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Hybridization patterns in two contact zones of grass snakes reveal a new Central European snake species

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Recent studies found major conflicts between traditional taxonomy and genetic differentiation of grass snakes and identified previously unknown secondary contact zones. Until now, little is known about gene flow across these contact zones. Using two mitochondrial markers and 13 microsatellite loci, we examined two contact zones. One, largely corresponding to the Rhine region, involves the western subspecies *Natrix natrix helvetica* and the eastern subspecies *N. n. natrix*, whereas in the other, more easterly, contact zone two lineages meet that are currently identified with *N. n. natrix* and *N. n. persa*. This second contact zone runs across Central Europe to the southern Balkans. Our analyses reveal that the western contact zone is narrow, with parapatrically distributed mitochondrial lineages and limited, largely unidirectional nuclear gene flow. In contrast, the eastern contact zone is very wide, with massive nuclear admixture and broadly overlapping mitochondrial lineages. In combination with additional lines of evidence (morphology, phylogeny, divergence times), we conclude that these differences reflect different stages in the speciation process and that *Natrix helvetica* should be regarded as a distinct species. We suggest a nomenclatural framework for presently recognized grass snake taxa and highlight the need for reconciling the conflicts between genetics and taxonomy.

Even though species delimitation became a Renaissance issue in zoology, with new approaches being developed for assessing species boundaries^{1–5}, the validity of many approaches largely depends on the underlying species concept. Currently, there are more than 30 species concepts distinguished, with an ever increasing number⁶. While the application of different concepts lead to a taxonomic inflation for some regions and some groups, the number of Central European vertebrate species remained generally stable, suggesting their diversity is well understood. An exception to that rule might be bats^{7,8}. With respect to Central European snakes, the number of recognized species did not change for more than a century^{9–15}, even though some southern European taxa, like *Elaphe sauromates*¹⁶, *Macroprotodon brevis* and *M. mauritanicus*^{17,18}, *Malpolon insignitus*¹⁹, *Natrix astreptophora*²⁰, *Vipera graeca*²¹, and *Zamenis lineatus*²², have been elevated to species status within the last two decades and a new species of viper (*Vipera walser*) has been recently discovered in the Alps²³.

Grass snakes (*Natrix natrix* sensu lato) are the most abundant and one of the most widely distributed snake species of the Palaearctic region^{24,25}. For a long time, little was known about their genetic and phylogeographic structuring. Based on external morphology, many subspecies have traditionally been recognized^{25,26}, suggestive of pronounced phylogeographic structuring. Indeed, in a pioneering nearly range-wide study using mitochondrial DNA (mtDNA), not less than 16 distinct genetic lineages were identified²⁶. However, the majority of these lineages conflicts with previously recognized subspecies and only two lineages match with morphologically defined taxa. In phylogenetic analyses, these lineages correspond to 16 terminal clades that cluster in three major

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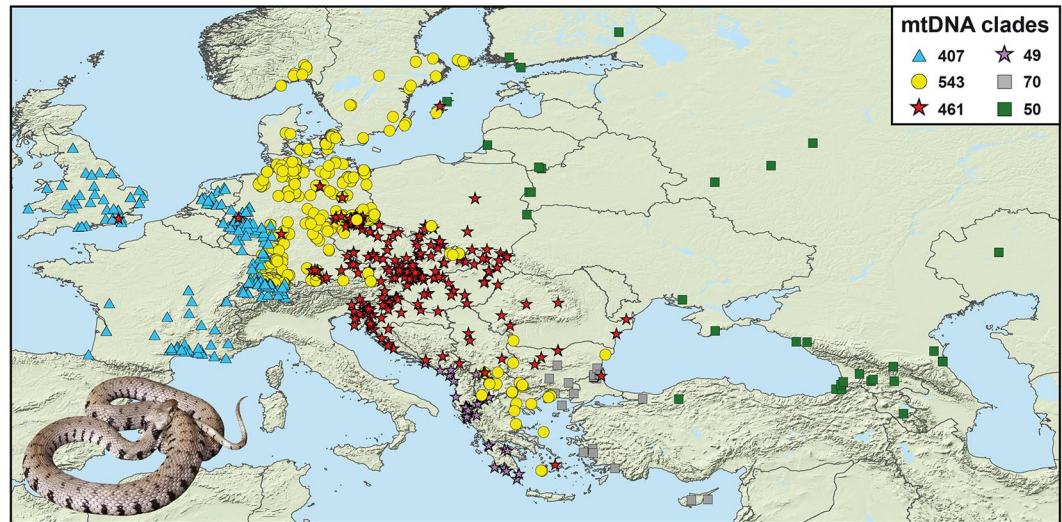


Figure 1. Distribution of mitochondrial lineages of 1,580 grass snakes used in this study. Total sample size of each clade shown in the legend. Eight allochthonous grass snakes with haplotypes of Italian lineages caught in southern Great Britain and Hesse, Germany, not shown. Map was created using ARCGIS 10.2 (<http://www.esri.com/arcgis>) and ADOBE ILLUSTRATOR CS6 (<http://www.adobe.com/products/illustrator.html>). Inset: *Natrix natrix helvetica* (Linz am Rhein, Germany); photo: Wolfgang Böhme.

clades (Supplementary Fig. S1). One of these major clades matches with the Ibero-Maghrebian taxon *astreptophora*, which had traditionally been recognized as a subspecies of *N. natrix*. In the face of virtually lacking gene flow with the geographically neighbouring taxon (*N. n. helvetica*) and concordant morphological and genetic evidence, Ibero-Maghrebian grass snakes have recently been split off as the distinct species *N. astreptophora*²⁰.

In agreement with earlier morphological investigations²⁷, the recent phylogeographic assessment of grass snakes²⁶ revealed a contact zone of two deeply divergent mitochondrial lineages for the Rhine region, with an unexpectedly clear-cut parapatric distribution pattern. The involved lineages match with what is currently identified with *N. n. helvetica* and *N. n. natrix*. Another unexpected discovery was that the distribution ranges of *N. n. natrix* in Central Europe and *N. n. persa* in the Balkan Peninsula comprise a previously unknown, more easterly located, contact zone of two distinct lineages, which conflict with morphological taxon delimitation and occur across the distribution ranges of *N. n. natrix* and *N. n. persa*²⁶. In contrast to the situation in the Rhine region, the haplotypes of these two eastern lineages occur in wide sympatry and represent lineages that are placed in phylogenetic analyses in the same major clade, while the mitochondrial lineage of *N. n. helvetica* belongs to another one of the three major clades (Supplementary Fig. S1). According to molecular clock calculations²⁸, the clade containing *N. n. helvetica* diverged from the eastern lineages 7.3–8.2 million years ago, while the two eastern lineages are with 5.1–5.9 million years significantly younger.

The present study aims at examining and comparing differentiation and gene flow across the two contact zones of genetic lineages of different age and phylogenetic hierarchy. For doing so, the previous sampling²⁶ was increased fourfold and mitochondrial DNA data (1,983 bp) were combined with evidence from 13 highly polymorphic nuclear microsatellite loci.

Materials and Methods

Sampling and laboratory procedures. The focus of the present study lies on two contact zones of distinct genetic lineages of grass snakes (Fig. 1), one in the Rhine region, involving *Natrix natrix helvetica* ('blue lineage') and the nominotypical subspecies ('yellow lineage') and another contact zone further in the east, which was identified for the first time in a previous study²⁶. This second contact zone runs across Central Europe to the southern Balkans and concerns two genetic lineages ('red' and 'yellow lineages'), which occur within the distribution ranges of *N. n. natrix* and *N. n. persa*. However, neither of these subspecies is congruent with the two genetic lineages, and both lineages occur within the range of either taxon and beyond, suggesting that a taxonomic revision is required²⁶. Only one of these lineages, the 'yellow lineage', occurs naturally in the Rhine region, i.e. in the contact zone with *N. n. helvetica*.

