoffier and Lischer 2010). The potential presence of null alleles was assessed with Micro-Checker (van Oosterhout et al. 2004).

STRUCTURE 2.3.4 (Pritchard et al. 2000; Falush et al. 2003) was used to identify genetic clusters and admixture. This software applies an unsupervised model-based and spatially non-explicit Bayesian approach to delineate genetic clusters by assuming Hardy–Weinberg and linkage equilibrium. Analyses were performed with the admixture model with correlated allele frequencies for $K = 1$ to 10 and 10 replicates per K. Each run comprised 1,000,000 MCMC iterations with a burn-in of 250,000. The most likely number of genetic clusters was determined using the ΔK method (Evanno et al. 2005) in StructureSelector (Li and Liu 2018). Results were visualised using the R packages *ggplot2* (Wickham 2016) and *dplyr* (Wickham et al. 2023).

The genotype of each sample was then classified according to its coefficient of cluster membership (Q value) using the hybrid simulations of Kindler et al. (2017). Snakes with ≥ 80% assignment to a single cluster were considered genetically pure; snakes with less than 80% were classified as hybrids. The geographic distribution of genetically pure and hybrid genotypes was also mapped using QGIS 3.4 (QGIS Development Team 2023).

3 | Results

3.1 | Mitochondrial Phylogeography

3.1.1 | Geographic Distribution of Mitochondrial Lineages

All samples from Poland were assigned to three mitochondrial clades in the NJ trees (Figures S1 and S2) and three corresponding clusters in the haplotype networks (Figures 2 and 3), the so-called yellow, red, and green lineages of *Natrix natrix* (see Kindler et al. 2013, 2017). Seventy-four samples represented the yellow lineage (clade 3 of Kindler et al. 2013, 2017), which is characteristic for the nominotypical subspecies, 150 samples represented the red lineage (clade 4 of Kindler et al. 2013, 2017), typical for *N. n. vulgaris*, and 39 samples represented the green lineage (clade 8 of Kindler et al. 2013, 2017), typical for *N. n. scutata* (Table S1). The present study provides for the first time a detailed picture of the geographic distribution of these mitochondrial lineages for Poland (Figure 4). The yellow lineage was found in the west and centre of Poland, the red lineage in the south and southeast, with two outliers at the Baltic coast, and most records of the green lineage were found in a less than 200-km-wide strip along the eastern border. However, there were also two records of the green lineage in southwestern Poland near the Czech border, in close proximity or even sympatry with haplotypes of the yellow and red lineages. Haplotypes of the latter two lineages widely co-occurred in the south of the country, while haplotypes of the red and green lineages overlapped elsewhere only slightly in the southeast of Poland.

3.1.2 | Haplotype Networks

Parsimony network analyses of the full-length sequences from Poland revealed several previously unknown haplotypes. The network for *cyt b* (Figure 2) contained a total of 186 haplotypes. Thirty-eight haplotypes were found in Poland of which 26 were new: y53–y59 (yellow lineage), r73–r86 (red lineage), and gn37–gn41 (green lineage). Haplotypes of the yellow and red lineages were separated by a minimum of 59 mutations; haplotypes of the yellow and green lineages, by a minimum of 53 mutations; and haplotypes of the green and red lineages, by a minimum of 48 mutations. The most frequent haplotype of the yellow lineage in Poland ($n = 27$) was the central haplotype y5, which has been previously found elsewhere. Six new haplotypes (y53–y58) differed by one or two mutations from y5. Four other previously

