Distribution, morphology and genetics of a newly discovered grass snake lineage (*Natrix helvetica* ssp.) in southern and western Switzerland

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Abstract

Eurasian grass snakes have recently been split into two species, which meet along the Rhine: *Natrix natrix* in Asia and East Europe and *Natrix helvetica* in Central and West Europe. Switzerland is occupied by *N. h. helvetica* (mitochondrial DNA lineage E), besides north-eastern cantons where *N. n. natrix* (mitochondrial lineage 3) is found. A different mitochondrial DNA (mtDNA) lineage, C, has been found in the cantons of Ticino and Valais but also north of the Alps in Lausanne, at the exact location where alien grass snakes (*N. n. persa*, mtDNA lineage 7) escaped from an outdoor enclosure in the 1970s. It was not known where lineage C naturally occurs, whether this includes Lausanne or if it also escaped from the outdoor enclosure. Due to contradicting results of genetic and morphological studies, it was unclear whether this lineage belongs to native *N. h. helvetica* or the Italian subspecies *N. h. lanzai*. Two mtDNA and thirteen microsatellite markers of wild and museum specimens were genotyped to unravel the distribution range and admixture between lineages C and E. Landmarks, 'shape PCA's and linear discriminant analysis were used to examine morphological differences between lineages. The 'shape PCA' is a recently developed method to examine shape (represented by principal component scores) in relation to size. Lineage C was found in Ticino and Valais and a contact zone with lineage E was discovered along Lake Geneva. Microsatellite analysis computed separate clusters in Ticino (C-TI), Valais (C-VS), the Swiss midland (E-ML) and the region around Geneva (E-GE). Admixture between clusters was high and F_{ST} values were similar between all of them. No morphological differences were found. A universal subspecies concept does not exist and many definitions would classify lineage C as a different subspecies because it has a different mtDNA lineage and microsatellite clusters. In my opinion, however, morphological differentiation should also be included in a subspecies concept. Therefore, due to the extensive gene flow in a wide contact zone, similar F_{ST} values and missing morphological differentiation, I support the assignment of lineage C to the subspecies *N. h. helvetica* and propose to include this clade in conservation measures as this subspecies is already threatened.

Introduction

"The species concept is one of the oldest and most fundamental in biology. And yet it is almost universally conceded that no satisfactory definition of what constitutes a species has ever been proposed." (Dobzhansky 1935). Taxonomy is indeed still struggling in finding the one definition of species, given the existence of numerous, often discordant species concepts (Dayrat 2005; De Queiroz 2007). The most accepted concepts are the biological species concept (BSC), phylogenetic species concept (PSC) and evolutionary species concept (ESC) (Frankham et al. 2012). While the BSC focuses on reproductive isolation (Mayr 1942), the PSC states species as terminal phylogenetic clusters, which are (genetically or morphologically) diagnosable (Cracraft 1989), and the ESC defines a species by genetic integrity and a distinct evolutionary fate (Wiley 1978). Similarly, there is no universal concept for subspecies either. Traditionally, subspecies were defined as aggregates of local populations within the range of the species depicting phenotypic variation in morphology (Mayr 1963). Its necessity has been questioned and discussed ever since (Wilson & Brown 1953; Mayr 1982; Cracraft 1983; Frost & Hillis 1990; Patten & Unitt 2002; Isaac et al. 2004; Hawlitschek et al. 2012; Van Nieukerken et al. 2016). While many authors in the field of herpetology reject the subspecies classification (Reiserer et al. 2013), ornithologists favor it for understanding evolutionary divergence and conservation purposes (Mayr

1970; Hawlitschek et al. 2012). This difference can be observed in current ratios of species to subspecies across taxa, which vary from 1:2 for mammals (Reeder et al. 2007), 1:2.2 for birds (Avibase data 2019, https://avibase.bsc-eoc.org [Lepage 2014]) and 1:0.3 for reptiles (Uetz 2010). Since the genetic revolution, authors often rely on genetics to determine subspecies. For Groves and Grubb (2011), subspecies are not absolutely [genetically] differentiated and not 100% diagnosable but they seem to represent real evolutionary interests. Hawlitschek et al. (2012) propose to recognize species when more than one line of evidence for evolutionary divergence (mtDNA, nDNA or/and morphology) is found and subspecies in case of only one line of evidence. With an integrative approach, Kindler & Fritz (2018) combine the ideas of Mayr (1942), Avise & Ball (1990), Moritz (1994), Braby et al. (2012) and Torstrom et al. (2014): "Accordingly, subspecies should represent distinct mtDNA lineages (except for cases of mitochondrial capture), and they should represent also distinct nuclear genomic clusters, i.e. they should be confirmed by two independent lines of genetic evidence. Ideally, but not necessarily, distinct subspecies should be diagnosable also morphologically. In contrast to species, […], subspecies differ from species in that they are capable of extensive gene flow, i.e. they are completely genetically compatible with other subspecies." Using such subspecies concepts, genetic analyses tend to eliminate traditional subspecies. In a genetic survey of 41 avian species, only 3% of subspecies represented distinct evolutionary entities (Zink 2004). Torstrom et al. (2014) showed that the number of morphologically determined subspecies among three groups of reptiles (Testudines, Squamata and Lacertilia) drastically reduced after genetic analysis. This supports Zink's conclusion (2004) that there are currently too many subspecies, which can lead to a lot of wasted time, effort and money when subspecies that do not represent evolutionary distinct entities, are the target of conservation measures. The same pattern of morphologically defined subspecies being contradicted by genetic analyses is apparent in grass snakes too, the target species of this study.

Grass snakes (formerly one species, *Natrix natrix*) have one of the widest distributions among terrestrial snakes in the Palaearctic region. They occur from the North African Maghreb region and the Iberian Peninsula through most of Europe and West Asia to the region of Lake Baikal in Central Asia (Bannikov et al. 1977; Kabisch 1999). Grass snakes are more or less associated with aquatic habitats because they feed mostly on amphibians (but also fish, small mammals or nestling birds; Bannikov et al. 1977; Engelmann et al. 1986; Gruber 1989; Kabisch 1999; Arnold & Ovenden 2002; Kreiner 2007). Traditionally, many subspecies among *N. natrix* have been described according to morphological features such as body proportions, colouration and size (Hecht 1930; Mertens 1947, 1957, 1966; Mertens & Wermuth 1960; Kramer 1970; Bannikov et al. 1977). However, subspecies delimitation was not consistent over time as Thorpe (1979) reduced the number of subspecies to four while other authors treated up to fourteen subspecies as valid (Nilson & Andrén 1981; Engelmann et al. 1986; Gruber 1989; Kabisch 1999; Arnold & Ovenden 2002; Kreiner 2007; Baier et al. 2009). Two of them have even been proposed as distinct species: *Natrix cetti* corresponding to *N. c. cetti* on Sardinia and *N. c. corsa* on Corsica (Vanni & Cimmaruta 2010) and *Natrix megalocephala* in western Transcaucasia (Orlov & Tuniyev 1987). However, genetic analyses were needed to validate their species status.

More recently, Kindler et al. (2013) provided a comprehensive phylogeography based on a nearly range-wide sampling of 410 grass snakes. The phylogenetic analysis using two mitochondrial DNA (mtDNA) markers revealed 16 well-supported terminal clades within three major, more inclusive clades from (i) the Iberian Peninsula and the North African Maghreb region, (ii)

West Europe including the Apennine Peninsula, Sicily and Corso-Sardinia and (iii) East Europe and Asia (S Figure 1). The terminal clades often conflicted with traditional, morphologically determined subspecies, not supporting their monophyly by the phylogenetic tree. Moreover, the distinctiveness of *N. cetti* and *N. megalocephala* could not be confirmed by the genetic analyses and hence, their subspecies status has been reinstated (Fritz et al. 2012; Kindler et al. 2013). However, the most basal terminal clade fits with the distribution of the subspecies *N. n. astreptophora,* which has recently been split off as a distinct species (*N. astreptophora*) in the face of virtually lacking gene flow with the geographically neighbouring taxon (*N. n. helvetica*) and concordant morphological and genetic evidence (Pokrant et al. 2016).

Kindler et al. (2017) increased the sampling of Kindler et al. (2013) fourfold and looked at both mtDNA and microsatellites (nuclear DNA) to investigate two European hybrid zones between (i) clade E in West Europe, matching with the distribution of what has been previously known as *N. n. helvetica*, and clade 3 in Central Europe, corresponding to the nominotypical subspecies, and (ii) clade 3 and clade 4, named as *N. n. persa,* in East Europe. They considered clade 3 and 4 as conspecific due to the lacking morphological differentiation, wide hybrid zone, large-scale gene flow, placement in the same major phylogenetic clade and a low F_{ST} value. However, they revealed *N. helvetica* (Lacepède 1789) to be a separate species considering the largely unidirectional gene flow from *N. n. helvetica* into the nominotypical lineage in a narrow contact zone, the morphological distinctness of *N. helvetica*, its placement in another deeply divergent clade than eastern grass snake lineages and the considerable age of its mitochondrial lineage. As this hybrid zone follows the Rhine valley, two species of grass snake are now found in Switzerland, the barred grass snake *N. helvetica* (Lacepède 1789) (*N. h. helvetica*, clade E in Kindler et al. [2013]) and the common grass snake *N. natrix* (Linnaeus 1758) (*N. n. natrix*, clade 3 in Kindler et al. [2013]). All subspecies belonging to the more inclusive clade of *N. helvetica* have been transferred to *N. helvetica* sspp. (Kindler et al. 2017).

During their sampling, Kindler et al. (2013) found a third lineage in Switzerland, clade C in the canton Ticino. The taxonomic status of this clade is still unclear, whether it belongs to the Swiss or one of the Italian subspecies of *N. helvetica*. According to Kindler et al. (2013), snakes from the distribution range of clade C (North Italy and adjacent Switzerland) have traditionally been classified as *N. h. helvetica*. This would mean, however, that the latter is a paraphyletic group (see phylogenetic tree of mtDNA lineages in Kindler et al. [2013]). A very recent study from Kindler & Fritz (2018) used microsatellite and PCA analyses to show that clade C cannot be differentiated from clade F and therefore strongly clusters with Italian lineages. Whether the subspecies concept favours morphological differences over genetic distinctness or vice versa defines in this case whether clade C belongs to the Swiss or Italian subspecies, even whether the Italian subspecies (*N. h. lanzai*) is valid.

Dubey et al. (2017), while surveying offspring of alien grass snakes originating from Western Croatia (*N. n. persa,* clade 7 in Kindler et al. 2013) which escaped an outdoor reptile park in Lausanne in the 1970s, found a clade C individual at the same location. This is very surprising as also Chèvre (2015) and Kindler & Fritz (2018) detected this clade only in the cantons of Valais and Ticino, and raises the question where its natural distribution ends. Dubey et al. (2017) assumed that this clade was also present in the reptile park, escaped, survived and reproduced in the region. However, it is also possible that the clade has passed the Alps and naturally occurs in Western Switzerland. Very recently, Glaw et al. (2019) report a post-glacial transalpine immigration of this clade into South-East Germany. As individuals of clade C and 7 have potentially

hybridized with local clade E, it is of utmost importance for conservation purposes to find out whether clade C is native in western Switzerland or has been introduced (through the reptile park).
The aim of the study is to fill these knowledge gaps by (i) uncovering the natural distribution of clade C in Switzerland and (ii) unravelling its taxonomic status. I took DNA samples from wild snakes and museum specimens and determined their mtDNA lineage to see where the respective clades occur. Several nuclear DNA markers (microsatellites) were used in a clustering analysis to estimate the amount of admixture between the clades. Additionally, I looked for distinctive morphological features between them using a 'shape PCA', linear discriminant analysis (LDA) and landmark analysis. Both morphological and microsatellite analyses were used to understand which subspecies clade C belongs to. This new knowledge will be of importance for the new Swiss Red List of Reptiles and conservation measures.

Materials and Methods

Study area and sampling

The study area comprised the cantons of Vaud, Valais (up to the Pfynwald between Leuk and Sierre) and Ticino as clade C has been detected in these cantons. The sampling scheme was habitat-specific, rather than random or homogenous, to catch as many snakes as possible and to get the most representative distribution. I focused on preferred habitats like ponds, rivers, lakes and previous sightings of grass snakes (database of Info Fauna CSCF & KARCH). Snakes were caught by hand, processed on site and released directly afterwards at the exact location of capture. Handling lasted between 20 to 45 minutes. DNA samples were taken with buccal swabs (dried and stored at -20°C) or scale clipping (one to four ventral scales stored in 70% alcohol). Only adult snakes were used in morphological analysis. Following Chèvre (2015) and Thorpe (1979), individuals with a total length of less than 50 cm were classified as juveniles because they show too strong allometric discrepancies. To reduce handling time and to have the possibility to retake measurements afterwards, various body parts of adult snakes were photographed. Some traits had to be measured in the field as they could not be photographed. Additionally, natural history museum collections were specifically searched for specimens originating from the study area, but individuals from the cantons of Bern and Geneva were also included. See S Table 1 for the list of samples.