In total, 1,603 samples (shed skins, saliva samples, tissues from roadkills and museum specimens) were used in the present study. No snakes were sacrificed for the present study. All sampling and methods were carried out in accordance with relevant guidelines, regulations and best ethical and experimental practice of the Senckenberg Nature Research Society. Two mitochondrial DNA fragments (partial ND4 gene plus adjacent DNA coding for tRNAs = ND4 + tRNAs, below termed for simplicity ND4, and cytochrome *b* gene = cyt *b*) were sequenced (866 bp and 1,117 bp, respectively). Mitochondrial sequences of 391 specimens were available from previous studies^{20, 26, 29} and merged with new data for 1,197 grass snakes. For 15 samples, mtDNA could not be sequenced. In addition, samples were genotyped at 13 polymorphic nuclear microsatellite loci. Laboratory procedures for mtDNA and microsatellites followed previous studies^{20, 26}. The microsatellite data of 1,484 samples included those

for 31 grass snakes from a previous study²⁰. For 119 samples, for which mtDNA sequences were available, no microsatellite data could be generated. Approximately 200 samples from Switzerland were studied in the laboratory of the University of Basel, whereas the majority of samples was processed in the laboratory of Senckenberg Dresden. Fragment lengths of the two data sets were calibrated using 31 samples processed in both laboratories. For detailed sample information, see Supplementary Table S1.

Mitochondrial sequence analyses and networks. Mitochondrial sequences were aligned using BIOEDIT 7.0.9.0³⁰, resulting in an 866-bp-long alignment of 1,550 ND4 sequences and an 1,117-bp-long alignment of 1,313 *cyt b* sequences. The mitochondrial lineage of each new sample was identified by running exploratory Maximum Likelihood (ML) analyses using RAXML 7.2.8³¹ including previously published data^{20,26,29}, the GTR + G model and a fast ML search with 100 bootstrap values. Then, for the lineages involved in the studied contact zones, alignments of each mtDNA block were examined using POPART (<http://popart.otago.ac.nz>) and the implemented parsimony network algorithm of TCS³². Based on haplotypes, uncorrected *p* distances (means) were calculated using MEGA 7.0.21³³ and the pairwise deletion option.

Genetic cluster analysis, inferring hybrid status and PCA. All 13 microsatellite loci were tested for Hardy-Weinberg equilibrium (HWE) and linkage equilibrium using ARLEQUIN 3.5.1.2³⁴. The presence of null alleles was examined using MICRO-CHECKER 2.2.3³⁵. There was no evidence for linkage disequilibrium, null alleles or a deviation from HWE. Microsatellite data were then analyzed with the unsupervised Bayesian clustering approach of STRUCTURE 2.3.4^{36,37} using the admixture model and correlated allele frequencies. STRUCTURE searches in the data set for partitions that are, as far as possible, in linkage equilibrium and HWE. The Monte Carlo Markov chains ran for 1 million generations, including a burn-in of 250,000 generations. Calculations were repeated ten times for *K*s ranging from 1 to 10. The optimal number of clusters was determined using the ΔK method³⁸ with the software STRUCTURE HARVESTER³⁹. STRUCTURE results were visualized using DISTRUCT 1.1⁴⁰.

In the southern Balkans and the Baltic Sea region, additional genetic lineages occur^{26,29}. These lineages are expected to contribute to nuclear genomic admixture, which is why a stepwise approach was applied to assess their genetic impact and to single out the extent of admixture between the red and yellow lineages. This approach takes into account that STRUCTURE is known to identify only the uppermost hierarchical level of genetic partitioning³⁸, i.e. the clusters reflect the most differentiated genetic units. Accordingly, a first calculation comprising all available samples resulted in two clusters, one corresponding to *helvetica* (blue lineage) and the other to all other lineages.

To explore which individuals represent *helvetica* hybrids, hybrid genotypes were modelled using HYBRIDLAB 1.0⁴¹. For doing so, 20 non-admixed representatives of each lineage occurring in the Rhine contact zone were selected (blue and yellow lineages) as pure parental genotypes. Using these data, 20 genotypes of each hybrid class (F_1 , F_2 and two backcrosses) were inferred and the simulated hybrid data were then subjected to STRUCTURE analyses, together with the data of the 20 pure grass snakes of each cluster, to obtain a threshold for *Q* values for distinguishing pure animals, hybrids and backcrosses. Based on this threshold, all genotypes with genetic impact of *helvetica* were removed and a second STRUCTURE run included then only the remaining samples. With this run, admixture of the red and yellow lineages with the other geographically neighbouring lineages (lilac, grey, and green lineages; Fig. 1) was explored. Then, all samples with an impact from other lineages > 5% were eliminated and the remaining data (only red and yellow genotypes and their hybrids) were subjected to another STRUCTURE run. This data set was also examined using HYBRIDLAB to distinguish pure and hybrid samples. For this HYBRIDLAB run, samples from geographically distant populations were selected that originated in regions where either only the red or the yellow lineage occurs (Scandinavia vs. Austria and Slovakia).

In addition, for the microsatellite data of the STRUCTURE clusters, population genetic diversity values, pairwise F_{ST} values and Analyses of Molecular Variance (AMOVAs) were calculated using CONVERT 1.31⁴², ARLEQUIN³⁴ and FSTAT 2.9.3.2⁴³. Admixed individuals were excluded.

Microsatellite data were also examined using Principal Component Analyses (PCA) as implemented in the R package ADEGENET⁴⁴ to assess the distinctiveness of the genetic lineages without underlying population genetic presumptions. Two different PCAs were run, one including genotypes of all samples of the blue, red and yellow lineages and their hybrids and another one with samples of the yellow and red lineage without influence of *helvetica* and the adjacent eastern lineages. Obviously introduced individuals were excluded.

Cline analyses. To examine gene flow across contact zones, cline analyses were calculated for microsatellite and mtDNA data using the R package HZAR⁴⁵. This software fits molecular genetic data to classical equilibrium cline models using the Metropolis-Hastings Markov chain Monte Carlo algorithm. Cline fitting was performed by adding geographical information of sampling sites to genetic information. To acknowledge for mountain ranges, two transects were selected. The transect for the contact zone in the Rhine region ran from southern France to northeastern Germany (1,200 km) and the transect for the eastern contact zone, from northern Germany to northern Hungary (1,000 km). The eastern transect was not extended to the southern Balkans because the grass snakes are there genetically impacted by other genetic lineages. Using ARCGIS 10.2 (<http://www.esri.com/arcgis>), samples were arbitrarily pooled by dividing each transect into segments of 10 km length. Samples within 50 km left and right of each segment were assigned to one sampling unit.

For microsatellite data, the mean proportion *Q* of cluster membership (as inferred by STRUCTURE) for each pooled collection site was then calculated. For mitochondrial data, the frequency of haplotypes of the blue lineage (western contact zone) or the yellow lineage (eastern contact zone) was used. These four data sets (mtDNA and microsatellites for each contact zone) were processed independently. Using a burn-in of 10,000 iterations, followed by additional 90,000 iterations, fifteen implemented models were fitted to the mean proportions of cluster membership or haplotype frequencies. The best cline model was then selected based on the lowest AIC score

(Supplementary Table S2) and the corresponding Maximum Likelihood clines. Observed frequency data were plotted over the associated fuzzy cline regions (95% credible cline regions).

Results

Mitochondrial phylogeography and haplotypes. Our fourfold sampling (Fig. 1 and Supplementary Fig. S2) confirmed and refined previous findings²⁶, in particular that the blue lineage (*Natrix natrix helvetica*) meets with the yellow lineage (currently identified with *N. n. natrix*) in a secondary contact zone in the Rhine region, and that another, more easterly and much wider, contact zone of the yellow and red lineages runs across Central Europe to the Balkans. The latter two lineages are distributed within the ranges of what is currently identified with the grass snake subspecies *N. n. natrix* and *N. n. persa*, but these lineages conflict with morphology-based taxonomy so that neither the distribution ranges of the lineages and subspecies nor morphology and genetics are congruent²⁶. Rather, each subspecies corresponds to several distinct mitochondrial lineages and some of these lineages are shared between the two subspecies. Thus, there is obviously a pronounced conflict between morphological variation and genetics. Whereas the geographical distribution of haplotypes of the blue and yellow lineages abuts, with virtually no sympatric occurrences (Supplementary Fig. S2), haplotypes of the yellow and red lineages widely overlap in Central Europe (Fig. 1), in a region corresponding to central, eastern and southern Germany, southern Poland, Austria, the Czech Republic, and Slovakia. However, further southeastwards, only haplotypes of the red lineage occur and in the southern Balkans, only haplotypes of the yellow lineage are recorded (Fig. 1).