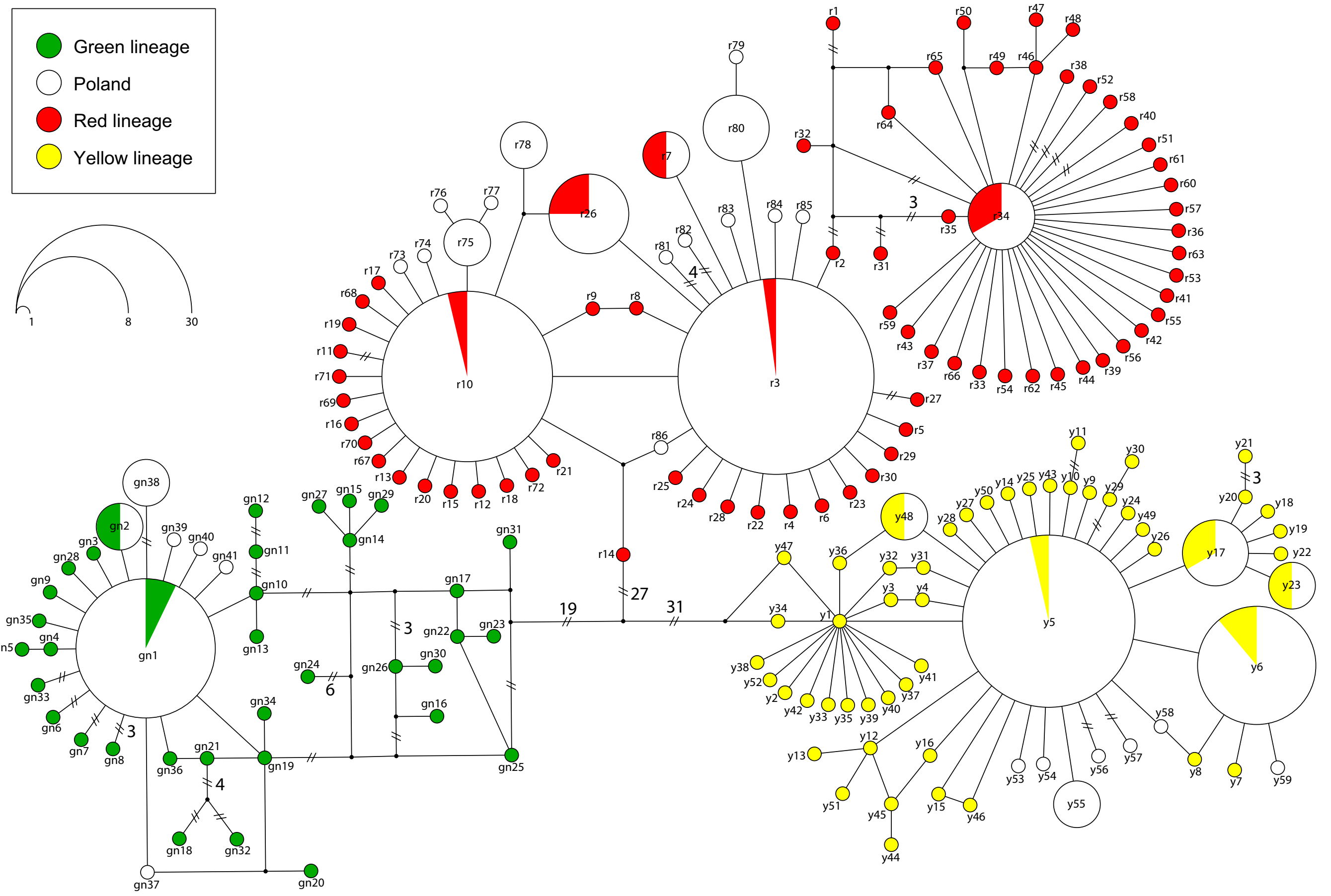

FIGURE 2 | Parsimony network for complete *cyt b* sequences of *Natrix natrix* from Poland, computed together with one representative of each previously identified haplotype of the respective lineages. Each colour represents a mitochondrial lineage. Coloured circles or slices correspond to previously identified haplotypes; white circles or slices are samples from Poland. Small black dots are missing node haplotypes. Symbol size reflects haplotype frequencies. Each line connecting two haplotypes corresponds to one mutation step; lines with hashmarks represent two mutation steps, higher numbers are indicated.

