A spotlight on the hybrid zone of grass snakes (*Natrix helvetica sicula* and *Natrix natrix*) in southern Bavaria – the Prien Valley

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Abstract. Based on dense sampling, we examined hybridization between *Natrix helvetica sicula* and *N. natrix* in a 24-km-long transect in the Prien Valley (southern Bavaria) using phenotypic data (six coloration and pattern traits), mtDNA sequences, and 13 polymorphic microsatellite loci. All three lines of evidence yielded concordant results. Genotypically pure or nearly pure *N. h. sicula* are restricted to the uppermost part of the valley in the mountains, whereas genotypically pure or nearly pure *N. natrix* occur downstream in the lowlands. The turnover between the two species takes place in a narrow stretch of only 4 km width in the central Prien Valley, where the flanking mountains spread out and the valley widens to the adjacent northern lowlands. We hypothesize that this extremely narrow hybrid zone is stabilized by different environmental preferences and selection against hybrids, acting in concert with competition and high-density blocking. Our study is the first investigation examining hybridization of the two grass snake species at a small spatial scale. The revealed transition zone is much narrower than previously modelled hybrid zones using cline analyses. This suggests that cline analyses are sensitive to sampling density. Melanistic grass snakes were restricted to the highest studied elevations, suggesting a correlation of elevation and pigmentation. Due to its linear structure, the Prien Valley offers a model area for future studies to examine the effect of environmental and climate change on a currently stable and narrow hybrid zone.

Key words. Squamata, Serpentes, Colubridae, Natricidae, Bavaria, Germany, hybridization.

Introduction

Until recently, grass snakes were thought to represent a single widespread species (*Natrix natrix* sensu lato) with numerous subspecies ranging from Northwest Africa through most of Europe to the Lake Baikal region in Central Asia (Kabisch 1999). However, recent studies using genetic methods led to the recognition of three distinct species of grass snakes (Pokrant et al. 2016, Kindler et al. 2017, Speybroeck et al. 2020): the red-eyed grass snake *Natrix astreptophora* (Seoane, 1884) from Northwest Africa, the Iberian Peninsula and adjacent France, the barred grass snake *Natrix helvetica* (Lacepède, 1789) from Great Britain, France, the Benelux countries, western Germany, Switzerland, Austria, the Italian Peninsula, Corsica, Sardinia, and Sicily, and the common grass snake *N. natrix* (Linnaeus, 1758) ranging from Europe east of the Rhine Region to Central Asia.

In Bavaria, grass snakes occur in all major river valleys and pond-rich areas. Also the foothills of the Bavarian Alps, offering suitable habitats with its lakeshores and bogs,

are densely populated (Hansbauer & Völkl 2019). Using genetic approaches, recent studies revealed the occurrence of both *N. helvetica sicula* (Cuvier, 1829) and *N. natrix* in Bavaria (Glaw et al. 2019, Asztalos et al. 2021a). These studies used two genetic marker systems that were found to be highly informative for the distinction of the different species and genetic lineages of grass snakes (Kindler et al. 2013, 2014, 2017, 2018a, Pokrant et al. 2016, Kindler & Fritz 2018, Schultze et al. 2019, 2020, Asztalos et al. 2021a): maternally inherited mitochondrial genes and biparentally inherited nuclear-genomic microsatellite loci. These independent markers systems allow, when used in combination, not only conclusions about taxonomic identity and phylogeography, but also about hybridization and gene flow.

The genetic investigations by Glaw et al. (2019) and Asztalos et al. (2021a) provided evidence that three mitochondrial lineages occur near the Inn River at the Bavarian–Tyrolean border in close proximity and local syntopy. Following the terminology of Kindler et al. (2017), these lineages are the so-called yellow and red lineages of

N. natrix (= lineages 3 and 4, respectively) and lineage C of *N. helvetica*. The latter lineage is characteristic for the subspecies *N. h. sicula* from northern Italy, suggestive of Holocene trans-Alpine dispersal (Glaw et al. 2019, Asztalos et al. 2021a). These genetic findings show that – in addition to the already known postglacial recolonization routes from southwestern Europe, southeastern Europe, and northern refugial areas (Kindler et al. 2018b) – a recolonization with grass snakes also took place across the Alps (Glaw et al. 2019, Asztalos et al. 2021a). A proposed trans-Alpine route runs from glacial refuges in northern Italy over the Brenner or Reschen Pass through the Inn Valley to southern Bavaria (Hellmich 1956, Glaw et al. 2019, Schmidtler 2019, Asztalos et al. 2021a).

Microsatellite and mitochondrial DNA (mtDNA) analyses unveiled for Sachrang and Lenggries (Bavaria) and adjacent Tyrol the presence of pure representatives of *N. h. sicula* together with hybrids, harboring haplotypes of all three mitochondrial lineages. Along the Inn River, in the District of Rosenheim (Landkreis Rosenheim, southern Bavaria), *N. h. sicula* is abruptly replaced by genetically pure *N. natrix* (Asztalos et al. 2021a).

The present study aims at high-resolution characterization of a transect within the contact zone of *N. natrix* and *N. h. sicula* in southern Bavaria using phenotypic data, mtDNA sequences and microsatellite loci. The transect corresponds to the valley of the Prien River and is well-defined by geographic features. The southernmost point is south of the village of Sachrang at the Bavarian–Tyrolean border. The transect follows then the Prien River downstream until it flows into the Chiemsee (Fig. 1), from where genetically pure *N. natrix* are known (Asztalos et al. 2021a).