While the vast majority of our 1,588 samples represents native grass snakes, there are some obvious cases of translocated individuals, like a few isolated records of the red lineage within the range of *N. n. helvetica* (Great Britain, Rhine region). One population in the Neander valley close to Düsseldorf, Germany, seems to consist exclusively of such allochthonous grass snakes and has been suspected to be introduced for a long time^{46, 47}. In addition to these non-native grass snakes, eight individuals of other mitochondrial lineages from the Mediterranean (Italy) were found in southern Great Britain and Hesse, Germany (not shown in Fig. 1). We cannot completely exclude that also our new record of a third mitochondrial lineage on the island of Gotland (green lineage; Fig. 1) refers to an introduced grass snake. However, when it is considered that grass snakes colonized Gotland via transoceanic dispersal²⁹ and that now all three lineages occurring all around the Baltic Sea have been recorded from Gotland, their natural occurrence seems possible there.

In parsimony network analyses, each lineage corresponded to a highly distinct haplotype cluster. For ND4 (Fig. 2, top), there are 12 haplotypes in the blue lineage, 43 haplotypes in the yellow lineage, and 33 haplotypes in the red lineage. Haplotypes of the yellow and red lineages differed by a minimum of 40 mutational steps. Haplotypes of the blue lineage (*N. n. helvetica*) were separated from the yellow haplotype cluster by at least 40 mutational steps and from the red cluster by a minimum of 54 steps. With only 12 haplotypes differing by maximally two mutation steps, there was distinctly less variation in the blue lineage compared to the two eastern lineages. In the yellow lineage, 43 haplotypes with maximally 15 mutations occurred; in the red lineage, 33 haplotypes differing by maximally 10 mutations.

For *cyt b* (Fig. 2, bottom) a similar pattern emerged. The blue lineage differed by a minimum of 73 mutations from the yellow lineage and by 64 mutations from the red lineage. The yellow and red haplotype clusters were connected by a minimum of 53 mutational steps. Compared to ND4, there was distinctly more variation observed, with 29 haplotypes in the blue lineage and 66 haplotypes in the red lineage. The yellow lineage comprised 42 haplotypes. Haplotypes of the blue lineage differed by a maximum of five mutations, haplotypes of the yellow lineage by a maximum of nine mutations and haplotypes of the red cluster by a maximum of 16 mutations. European Nucleotide Archive (ENA) accession numbers for haplotypes are listed in Supplementary Table S3.

For ND4, the blue lineage differed on average from the yellow lineage by an uncorrected *p* distance of 5.03% and from the red lineage, by 5.88%; the divergence between the yellow and red lineages was 5.16%. For *cyt b*, mean *p* distances between the blue, yellow and red lineages were 6.90% and 6.39%, respectively; the yellow and red lineages differed by 5.48%.

Genotyping and admixture. The 13 studied microsatellite loci were highly polymorphic, with allele numbers ranging from 12 to 39 per locus (Supplementary Table S4) and a total allele number of 285. For the complete data set including *Natrix natrix helvetica* and all five eastern lineages, the ΔK method suggested two as the optimal number of clusters (Supplementary Fig. S3a). One of these clusters represented *helvetica*, and the other contained all eastern lineages (Fig. 3a). The assignment of the eastern lineages to only one cluster matched with the close phylogenetic relationship of their mtDNA lineages²⁶. According to an AMOVA using microsatellite data, 59.84% of the molecular variance occurred within and 40.16% between the two clusters, corresponding to an F_{ST} value of 0.40. The high distinctiveness of both clusters was also supported by a large number of private alleles, especially for the eastern cluster (Supplementary Table S5).

In general, the *helvetica* cluster matched well with the mitochondrial clade (Fig. 3). Genotypic introgression occurs largely unidirectional from *helvetica* into the eastern cluster. Most hybrids between *helvetica* and the eastern cluster originate from northern Switzerland, where the sampling is very comprehensive and dense, with approximately 200 samples from the contact zone. However, this dense sampling also revealed that the contact zone is narrow, with hybrid signatures occurring only in a maximally 50-km-wide strip (Fig. 3a, right). The allochthonous population of grass snakes of the red lineage in the Neander valley is clearly assigned to the eastern cluster, in agreement with mitochondrial haplotypes (Fig. 3a, left), and without admixture with neighbouring *helvetica* populations.

The second STRUCTURE run (Fig. 3b) included the eastern lineages without impact of *helvetica*. Based on the HYBRIDLAB results (Supplementary Table S6), only samples with an eastern cluster membership of at least 95% were considered in that analysis, for which $K=2$ was again the best solution (Supplementary Fig. S3b).