DNA extraction and purification

Tissue samples (swab tips, scales, liver or muscle tissue in case of museum specimens) were incubated with ATL buffer and proteinase K (Qiagen) in a heat block for 16-20 hours at 56°C. Scales were previously placed in water for 24 hours to remove alcohol. After digestion, liquid from swab tips was extracted using a centrifuge. The DNA of the digested tissue was purified following the protocol "Purification of total DNA from Animal Tissues (Spin-Column Protocol)" (DNeasy Blood & Tissue Kit, Qiagen) using a Qiagen robot.

Mitochondrial DNA sequencing

To assign samples to clades, the cytochrome *b* gene (cyt *b*) and the NADH dehydrogenase subunit 4 gene with adjacent tRNA coding regions (ND4), both of which have been previously used in grass snake studies (Fritz et al. 2012; Kindler et al. 2013; Chèvre 2015; Kindler et al. 2017;

Glaw et al. 2019), were used. PCR mixtures contained 3 µl of extracted DNA, 2 µl (10 µM) forward and reverse primer resp. (similar to Kindler et al. 2013, 2017), 12.5 µl GoTuck (G2 hot start green mastermix, Promega corporation) and 5.5 µl nuclease-free water (see S Table 2 for primer information and PCR conditions). PCR products were sequenced at LGC Genomics GmbH (Berlin, Germany). Sequences were processed with CodonCode Aligner (https://www.codoncode.com) and compared with GenBank sequences to determine the genetic clade (e.g. C, E or other).

Microsatellite genotyping

Microsatellite markers were used to estimate the amount of admixture between the different clades and to test whether they form separate genetic clusters. The same thirteen microsatellites were used as in previous studies (Chèvre 2015; Kindler et al. 2017). Several primers were mixed together in multiplex-PCRs. Reaction volumes contained 2 µl DNA, 5 µl Type-it mastermix (Microsatellite PCR Kit, Qiagen), specific volumes of primers and enough nuclease-free water to fill up to 10 µl final volume. Forward primers were marked with a fluorescent dye. Details of reaction volumes, dyes and PCR conditions are provided in S Table 3 and S Table 4. PCR products were analysed with an AB3130xl Genetic Analyser (Applied Biosystems) at the Zoological Institute of the University of Basel. The Microsatellite Plugin in GENEIOUS PRIME 2020.0.3 (https://www.geneious.com) was used to visualize peaks and determine allele lengths.

Microsatellite analyses

A Bayesian clustering method based on Monte Carlo Markov chain (MCMC) in the software STRUCTURE (Pritchard et al. 2000) version 2.3.4 (Pritchard & Wen 2003) was used to calculate the optimal number of microsatellite clusters (K). After a burn-in of 100,000 generations, MCMCs were run for 200,000 iterations, ten times per K between one and ten. The analysis was run three times, always with the admixture model but different settings for alpha: (i) default seetings, (ii) with option 'separate α for each population' to improve the analysis against uneven sample sizes among populations (Puechmaille 2016; Wang 2017) and (iii) with option 'separate α for each population' but without 'use a uniform prior for α'. The optimal number of K was determined using both the ΔK method (Evanno et al. 2005) with the STRUCTURE harvester software (Earl & Von Holdt 2012) and the method of Pritchard & Wen (2003). The proportion of ancestry to clusters was extracted from the best STRUCTURE run (highest likelihood) with the optimal K and used as both the determinant of cluster affiliation and the measure of admixture. F_{ST} values between clusters were calculated in the software FSTAT (Goudet 1995) version 2.9.3 (Goudet 2001) using only individuals with a proportion ≥ 80% to a specific cluster.

Morphological measurements

Pictures of several body parts were taken in the field. The dorsal and right lateral side of the head were each photographed twice with much emphasis on having the pictures standardized as they will be used for landmarking. To measure the other variables, the ventral view of the head, left lateral side of the head, both sides of the nuchal and lunar marking, middle of the body and the cloaca (for sexing) were photographed. Pictures were taken with a Nikon D5100. The focal length of the camera was held constant at 55 mm and the manual focus was fixed during the entire study. Like this, the distance between the camera and the snake could be equalized by adjusting the distance until the eye appears in focus and, therefore, also the scale

was the same for all pictures. One picture of a ruler was taken after photographing a specimen to get the exact scale. Snakes were hold in a white photo box with non-reflecting, neutral walls and background so that the flashlight would not create reflections on the scales. The perpendicularity of the pictures was controlled by eye: the dorsal or ventral side of the head are not visible on a lateral picture and, on a dorsal one, the eyes are equally big and both posterior tips of the last supralabials are visible. In addition, M. Chèvre provided pictures of 21 *N. h. helvetica* (mtDNA clade E) and 20 *N. n. natrix* (mtDNA clade 3) from North-East Switzerland used in his MSc (Chèvre 2015). Clade 3 serves as a reference in subsequent morphological analyses, meaning that differences between microsatellite clusters bigger or equal than between clusters 3 and E most likely indicate separate species.

The selection of morphological variables strongly followed Chèvre (2015) who used variables from Thorpe (1979) that show strong geographic variation. Measured variables were snout-vent length (SVL), tail length (TL), body weight (BW), head length (HL), head width (HW), number of ventral scales (VS), relative position of the reduction from 19 to 17 dorsal scale rows (RelRedPos), number of subcaudal scales (SCS), contact between the temporal and post-ocular scale (TPOS), number of post-temporal scales (PTS), number of gular scales (GS), lateral blotch size (LBS), lateral blotch length (LBL), lateral blotch width (LBW), nuchal marking size (NMS), nuchal marking width (NMW), upper curvature of the nuchal marking (NMUC), lower curvature of the nuchal marking (NMLC) and distance between the nuchal marking and the parietal scales (NMPS) (see S Table 5 for variable description). SVL, TL, BW, VS, RelRedPos and SCS were measured in the field, while the other variables could be determined on the pictures.

Additionally, a landmark analysis on both the right and dorsal side of the head was performed to detect changes in head and scale shape. Mostly, junctions of scales were chosen as landmark positions as they are well-defined across individuals (S Figure 2). Landmarks were placed on pictures in the software TPSDIG2 (Rohlf 2017) version 2.30 and its coordinates were saved in tps files created by the software TPSUTIL32 (Rohlf 2013) version 1.74. Mean landmark coordinates were calculated for the replicated pictures per specimen and each side (dorsal and lateral). As M. Chèvre provided one picture per individual, they were duplicated. Pictures were drawn randomly amongst all specimens and replicates to prevent observer drift. Hereinafter, I will differentiate between 'morphological analysis' (involving the variables following Chèvre [2015]) and 'landmark analysis' to make clear what variables the analysis or results are about. In both analyses, only individuals with a proportion of ancestry ≥ 80% to a specific cluster were used. Pictures from Chèvre (2015) belong to specimens with a proportion of ancestry ≥ 95% to cluster E or 3 (calculated in his own STRUCTURE analysis) because his big sample size allowed to select enough individuals of that high 'purity'.

Landmark analysis

Preparation of landmark data was performed in the statistical software R (R core team 2017) version 3.4.1. To check whether landmark coordinates significantly differed between pictures of the same specimen, paired t-tests were used. Pictures from Chèvre (2015) were checked separately, as the only source of error for these pictures was the precision of placing landmarks. On the other hand, pictures from field and museum individuals also suffered from the error of photographing (possible shift in distance and/or angle across pictures). Afterwards, the function *estimate.missing()* from the package GEOMORPH (Adams et al. 2019) was used to interpolate

missing landmarks with the thin-plate spline method. Then, mean values were calculated for each landmark and specimen.

The analysis of the mean landmark coordinates was performed in MORPHOJ (Klingenberg 2011) version 1.07a, similar to Sidlauskas et al. (2011). First, a Procrustes Fit was applied to correct for size, rotation and position of the landmark configurations. Allometric correction was then performed using a linear regression with the log centroid size as explanatory variable and the Procrustes coordinates as response variable. The regression included a permutation test with 10,000 rounds and pooled regression within clusters. A Canonical Variate Analysis (CVA) was used to examine shape changes rather than a Principal Component Analysis (PCA) as microsatellite analysis provided cluster affiliation. The CVA was run with the residuals of the allometry correcting regression and a permutation test for pairwise distances of 10,000 iterations. Scatterplots of CV scores were checked for clustering of groups and wireframe graphs were used to visualize shape changes. The starting and target shape of wireframe graphs were placed next to each other, as suggested by Klingenberg (2013), to be able to objectively examine shape changes. Besides visually plotting the shape differences, the CVA also runs both Mahalanobis and Procrustes permutation tests (10,000 iterations) to check for the significance of potential shape changes.

Morphological analysis

A new tool to analyse ratios in a multivariate space of PCA and linear discriminant function (LDA) has been developed by Baur & Leuenberger (2011). Their so called 'shape PCA' enables the examination of shape changes depending on size and the 'PCA ratio spectrum' allows the interpretation of PCs in terms of ratios and shows the most discriminating ratio. This approach has been used in several studies to find morphological differences among taxa (László et al. 2013; Baur et al. 2014; Huber & Baur 2016; Gebiola 2017). The whole analysis was performed in the statistical software R. First, variables were divided into three groups: distance measures (SVL, TL, HL and HW), scale counts (VS, SCS, PTS and GS) and marking measures (LBS, LBL, LBW, NMS, NMW, NMUC, NMLC and NMPS). The package MICE (Van Buuren & Groothuis-Oudshoorn 2011) was used to replace missing values through multiple imputation within variable groups after excluding individuals with > 25% NAs. A shape PCA was run for each of the variable groups separately. If a separation was visible, the PCA ratio spectrum was inspected to get the best diagnostic variable ratio. Additionally, a linear discriminant analysis including all variable groups and RelRedPos, TPOS and BW was used to check how good these clusters were distinguishable. The function *lda()* from the package MASS (Venables & Ripley 2002) was used with equal priors and CV=TRUE. This was repeated but without cluster 3 to be able to discriminate more precisely between C and E.

Results

Mitochondrial DNA clade distribution

Figure 1 reveals the distribution of mtDNA lineages in southern Switzerland (dataset in S Table 1). The cantons of Ticino and Valais are solely occupied by clade C whereas cantons north of the Alps harbour mainly clade E. A contact zone appears to be located between Lausanne and Montreux in the canton of Vaud. However, there are several locations north of the Alps (Allaman, Lausanne and Emmental) where a few clade C individuals have also been found. Some

N. n. persa (clade 7 in Kindler et al. [2013]) were caught in Lausanne too as expected based on Dubey et al. (2017).

Microsatellite clusters

According to Pritchard & Wen (2003), the optimal value for K has the highest likelihood L(K), which is here K=5 (S Figure 3, top left). The ΔK method (Evanno et al. 2005) returns K=2 and K=5 with the highest ΔK values (S Figure 3, bottom right). It has been shown recently that the ΔK method has a strong bias to return K=2 as the optimal result and, therefore, one should only accept this result if it makes sense in the biological context (Janes et al. 2017). As three distinct mtDNA lineages (E, C, 7) occur in the study area, I expect at least three microsatellite clusters and would thus consider the second large peek, K=5, as the 'true' value of K, which coincides with the former method. Results are consistent across all settings for alpha. The results with default settings are used hereinafter because they represent the simplest model and have the highest ΔK value.

Figure 2 shows the distribution of the five microsatellite clusters (dataset in S Table 1). Snakes of mtDNA clade C are divided into the green cluster in Ticino and northern Italy (C-TI) and the red cluster in Valais (C-VS). Snakes of mtDNA clade E belong to the blue cluster in the midland (E-ML) and the yellow cluster along Lake Geneva (E-GE). The fifth, grey cluster (7) includes the snakes of mtDNA clade 7 around Lausanne. Admixed individuals (between E-GE, E-ML and C-VS) occur mostly around Lake Geneva and the adjacent valley south of Montreux, while the other parts of Switzerland are almost solely occupied by its specific cluster. F_{ST} values are very similar between clusters except the slightly lower values for E-GE/E-ML and for E-ML/7, whereas the highest value was observed between C-VS/E-GE (S Table 6).