Materials and methods
Study area

The study area (Fig. 1, Table 1) is located at the foot of the Bavarian Alps in the District of Rosenheim (Landkreis Rosenheim). Previous studies (Glaw et al. 2019, Asztalos et al. 2021a) detected *Natrix natrix* and *N. h. sicula* in this region along the river Inn. At the Bavarian–Tyrolean border, the course of the Inn River bends to the north. In the
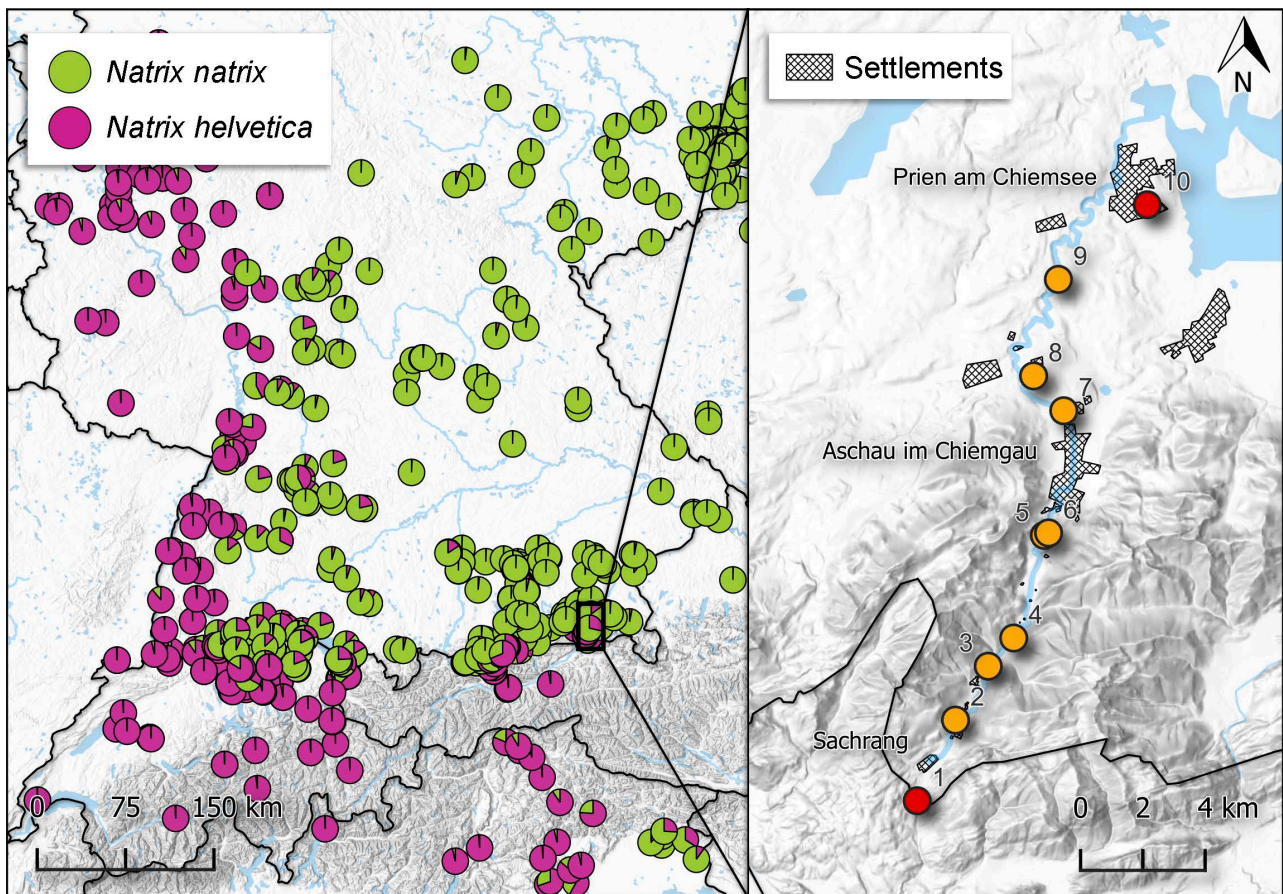

Figure 1. Left: General distribution of *Natrix helvetica* and *N. natrix* in Germany, Austria, northern Italy, Switzerland, and the Czech Republic, with pie charts corresponding to microsatellite genotypes; purple – *N. helvetica* ancestry, green – *N. natrix* ancestry. Right: Sampling sites in the Prien Valley (see Table 1 for details). The sites of 41 samples taken for this study are marked by orange dots, and those of the eight samples studied by Asztalos et al. (2021a) are indicated by red dots.

Table 1. Sampling sites in the Prien Valley. Site numbers refer to Figure 1.

Site	Altitude	Latitude	Longitude	Reference
1 Pond near Café SusAl, Sachrang	722 m	47.6792	12.2567	Asztalos et al. (2021a)
2 Barn south of Huben	742 m	47.7024	12.2731	This study
3 River bank near Grattenbach	708 m	47.7179	12.2871	This study
4 Stein 12	698 m	47.726	12.298	This study
5 Einfang	644 m	47.7555	12.3111	This study
6 Bach, fish farm	639 m	47.7562	12.3129	This study
7 Hayler bridge	593 m	47.7913	12.3194	This study
8 Barn near A8	618 m	47.8013	12.3065	This study
9 Curling rink, Vachendorf	570 m	47.829	12.3171	This study
10 Prien-Ernsdorf	557 m	47.8504	12.3551	Asztalos et al. (2021a)

extension of its previous flow direction toward the Chiemsee lies our study area in the Prien Valley.

The Prien Valley (also known as Aschauer Valley; Figs 1, S1, S2) is the westernmost transversal valley across the Chiemgau Mountains. The Prien Valley is separated in the west from the Inn Valley by the mountain chain formed by the Kranzhorn, Heuberg, Hochries, Zinnenberg, and Spitzstein Mountains. The Prien River, which has its source on the southeastern Spitzstein Mountain, gives its name to the valley (Kabisch & Kästle 2014). Geigelstein and Kampenwand segregate it from the Marquardstein Valley in the east. The Prien Valley stretches from the southern border of the Chiemgau Alps to the Chiemsee Plain. Until approximately 10,000 years ago, the valley was traversed by a branch of the Inn Glacier, the so-called Aschauer Glacier (Siebert 2000, cited in Kabisch & Kästle 2014).

Our study region reaches from the Austrian–German border near the valley's watershed south of Sachrang (721 m a.s.l., 47°40'45" N, 12°15'24" E) and along the Prien River to the Chiemsee (520 m a.s.l., 47°52'30" N, 12°21'26" E), a large freshwater lake, in which the Prien River flows. At the upstream watershed, the Prien River and the Walchental Creek (Walchentaler Bach), which flows into Inn River, are separated by only 250 m (Lenk 1901). Compared to the open areas near Aschau, to the north of our study region (Fig. 1), the upstream parts of the valley differ by higher altitudes and lower temperatures. According to a local from Huben in the Prien Valley, the vegetation period in the mountainous parts of the valley starts on average 10–14 days later than in Aschau (Vordermayer pers. comm. 2023).

One of the peculiarities of the Prien River is its extremely variable amount of water (Fig. S3). Depending on precipitation, snowmelt and dry periods, flowing water is only present for a few weeks or months per year. Often there are only remnant pools along the river as the surface water seeps away and runs underground. Fishes and other limnic species accumulate in these pools and provide an ideal source of food for grass snakes (Kabisch & Kästle 2014).