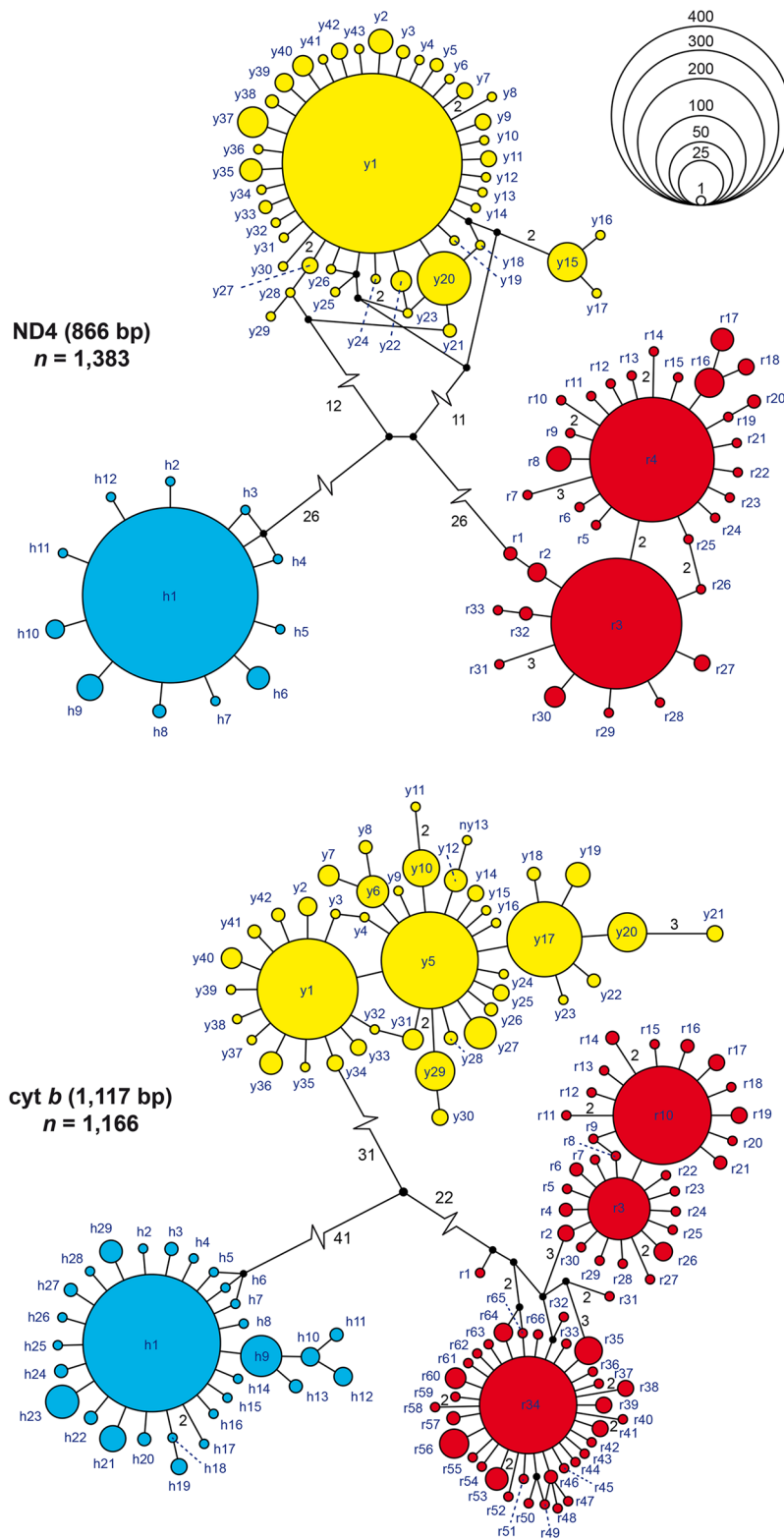


Figure 2. Parsimony networks of mtDNA sequences. Symbol sizes reflect haplotype frequencies. Small black circles are missing node haplotypes; each line connecting two haplotypes corresponds to one mutation step, if not otherwise indicated by numbers. Haplotype colours correspond to lineages, i.e. *Natrix natrix helvetica* (h) in blue; eastern lineages in yellow (y) and in red (r).

One cluster (pink) embraced the yellow and red lineages and another one (brown) the grey, lilac and green lineages. Many southern samples of the yellow and red lineages, especially from Slovenia, Croatia and the southern

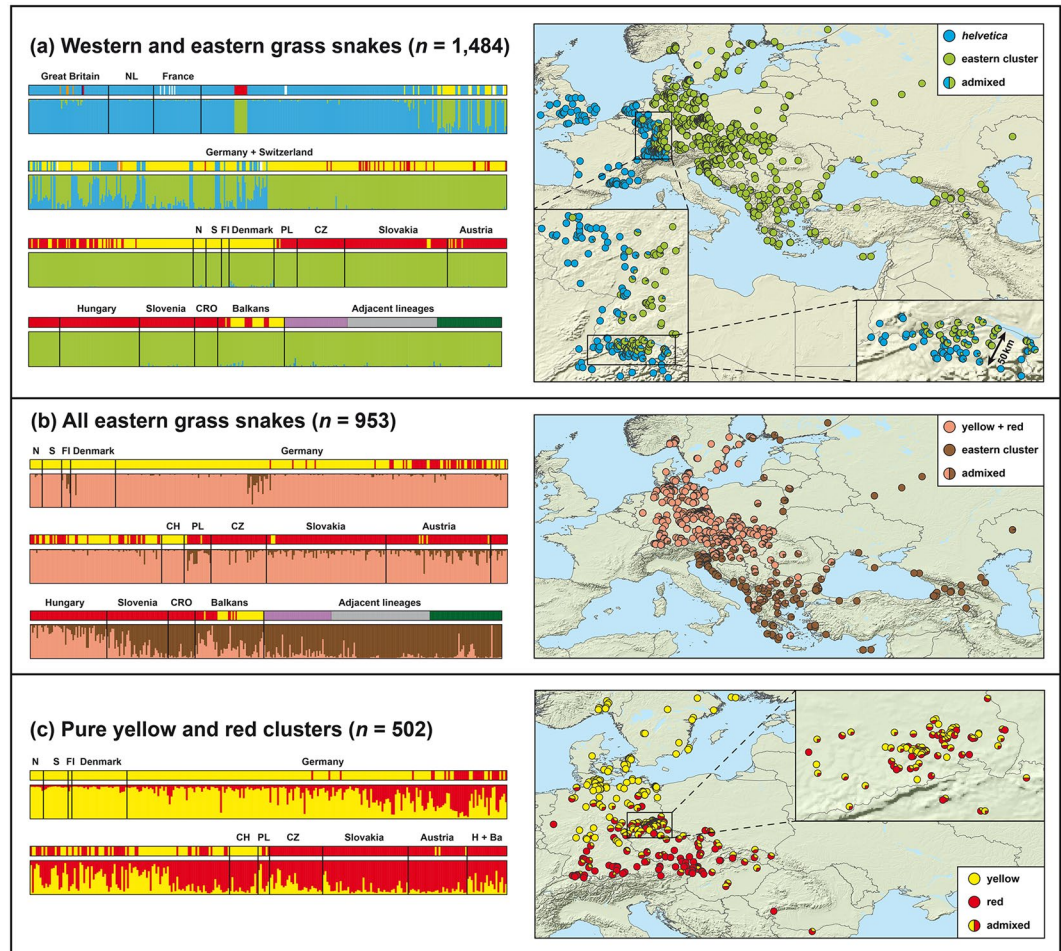


Figure 3. Genotypic structuring of grass snakes. On the left, the mitochondrial lineage of each sample is shown above the STRUCTURE diagrams, with haplotypes of *Natrix natrix helvetica* indicated in blue and haplotypes of the eastern lineages in colours corresponding to Fig. 1 (yellow, red, lilac, grey, green; white = missing data). In (a), orange and dark blue corresponds to non-native snakes (Italian lineages). Samples in STRUCTURE diagrams are arranged within each country from west to east (a) or from north to south (b,c). In STRUCTURE diagrams, an individual sample is represented by a vertical bar reflecting its inferred ancestry. In (a), the blue cluster corresponds to *N. n. helvetica* and the light green cluster to all other lineages. The isolated red/light green block (first row) represents the allochthonous population from the Neander valley, Germany. In (b), samples with genetic impact of *helvetica* are excluded. The pink cluster corresponds to samples from the yellow and red lineages. Brown percentages indicate genetic impact of adjacent lineages (lilac, grey, green). In (c) only samples from the yellow and red lineages and their hybrids, without genetic signatures of other lineages, were processed. Country abbreviations: Ba – Balkans (Albania, Bosnia and Herzegovina, Montenegro, Serbia, Kosovo, Former Yugoslav Republic of Macedonia, Romania, Bulgaria, and Greece), CH – Switzerland, CRO – Croatia, CZ – Czech Republic, FI – Finland, H – Hungary, N – Norway, NL – Netherlands, PL – Poland, S – Sweden. Maps were created using ARCGIS 10.2 (<http://www.esri.com/arcgis>) and ADOBE ILLUSTRATOR CS6 (<http://www.adobe.com/products/illustrator.html>).

Balkans, showed a high degree of admixture with the brown cluster. This admixture area largely corresponds to those regions where only haplotypes of the yellow and red lineages are present.

Samples with admixed ancestry with the brown cluster >5% were excluded from the third STRUCTURE analysis (Fig. 3c), for which the optimal number of clusters was again $K = 2$ (Supplementary Fig. S3c). Now, one cluster corresponded to the yellow and the other to the red lineage. According to the HYBRIDLAB results (Supplementary Table S6), grass snakes with at least 80% cluster membership were treated as ‘pure yellow’ and with at least 83% as ‘pure red’. Introgression was common in both directions, indicating massive gene flow across hundreds of kilometres (Fig. 3c, right), including regions where exclusively or predominantly mitochondrial haplotypes of one lineage are present. For the yellow and red clusters, 89.18% of the molecular variance occurred within, and only 10.82% between the two clusters, equalling an F_{ST} value of 0.11 (Supplementary Table S5).