In the subsequent morphological and landmark analysis, clusters E-ML and E-GE were merged and referred to as cluster E while C-VS and C-TI were differentiated. C-VS and C-TI are strongly separated by mountainous regions whereas E-ML and E-GE are in contact and admixing. Additionally, the number of samples for E-GE is very limited.

Landmark differences

Paired t-tests showed that the errors of photographing and measuring between pictures of a specimen were not significant for any landmark (field and museum specimens: df = 26, lowest p value > 0.15; M. Chèvre's specimens: df = 39, lowest p value > 0.96).

Some landmarks were removed because the sample size was too small for 27x2 coordinate variables. Landmark 8 was removed because the temporal scale was sometimes split into two scales and/or small so it did not reach the 7^{th} supralabial scale. Landmarks 12, 14, 16 and 18 were excluded as they all have other landmarks in close proximity. Lastly, landmarks 21, 24 and 27 were removed because they are at the edge and might already be influenced by the curvature of the head. Therefore, only nineteen landmarks (1-7, 9-11, 13, 15, 17, 19, 20, 22, 23, 25, 26) were used finally.

Mahalanobis and Procrustes permutation tests compute significant morphological differences between all clusters (Table 1). However, Figures 3 and 5 show that there is always some overlap between clusters except for clade 3 at lateral landmarks. Moreover, there are no clear shape differences visible in the wireframe graphs (Figures 4 and 6).

Morphological differences

PC scores of microsatellite clusters overlap both in size and shape for each variable group (Figure 7). No distinction between clusters is possible. Only cluster 3 is more or less separable by marking measures, which are smaller and of a different shape. The LDA shows clusters to be indistinguishable too (Table 2). Cluster 3 is most separable with a percentage of correctly allocated individuals of 86.7%. The percentage is < 70% for all other clusters. S Table 7 shows raw data (mean and standard deviation) of every morphological variable for mtDNA clades E and C for visual inspection.

Discussion

After the discovery of an alien mtDNA lineage (clade C in Kindler et al. [2013]) of grass snake *Natrix helvetica* in Lausanne, it was not known whether this clade naturally migrated so far or has been introduced. Dubey et al. (2017), who studied the presence of different alien grass snakes *Natrix natrix persa* (clade 7 in Kindler et al. [2013]) at the same location, assumed that clade C has also escaped from an outdoor enclosure of a reptile park in the 1970s, together with the ancestors of these *N. n. persa*. Our study showed that clade C actually occupies a bigger area of Switzerland than expected. From the cantons of Ticino and Valais, its distribution extends to the canton of Vaud, along Lake Geneva, where it is in sympatry with clade E. Microsatellites match this pattern. Both mtDNA lineages C and E include two microsatellite clusters. While the clusters of clade C are allopatrically separated, clusters of clade E show more of a parapatric distribution. Regions north of Lake Geneva plus north-western Valais appear to be a contact zone where clade E (clusters E-GE and E-ML) and clade C (cluster C-VS) naturally occur and hybridize. Therefore, snakes of clade C should no longer be considered as 'aliens' in Lausanne, as supposed by Dubey et al. (2017).

The situation near Lausanne is, however, still unresolved. The alien population of *N. n. persa* is not only sustainable and reproducing but also able to admix with both local clades. I report two introgression events - two individuals with mtDNA lineage C and microsatellites from both clusters 7 and E. Alien snakes should be removed from the region before they might spread and further interfere in the gene pool of *N. h. helvetica* which is already classified as "vulnerable" in the Swiss Red List of Reptiles back in 2005 (Monney & Meyer 2005).

The landmark analysis poses a contradicting pattern. Although the statistics are highly significant and the CV score plots show not a perfect but good separation of clusters, wireframe graphs depicting shape changes look the same, even when amplified by a factor of three. I assume, clusters are indeed variable and distinguishable in head and scale shape, at a level, however, which is not visible by eye. This is similar to Chèvre (2015) who found significant Mahalanobis distances between species (*N. natrix* vs. *N. helvetica*) but species discrimination success was only 55% - 85%. In addition, shape PCAs with classic morphological variables showed no separation of clusters. Identification seems only possible for clade 3 (*N. n. natrix*, Kindler et al. [2013]) using marking measures, which are the same traits that proved most diagnostic in Chèvre (2015). According to Table 1 in Kindler et al. (2013), also Thorpe (1979), Orlov & Tuniyev (1999), Kabisch (1999) and Kreiner (2007) assigned both clades C and E to what is now regarded as *N. h. helvetica*.

It was also unknown whether clade C constitutes another haplotype of the native subspecies *Natrix helvetica helvetica* (clade E in Kindler et al. [2013]) or belongs to a different subspecies, which may be in need of conservation measures in Switzerland. Clades C and E do not represent

distinct species as morphological discrimination is not possible, they belong to the same more inclusive clade (Kindler et al. 2013) and extensive gene flow occurs in a wide hybrid zone - in contrast to *N. natrix* and *N. helvetica* (Chèvre 2015; Kindler et al. 2017). Whether or not both clades are assigned to the same subspecies depends on the underlying theoretical concept. According to the traditional idea, both clades have to be lumped together due the nonexistence of diagnostic, morphological characters. Subspecies concepts focusing more on genetics, however, would consider them as separate subspecies. According to Groves and Grubb (2011), clade C would be considered as a different subspecies and Hawlitschek et al. (2012) would even name it a different species. All requirements of Kindler & Fritz's subspecies concept (2018) are fulfilled and their microsatellite analysis showed clade C to be more closely related to Italian lineages. Therefore, the alternative is that clade C belongs to *N. h. lanzai*. In my opinion, however, subspecies should have enough morphological differences so that one can visually discriminate them, in addition to a certain genetic distinctness. Focusing only on genetic data and ignoring ecological data seems to be inappropriate for the determination of evolutionary significant units according to Crandall et al. (2000). Additionally, clusters C-VS, E-GE and E-ML admix equally among each other in the contact zone and F_{ST} values are similar between and within clades, which are other indicators that clades C and E belong to the same taxon. I therefore support the acknowledgement of clade C as a new haplotype of *N. h. helvetica* in Switzerland and propose to incorporate this clade in conservation measures as *N. h. helvetica* is already threatened.

The distribution and contact zone of clades C and E can also be explained in a biogeographical context. Kindler et al. (2018) showed that, during the last glaciation in Europe, clade E has survived this period in an extra-Mediterranean refuge in southern France. From there, it has colonized the rest of France, Britain and Central Europe until it met with clade 3 along the Rhine. Clade C, on the other hand, has outlived the glaciation in a separate 'microrefugium' in northeastern Italy and adjacent Switzerland (Kindler et al. 2013). This distinct refuge is further supported by an endemic species and endemic mitochondrial lineages of several amphibian and reptile species (Kindler et al. 2013). After the ice melted, clade C spread over the Alps to the canton of Valais and Vaud, where it is in secondary contact with clade E. This scenario seems very plausible as *N. helvetica* is generally known to occur at high altitudes (up to 1800 m.a.s.l. according to data from Info Fauna CSCF & KARCH) and Glaw et al. (2019) indeed report a transalpine immigration. I cannot say, however, whether both clades initially met along Lake Geneva. It is also possible that one has been, and maybe still is, outcompeting and displacing the other, which would mean that the contact zone might still be moving.

In consequence of clades C and E being the same subspecies, I suggest revaluating the taxonomic status of mtDNA lineage D (Kindler et al. 2013) because it seems unlikely that a new subspecies has emerged between two haplotypes of another subspecies in the phylogenetic tree (Kindler et al. 2013). Clade D is currently ranked among *N. h. lanzai*, which has been described as a new subspecies because it is distinct from *N. h. helvetica* "especially by bigger lateral blotches" (Kramer 1970). However, his description of *N. h. lanzai* fits very well with clade C, which is considered as *N. h. helvetica*, and individuals with large lateral blotches have been found for both clades C and E in this study. Indeed, clade E appears to have a huge variation in lateral blotches size and completely covers the top range of clade C (see Figure 8 bottom for marking measures). Moreover, Kramer (1970) used a very small and biased sample representing clade E. Other morphological studies also doubt the validity of *N. h. lanzai* (Thorpe 1979,

1980, 1984a,b) while a genetic study supports it (Kindler & Fritz 2018). Apparently, the same conflict between morphology and genetics applies for Italian grass snakes too.
The case of European grass snakes demonstrates what conflict arises when several concepts focusing on either morphology or genetics exist. Depending on the subspecies concept, Switzerland would have one subspecies more or Europe would lose one. The same conflict is found all over the animal kingdom and in plants (reptiles, Losos et al. 2012, Gauthier et al. 2012, Torstrom et al. 2014; birds, Zink 2004; fishes, Marghali et al. 2014; bees, Gruber et al. 2013; tapeworms, Bazsalovicsová et al. 2014; amphipods, Finston et al. 2004; plants, Ng 2019). In order to stop the ongoing revision of taxa, scientists need to come up with a more widely applicable subspecies concept. A universal concept is difficult if not impossible and probably too inaccurate due to the very different i.a. biogeographical context of different regions. The problem of genetics often refuting traditional interpretations, however, could be solved. I propose an integrative approach, including both morphological and genetic data, as discussed by many other authors (Crandall et al. 2000; Jenner 2004; Wiens 2004; Pisani et al. 2007; Winker 2009; Patten & Remsen Jr 2017). Subspecies should be morphologically diagnosable, as constituted by the traditional idea, but also represent evolutionary significant units by depicting distinct mitochondrial lineages or nuclear DNA clusters. In contrast to species, extensive gene flow may occur between subspecies.

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Figures and Tables

Figures

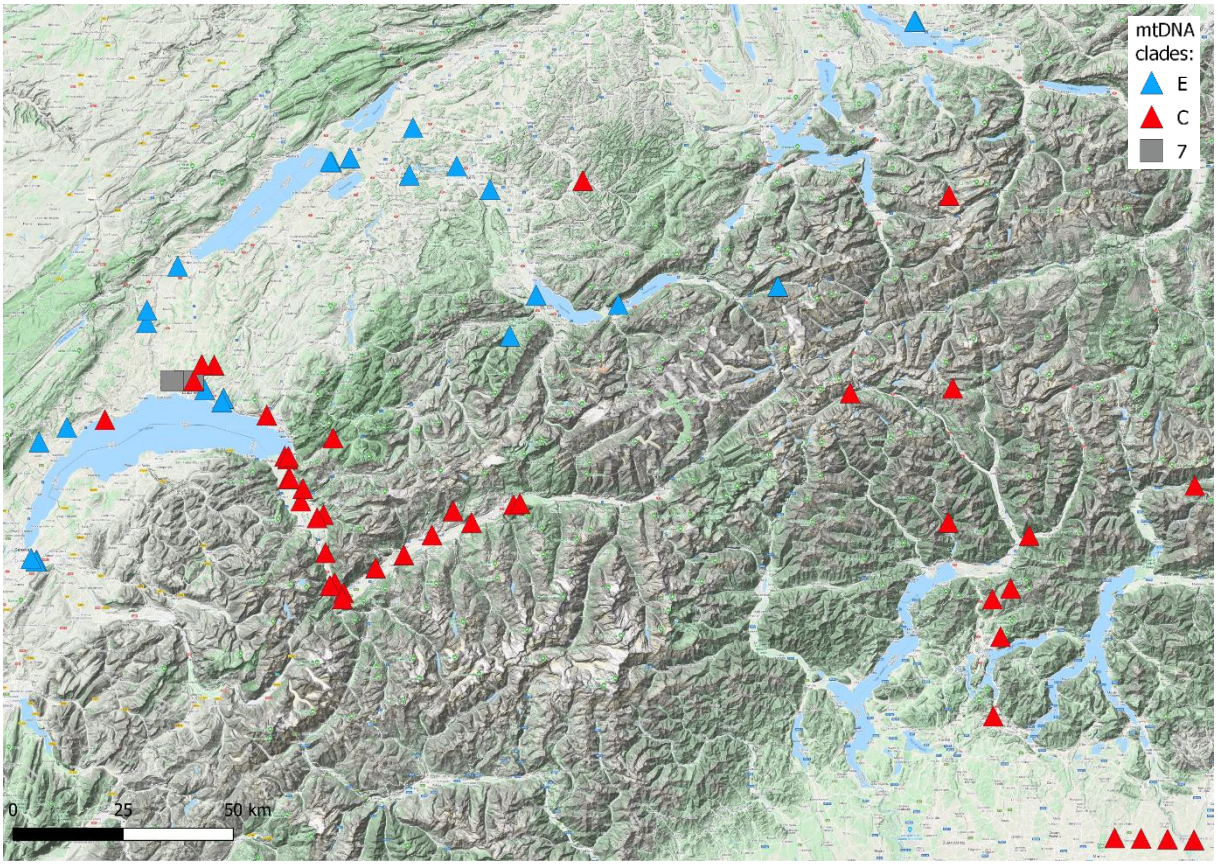


Figure 1 Distribution of mtDNA clades of *Natrix helvetica* in southern and western Switzerland. Symbols and colors correspond to Kindler et al. (2013). Clade 7 individuals are located near the old vivarium in Lausanne but are shifted to improve visibility. The four samples in the bottom right corner originate from North Italy outside of the range of this map. The map was created using QGIS 3.6 (2019) and Google Maps terrain layer (Google n.d.).