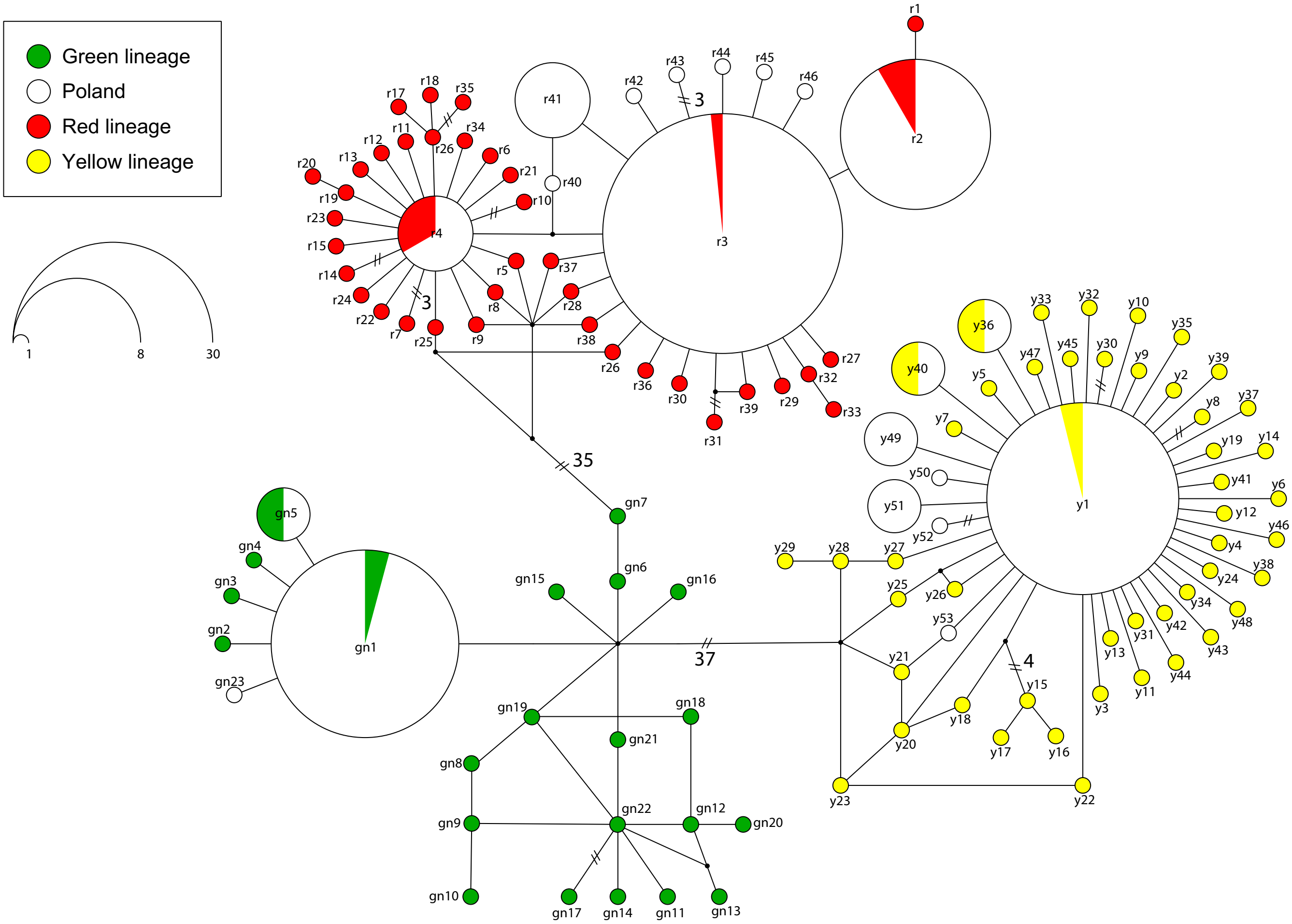

FIGURE 3 | Parsimony network for 142 complete *ND4 + tRNAs* sequences of *Natrix natrix* from Poland, computed together with one representative of each previously identified haplotype of the respective lineages. Each colour represents a mitochondrial lineage. Coloured circles or slices correspond to previously identified haplotypes; white circles or slices are samples from Poland. Small black dots are missing node haplotypes. Symbol size reflects haplotype frequencies. Each line connecting two haplotypes corresponds to one mutation step; lines with hashmarks represent two mutation steps, higher numbers are indicated.

identified haplotypes (y6, y17, y23, y48) were also found in Poland and differed by one or two mutations from y5. The additional new haplotype y59 differed by one mutation from y6, the latter was with eight records the second most frequent haplotype in Poland. In the red lineage, most individuals were assigned to the previously described central haplotypes r3 and r10, with 50 and 29 records in Poland, respectively. Most other haplotypes of the red lineage found in Poland differed by one to four mutations from one of these haplotypes, including all newly identified and two previously identified haplotypes. An exception was the previously identified third central haplotype r34, which was also recorded from Poland. It differed by eight mutations from r3. Within the green lineage, gn1 was with 18 records the most frequent haplotype from Poland; five new and one previously identified haplotypes represented by one or two records differed by one or two mutations from gn1.

The network for *ND4 + tRNAs* (Figure 3) consisted of 122 haplotypes. A total of 21 haplotypes were found in Poland, 13 of which were new: gn23, r40–r46, and y49–y53. Among these, five haplotypes represented the yellow lineage, seven the red lineage, and one the green lineage. The green lineage was separated from the yellow and red lineages by at least 37 and 39 mutational steps, respectively. The most frequent haplotype of the yellow lineage was the central haplotype y1 with 28 records in Poland. Two previously identified haplotypes (y36, y40) found in Poland and all new haplotypes of the yellow lineage differed by one or two mutations from y1. The haplotypes for *ND4 + tRNAs* previously described as endemic to the southern Balkan Peninsula (y15–18, y21, y23; Kindler et al. 2018) were not found in Poland. Within the red lineage, the central haplotype r3 was most frequent in Poland ($n = 70$), and the remaining nine haplotypes from Poland (7 new, 2 previously identified) differed by one to three mutations from r3. For the green lineage only three haplotypes were identified in Poland: the previously described haplotypes gn1 and gn5 and the new haplotype gn23; gn5 and gn23 differed by one mutation from gn1.