The PCAs using microsatellite data (Fig. 4) are in line with the STRUCTURE analyses in that the blue lineage (*N. n. helvetica*) was highly distinct from the yellow and red lineages, with some mismatches reflecting mitochondrial introgression mainly from *helvetica* into the eastern group. In contrast, the eastern lineages showed weak

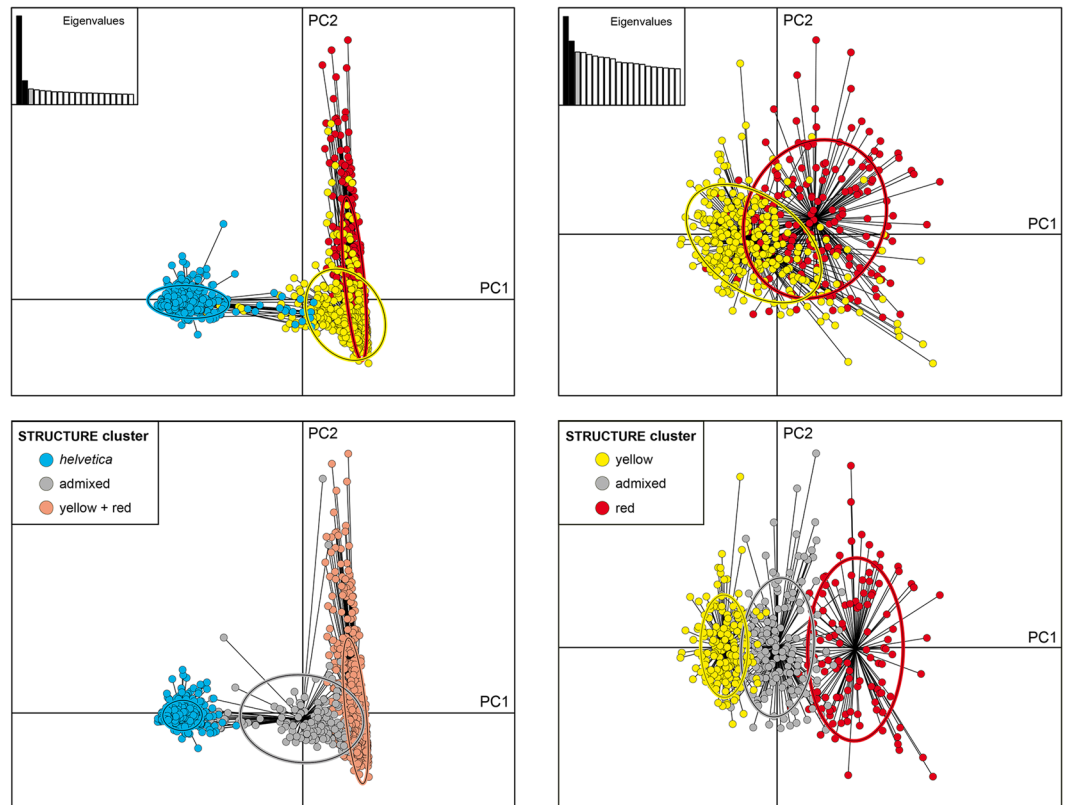


Figure 4. PCA axes 1–2 for microsatellite data. Samples are coloured according to mitochondrial lineages (top) or STRUCTURE clusters (bottom). Admixed individuals were identified according to HYBRIDLAB results. PCAs for the yellow and red lineages correspond to the samples from Fig. 3c. Non-native samples were excluded. The oval outlines represent 95% confidential intervals. For *helvetica* and the eastern lineages (left) the x axis explains 16.6% and the y axis 4.5% of variation. For the eastern lineages (right) the x axis explains 3.8% and the y axis 2.9% of variation. Analyses along axes 1–3 produced nearly identical results (see Supplementary Fig. S4).

differentiation and massive overlap. The PCAs corroborate furthermore that our definition of admixed individuals is appropriate because hybrids were intermediate also in the PCA, independently from population affiliation, HWE or linkage equilibrium.

Cline analyses. The cline analyses revealed completely different patterns in the two contact zones (Fig. 5). For the western contact zone (contact zone I), the cline is concordant and very steep for both marker systems. For microsatellites, the cline centre was estimated to be located 639.9 km (95% confidence interval: 635.1–644.2 km) north-east from the starting point in southern France with a cline width of 39.4 km (24.4–59.6 km). For mitochondrial data, the cline centre was revealed almost at the same point, at 637.2 km (632.9–641.2 km), with a similar cline width of 37.5 km (27.3–53.6 km). A much smoother cline was found for the contact zone of the yellow and red lineages (contact zone II). The cline centre for microsatellites was located 518.7 km (454.5–590.4 km) distant from the reference site in northern Germany with a considerable cline width of 677.1 km (404.5–1,009.6 km). The width for the mtDNA cline was with 358.3 km (261.5–489.7 km) approximately half as wide as the microsatellite cline. The location of the centre was nearly identical at 503.8 km (474.6–537.0 km) from the reference site.

Discussion

Hybrid zones are regions in which genetically distinct populations meet and produce hybrid offspring⁴⁸. They can be interpreted as ‘windows on the evolutionary process’⁴⁹ and ‘natural laboratories for evolutionary studies’⁵⁰. Hybridization can provide important insights into divergence and speciation processes^{50–56} and, thus, contributes to a better understanding of evolution. Based on extensive sampling of approximately 1,600 grass snakes and using a powerful data set of 13 microsatellite loci and two mitochondrial markers, this study presents a fine-scale analysis of gene flow across two secondary contact zones of grass snake lineages.

There are significantly different patterns of gene flow. While gene flow in the contact zone of *N. n. helvetica* and the ‘yellow lineage’ is limited and largely unidirectional from *helvetica* into the ‘yellow lineage’, with a cline width of less than 50 km in the contact zone, gene flow in the contact zone of the ‘yellow’ and ‘red lineages’ is extensive and in both directions. This contact zone is wide (cline widths for microsatellites and mtDNA approx. 680 km and 360 km), and the ‘yellow’ and ‘red lineages’ seem to be panmictic there (Fig. 3).

Our data provide evidence for hybridization in the contact zone of *N. n. helvetica* and the yellow lineage. The extent of admixture there clearly exceeds the negligible gene flow between *helvetica* and *N. astreptophora*