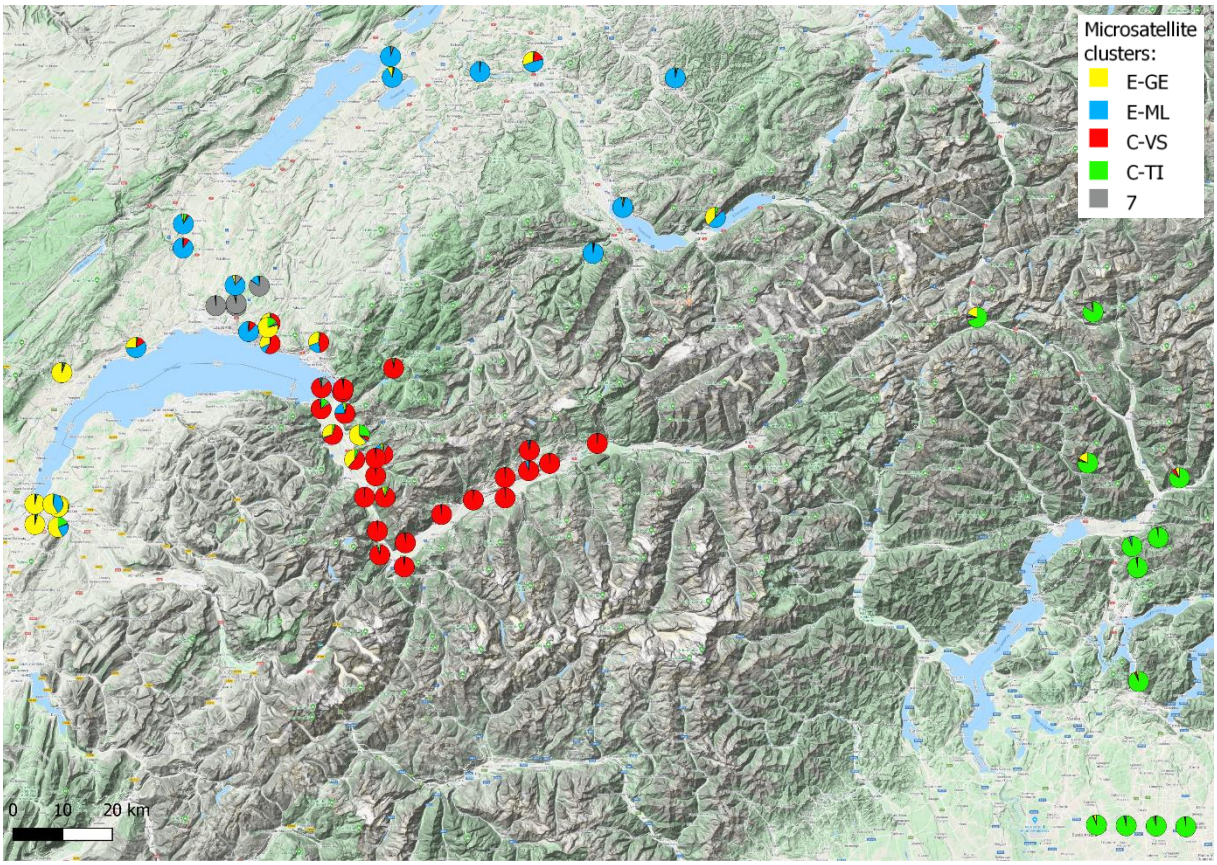


Figure 2 Proportion of ancestry to microsatellite clusters of *Natrix helvetica*, computed by STRUCTURE. Diagrams are slightly shifted from the sampling location to make more diagrams visible. The four samples in the bottom right corner originate from North Italy outside of the range of this map. The map was created using QGIS 3.6 (2019) and Google Maps terrain layer (Google n.d.).

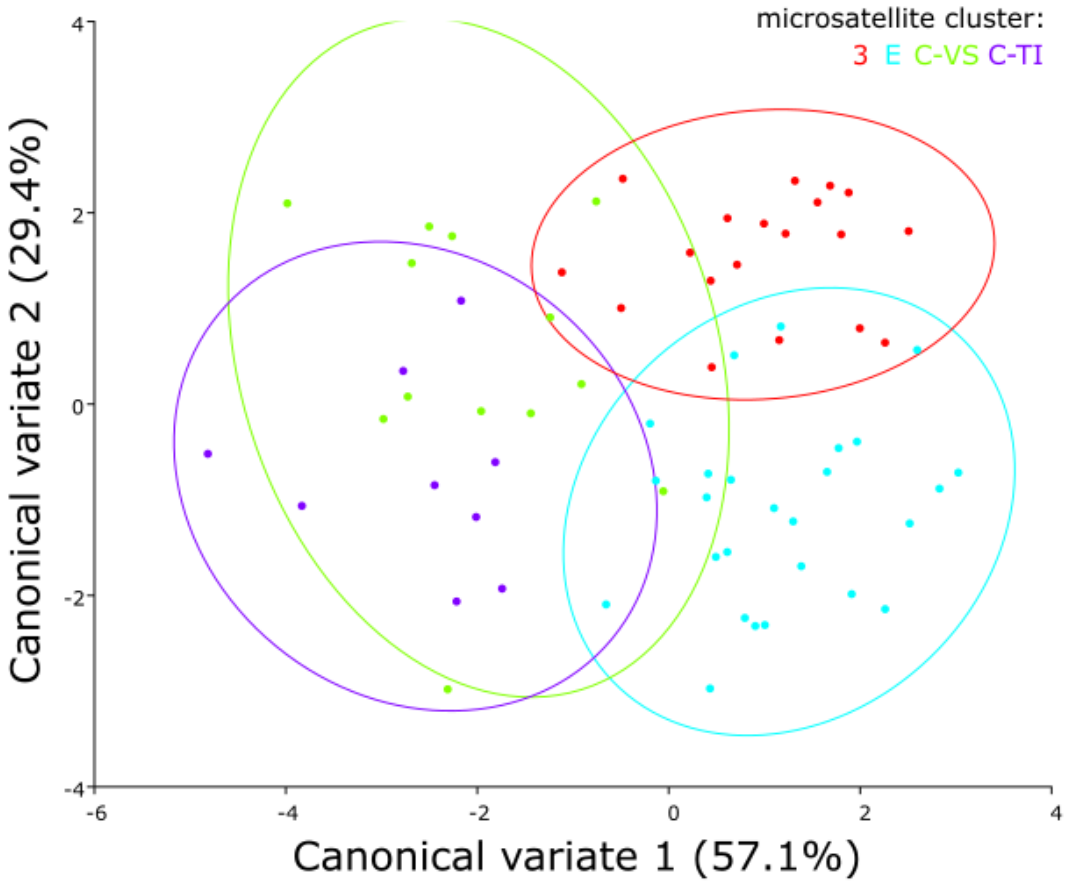


Figure 3 Canonical variate analysis (CVA) of dorsal landmark coordinates of *Natrix spp.* based on microsatellite clusters (*Natrix natrix*: 3; *Natrix helvetica*: E, C-VS, C-TI). Only individuals with a proportion of ancestry to one cluster ≥ 80% were used. Circles represent 95% confidence ellipses.

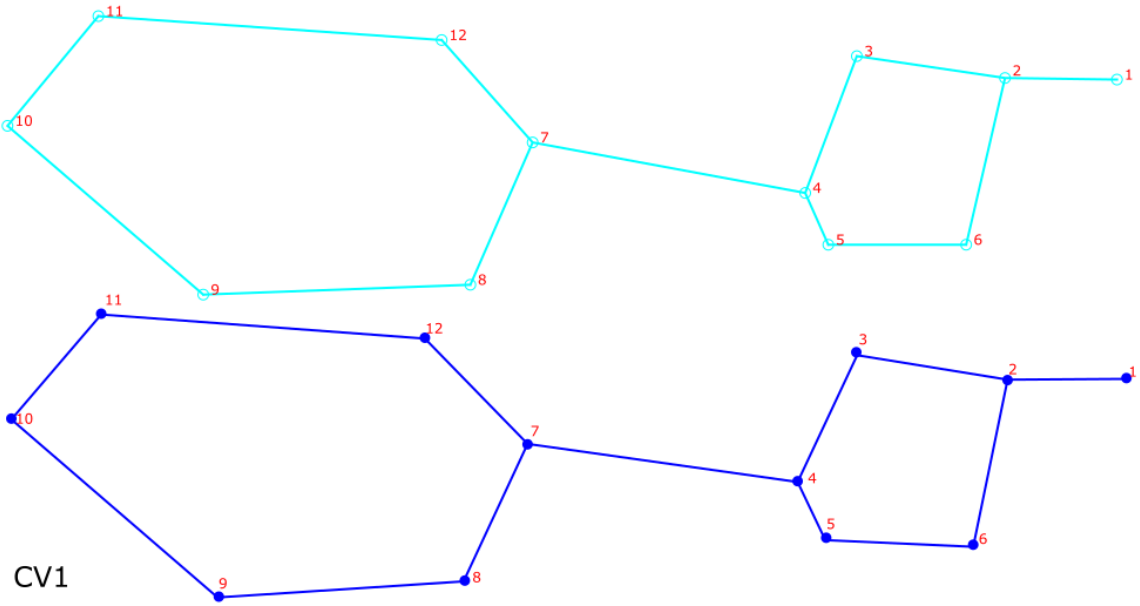


Figure 4 Wireframe graph showing the shape changes along the canonical variate 1 (CV1) for dorsal landmarks. Light blue shows the starting shape (mean landmark coordinates) and dark blue the target shape (shape shift along CV1). The lower the scores of a specimen for CV1, the more does its shape resemble the light blue shape, the higher, the more does its shape resemble the dark blue shape. Accordingly, dorsal landmarks of microsatellite clusters C-VS and C-TI more closely resemble the light blue configuration, while E and 3 are more similar to the dark blue one (see Figure 3 for CV scores). Shape changes from light to dark blue are increased threefold.

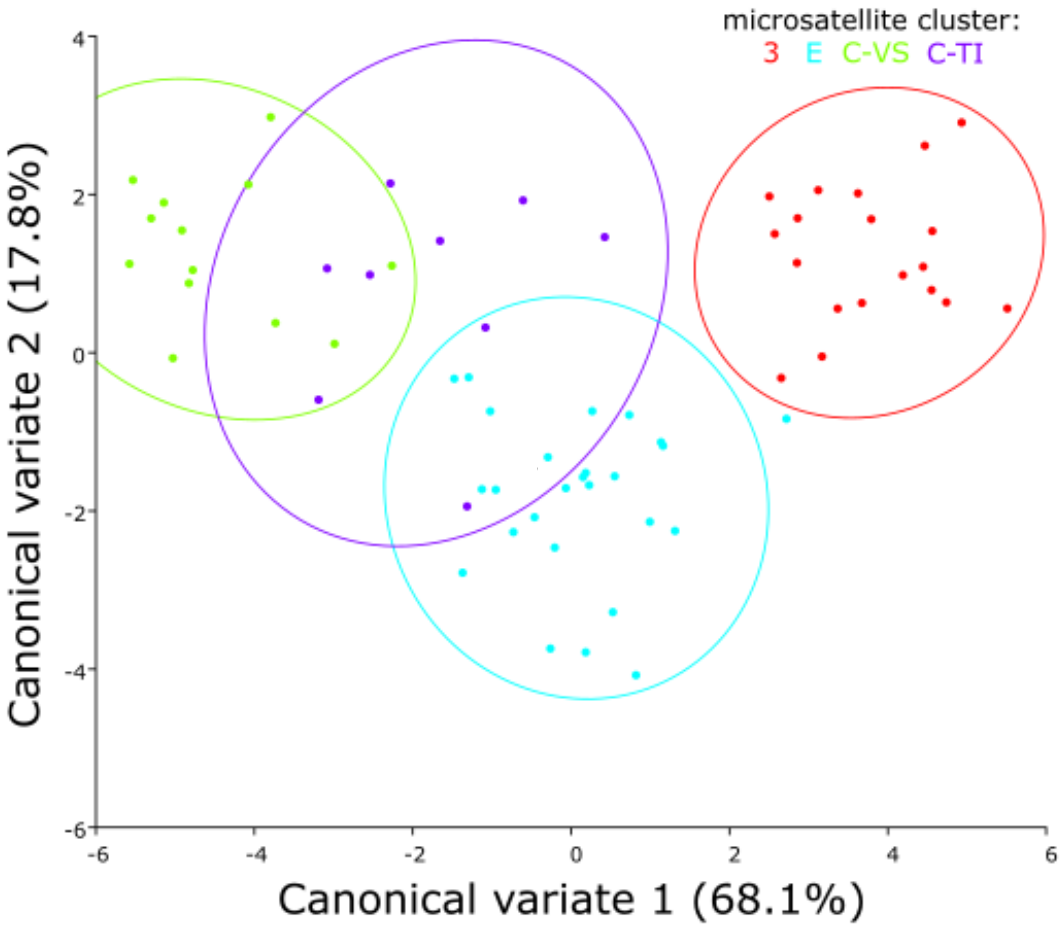


Figure 5 Canonical variate analysis (CVA) of lateral landmark coordinates of *Natrix spp.* based on microsatellite clusters (*Natrix natrix*: 3; *Natrix helvetica*: E, C-VS, C-TI). Only individuals with a proportion of ancestry to one cluster ≥ 80% were used. Circles represent 95% confidence ellipses.