3.2 | Microsatellite Clusters, Subspecies Identity and Admixture

Microsatellite data were available for 247 grass snakes from Poland. The 13 microsatellite loci were highly polymorphic, with allele counts ranging from 8 to 30 per locus, resulting in a total of 182 alleles identified across all loci for the Polish

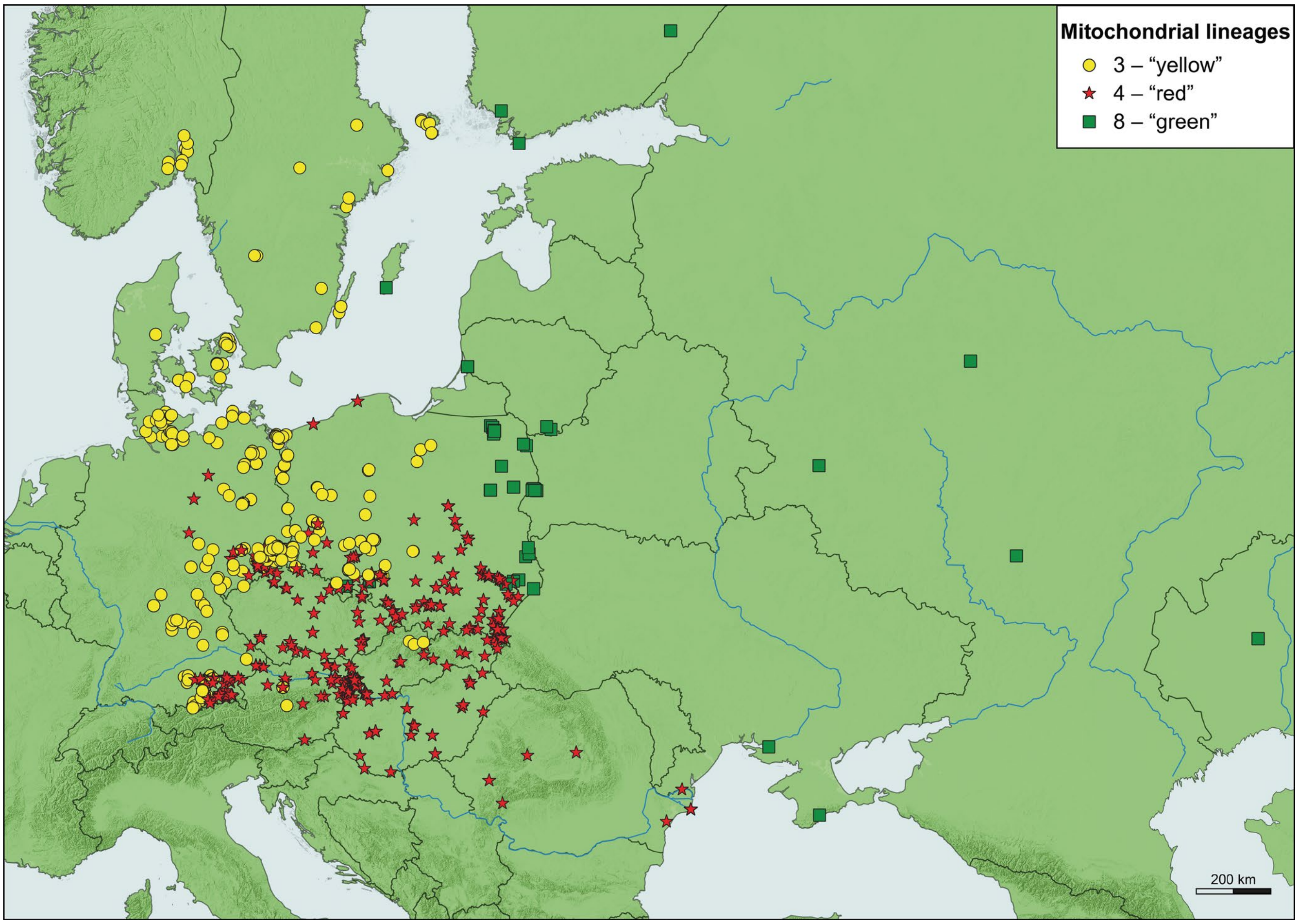

FIGURE 4 | Geographic distribution of haplotypes of the three mitochondrial lineages of *Natrix natrix* ($n = 982$) in Poland and adjacent countries.

material (Table S3). There were no significant deviations from HWE, nor was there any evidence of linkage disequilibrium or the presence of null alleles.

Bayesian cluster analysis for Poland and neighbouring countries was performed using STRUCTURE 2.3.4 and microsatellite data from 960 individuals (Figure 5; Table S1). The optimal number of clusters ($K = 3$) was determined using the ΔK method implemented in StructureSelector (Figure S3). If admixed individuals were disregarded, each of the three clusters corresponded to one mitochondrial lineage: the yellow cluster to lineage 3, the red cluster to lineage 4, and the green cluster to lineage 8. Depending on the geographic region, a high degree of admixture occurred.