Sampling and phenotype data

Fieldwork was performed for 17 days from May to July 2022 by AN under predominantly sunny and warm conditions. Grass snakes were found by inspecting vegetation along trails and slopes of the Prien River, by looking underneath metal or wood sheets, and by checking ponds and their vicinity. Snakes were caught by hand and coordinates were recorded for each individual using a Garmin GPS-MAP 65s handheld GPS device. QGIS 3.24.1 (http://qgis.osgeo.org) was used for referencing the sampling sites in the Prien Valley to elevation data above sea level (a.s.l.). In addition to morphological data, buccal mucosa swabs were taken, and snout–vent length and tail length were measured by carefully stretching the snake on a folding ruler according to Bennett (1999). Photos of each snake in dorsal and ventral view, as well as from the head in lateral view, were taken using a smartphone (Samsung Galaxy S21 5G). These photos were used to score six species-diagnostic coloration and pattern traits (Fig. 2, Table 2) based on Kabisch (1999), Glaw et al. (2019), and Oefele (2019) using five character states for each trait (1 = typical character state of *N. helvetica*, 2 = slightly intermediate character state, but mainly *N. helvetica*, 3 = intermediate, 4 = slightly intermediate character state, but mainly *N. natrix*, 5 = typical character state of *N. natrix*). Using these values, percentages of phenotypic identity were calculated. For doing so, the mean of the values for one individual was subtracted from the highest possible score (30). Then, the resulting value was divided by the difference between the potential minimum and maximum scores, resulting in a percentage representing the species relative identity. Melanistic snakes were excluded from morphological scoring. Since the morphological traits are species-specific, we use below where appropriate "*N. helvetica*" without subspecies epithet for making communication easier. After sampling, all snakes were released at the site of their discovery. To ensure that no individual was sampled twice, the images were subsequently compared. Phenotypic identities were mapped as pie charts using QGIS 3.2.4.1 and ARCGIS Online (Esri).

Table 2. Morphological traits for differentiating the two grass snake species. For each trait a scoring system from 1 to 5 was used; melanistic individuals were not scored. Letters refer to Figure 2.

	Natrix natrix	*Natrix helvetica*
Light lunar markings (a)	Well pronounced with distinct borders	Weakly pronounced with blurred borders or entirely lacking
Color of lunar markings (a)	Mostly yellow, sometimes orange	Pale yellow or white
Dark nuchal markings (b)	Curved far into the direction of the parietal scales, often touching medially	Little to no curvature, not touching each other
Length of dark nuchal markings (b)	Narrow, not longer than lunar markings	Wide, longer than lunar markings
Occipital markings (c)	Present, dark	Absent
Supralabials (d)	Distinct dark markings, connecting at least two scales	No or narrow dark markings on scale edges

Altogether 49 grass snakes from 10 sites along a transect of 24 km length were examined (Fig. 1, Table 1, Appendix). This included data for eight individuals studied by Asztalos et al. (2021a) and newly collected material (36 buccal swabs, 4 shed skins, 1 tissue from a dead individual). In the following, samples and individuals are identified by their MTD T collection numbers (MTD T = Museum of Zoology, Senckenberg Dresden, Tissue Collection).
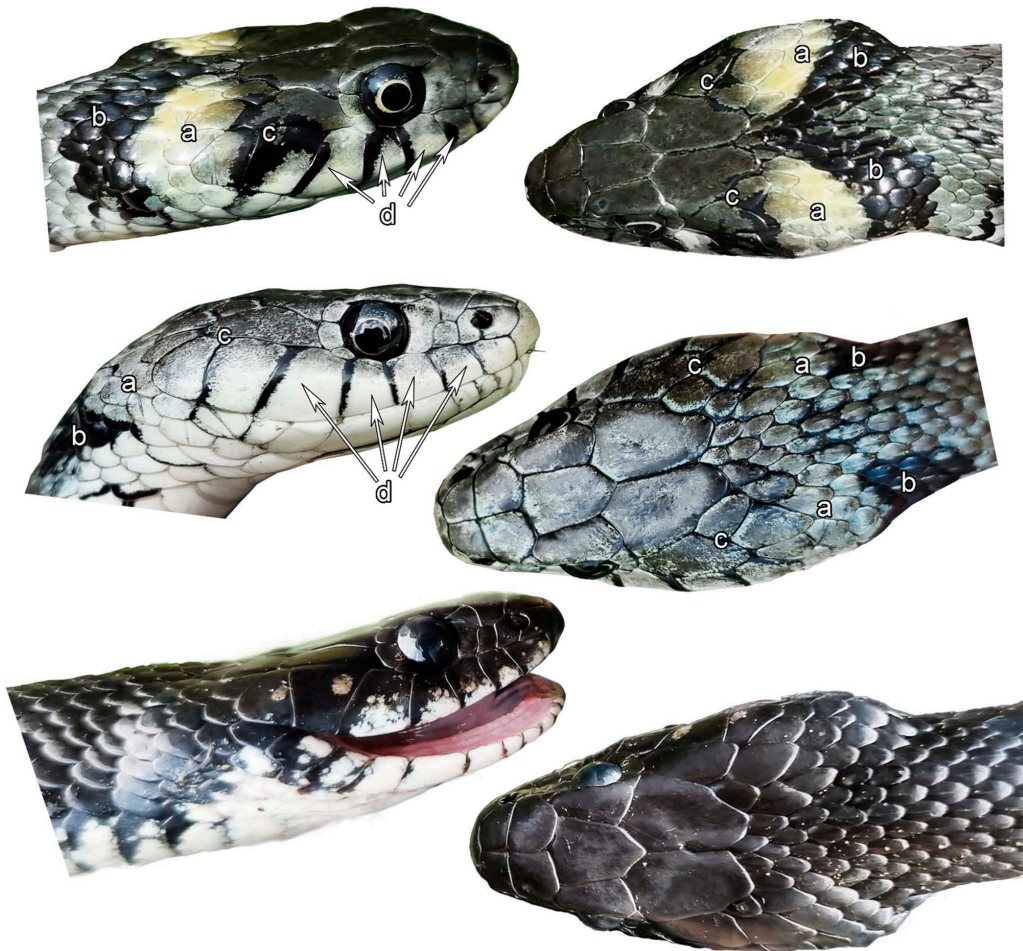

Figure 2. Head coloration and pattern. Lateral (left) and dorsal views (right) of the heads of a male *Natrix natrix* (top, MTD T 24496, snout–vent length: 79 cm, tail length: 16.5 cm), a male *N. helvetica* (center, MTD T 24474, snout–vent length: 68 cm, tail length: 16 cm), and a melanistic *N. helvetica* (bottom, MTD T 24491 snout–vent length: 83 cm, tail length: 16 cm). Lunar markings (a), nuchal markings (b), occipital markings (c), and supralabials (d) are highlighted.

Molecular genetics

Laboratory work was carried out in the molecular laboratory of Senckenberg Dresden. Two genomic marker systems, used in previous publications on grass snakes (Kindler et al. 2013, 2014, 2017, 2018a, Pokrant et al. 2016, Kindler & Fritz 2018, Schultze et al. 2019, 2020, Asztalos et al. 2021a) were applied: mtDNA sequences and length polymorphisms of 13 polymorphic microsatellite loci. As mitochondrial marker, the cytochrome *b* (cyt *b*) gene was used.