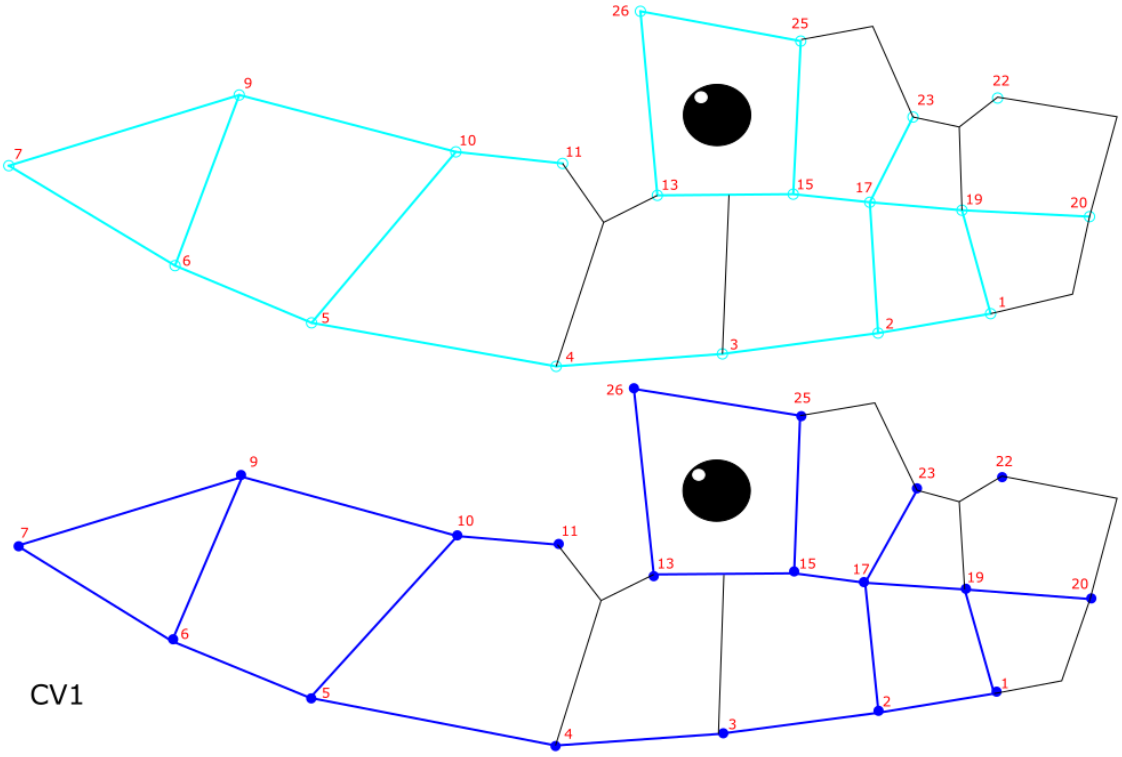


Figure 6 Wireframe graph showing the shape changes along the canonical variate 1 (CV1) for lateral landmarks. Light blue shows the starting shape (mean landmark coordinates) and dark blue the target shape (shape shift along CV1). The lower the scores of a specimen for CV1, the more does its shape resemble the light blue shape, the higher, the more does its shape resemble the dark blue shape. Accordingly, lateral landmarks of microsatellite clusters resemble the light blue configuration most to least as follows: C-VS > C-TI > E > 3 (see Figure 5 for CV scores). Shape changes from light to dark blue are increased threefold.

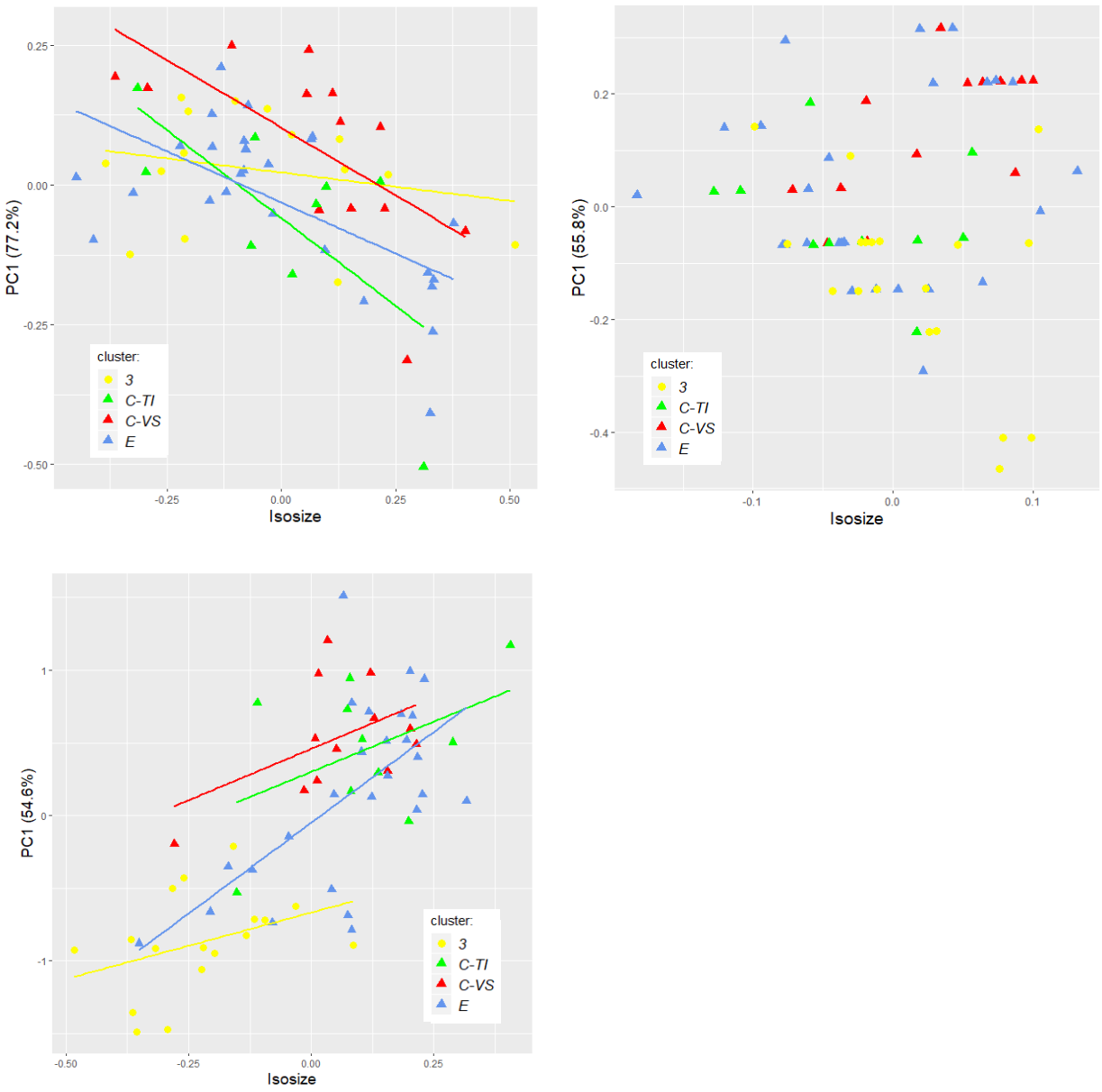


Figure 7 Shape PCAs of distance measures (top left), scale counts (top right) and marking measures (bottom) of *Natrix spp.* based on microsatellite clusters (*Natrix natrix*: 3; *Natrix helvetica*: E, C-VS, C-TI). Only individuals with a proportion of ancestry to one cluster ≥ 80% were used.

Tables

Table 1 Statistical test results of corresponding Canonical Variate Analysis (CVA) of lateral and dorsal landmarks (Figure 3 and 5), describing shape differences between microsatellite clusters of *Natrix natrix* (3) and *Natrix helvetica* (E, C-VS and C-TI). 10,000 permutations were run for each test.

Lateral landmarks							
Mahalanobis distances among groups				Procrustes distances among groups			
	3	E	C-VS		3	E	C-VS
E	4.8305			E	0.0308		
C-VS	8.2776	5.6166		C-VS	0.0380	0.0285	
C-TI	6.5247	4.5882	5.2595	C-TI	0.0431	0.0324	0.0237
P values from permutation tests				P values from permutation tests			
	3	E	C-VS		3	E	C-VS
E	<.0001			E	<.0001		
C-VS	<.0001	<.0001		C-VS	<.0001	0.0001	
C-TI	<.0001	<.0001	<.0001	C-TI	<.0001	0.0005	0.0783
Dorsal landmarks							
Mahalanobis distances among groups				Procrustes distances among groups			
	3	E	C-VS		3	E	C-VS
E	2.7561			E	0.0232		
C-VS	3.5488	3.7053		C-VS	0.0236	0.0268	
C-TI	4.4217	4.1624	2.9620	C-TI	0.0375	0.0389	0.0264
P values from permutation tests				P values from permutation tests			
	3	E	C-VS		3	E	C-VS
E	<.0001			E	<.0001		
C-VS	<.0001	<.0001		C-VS	0.0003	<.0001	
C-TI	<.0001	<.0001	0.0001	C-TI	<.0001	0.0254	<.0001