A total of 540 individuals were classified as genotypically pure based on the microsatellite data. Among these, 205 individuals represented *N. n. natrix* (yellow cluster), 210 individuals represented *N. n. vulgaris* (red cluster), and 125 individuals represented *N. n. scutata* (green cluster). The remaining 420 grass snakes had admixed nuclear genotypes (Figure 5; Table S1). Grass snakes with genotypes of *N. n. scutata* or admixed individuals with a high percentage of ancestry of *N. n. scutata* occurred across the entire country of Poland, and many of them showed mitonuclear discordance in that nuclear genotypes of *N. n. scutata* were combined with mitochondrial haplotypes of the yellow or red lineages (compare Figures 4 and 5). Except for three genotypically pure representatives of *N. n. natrix* from the Baltic coast at the Polish-German border (Uznam: 2, Wolin: 1; Table S1), all other grass snakes from Poland were either genotypically pure *N. n. scutata* (108) or admixed (136), including additional admixed snakes from Uznam and Wolin. In admixed snakes, a westward and southwestward increase of nuclear signatures of *N. n. natrix* and *N. n. vulgaris* is evident (Figure 5; Table S1).

4 | Discussion

This study is the first comprehensive phylogeographic investigation of the common grass snake (*Natrix natrix*) in Poland, filling a major gap left by earlier studies. The integration of both mitochondrial and nuclear genomic markers with an extended sampling reveals a complex and unexpected pattern shaped by postglacial recolonisation dynamics, secondary contact between distinct genetic lineages, and extensive admixture. In the face of a previously published morphological assessment including many iNaturalist records for Poland (Fritz and Ihlow 2022), the wide westward-reaching nuclear signatures of *N. n. scutata* (green cluster) were surprising. Fritz and Ihlow (2022) found in their sample of iNaturalist photos of 201 grass snakes from Poland only seven individuals with orange occipital crescents, as typical for *N. n. scutata*, and six of them were from the immediate eastern border regions. According to our results, the territory of Poland represents a single major contact and hybrid