Laboratory procedures followed established work flows as detailed in Kindler et al. (2013, 2017) and Asztalos et al. (2021a). Cyt *b* sequences were edited using BIOEDIT 7.05.2 (Hall 1999) and PHYDE 0.9971 (Müller et al. 2010). To identify these sequences with previously characterized mitochondrial haplotypes, the new sequences were aligned in BIOEDIT with one representative of each known haplotype of lineages C and E of *N. helvetica* and lineages 3 and 4 of *N. natrix* (Kindler et al. 2013, 2014, 2017, 2018a, Kindler & Fritz 2018, Schultze et al. 2019, 2020, Asztalos et al. 2021a), resulting in an alignment of 1117 bp length that included 210 cyt *b* sequences. Then, a parsimony network was drawn using TCS 1.23 (Clement et al. 2000) and an arbitrary connection limit of 100 mutation steps. TCSBU (Múrias dos Santos et al. 2016) was used to visualize the haplotype network for the alignment of 1117-bp-long cyt *b* sequences. For nine samples, the cyt *b* gene could not be sequenced completely, so that three shorter alignments of 1063 bp, 724 bp, and 541 bp length were created. Haplotype networks for these alignments allowed the identification of the mitochondrial lineage for the new samples, but not of the individual haplotype.

Microsatellite data for 1200 *N. natrix* and *N. helvetica* from northern Italy, Switzerland, Austria, Germany, and the Czech Republic from previous studies (Kindler et al. 2013, 2014, 2017, 2018a, Kindler & Fritz 2018, Schultze et al. 2019, 2020, Asztalos et al. 2021a) were combined with the data of the 41 samples from the Prien Valley. This dataset included genotypes of *N. h. helvetica* and *N. h. sicula*. Since Asztalos et al. (2021a) detected for some loci in this data set null alleles, unsupervised Bayesian cluster analyses were ran using STRUCTURE 2.3.4 (Pritchard et al. 2000, Falush et al. 2003) that account for the presence of null alleles. Further settings for the STRUCTURE runs were the same as in Asztalos et al. (2021a), i.e., all calculations were repeated 10 times for each K ranging from 1 to 10 using a Monte Carlo Markov chain of 1,000,000 generations with a burn-in of 250,000. For each K the run with the highest Ln probability was selected and the same optimal number of K as in Asztalos et al. (2021a) was assumed, i.e., K = 2, with one cluster corresponding to *N. helvetica* and the other to *N. natrix*. However, only calculations using the admixture model and correlated allele frequencies were run. Based on the genotypes of 20 pure representatives of each species from our data set, Asztalos et al. (2021a), calculated thresholds for species identities using HYBRIDLAB 1.0 (Nielsen et al. 2006) resulting in 98% cluster assignment for pure *N. natrix* and 96% cluster as-signment for pure *N. helvetica*. These thresholds were also applied for the present study. STRUCTURE results were visualized using the R package POPHELPER 2.3.1 (Francis 2017) and mapped using QGIS 3.24.1.

Results
Phenotype

Out of 37 snakes studied morphologically, 10 were melanistic (Figs 2 and 3, Appendix). In the southern Prien Valley, traits of *N. helvetica* prevail, while in the north those of *N. natrix* are much more frequent. Most intermediate individuals are from the central part of the valley. Melanistic grass snakes were quite frequent in the south. Near Huben and Grattenbach (sites 2 and 3, Fig. 1) 62.5% of the grass snakes were melanistic (10 out of 16 grass snakes with known phenotype).

Molecular genetics

None of our sequences corresponded to a previously unknown haplotype and the parsimony network for complete cyt *b* sequences was identical to the one presented in Asztalos et al. (2021a). Three distinct mtDNA lineages were identified in the study area, lineage C, characteristic for *N. h. sicula* (Schultze et al. 2020), and the so-called yellow and red lineages of *N. natrix* (lineages 3 and 4; Kindler et al. 2017). Twenty-one individuals harbored haplotype c13 of lineage C, which is the only haplotype recorded before in Bavarian *N. h. sicula* (Asztalos et al. 2021a). Another five individuals represented by shorter cyt *b* sequences also belonged to lineage C. Twenty further individuals corresponded to the red lineage of *N. natrix*, and among them, the 13 samples with complete cyt *b* sequences had haplotype r10. Haplotypes c13 and r10 differ by 65 mutational steps. One record from Asztalos et al. (2021a) from the uppermost Prien Valley represents an undetermined haplotype of the yellow lineage of *N. natrix* (Appendix).

Lineage C clearly prevails in the south of the Prien Valley (Fig. 4), even though there is also one record each of the yellow and red lineages (Asztalos et al. 2021a, Appendix). Both lineage C and lineage 4 occur just south of Aschau (from Stein to Einfang, sites 4 and 5 in Fig. 1), i.e., in the central course of the river where the bordering mountains open to the adjacent northern lowlands (Fig. 1). In the northern part of the river, downstream from Aschau, only grass snakes with mtDNA of the red lineage were found.

Unsupervised STRUCTURE analyses yielded results (Fig. 5) that largely agreed with the morphological assessment of the phenotypic identity of the studied grass snakes (Fig. 3, Appendix) and the distribution of mitochondrial haplotypes (Fig. 4). Seventeen pure individuals of *N. helvetica* and three pure individuals of *N. natrix* were identified; 28 snakes were found to be admixed (Appendix). Pure or largely pure *N. helvetica* were confined to the southern part of the Prien Valley. In the central valley,

the genetic impact of *N. natrix* increases abruptly and even though many hybrid individuals were identified, most of them are genotypically close to pure *N. natrix*.