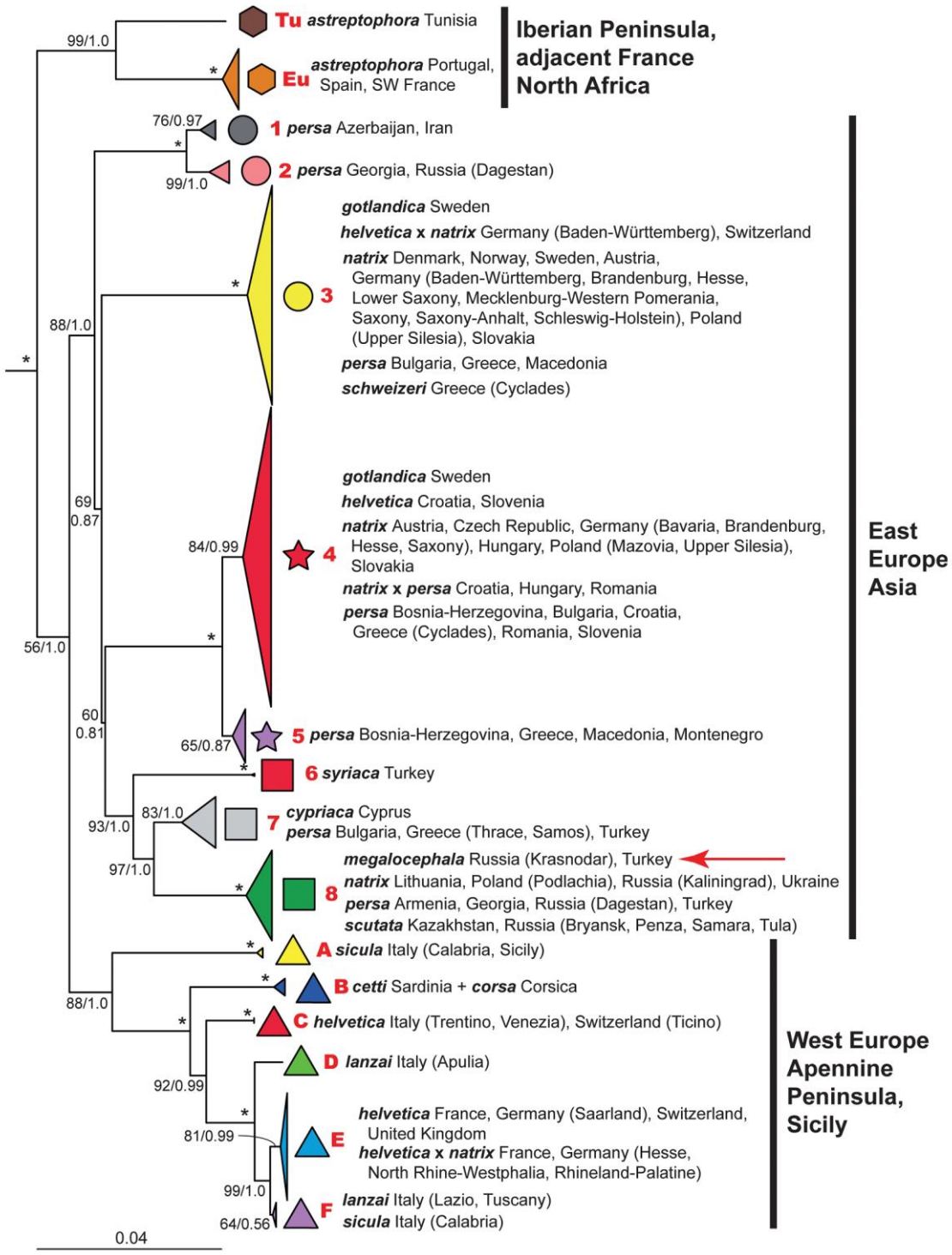
Table 2 Allocation of specimens to microsatellite clusters of *Natrix natrix* (3) and *Natrix helvetica* (E, C-VS and C-TI) based on all morphological variables by a linear discriminant analysis (LDA). The table shows which cluster (columns) specimens of a certain cluster (rows) were assigned to by the LDA. Subtables present results for LDA with (left) and without (right) cluster E individuals. %: percentage of correctly classified individuals per cluster.

	3	E	C-VS	C-TI	%
3	13	2	0	0	86.7
E	2	12	6	4	50
C-VS	0	3	9	1	69.2
C-TI	0	3	2	3	37.5

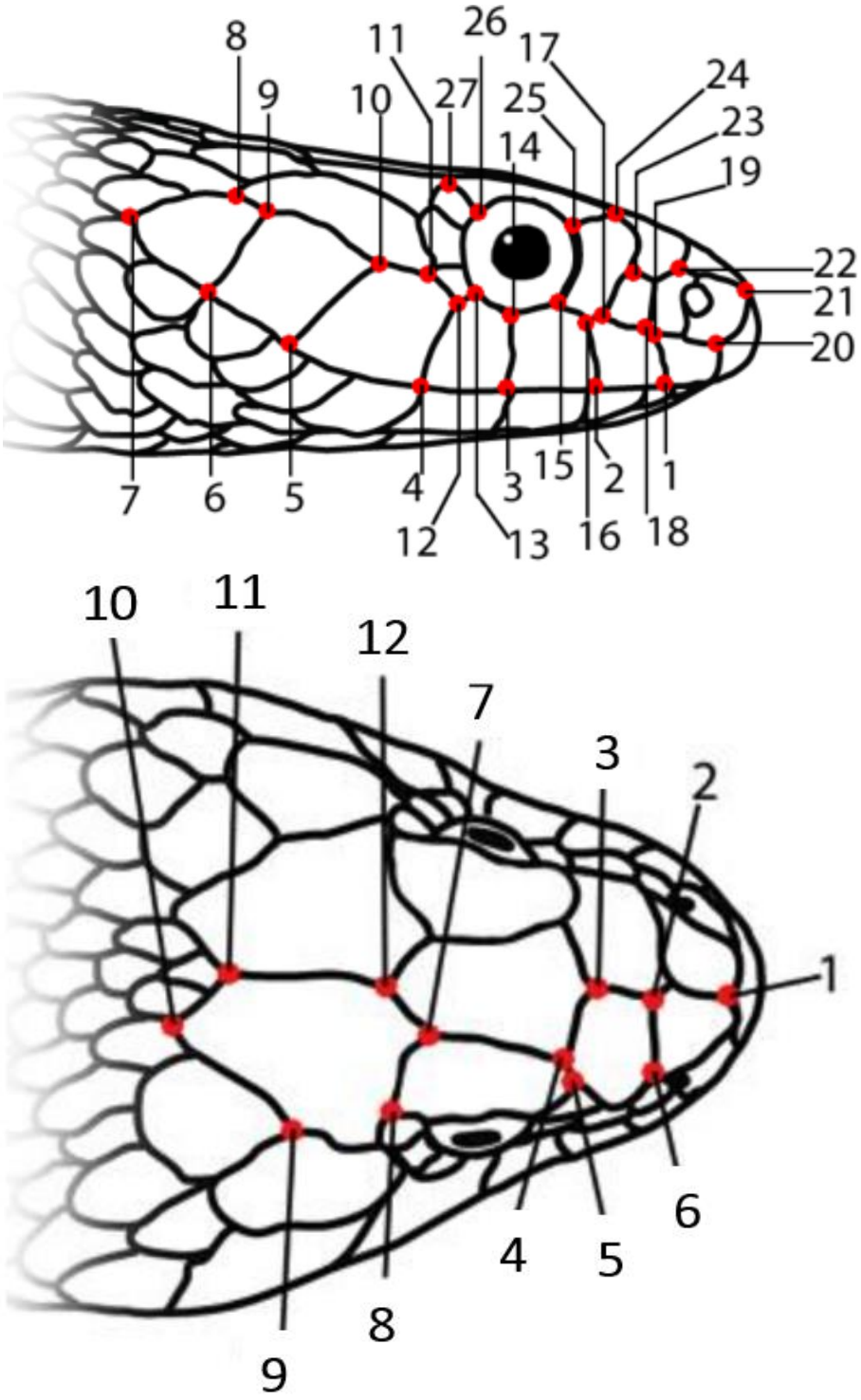
	E	C-VS	C-TI	%
E	13	5	6	54.2
C-VS	3	8	2	61.5
C-TI	4	1	3	37.5

Supplementary

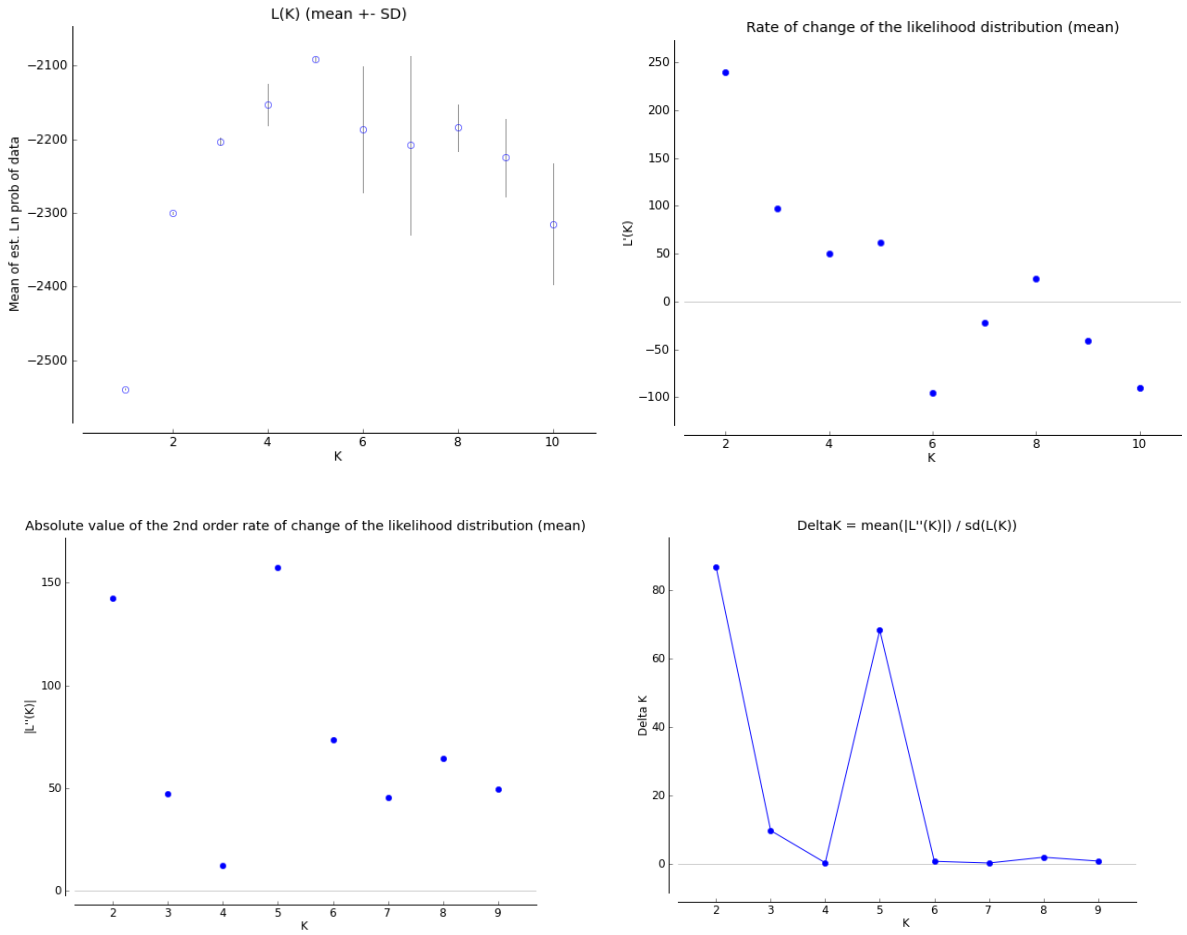
S Figures



S Figure 1 Mitochondrial phylogeny of grass snakes inferred from Maximum Likelihood analyses using 1984-bp mtDNA (ND4+tRNAs, cyt b) of 407 samples of *Natrix natrix* and three samples of *N. megalocephala*. Terminal clades collapsed to cartoons. Outgroups (*N. maura*, *N. tessellata*, *Nerodia sipedon*) removed for clarity; for a complete tree, see Fig. S1. Numbers along nodes indicate branch support under Maximum Likelihood (1000 bootstrap replicates) and Bayesian analyses (posterior probabilities). Asterisks indicate maximum support under both tree-building methods. Clade symbols correspond to Fig. 1; red letters and numbers preceding taxon names refer to the text and Table 1. Red arrow highlights placement of *Natrix megalocephala* among *N. natrix*. (Kindler et al. 2013)



S Figure 2 Landmark positions. All landmarks were placed at junctions of certain scales except for number 7 and 8 on the lateral side of the head. Landmark 7 is the most posterior tip of the last supralabial scale and landmark 8 the most posterior point of contact between the temporal scale and a supralabial scale.



S Figure 3 Structure harvester output calculating the optimal number of microsatellite clusters (K). According to Pritchard & Wen (2003), the optimal value for K has the highest likelihood (L(K), top left), which is here K=5. The ΔK method (Evanno et al. 2005) returns the optimal value with the highest ΔK value (bottom right). As Janes et al. (2017) discovered a strong bias of this method to compute K=2 as the best result and I expected at least three clusters (due to the presence of three mtDNA lineages), I consider the second large peek, K=5, as the 'true' value of K.

S Tables

S Table 1 Database of studied grass snakes. Sample origin: [1] A. Schild field sample, [2] G. Meier field sample, [3] E. Gallice field sample, [4] T. Gil field sample, [5] S. Dubey field sample, [6] Naturhistorisches Museum Bern, [7] Museo cantonale di storia naturale Lugano, [8] Naturmuseum Sitten, [9] M. Chèvre (2015), [10] S. Ursenbacher private collection; Proportion of ancestry to microsatellite clusters E-GE, E-ML, C-VS, C-TI and 7, returned by STRUCTURE; C: Canton.