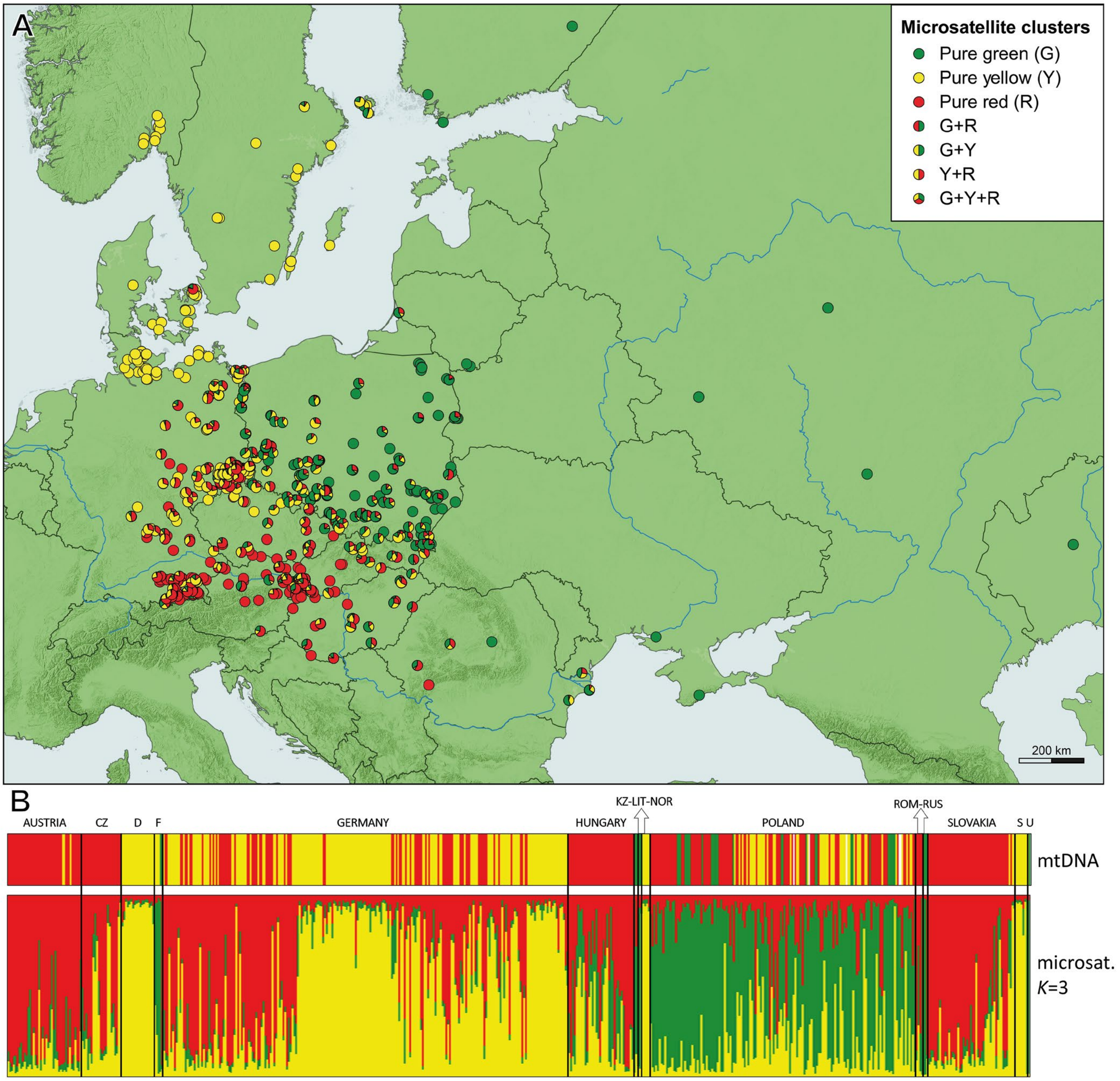

FIGURE 5 | Genotypic structuring of *Natrix natrix* in the study region ($n = 960$). (A) Geographic distribution of pure and admixed genotypes of *N. n. natrix* (yellow cluster), *N. n. vulgaris* (red cluster), and *N. n. scutata* (green cluster). (B) Mitochondrial identity (top) and genotypic identity (bottom) of individual grass snakes. Each individual corresponds to a vertical bar with coloured segments reflecting its percentage of ancestry (cluster membership) inferred by STRUCTURE. Colour coding as in previous figures except for white (missing mtDNA data for respective samples). CZ, Czech Republic; D, Denmark; F, Finland; KZ, Kazakhstan; LIT, Lithuania; NOR, Norway; ROM, Romania; RUS, Russia; S, Sweden; U, Ukraine.

zone of the three subspecies *N. n. natrix*, *N. n. vulgaris*, and *N. n. scutata*, with signatures of wide-reaching nuclear genomic introgression. The mismatched distribution of mitochondrial haplotypes (Figure 4) and microsatellite genotypes (Figure 5) reflects philopatry of females and mainly male-mediated gene flow, as also revealed for other parts of the distribution range of *N. natrix* (Schultze et al. 2019) and the closely related species *N. helvetica* (Schultze et al. 2020). The distribution pattern in Poland is evidence for a dynamic and moving postglacial hybrid zone. The mitochondrial haplotypes reflect the older substrate of local grass snake populations which are overrun by immigrating males with a different genetic identity. This is consistent with the results of the literature survey and simulation study by Currat et al. (2008), who showed that mitochondrial introgression occurs mainly from the local taxon into the invading lineage.

According to the demographic reconstructions published by Kindler et al. (2018), the nominotypical subspecies *N. n. natrix* (yellow microsatellite cluster, yellow mtDNA lineage) survived the last glaciation somewhere in Central Europe, trapped between the Alpine glaciers and the northern ice sheet, whereas *N. n. vulgaris* (red microsatellite cluster, red mtDNA lineage) is a postglacial invader from the Balkan Peninsula that successively swamped the original populations of *N. n. natrix*. As a result of this process, the grass snake populations in southern Germany and adjacent Switzerland show mitonuclear discordance. Their nuclear genomic identity corresponds largely to *N. n. vulgaris*, combined with 'yellow' mtDNA haplotypes of *N. n. natrix* (Asztalos, Glaw, et al. 2021). Our new data for Poland confirm and complete this picture, with the addition of the unexpectedly wide-reaching impact of *N. n. scutata* (green microsatellite cluster, green mtDNA lineage). As in previous studies focusing on other European regions (Kindler et al. 2017, 2018; Schultze et al. 2019, 2020; Asztalos, Ayaz, et al. 2021; Asztalos, Glaw, et al. 2021), we did not find the few 'yellow' haplotypes (*ND4 + tRNAs*) previously described as endemic for the southern Balkan Peninsula (y15–18, y21, y23; Kindler et al. 2018), supporting that these disjunct southern Balkan records are a relict from a preglacially continuous distribution range of the yellow lineage, extending then deep into the Balkan Peninsula (Kindler et al. 2018). Based on microsatellite analyses reflecting their nuclear genomic identity (Asztalos, Ayaz, et al. 2021) and coloration traits (Fritz and Ihlow 2022), the southern Balkan populations harbouring 'yellow' haplotypes differ from *N. n. natrix* and represent the subspecies *Natrix natrix moreotica* (Bedriaga, 1882), indicating complete genetic amalgamation with neighbouring populations with distinct mitochondrial lineages.