Spatial and altitudinal species distribution

Along the 24-km-long study transect, both pure representatives of *N. h. sicula* and *N. natrix* have been recorded along with their hybrids. In the central course of the Prien River, a sharp break with respect to morphology, mitochondrial and genotypic identity occurs between the two species. In the upper part of the valley, embedded between mountain chains, *N. h. sicula* and hybrids with little impact from *N. natrix* prevail. South of Aschau, in the central part of the river course where the mountains open to the lowlands, the genetic identity abruptly changes within a narrow hybrid zone. Further north, close to the Chiemsee, occur pure *N. natrix* (Figs 3–5). This situation is also reflected by a different altitudinal distribution (Fig. 6), with *N. h. sicula* being dominant at higher elevations and *N. natrix* at lower elevations. *Natrix helvetica* cluster membership positively correlates with elevation (r = 0.84***, p = 4.756 × 10^{-14}).
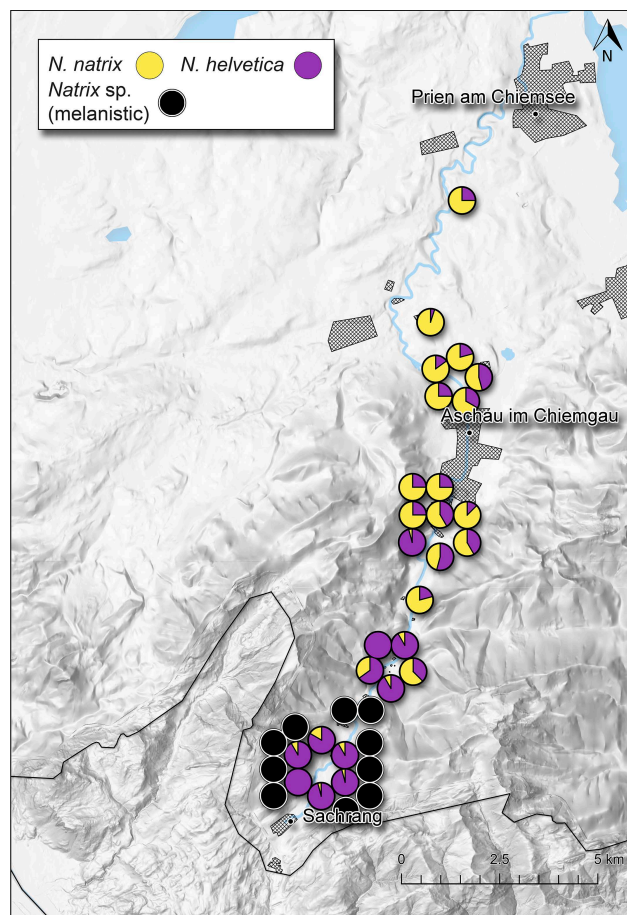

Figure 3. Phenotypes of 37 grass snakes from the Prien Valley.

Discussion

This study is the first investigation examining hybridization of *N. h. sicula* and *N. natrix* at a small spatial scale. Our study region lies within the recently identified contact zone of the two species in southernmost Bavaria, which was previously only studied at a larger geographic scale (Glaw et al. 2019, Asztalos et al. 2021a). Thus, no information about the exact spatial shift from one species to the other was available before.

We used three different lines of evidence to determine the identity of the studied grass snakes: phenotype, mtDNA sequences and microsatellite loci. Since mtDNA is inherited only in the maternal line (Ladoukakis & Zouros 2017), mismatches with the biparentally inherited microsatellite loci are expected. However, we found no cases of mito-nuclear discordance in our genotypically pure representatives of the two species. Among the hybrids were individuals harboring mitochondrial haplotypes of both *N. h. sicula* and *N. natrix*, providing evidence for gene flow in both directions, in accordance with a previous study (Asztalos et al. 2021a). The record of two hybrid grass snakes harboring haplotypes of the red and the yellow mtDNA lineages in the uppermost Prien Valley (Asztalos et al. 2021a) is remarkable as all other grass snakes there yielded mtDNA lineage C and were genotypically pure *N. helvetica* or showed only little genetic impact of *N. natrix*. In particular, the single record of a haplotype of the yellow lineage is unexpected, even though this lineage is known from elsewhere in southern Bavaria (Asztalos et al. 2021a). We can only speculate about this finding, but one possibility is active or passive translocation of allochthonous snakes to this artificial reservoir, which was constructed several decades ago. It is known that grass snakes are accidentally transported with building material (e.g., Ahnelt et al. 2021), and this could be responsible for the unexpected records in the south of our study region.

Using six coloration and pattern traits, we determined the phenotypic identities of the studied grass snakes. Although some of the phenotypic identities differ from the species identity according to microsatellite genotypes, scoring of morphological traits identified the *Natrix* species and the hybrids in most cases accurately (Appendix). This is in line with another recent publication using coloration and pattern of a large data set of grass snakes from iNaturalist photos for comparison with independent molecular data sets (Fritz et al. 2023).

Our present study confirms that pure *N. natrix* and *N. helvetica* occur and hybridize in the Prien Valley along our 24-km-long transect. At the southernmost study sites, in the uppermost Prien Valley, genotypically pure or largely pure *N. helvetica* were recorded, and a sharp transition to predominantly *N. natrix* genotypes occurs in the central valley south of Aschau, where the flanking mountains spread out and the valley widens to the adjacent northern lowlands (Fig. 5), i.e., between Stein (site 4) and Bach (site 6, Fig. 1, Table 1), matching a distance of approximately 4 km. This break is also reflected by the concordant change

of the phenotypic identities of the grass snakes (Fig. 3) and the distribution of mtDNA lineages (Fig. 4). This narrow hybrid zone is remarkable since *N. helvetica* individuals are most likely regularly carried downstream during major flooding events and grass snakes (especially males) are known to have large home ranges and to migrate over long distances (MADSEN 1984, WISLER et al. 2008, READING & JOFRÉ 2009).

Hybrid zones between *N. helvetica* and *N. natrix* have been examined by a number of previous studies (KINDLER et al. 2017, SCHULTZE et al. 2019, 2020, ASZTALOS et al. 2021a, FRITZ et al. 2023), but at a larger geographical scale and using less dense sampling. Most of these studies used cline analyses for inferring the width of the hybrid zones. For Lake Constance (KINDLER et al. 2017), the Upper Rhine Valley (SCHULTZE et al. 2019), the Rhein Main Region (FRITZ et al. 2023), and northeastern Italy (SCHULTZE et al. 2020), hybrid zones from less than 50 km to 120 km width were inferred. Using the same genetic markers as in the present study, FRITZ et al. (2023) calculated for the Al-

pine hybrid zone a width of up to 86 km. Based on phenotype data from iNaturalist photos, however, these authors estimated the width at only approximately 40 km. This discrepancy could be related to the denser coverage of their study region by photos compared to the genetic sampling. This notion is supported by the even narrower hybrid zone in the Alpine Prien Valley, which is covered by an unprecedentedly dense sampling, and argues for caution with cline analyses that seem to be susceptible to sampling density.