ID	Sex	Clade	E-GE	E-ML	C-VS	C-TI	7	C	Community
AS001[1]	M	E	0.076	0.885	0.012	0.018	0.01	VD	Cudrefin
AS002[1]	F	C	0.007	0.027	0.801	0.116	0.05	VD	Noville
AS003[1]	M	C	0.385	0.014	0.516	0.057	0.028	VD	Aigle
AS004[1]	J	C	0.616	0.033	0.065	0.275	0.012	VD	Aigle
AS005[1]	M	E	0.006	0.939	0.011	0.007	0.037	VD	Cudrefin
AS006[1]	J	C	0.005	0.006	0.979	0.006	0.004	VD	Lavey-Morcles
AS007[1]	F	C	0.003	0.005	0.984	0.004	0.004	VS	Dorénaz
AS008[1]	F	C	0.007	0.008	0.972	0.008	0.005	VS	Martigny
AS009[1]	F	C	0.006	0.006	0.976	0.006	0.006	VS	Martigny
AS010[1]	M	C	0.003	0.005	0.985	0.003	0.003	VS	Chamoson
AS011[1]	J	C	0.004	0.005	0.983	0.005	0.004	VS	Sitten
AS012[1]	M	C	0.003	0.004	0.986	0.003	0.003	VS	Sitten
AS013[1]	M	C	0.004	0.006	0.979	0.008	0.004	VS	Salgesch
AS014[1]	J	C	0.003	0.003	0.988	0.003	0.003	VS	Siders
AS015[1]	M	C	0.004	0.048	0.93	0.007	0.011	VS	Ayent
AS016[1]	J	C	0.004	0.026	0.944	0.009	0.016	VS	Ayent
AS017[1]	J	C	0.299	0.02	0.635	0.04	0.005	VD	Yvorne
AS018[1]	J	C	0.035	0.218	0.704	0.024	0.019	VD	Yvorne
AS019[1]	F	C	0.011	0.009	0.954	0.013	0.014	VD	Noville
AS020[1]	F	C	0.011	0.019	0.948	0.017	0.005	VD	Noville
AS021[1]	J	C	0.306	0.221	0.446	0.022	0.006	VD	Vevey
AS022[1]	J	E	0.172	0.247	0.555	0.011	0.015	VD	Grandvaux
AS023[1]	M	E	0.319	0.103	0.552	0.01	0.017	VD	Grandvaux
AS024[1]	F	C	0.013	0.014	0.948	0.016	0.01	VD	Château-d'Oex
AS025[1]	J	E	0.013	0.858	0.117	0.007	0.006	VD	Belmont-sur-Lausanne
AS026[1]	F	C	0.052	0.007	0.875	0.042	0.024	VD	Lavey-Morcles
AS027[1]	J	C	0.192	0.016	0.626	0.14	0.025	VD	Noville
AS028[1]	J	C	0.006	0.006	0.977	0.007	0.004	VD	Noville
AS029[1]	J	7	0.004	0.009	0.004	0.005	0.978	VD	Lausanne
AS030[1]	J	C	0.012	0.139	0.008	0.009	0.831	VD	Le Mont-sur-Lausanne
AS031[1]	J	E	0.008	0.886	0.09	0.006	0.01	VD	La Sarraz
AS032[1]	J	C	0.007	0.009	0.004	0.006	0.974	VD	Lausanne
AS033[1]	J	E	0.029	0.875	0.028	0.059	0.009	VD	Arnex-sur-Orbe
AS034[1]	J	C	0.174	0.012	0.018	0.781	0.016	TI	Lavertezzo
AS035[1]	NA	C	0.021	0.004	0.005	0.838	0.132	TI	Blenio
AS036[2]	J	C	0.017	0.007	0.008	0.957	0.011	TI	Isone
AS037[2]	J	C	0.013	0.004	0.004	0.974	0.005	TI	Isone
AS038[2]	J	C	0.009	0.008	0.008	0.944	0.03	IT	Mosso
AS039[2]	J	C	0.006	0.006	0.012	0.971	0.005	TI	Camignolo
AS040[3]	J	E	0.573	0.228	0.005	0.185	0.009	GE	Chêne-Bougeries
AS041[3]	J	E	0.98	0.008	0.004	0.004	0.004	GE	Thônex
AS042[3]	J	E	0.979	0.008	0.004	0.005	0.004	GE	Chêne-Bougeries
AS043[3]	J	E	0.955	0.022	0.006	0.01	0.007	GE	Chêne-Bougeries

AS044[4]	J	E	NA	NA	NA	NA	NA	GE	Chêne-Bougeries
AS045[4]	J	E	0.959	0.018	0.006	0.011	0.006	GE	Chêne-Bougeries
AS046[4]	J	E	0.581	0.392	0.007	0.014	0.006	GE	Chêne-Bougeries
AS047[5]	F	E	0.94	0.015	0.003	0.02	0.022	VD	Trélex
AS048[5]	M	C	0.261	0.581	0.133	0.017	0.008	VD	Allaman
AS049[2]	F	C	0.192	0.03	0.022	0.69	0.065	TI	Airolo
NMBE 1016027[6]	F	E	NA	NA	NA	NA	NA	BE	Mühleberg
NMBE 1021790[6]	M	C	NA	NA	NA	NA	NA	BE	Langnau im Emmental
NMBE 1048883[6]	J	E	NA	NA	NA	NA	NA	BE	Aarberg
NMBE 1052697[6]	NA	E	NA	NA	NA	NA	NA	BE	Ringgenberg
NMBE 1055597[6]	NA	E	NA	NA	NA	NA	NA	BE	Diemtigen
NMBE 1055598[6]	F	E	NA	NA	NA	NA	NA	BE	Allmendingen
NMBE 1055599[6]	J	E	NA	NA	NA	NA	NA	BE	Spiez
NMBE 1055681[6]	M	E	NA	NA	NA	NA	NA	BE	Walperswil
NMBE 1055998[6]	J	E	NA	NA	NA	NA	NA	BE	Wohlen bei Bern
V 1095[7]	F	C	NA	NA	NA	NA	NA	IT	Cameri
V 1121[7]	J	C	NA	NA	NA	NA	NA	IT	Cameri
VT 1327[7]	J	C	NA	NA	NA	NA	NA	TI	Lugano
VT 1332[7]	F	NA	NA	NA	NA	NA	NA	TI	Agno
VT 1326[7]	M	NA	NA	NA	NA	NA	NA	TI	NA
VT 2917[7]	F	NA	NA	NA	NA	NA	NA	TI	Bedigliora
VT 5192[7]	F	NA	NA	NA	NA	NA	NA	TI	Alto Malcantone
VT 1324[7]	F	NA	NA	NA	NA	NA	NA	TI	NA
VT 1317/1[7]	J	NA	NA	NA	NA	NA	NA	TI	NA
VT 2340[7]	J	C	NA	NA	NA	NA	NA	TI	Rancate
VT 1325/1[7]	M	NA	NA	NA	NA	NA	NA	TI	NA
VT 1325/2[7]	M	NA	NA	NA	NA	NA	NA	TI	NA
VT 2421[7]	F	NA	NA	NA	NA	NA	NA	TI	Genestrerio
VT 1331[7]	F	C	NA	NA	NA	NA	NA	TI	Lumino
CCTC 2000539[8]	F	C	NA	NA	NA	NA	NA	VS	Saillon
CCTC 2000540[8]	J	C	NA	NA	NA	NA	NA	IT	Verrès
CCTC 2010517[8]	F	C	NA	NA	NA	NA	NA	VS	Vernayaz
CCTC 9631[8]	M	C	NA	NA	NA	NA	NA	VS	Martigny
BAL02[9]	M	E	NA	NA	NA	NA	NA	SG	Widnau
BAL04[9]	M	E	NA	NA	NA	NA	NA	SG	Widnau
DIE02[9]	F	E	NA	NA	NA	NA	NA	SG	Diepoldsau
DUB01[9]	F	E	NA	NA	NA	NA	NA	ZH	Volketswil
DUB02[9]	M	E	NA	NA	NA	NA	NA	ZH	Volketswil
DUB03[9]	F	E	NA	NA	NA	NA	NA	ZH	Volketswil
DUB04[9]	F	E	NA	NA	NA	NA	NA	ZH	Volketswil
GIP03[9]	F	E	NA	NA	NA	NA	NA	AG	Leuggern
GIP04[9]	F	E	NA	NA	NA	NA	NA	AG	Leuggern
GIP05[9]	F	E	NA	NA	NA	NA	NA	AG	Leuggern
KGN0[9]	F	E	NA	NA	NA	NA	NA	AG	Böttstein
KLI00[9]	F	E	NA	NA	NA	NA	NA	AG	Klingnau
ROH01[9]	F	E	NA	NA	NA	NA	NA	AG	Aarau
ROH02[9]	M	E	NA	NA	NA	NA	NA	AG	Aarau
ROH04[9]	F	E	0.01	0.975	0.005	0.005	0.006	AG	Aarau

STA01[9]	F	E	0.011	0.951	0.007	0.011	0.019	ZH	Hombrechtikon
STA02[9]	M	E	NA	NA	NA	NA	NA	ZH	Hombrechtikon
ZHC01[9]	M	E	0.382	0.483	0.018	0.046	0.071	ZH	Zürich
ZHC02[9]	M	E	0.008	0.97	0.007	0.006	0.009	ZH	Zürich
ZHN01[9]	M	E	0.011	0.962	0.011	0.009	0.007	ZH	Zürich
ZHN02[9]	M	E	0.015	0.954	0.014	0.012	0.006	ZH	Zürich
AAD00[9]	F	3	0.021	0.941	0.011	0.007	0.02	TG	Aadorf
BAD01[9]	F	3	0.304	0.488	0.18	0.016	0.013	AG	Bad Zurzach
BAD02[9]	M	3	0.044	0.007	0.007	0.936	0.005	AG	Bad Zurzach
BAD06[9]	M	3	0.008	0.006	0.006	0.975	0.005	AG	Bad Zurzach
FCH02[9]	F	3	NA	NA	NA	NA	NA	ZH	Flaach
FCH04[9]	M	3	0.008	0.029	0.007	0.923	0.034	ZH	Flaach
KLO02[9]	M	3	NA	NA	NA	NA	NA	ZH	Rümlang
KLO07[9]	F	3	NA	NA	NA	NA	NA	ZH	Rümlang
MAR01[9]	M	3	NA	NA	NA	NA	NA	ZH	Rheinau
MAR02[9]	F	3	NA	NA	NA	NA	NA	ZH	Rheinau
NEB02[9]	F	3	NA	NA	NA	NA	NA	SG	St. Margrethen
NEB04[9]	M	3	0.028	0.012	0.023	0.926	0.01	SG	St. Margrethen
NEE03[9]	M	3	NA	NA	NA	NA	NA	ZH	Höri
NWN01[9]	M	3	NA	NA	NA	NA	NA	TG	Kemmental
NWN02[9]	F	3	NA	NA	NA	NA	NA	TG	Kemmental
OBE04[9]	M	3	0.061	0.015	0.056	0.841	0.026	SG	Oberuzwil
RIM00[9]	F	3	0.005	0.014	0.973	0.004	0.004	AG	Rietheim
SHF01[9]	F	3	0.009	0.018	0.005	0.961	0.007	SH	Schaffhausen
SHO02[9]	M	3	0.03	0.023	0.93	0.011	0.006	SH	Hallau
SHO03[9]	F	3	0.007	0.008	0.974	0.006	0.005	SH	Hallau
NN1017[10]	NA	C	NA	NA	NA	NA	NA	NA	NA
NN1041[10]	NA	7	0.005	0.007	0.006	0.007	0.975	NA	NA
NN1042[10]	NA	7	0.01	0.023	0.011	0.006	0.95	NA	NA
NN1049[10]	NA	C	NA	NA	NA	NA	NA	NA	NA
NN1053[10]	NA	C	0.046	0.838	0.007	0.008	0.1	NA	NA
NN1054[10]	NA	C	0.038	0.109	0.77	0.019	0.064	NA	NA
NN1055[10]	NA	NA	0.013	0.027	0.833	0.016	0.112	NA	NA
NN1056[10]	NA	E	0.788	0.021	0.006	0.155	0.03	NA	NA
NN1057[10]	NA	E	NA	NA	NA	NA	NA	NA	NA
NN1058[10]	NA	C	0.005	0.007	0.976	0.007	0.004	NA	NA
NN1060[10]	NA	C	0.005	0.008	0.977	0.005	0.005	NA	NA
NN1061[10]	NA	C	NA	NA	NA	NA	NA	NA	NA
NNB01[10]	NA	E	NA	NA	NA	NA	NA	NA	NA
NNB02[10]	NA	E	NA	NA	NA	NA	NA	NA	NA
NNB03[10]	NA	E	NA	NA	NA	NA	NA	NA	NA
NNB04[10]	NA	E	NA	NA	NA	NA	NA	NA	NA
NNB05[10]	NA	E	NA	NA	NA	NA	NA	NA	NA
NNB06[10]	NA	C	NA	NA	NA	NA	NA	NA	NA
NNB07[10]	NA	E	NA	NA	NA	NA	NA	NA	NA

S Table 2 Details of the primers used in this study for the mtDNA and their respective PCR conditions. Abbreviations: c = number of cycles, d = denaturing, a = annealing, e = extension, fe = final extension. (Chèvre 2015)

Gene fragment	Primer	Primer sequence (5' → 3')	Source reference	PCR conditions				
				c	d	a	e	fe
					45 sec	45 sec	60 sec	10 min
ND4+tRNAs	ND4ab	f - CACCTATGACTACCAAAAGCTCATGTAGAAGC	Guicking et al. (2006)	40	94°C	55°C	72°C	72°C
	tRNA-leu	r - CATTACTTTTACTTGGATTTGCACCA	Guicking et al. (2006)					
Cyt *b* (total)	L14724NAT	f - GACCTGCGGTCCGAAAAACCA	Guicking et al. (2006)	40	94°C	55°C	72°C	72°C
	Thrsnr2	r - CTTTGGTTTACAAGAACAATGCTTTA	Guicking et al. (2006)					

Y = C or T

R = A or G

S Table 3 Details of the 14 microsatellites used in this study and their respective PCR conditions. Abbreviations: N_A = number of alleles, m = multiplex-PCR, p = primer concentration (µM), c = number of cycles, id = initial denaturation, d = denaturing, a = annealing, e = extension, fe = final extension. Note that N_A belongs to Chèvre (2015), Nsµ2 is a separate multiplex-PCR but sequenced together with multiplex-PCR 2 and Nsµ3 was abandoned in this study. Table from Chèvre (2015).