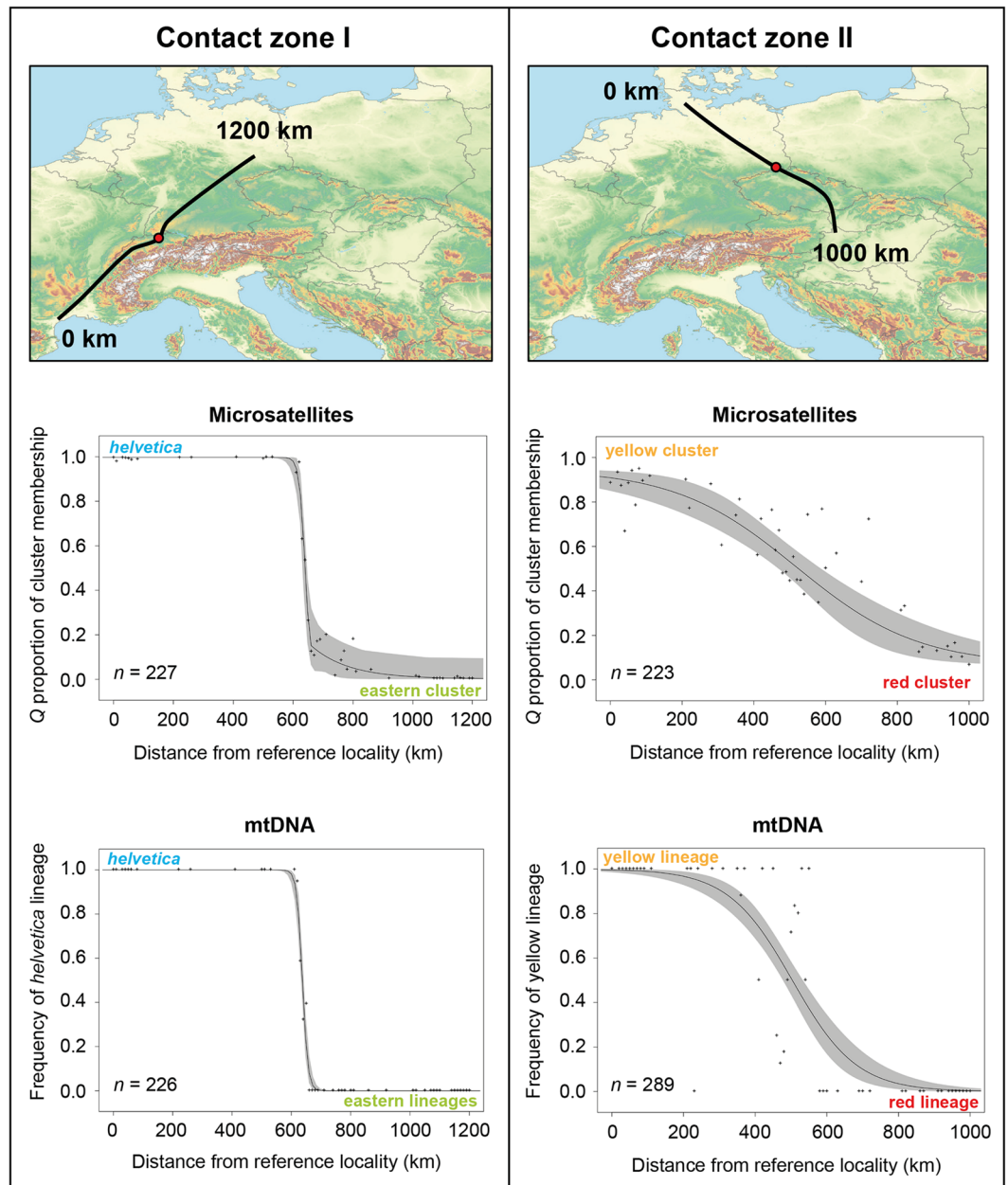


Figure 5. Cline analyses of mitochondrial DNA and microsatellite data. Transects (top) through the two different contact zones of grass snake lineages (*helvetica*/eastern lineages – left; yellow/red lineages – right) and associated Maximum Likelihood clines for microsatellites (centre) and mtDNA (bottom). Grey: fuzzy 95% credible cline region. Red points (top) indicate cline centres. Maps were created using ARCGIS 10.2 (<http://www.esri.com/arcgis>) and ADOBE ILLUSTRATOR CS6 (<http://www.adobe.com/products/illustrator.html>).

in southwestern France. The latter taxon is now regarded as a distinct species²⁰. Nevertheless, there also seems to be a barrier against gene flow between *helvetica* and eastern grass snakes. Their contact zone is characterized by steep clines for both marker systems (Fig. 5, left). In addition, the two clusters are separated by a high F_{ST} value of 0.40 for microsatellites. Due to the parapatric distribution of mitochondrial haplotypes along the contact zone (Supplementary Fig. S2) combined with mainly unidirectional genotypic introgression (Fig. 3), it can be concluded that gene flow is mainly mediated by *helvetica* males. Asymmetrical introgression is not unusual in hybridizing taxa⁵⁷. According to our STRUCTURE and HYBRIDLAB analyses, F_1 hybrids between *helvetica* and eastern grass snakes are rare. Parental genotypes in the contact zone, together with backcrosses, correspond to a bimodal hybrid zone and an advanced speciation process⁵⁸. Other examples for such bimodal hybrid zones in Europe include, for instance, fire-bellied toads (*Bombina bombina*, *B. variegata*^{59,60}), crested and marbled newts (*Triturus cristatus*, *T. marmoratus*^{61,62}; *T. carnifex*, *T. cristatus*, *T. dobrogicus*⁶³), pond turtles (*Emys orbicularis*, *E. trinacris*⁵⁵), and wall lizards (*Podarcis bocagei*, *P. carbonelli*⁶⁴), taxa which are all regarded as distinct species.

Current taxonomy	Proposed taxonomy
<i>Natrix astreptophora</i>	<i>Natrix astreptophora</i>
<i>Natrix natrix natrix</i>	<i>Natrix natrix natrix</i>
<i>Natrix natrix cypriaca</i>	<i>Natrix natrix cypriaca</i>
<i>Natrix natrix fusca</i>	<i>Natrix natrix fusca</i>
<i>Natrix natrix gotlandica</i>	<i>Natrix natrix gotlandica</i>
<i>Natrix natrix persa</i>	<i>Natrix natrix persa</i>
<i>Natrix natrix schweizeri</i>	<i>Natrix natrix schweizeri</i>
<i>Natrix natrix scutata</i>	<i>Natrix natrix scutata</i>
<i>Natrix natrix syriaca</i>	<i>Natrix natrix syriaca</i>
<i>Natrix natrix helvetica</i>	<i>Natrix helvetica helvetica</i>
<i>Natrix natrix cetti</i>	<i>Natrix helvetica cetti</i>
<i>Natrix natrix corsa</i>	<i>Natrix helvetica corsa</i>
<i>Natrix natrix lanzai</i>	<i>Natrix helvetica lanzai</i>
<i>Natrix natrix sicula</i>	<i>Natrix helvetica sicula</i>

Table 1. Current and proposed taxonomy for grass snakes.

Generally, a steep cline across a narrow hybrid zone suggests lower hybrid fitness and selection against hybrids^{48, 65–67}, corresponding to intrinsic isolating mechanisms, which may also include assortative mating.

A completely different pattern is represented by the contact zone of the two eastern grass snake lineages, the ‘red’ and the ‘yellow lineage’. Virtually all individuals are admixed there, indicating a unimodal hybrid zone. Cytonuclear discordance is frequent, in particular, ‘red’ or mainly ‘red’ genotypes are often combined with ‘yellow’ haplotypes (Fig. 3c). The contact zone covers a broad geographical area and is characterized by smooth wide clines (Fig. 5, right); the F_{ST} value of 0.11 (microsatellites) of the involved lineages is low. Similar, also geographically wide-ranging, admixture among distinct taxa is observed for instance in European pond turtles (*Emys orbicularis galloitalica*, *E. o. hellenica*⁵⁵) and rabbits (*Oryctolagus cuniculus cuniculus*, *O. c. algirus*⁶⁸). Enigmatic is the more or less exclusive presence of mitochondrial haplotypes of the ‘red lineage’ in the central part of the contact zone and the more or less exclusive presence of ‘yellow haplotypes’ in the southernmost part (Fig. 1), despite massive nuclear admixture (Fig. 3). Unlike in the very north, where the exclusive presence of yellow haplotypes can be easily explained by early Holocene colonization and subsequent high-density blocking⁶⁹, the absence of one mitochondrial lineage in the hybrid zone could be related to selective pressure against one mitochondrial lineage; a finding requiring further research. The fact that additional genetic lineages are involved in these parts of the contact zone further complicates the matter.

In summary, we found good agreement between the studied mitochondrial lineages and nuclear genotypes of grass snakes. Using 13 highly polymorphic microsatellite loci, distinct clusters were revealed that correspond to previously identified mitochondrial lineages²⁶. However, we discovered very different gene flow patterns, with steep clines and a narrow contact zone for *N. n. helvetica* and the ‘yellow lineage’ and a wide contact zone with smooth clines for the ‘yellow’ and ‘red lineages’. According to mtDNA, the involved lineages are of different phylogenetic hierarchy and age (Supplementary Fig. S1): *Natrix natrix helvetica* belongs to another major clade than the ‘yellow’ and ‘red lineages’, which are placed in phylogenetic analyses into the same major clade²⁶. *Natrix natrix helvetica* diverged from the two eastern lineages 7.3–8.2 million years ago, whereas the yellow and red lineages split only 5.1–5.9 million years ago²⁸. With respect to nuclear genotypes, *N. n. helvetica* and the ‘yellow lineage’ differ by an F_{ST} value of 0.40, while the ‘yellow’ and the ‘red lineage’ differ by 0.11. When these values are compared to the F_{ST} value of 0.27 for *N. n. helvetica* and *N. astreptophora* (based on the same loci²⁰), this together with the limited gene flow in the narrow contact zone raises the question whether *N. n. helvetica* represents a distinct species and not only a subspecies.