Our results suggest that two massive postglacial range expansions, one from the southeast (*N. n. vulgaris*) and another from the east (*N. n. scutata*), encroached into the Central European range of *N. n. natrix*, and all three lineages met in Poland (Figure 6). The relatively wider presence of 'red' mitochondrial haplotypes (Figure 4) compared to 'green' ones implies that *N. n. vulgaris* arrived earlier than *N. n. scutata*, with the males in both cases leading the immigration front due to their larger home ranges (see Schultze et al. 2020). Consequently, the nuclear genomic signatures of the postglacial invaders reach much deeper into the former range of *N. n. natrix* (Figure 5) than the respective mitochondrial lineages. This is also in line with the observation that the green mitochondrial lineage is represented by only a few haplotypes in Poland (Figure 4; see also Simonov et al. 2024 for adjacent countries in the east with a similar low haplotype diversity). This suggests that the females of *N. n. scutata* that left their mitochondrial footprints represent the forefront of the slower female immigration wave, reflecting a typical founder effect (see Waters et al. 2013). With respect to the immigration of *N. n. vulgaris* it remains unclear whether this subspecies expanded its range from the Balkan refugium only across the Carpathian Basin, among others using the Moravian Gate and low mountain passes, or whether it additionally circumvented the mountains in the east (Figure 6). The few samples from east of the Carpathians (Danube Delta) yielded red mitochondrial haplotypes combined with a strong genotypic impact of *N. n. scutata* (Figures 4 and 5), allowing no firm conclusion.

In the face of the described phylogeographic pattern, one is inclined to ask why the nominotypical subspecies did not expand its range in Central Europe eastward and is instead genetically swamped by the other two subspecies. However, *N. n. natrix* expanded its range in the Holocene—but to the north (Figure 6). The current Scandinavian and northern Central European populations occur in regions that were covered by the northern ice sheet during the last glaciation and, thus, must have been colonised in the Holocene after the retreat of the glacier, which started in the Late Weichselian only 15,000 years ago (see reconstructions in Siegert and Dowdeswell 2004). In contrast to

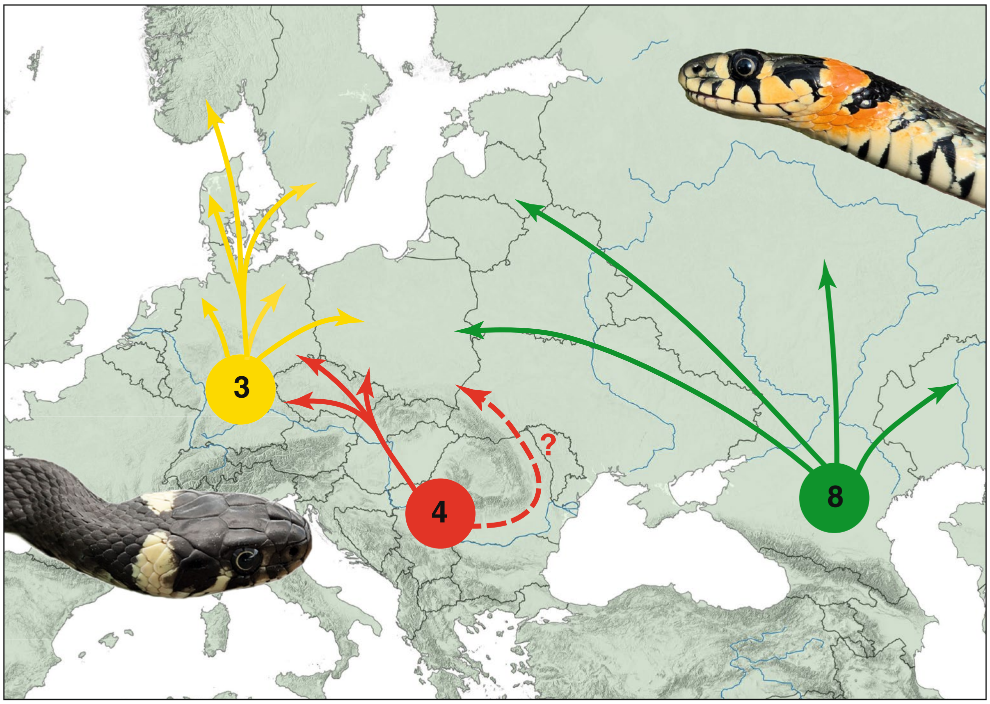

FIGURE 6 | Hypothetical postglacial dispersal routes of common grass snake subspecies as reflected by their mitochondrial lineages (*Natrix natrix natrix*: Yellow lineage = 3, *N. n. vulgaris*: Red lineage = 4, *N. n. scutata*: Green lineage = 8). The location of the putative glacial refuges (coloured circles) is approximate. Insets show typical representatives of *N. n. natrix* (Öland, Sweden; left photo: H. Bringsøe) and *N. n. scutata* (Haidary, Kharkiv Oblast, Ukraine; photo: O. Zinenko).

the other two subspecies, *N. n. natrix* seems to behave less expansively with respect to the more southern regions of Central Europe. One attractive hypothesis explaining this could be that *N. n. natrix*, as a glacial survivor in Central Europe, is better adapted to the temperate northern environments and expanded therefore northward, while its populations remained stationary in more southern regions, which increasingly warmed during the Holocene. This preference for a temperate climate could also have contributed to the success of the immigrating subspecies, which are better adapted to the changing climatic conditions in southern Central Europe.