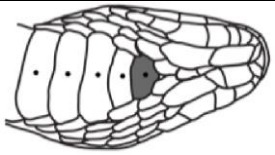
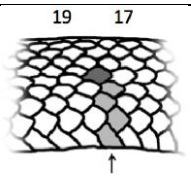
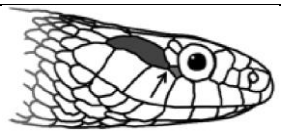
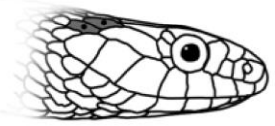
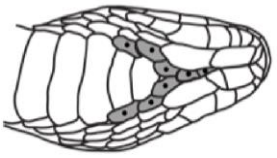
Locus	Primer sequence (5'→3')	Repeated motif	Range (bp)	N_A	Fluo. dye	Source reference	PCR conditions							
							m	p	c	id	d	a	e	fe
										5 min	30 sec	90 sec	30 sec	30 min
Natnat09	f-TGTAAATAACACTGTACCATTTTGG* r-TGACTGGGCAACAGAAAAGC	$(AC)_{22}$	96 - 146	16	VIC	Meister et al. (2009)	1	10	32	95°C	95°C	56°C	72°C	60°C
Natnat05	f-TCTGCACTGGGGATAGGAAG* r-GTCCCTTTTTCAGTGCTGTTG	$(GT)_{16}$	138 - 186	13	6-FAM	Meister et al. (2009)	1	10	32	95°C	95°C	56°C	72°C	60°C
µNt8new	f-GTATCGTCCTTCCAGACAAG* r-GCAAATCAAATAAATCTCACTGG	$(AC)_{15}$	82 - 104	7	PET	Gautschi et al. (2000), Meister et al. (2009)	1	10	32	95°C	95°C	55°C	72°C	60°C
µNt3	f-GGCAGGCTATTGGAGAAATG* r-GGCAAAACTCCAGGTGCTAC	$(AC)_{16}$	116 - 146	9	PET	Gautschi et al. (2000)	1	10	32	95°C	95°C	63°C	72°C	60°C
µNt7	f-TTTGAAAGGAGAATGAATCGTG* r-CGCGAGGAATCAGAATGAAC	$(AC)_{17}$	170 - 210	16	VIC	Gautschi et al. (2000)	1	5	32	95°C	95°C	58°C	72°C	60°C
Hb30	f-CCCACTGGCTCATTTCAAGT* r-CCACATTTGCATCGGAGTG	$(CA)_{14}$	238 - 264	12	6-FAM	Burns & Houlden. (1999)	1	10	32	95°C	95°C	59°C	72°C	60°C
Natnat11	f-GGCTGTTTTCCCAGTGAAGC* r-GGTCTGGGGAAAAAGAAAGG	$(GA)_{13}$	104 - 122	7	NED	Meister et al. (2009)	1	2.5	32	95°C	95°C	56°C	72°C	60°C
Natnat06	f-AATGGCATTCTCTCCAGCTC* r-ACCCATATCCGTATCCATATCC	$(GT)_{21}$	160 - 192	13	PET	Meister et al. (2009)	2	10	32	95°C	95°C	56°C	72°C	60°C
Eobµ1	f-ATCAGTAGGAGTGAGAGCAACT* r-CTGCATACTCTTCCAGAACC	$(TG)_{21}$	130 - 138	5	VIC	Blouin-Demers & Gibbs (2003)	2	10	32	95°C	95°C	55°C	72°C	60°C
Eobµ13	f-TGATCTGAGTCTCTTTCTGG* r-CAATTCAAATCCATTGGTTT	$(AC)_{20}$	136 - 164	9	6-FAM	Blouin-Demers & Gibbs (2003)	2	10	32	95°C	95°C	55°C	72°C	60°C
3TS	f-GGTCACTTAAATACAACGAAATTGGTTAGCT* r-CGGACAGCTCTGGCTCCCTTG	TAGA	198 - 268	15	VIC	Garner et al. (2002)	2	10	32	95°C	95°C	58°C	72°C	60°C
Tbu_A09	f-CATCTCAACCAAAGTCGCTTC* r-GGATGTTGTGGGGTGTTTTC	$(AC)_7$	110 - 138	12	NED	Sloss et al. (2012)	2	10	32	95°C	95°C	55°C	72°C	60°C
Nsµ2	f-TCCTCTTTGGCAGAGTAATAGT* r-AGCCGAGAACACACTAGTAAGT	$(AC)_{18}$	326 - 472	42	PET	Prosser et al. (1999)		10	37	95°C	95°C	55°C	72°C	60°C
Nsµ3	f-CTGACTCACTTCTGACCCTAAT* r-AATATTTGCTTGGCTCAAAC	$(ATCT)_{14}$ $AT(CA)_{20}$	(Abandon)		NED	Prosser et al. (1999)		10	37	95°C	95°C	51°C	72°C	60°C

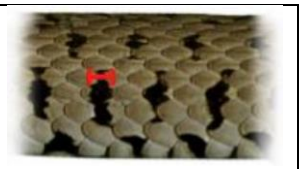
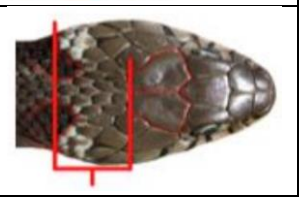
*Fluorescent-labelled primer

S Table 4 Composition of multiplex-PCR (MP) mixtures. Volumes are given in [µl]. Colours indicate fluorescent dyes. PCR conditions are as described in S Table 3 except for annealing temperatures (MP 1: 54°C; MP 2: 55°C; MP 4: 54°C).

Multiplex-PCR 1		Multiplex-PCR 2		Multiplex-PCR 4	
0.1	Natnat09-f	0.2	Natnat6-f	0.4	Nsµ2-f
0.1	Natnat09-r	0.2	Natnat6-r	0.4	Nsµ2-r
0.1	Natnat05-f	0.1	Eobµ1-f		
0.1	Natnat05-r	0.1	Eobµ1-r		
0.15	µN8new-f	0.4	Eobµ13-f		
0.15	µN8new-r	0.4	Eobµ13-r		
0.1	µNt3-f	0.1	Tbu-A09-f		
0.1	µNt3-r	0.1	Tbu-A09-r		
0.1	µNt7-f	0.2	Nsµ3-f		
0.1	µNt7-r	0.2	Nsµ3-r		
0.6	HB30-f	0.3	3TS-f		
0.6	HB30-r	0.3	3TS-r		
0.1	Natnat11-f				
0.1	Natnat11-r				
0.5	H20	0.4	H20	2.2	H20
5	Mastermix	5	Mastermix	5	Mastermix
2	DNA	2	DNA	2	DNA
10	Total	10	Total	10	Total

S Table 5 Description of morphological variables measured. Illustrations from Chèvre (2015).

Variable	Code	Notes	Unit	Illustration
Snout-vent length	SVL	From tip of snout to cloaca. Snake was hold next to a ruler to measure	cm	
Tail length	TL	From cloaca to end of tail; only for intact tail. Snake was hold next to a ruler to measure	cm	
Body weight	BW	Using a kitchen scales (precision: ± 0.5 g). Not for museum specimens	g	
Head length	HL	From tip of snout to inflexion point at the end of the mandible. Mean of lengths to left and right mandible	cm	
Head width	HW	Distance between posterior tips of last supralabial scale	cm	
Number of ventral scales	VS	Ventral scales are wider than long, from head to cloaca		
Relative position of the reduction from 19 to 17 dorsal scale rows	RelRedPos	% of the number of ventral scales to the position of reduction (starting from the head) from the total number of ventral scales		
Number of paired sub-caudal scales	SCS	From cloaca to tip of tail		
Number of contacts between temporal and lower post-ocular scale	TPOS	Sum of both sides -> 0, 1 or 2		
Number of post-temporal scales	PTS	Sum of both sides		
Number of gular scales	GS			
Lateral blotches size	LBS	Number of scales filled by the blotch		
Lateral blotches length	LBL	Number of scale widths (NOT number of scale rows)		

Lateral blotches width	LBW	Number of scale lengths (NOT number of scale rows)		
Nuchal marking size	NMS	Number of scales filled by the marking		
Nuchal marking width	NMW	Number of scale lengths at widest point (NOT number of scale rows)		
Upper curvature of nuchal marking	NMUC	Number of scales exceeding midpoint of marking (dorsal)		
Lower curvature of nuchal marking	NMLC	Number of scales exceeding midpoint of marking (ventral)		
Distance between nuchal marking and parietal scales	NMPS	Number of scale lengths between midpoint of nuchal marking and posterior tips of parietal scales		

S Table 6 Pairwise F_{ST} values between microsatellite clusters of *Natrix helvetica* (E-GE, E-ML, C-VS and C-TI) and *Natrix natrix* (7). Values were calculated with FSTAT (Goudet 1995) version 2.9.3 (Goudet 2001) including only individuals with a microsatellite assignment to one cluster ≥ 80%.

E-GE	E-ML	C-VS	C-TI	7	
0.000					E-GE
0.150	0.000				E-ML
0.282	0.180	0.000			C-VS
0.197	0.196	0.197	0.000		C-TI
0.199	0.139	0.188	0.194	0.000	7

S Table 7 Mean and standard deviation (SD) of different morphological traits for Swiss grass snakes of mtDNA lineage E and C (Kindler et al. 2013). More information on the variables is found in S Table 4.

Variable	Mean E	SD E	Mean C	SD C
Snout-vent length [cm]	60.81	± 16.30	64.00	± 14.20
Tail length [cm]	14.03	± 2.79	15.70	± 2.75
Body weight [g]	139.40	± 118.10	178.00	± 128.54
Head length [cm]	2.80	± 0.71	2.92	± 0.72
Head width [cm]	1.57	± 0.48	1.55	± 0.43
Number of ventral scales	172.66	± 4.96	173.83	± 3.69
Relative position of the reduction from 19 to 17 dorsal scale rows	0.53	± 0.04	0.52	± 0.08
Number of paired subcaudal scales	60.44	± 6.49	64.68	± 8.68
Number of contacts between temporal and lower post-ocular scale	1.52	± 0.80	1.58	± 0.72
Number of post-temporal scales	4.66	± 1.04	4.75	± 0.90
Number of gular scales	8.19	± 1.42	7.83	± 0.82
Lateral blotches size	1.37	± 0.66	1.57	± 0.71
Lateral blotches length	2.33	± 1.15	2.58	± 0.81
Lateral blotches width	0.66	± 0.16	0.62	± 0.24
Nuchal marking size	26.35	± 4.26	24.98	± 4.45
Nuchal marking width	4.11	± 0.49	3.87	± 0.50
Upper curvature of nuchal marking	0.94	± 0.41	0.73	± 0.34
Lower curvature of nuchal marking	1.27	± 0.34	1.34	± 0.38
Distance between nuchal marking and parietal scales	3.44	± 0.42	3.54	± 0.52