The present occurrence of *N. h. sicula* in southern Bavaria is understood as the result of a Holocene range expansion from the south (GLAW et al. 2019, ASZTALOS et al. 2021a). After the end of the Würm glaciation 12,000
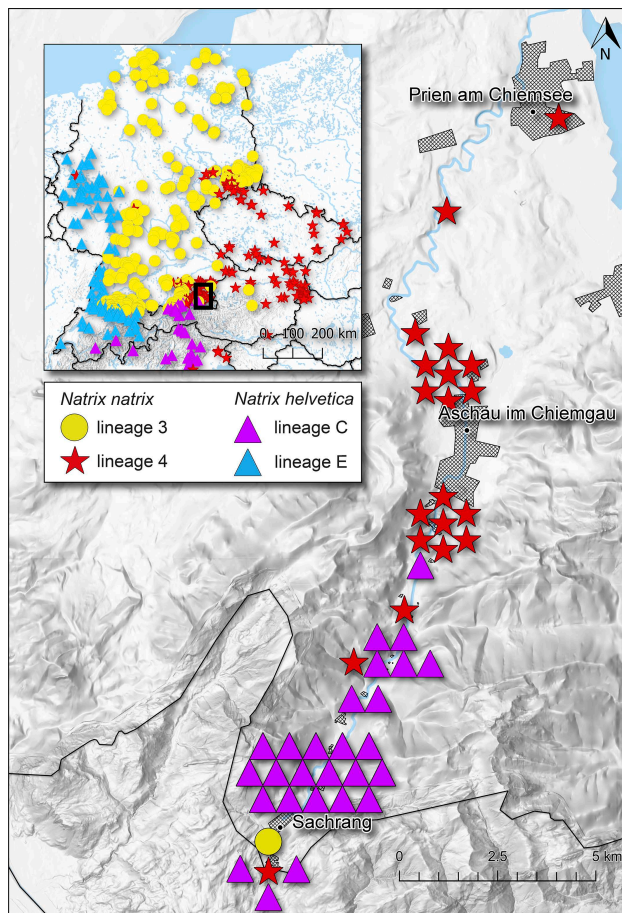

Figure 4. Distribution of mitochondrial haplotypes of 47 grass snakes in the Prien Valley. The inset (top left) shows the distribution of the haplotypes of 1241 grass snakes from Germany, Austria, northern Italy, Switzerland, and the Czech Republic. The black rectangle corresponds to the main map.
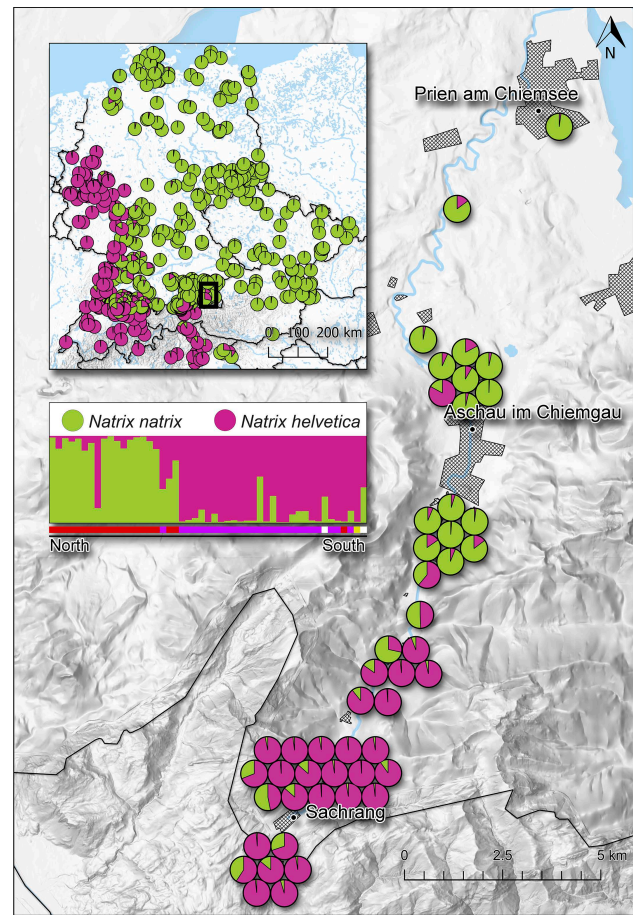

Figure 5. Distribution of microsatellite genotypes (STRUCTURE cluster memberships) of 49 grass snakes in the Prien Valley. The upper inset shows the distribution of the microsatellite genotypes of 1241 grass snakes from Germany, Austria, northern Italy, Switzerland, and the Czech Republic. The black rectangle corresponds to the main map. The lower inset shows STRUCTURE barplots (top) and mitochondrial identity (bottom) of the 49 Prien Valley snakes, with samples sorted from north to south. Barplot colors for microsatellite genotypes correspond to those in the map: purple – *Natrix helvetica* ancestry, green – *N. natrix* ancestry. Barplot colors for mitochondrial identity of *N. helvetica*: dark purple – lineage C, *N. natrix*: yellow – yellow lineage (lineage 3), red – red lineage (lineage 4), white – unknown.

years ago, most amphibian and reptile species were extinct in Central Europe and recolonized what is now Germany from their glacial refugia, which were typically in the south (Schmidtler 2019). However, for the yellow lineage of *N. natrix*, glacial refuges have been inferred both for the Balkan Peninsula and the Central European region between the Alpine glaciers and the northern ice sheet, whereas the red lineage of *N. natrix* is regarded as a very recent Holocene colonizer from the Balkan Peninsula. A model using an Approximate Bayesian Computation approach inferred that the red lineage only invaded Central Europe 3000 years before present (Kindler et al. 2018b), suggesting a rapid and highly dynamic admixture process
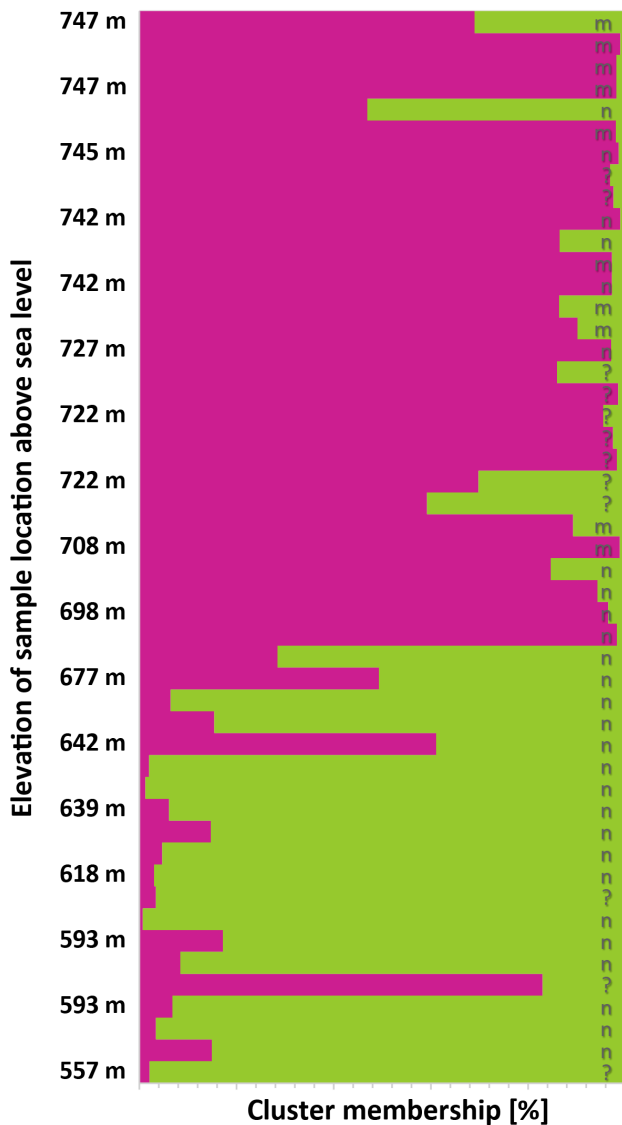