Even though the vast majority of our samples match the described scenario, there are a few records of grass snakes with conflicting genetic identity. This concerns in Poland two records of the red mitochondrial lineage at the Baltic coast, other isolated northern and western records of the red lineage in Germany (Kindler et al. 2017, see also our Figure 4), or the record of a grass snake with a microsatellite genotype corresponding mainly to *N. n. vulgaris* on the Danish island of Zealand (Figure 5). Also, the two records of the green mitochondrial lineage in southwestern Poland could be mentioned here (Figure 4). However, there are many cases of intentional and unintentional translocations of grass snakes, including cases of long-distance transport (France: Asztalos et al. 2020; Germany and Great Britain: Kindler et al. 2017; Ahnelt et al. 2021; Italy: Schultze et al. 2020; Netherlands: van Riemsdijk et al. 2020; Asztalos, Wielstra, et al. 2021; Switzerland: Dubey et al. 2017; Schild et al. 2024). For instance, combining genetic evidence and local knowledge, Ahnelt et al. (2021) inferred that some of the founders of the thriving population of non-native grass snakes on Sylt (northern Germany) have been transported to the island from the Hungarian lowlands with reed for thatching houses. We believe that the few grass snakes with mismatched genetic identity in our dataset most likely represent such cases of translocated non-native grass snakes.

5 | Conclusions

Our study revealed the unexpected finding that the entire territory of Poland represents a contact and hybrid zone of three subspecies of grass snake, namely *N. n. natrix*, *N. n. scutata*, and *N. n. vulgaris*. The presence of mitonuclear discordance in most of the area provides evidence for a moving hybrid zone, with genetic signatures of *N. n. scutata* and *N. n. vulgaris* expanding from the east and southeast westwards into the distribution range of the nominotypical subspecies. In this contact zone, the admixture process is spearheaded by male-mediated gene flow, reflecting the larger home range of male grass snakes, and successive immigration of the less vagile females. Our investigation supports that *N. n. natrix* was a glacial survivor in Central Europe. This taxon seems to be adapted to a temperate climate, and its genetic identity is being dissolved by a westward moving hybrid zone with the immigrating eastern and southeastern subspecies *N. n. scutata* and *N. n. vulgaris*. It remains unclear whether *N. n. vulgaris* reached Central Europe in the Holocene only using a western route or whether a second wave circumvented the Carpathians in the east. Further sampling from eastern Romania, Moldova, and western Ukraine is needed to clarify this.

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Data Availability Statement

The data that support the findings of this study are openly available in ENA at https://www.ebi.ac.uk/ena/browser/home.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** zsc70034-sup-0001-DataS1.zip. Material studied and previously published mtDNA sequences and genotypes of *Natrix natrix*. STRUCTURE cluster membership is colour-coded as in Figure 5. Grey corresponds to admixed individuals. Partial haplotypes bear asterisks; haplotypes in blue are new. **Table S2:**

Primers used for amplification and sequencing of mitochondrial genes (*cyt b*, *ND4+tRNAs*), primer combinations, and thermocycling conditions. When necessary, internal primers (Fi, Ri) were used to amplify and sequence partial fragments. **Table S3:** Microsatellite loci arranged in multiplex set used in this study. Forward primers are fluorescent labelled. Allele size range (*ASR*) and number of alleles (*nA*) correspond to the global dataset of *Natrix* as well as the new generated data for Poland (P). **Table S4:** Haplotypes of lineages 3, 4, and 8 of *Natrix natrix* for *cyt b* and *ND4+tRNAs* with their European Nucleotide Archive (ENA)/GenBank accession numbers. **Figure S1:** Neighbor-Joining tree using all cyt b sequences of *Natrix natrix* from Poland, rooted with *N. tessellata*. Numbers along nodes indicate branch support, terminal numbers are sample codes (see Table S1). Support values are only shown for deeper nodes. Some weakly supported or divergent terminals within the three clades are short low-quality sequences. For previously published sequences, the GenBank/ENA accession numbers are also given. Figure can be enlarged for viewing numbers. **Figure S2:** Neighbor-Joining tree using all *ND4+tRNAs* sequences of *Natrix natrix* from Poland, rooted with *N. tessellata*. Numbers along nodes indicate branch support, terminal numbers are sample codes (see Table S1). Support values are only shown for deeper nodes. Some weakly supported or divergent terminals within the three clades are short lowquality sequences. For previously published sequences, the GenBank/ENA accession numbers are also given. Figure can be enlarged for viewing numbers. **Figure S3:** Values for ΔK for STRUCTURE analyses. Maximum value (3) highlighted.