Species conceptualization and delimitation is a complicated issue. In particular, it is difficult to distinguish whether observed differences are on the species or population level⁷⁰. Moreover, different species concepts can lead to different conclusions about species status, and there are currently more than 30 species concepts used⁶. However, to bypass conflicts between different species concepts, it has been suggested to unite their common elements and to characterize species primarily as independent evolutionary lineages^{71, 72}. Morphology, reproductive isolation, ecological niches or reciprocal monophyly are understood as different lines of evidence for species status⁷¹.

It is important that these lines of evidence emerge at different times in the speciation process⁷¹. Regarding reproductive isolation, species boundaries are known to be ‘semipermeable’^{49, 73, 74} and approximately 10% of animal species are known to hybridize⁷⁵. Speciation may occur despite continuous gene flow^{76–81} and high abundances of hybrids within a hybrid zone are not uncommon⁴⁸. Obviously, reproductive isolation in nature is a matter of degree⁸², with the complete lack of interbreeding and hybridization representing only the most extreme condition⁶. Hybridization between sister species in narrow contact zones is also known from other European snake species, like *Vipera aspis* and *V. latastei* in Spain⁸³. Therefore, the limited gene flow between *N. n. helvetica* and the ‘yellow lineage’ fits in that pattern and is not contradicting species status.

Considering the largely unidirectional gene flow from *N. n. helvetica* into the ‘yellow lineage’ in a narrow contact zone, the morphological distinctness of *N. n. helvetica*²⁵, its placement in another deeply divergent clade than

eastern grass snake lineages²⁶, and the considerable age of its mitochondrial lineage²⁸ (Supplementary Fig. S1), we propose to elevate this taxon to full species level and to recognize *Natrix helvetica* (Lacépède, 1789) as a distinct species.

In contrast, we regard the ‘yellow’ and ‘red lineages’ as conspecific, representing a less advanced stage in the speciation process. This assessment is supported by their lacking morphological differentiation, their wide hybrid zone with panmictic large-scale gene flow, the placement of their younger mitochondrial lineages in the same more inclusive clade^{26,28} (Supplementary Fig. S1) and a low F_{ST} value compared to the differentiation of *N. helvetica*. Our STRUCTURE analyses (Fig. 3b) also provide evidence that the ‘yellow’ and ‘red lineages’ admix on broad scale with other lineages from the same clade in the southern Balkans²⁶, supporting their conspecificity. These lineages are currently identified in the Balkans with *N. n. persa*, and some of them with *N. n. natrix* in more northerly regions^{14,25,26}.

Nomenclaturally, the recognition of *N. helvetica* as a full species necessitates that all nominal subspecies assigned to the same major clade in phylogenetic analyses²⁶ have to be transferred to *N. helvetica*, resulting in a revised taxonomy (Table 1). Fortunately, the many previously described mismatches between morphologically defined taxa and genetic lineages²⁶ refer only to taxa within, but not across, the newly delimited species, so that the suggested taxonomy does not contribute to further nomenclatural confusion, but reflects deep genetic divergences and discontinuities much better than before. However, we wish to underline that further research is needed for reconciling the conflicts between genetics and morphology of the individual subspecies within each of the three grass snake species.

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Author Contributions

C.K. performed the lab work, raw data editing, genetic analyses, created the figures and wrote the manuscript. U.F. conceived and designed the study, discussed the data and text, and revised the manuscript. M.V. helped with calculations. M.C. and S.U. conducted sequencing and fragment length analysis of Swiss samples. W.B., A.H. and D.J. contributed many samples. All authors critically read the manuscript.

Additional Information

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Hybridization patterns in two contact zones of grass snakes reveal a new Central European snake species

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Scientific Reports

Supplementary Information

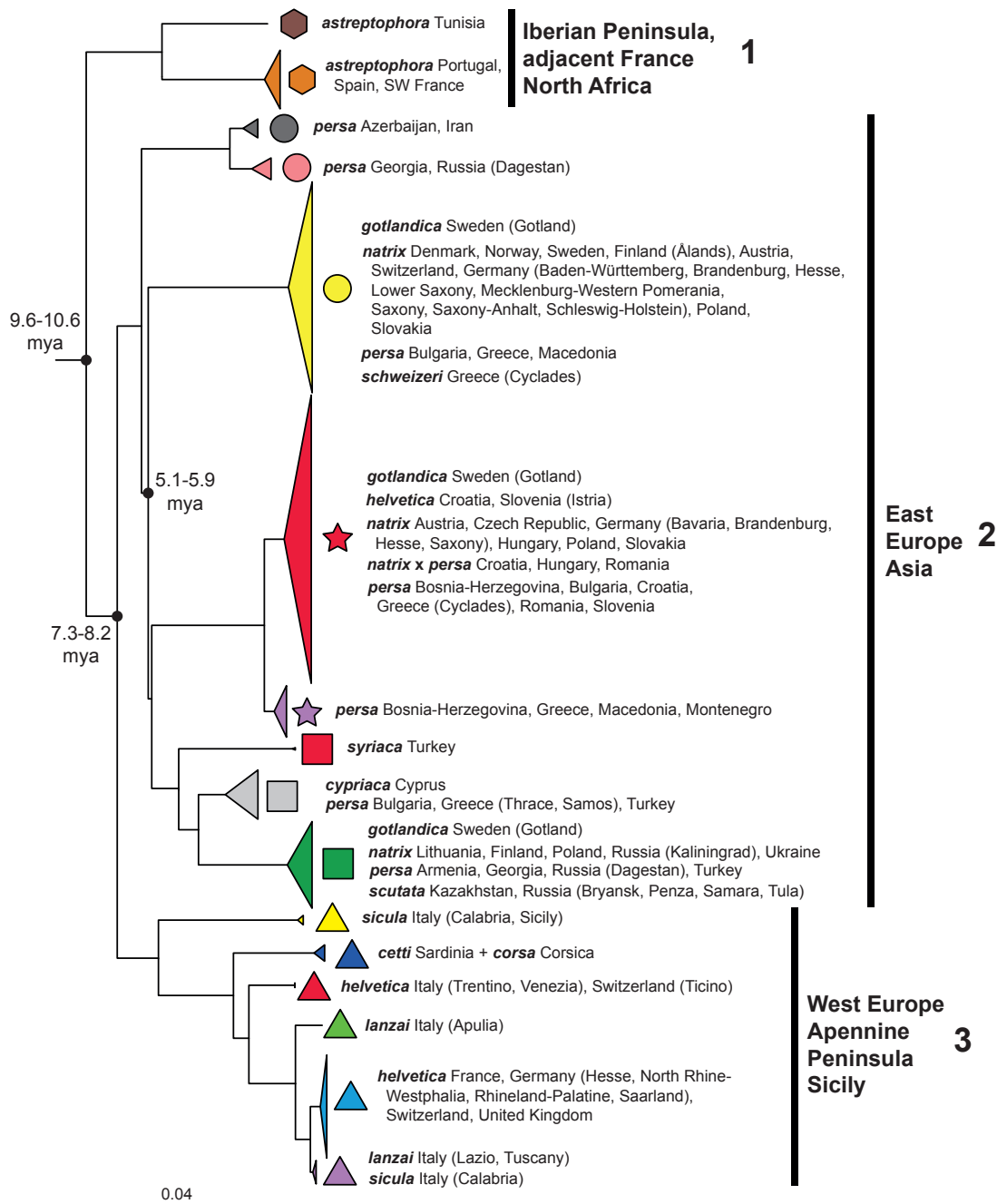


Figure S1. Simplified mitochondrial phylogeny for grass snakes (from Kindler *et al.* 2013, 2014 based on 1,984 bp of ND4 and *cyt b*). The three major clades 1-3 are highlighted; numbers at nodes indicate split ages according to Fritz *et al.* (2012). The new record for Gotland, Sweden, is added (clade with green square). Note the many mismatches between morphologically defined taxa and mitochondrial clades. The traditional identification of grass snakes from Istria (Kabisch 1999; Kreiner 2007) is in error and also conflicts with morphology (unpubl. data). The figure was created using ADOBE ILLUSTRATOR CS6 (<http://www.adobe.com/products/illustrator.html>).