Figure 6. Barplot showing the ancestry of the 49 grass snakes from the Prien Valley sorted by altitude (Y-axis not metrical, height indicated for every third individual). Purple: *Natrix helvetica* ancestry, green: *N. natrix* ancestry. Phenotypes are indicated by letters (m: melanistic, n: normal, ?: unknown).

– in sharp contrast to obviously quite stable postglacial hybrid zones of *N. helvetica* and *N. natrix*.

It has been suggested that *N. h. sicula* used the Brenner and/or the Reschen Passes for its northward range expansion from a refuge in northern Italy (Glaw et al. 2019, Schmidtler 2019, Asztalos et al. 2021a). After the Holocene retreat of the glaciers at the end of the last glaciation (Seguinot et al. 2018), it became possible for *N. natrix* to colonize the northern Alps along the Inn River and its side valleys. Most likely, the original population of common grass snakes corresponded then to the nominotypical subspecies *N. n. natrix* (genotypically pure representatives of the yellow nuclear-genomic cluster, harboring mitochondrial haplotypes of the yellow lineage; see Kindler et al. 2017, Fritz & Schmidtler 2020, Asztalos et al. 2021a, b). These grass snakes became later genetically swamped by the immigration of *N. n. vulgaris* (red lineage) from the southeast, but with increasing distance to the immigration front in South Germany, the purity of *N. n. natrix* populations increases (compare the maps in Asztalos et al. 2021a, b), providing evidence for a northwestward moving hybridization wave leading to the amalgamation of the two subspecies. The exclusive presence of mtDNA haplotypes of the yellow lineage in Baden-Württemberg combined with nuclear genotypes of *N. n. vulgaris* provides evidence that gene flow is first mainly mediated by males, resulting in the survival of the mitochondrial signatures of the original residential population (yellow lineage, *N. n. natrix*). In Bavaria, both mtDNA haplotypes of the yellow and the red lineages have been recorded, suggestive of a continued northwestward expansion of *N. n. vulgaris* (including females).

The retreating Alpine glaciers also opened migration routes for *N. h. sicula* from their refuge in northern Italy, resulting in the establishment of a contact zone with southward expanding *N. natrix*. As it is unlikely that two species have the same fitness when they meet up in their secondary contact zone (Arntzen et al. 2017), it can be speculated that *N. h. sicula* is better adapted to the Alpine climate and *N. natrix* to lowland conditions. This may have contributed to a stabilization of their hybrid zone, despite continued temperature changes in the Holocene (Schönwiese 1995, Ilyashuk et al. 2011), in contrast to the swamping hybridization wave of *N. n. vulgaris* running across the range of *N. n. natrix*. Further factors that could contribute to the stabilization of the hybrid zone of *N. h. sicula* and *N. natrix* are selection against hybrids and high-density blocking combined with competitive exclusion (Waters et al. 2013), i.e., that the high density of *N. natrix* in the pre-Alpine lowlands prevents the further spread of *N. h. sicula* out of the Alpine valleys due to their ecological similarity and vice versa.

The presence of melanistic grass snakes south of Huben (site 2, Fig. 1) matches observations by Kabisch & Kästle (2014), who also recorded both normal and melanistic snakes there. In their study, Huben was the lowest site where melanistic grass snakes were observed. In the present study, melanistic snakes were even encountered lower,

near Grattenbach (site 3, Fig. 1). However, beyond Grattenbach no melanistic grass snakes could be found at lower altitudes (Fig. 6). Our observations are in line with those of Glaw et al. (2019). They reported many melanistic individuals near Sachrang, which is the southernmost and highest site of our studied transect. All of these observations suggest a correlation of elevation and pigmentation. Melanistic grass snakes could be favored by lower environmental temperatures (Andrén & Nilson 1981, Clusella Trullas et al. 2007, Hodges 2018, Bruni et al. 2022, Fănaru at al. 2022). This is supported by the observation that in *N. natrix* the frequency of dark body coloration, including melanistic snakes, increases from south to north and from west to east (Fritz & Ihlow 2022), i.e., from milder and more oceanic climates to colder and more continental climates.

Kabisch & Kästle (2014) reported repeated sightings of melanistic grass snakes at the Alpine pasture border at the Zinnenberg, which is the highest known site for grass snakes in Germany (approx. 1500 m a.s.l.). Based on the distribution of the two species in the Prien Valley, it seems likely that these records refer to barred grass snakes. Unfortunately, during three visits to the Zinnenberg, we found no grass snakes, but several melanistic European adders (*Vipera berus*).

Our study revealed an extremely narrow transition between *N. h. sicula* and *N. natrix*, suggesting that the hybrid zone is stabilized by different environmental preferences and selection acting in concert with competition and high-density blocking. This massively contrasts with the patterns described for the conspecific *N. natrix* lineages (Kindler et al. 2017, Asztalos et al. 2021a), the subspecies *N. n. natrix* and *N. n. vulgaris*, which amalgamate completely in a northwestward moving hybrid belt, leading to the loss of their subspecific identity in some regions (Asztalos et al. 2021a, b). This exemplifies that it has to be kept in mind that hybrid zones are expected to move (compare the recent review by Wielstra 2019), and dynamics are expected to increase with changing environment. Anthropogenic climate change and habitat alterations will lead to range expansions and contractions as well as local extinctions. Hybrid zones therefore should be sampled with change in mind, and not seen as equilibrium situations (Taylor et al. 2015). Furthermore, the current distribution patterns of *N. helvetica* and *N. natrix* could be altered by the translocation of grass snakes across hybrid zone boundaries (Schultze et al. 2019), including the introduction of alien taxa causing genetic pollution as known from the Netherlands (Asztalos et al. 2021c). Thus, our present study only shows a snapshot of the contact zone of *N. h. sicula* and *N. natrix* in the Prien Valley. However, due to the linear structure of the valley and its elevation gradient, it offers a model area for future studies on the effect of environmental change on a currently stable and narrow hybrid zone. Detailed studies along other transects across the contact zone would surely contribute additional insights, in particular regarding other geographical settings.

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Supplementary data

The following data are available online:

Supplementary Figure S1. View of the Prien Valley from Innerwald in north-northeast direction and view on Innerwald in the Prien Valley, looking from west to east.

Supplementary Figure S2. View looking north on Mount Zinnenberg and the ridge bordering the Prien Valley to the west.

Supplementary Figure S3. Course of the Prien River near Innerwald with three weeks difference.

Appendix

Studied grass snakes (*Natrix helvetica sicula*, *N. natrix*) from the Prien Valley, Bavaria. Haplotypes of partial cyt *b* sequences which could be not exactly determined bear an asterisk. ENA accession numbers for haplotypes c13 and r10 are LR991913 and LT839309, respectively. The microsatellite identity is based on the modeled thresholds of 98% cluster membership for *N. helvetica* and 96% for *N. natrix* (Asztalos et al. 2021a). Phenotype: m = melanistic.

		Microsatellite genotype			Phenotype		Coordinates			
MTD T	mtDNA haplotype	*helvetica*	*natrix*	Identity	*helvetica*	*natrix*	Latitude	Longitude	Site	Reference
20497	c13	98.3%	1.7%	*helvetica*	?	?	47.6792	12.2567	1	Asztalos et al. (2021a)
20498	c13	97.5%	2.5%	*helvetica*	?	?	47.6792	12.2567	1	Asztalos et al. (2021a)
22155	-	59.2%	40.8%	Hybrid	?	?	47.6792	12.2571	1	Asztalos et al. (2021a)
22161	r*	95.5%	4.5%	Hybrid	?	?	47.6792	12.2567	1	Asztalos et al. (2021a)
22170	c*	98.6%	1.4%	*helvetica*	?	?	47.6792	12.2567	1	Asztalos et al. (2021a)
22179	y*	86.0%	14.0%	Hybrid	?	?	47.6792	12.2567	1	Asztalos et al. (2021a)
22153	-	69.8%	30.2%	Hybrid	?	?	47.6805	12.2581	1	Asztalos et al. (2021a)
24465	c13	98.1%	1.9%	*helvetica*	m	m	47.7020	12.2724	2	This study
24477	c13	97.1%	2.9%	*helvetica*	96%	4%	47.7023	12.2745	2	This study
24469	c13	86.4%	13.6%	Hybrid	m	m	47.7024	12.2732	2	This study
24476	c*	86.5%	13.5%	Hybrid	100%	0%	47.7024	12.2731	2	This study
24466	c13	90.2%	9.8%	Hybrid	m	m	47.7025	12.2731	2	This study
24470	c13	97.6%	2.4%	*helvetica*	?	?	47.7025	12.2730	2	This study
24471	c13	96.9%	3.1%	*helvetica*	?	?	47.7025	12.2730	2	This study
24472	c13	97.3%	2.7%	*helvetica*	92%	8%	47.7025	12.2730	2	This study
24473	c*	97.3%	2.7%	*helvetica*	m	m	47.7025	12.2730	2	This study
24474	c13	98.7%	1.3%	*helvetica*	96%	4%	47.7025	12.2729	2	This study
24475	c*	46.9%	53.1%	Hybrid	83%	17%	47.7025	12.2728	2	This study
24491	c13	98.2%	1.8%	*helvetica*	m	m	47.7025	12.2728	2	This study
24492	c13	98.2%	1.8%	*helvetica*	m	m	47.7025	12.2728	2	This study
24501	c13	99.0%	1.0%	*helvetica*	m	m	47.7025	12.2729	2	This study
24502	c13	69.0%	31.0%	Hybrid	m	m	47.7025	12.2728	2	This study

MTD T	mtDNA haplotype	Microsatellite genotype			Phenotype		Coordinates		Site	Reference
		helvetica	*natrix*	Identity	*helvetica*	*natrix*	Latitude	Longitude		
24503	c13	99.0%	1.0%	*helvetica*	92%	8%	47.7025	12.2730	2	This study
24487	c13	98.9%	1.1%	*helvetica*	m	m	47.7179	12.2871	3	This study
24488	c13	89.3%	10.7%	Hybrid	m	m	47.7179	12.2870	3	This study
24467	c13	96.5%	3.5%	helvetica	92%	8%	47.7260	12.2980	4	This study
24468	c13	94.3%	5.7%	Hybrid	65%	35%	47.7260	12.2980	4	This study
24484	c*	84.7%	15.3%	Hybrid	92%	8%	47.7260	12.2980	4	This study
24500	c13	98.3%	1.7%	helvetica	100%	0%	47.7261	12.2980	4	This study
24529	r10	28.4%	71.6%	Hybrid	38%	63%	47.7262	12.2977	4	This study
24489	r10	49.3%	50.7%	Hybrid	21%	79%	47.7381	12.3028	5	This study
24494	c13	61.1%	38.9%	Hybrid	96%	4%	47.7548	12.3104	5	This study
24490	r10	15.4%	84.6%	Hybrid	25%	75%	47.7555	12.3111	5	This study
24493	r10	6.4%	93.6%	Hybrid	54%	46%	47.7555	12.3111	5	This study
24495	r10	1.2%	98.8%	*natrix*	13%	88%	47.7561	12.3131	6	This study
24496	r10	2.0%	98.0%	*natrix*	42%	58%	47.7561	12.3131	6	This study
24497	r10	4.7%	95.3%	Hybrid	25%	75%	47.7562	12.3129	6	This study
24498	r10	14.7%	85.3%	Hybrid	25%	75%	47.7562	12.3129	6	This study
24499	r10	6.1%	93.9%	Hybrid	42%	58%	47.7563	12.3130	6	This study
24478	r10	3.4%	96.6%	Hybrid	15%	85%	47.7913	12.3193	7	This study
24479	r*	6.8%	93.2%	Hybrid	33%	67%	47.7913	12.3193	7	This study
24480	r*	83.0%	17.0%	Hybrid	?	?	47.7913	12.3194	7	This study
24481	r10	8.4%	91.6%	Hybrid	25%	75%	47.7913	12.3194	7	This study
24483	r10	17.2%	82.8%	Hybrid	21%	79%	47.7913	12.3194	7	This study
24485	r*	0.7%	99.3%	*natrix*	46%	54%	47.7913	12.3193	7	This study
24486	r*	3.4%	96.6%	Hybrid	?	?	47.7913	12.3193	7	This study
24482	r*	3.0%	97.0%	Hybrid	5%	95%	47.8013	12.3065	8	This study
24504	r10	14.9%	85.1%	Hybrid	25%	75%	47.8290	12.3171	9	This study
22166	r*	2.2%	97.8%	Hybrid	?	?	47.8504	12.3551	10	Asztalos et al. (2021a)