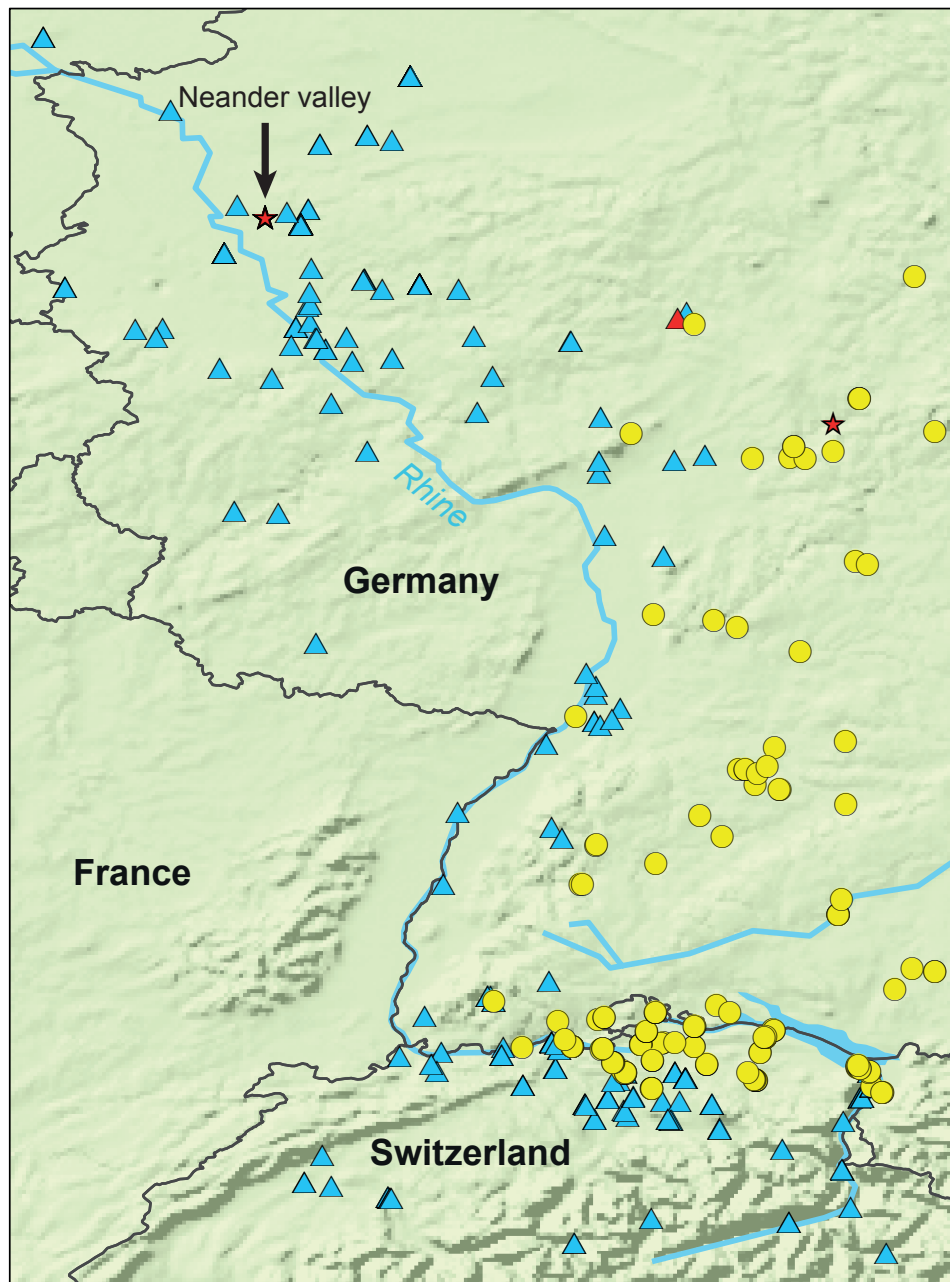
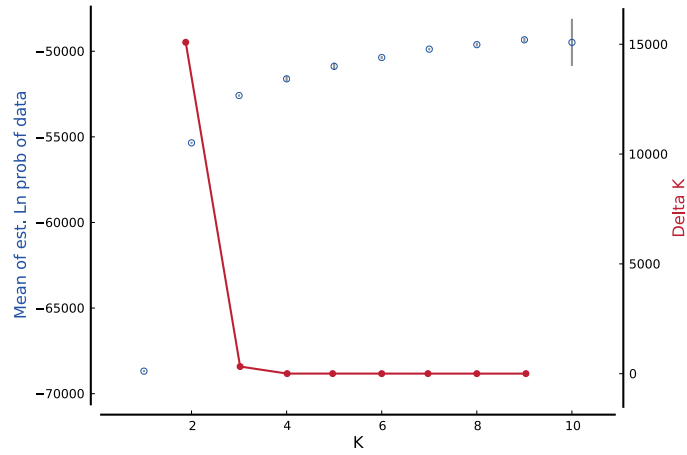
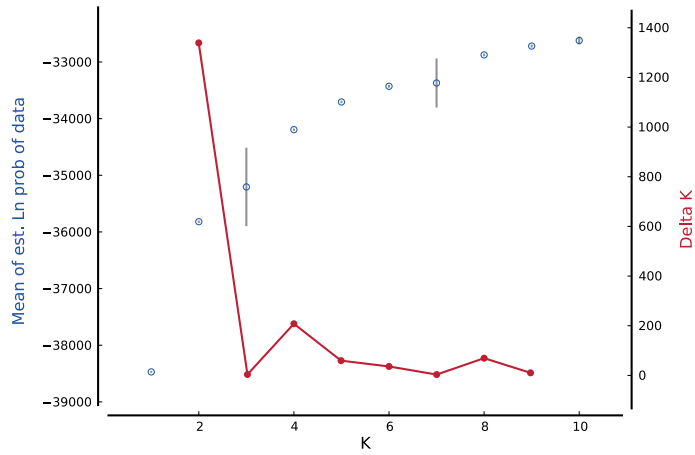


Figure S2. Distribution of mitochondrial haplotypes in the western contact zone. Symbols correspond to Figures 1 and S1. Arrow highlights allochthonous population of the 'red lineage' in the Neander valley. Red triangle represents a non-native lineage from northern Italy. Map was created using ARCGIS 10.2 (<http://www.esri.com/arcgis>) and ADOBE ILLUSTRATOR CS6 (<http://www.adobe.com/products/illustrator.html>).

(a) *helvetica* and all eastern lineages



(b) All eastern lineages without impact of *helvetica*



(c) Yellow and red lineage without impact of adjacent lineages

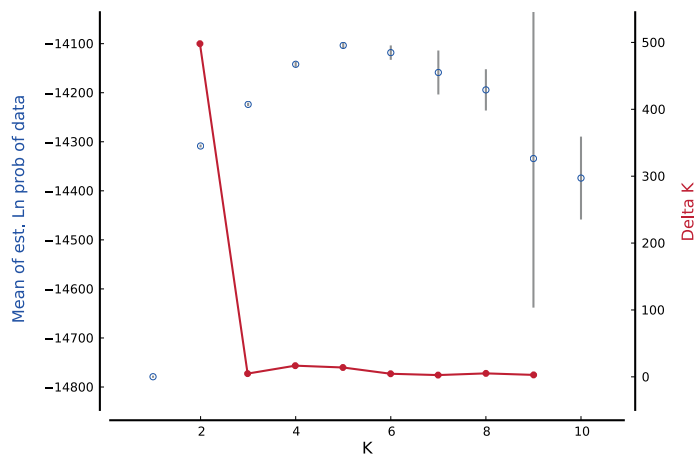


Figure S3. ΔK values and posterior probabilities for STRUCTURE runs for three data sets using STRUCTURE HARVESTER (Earl & vonHoldt, 2012). The modal value is always at $K=2$. $K=1$ could be excluded because of the higher posterior probabilities for $K=2$.

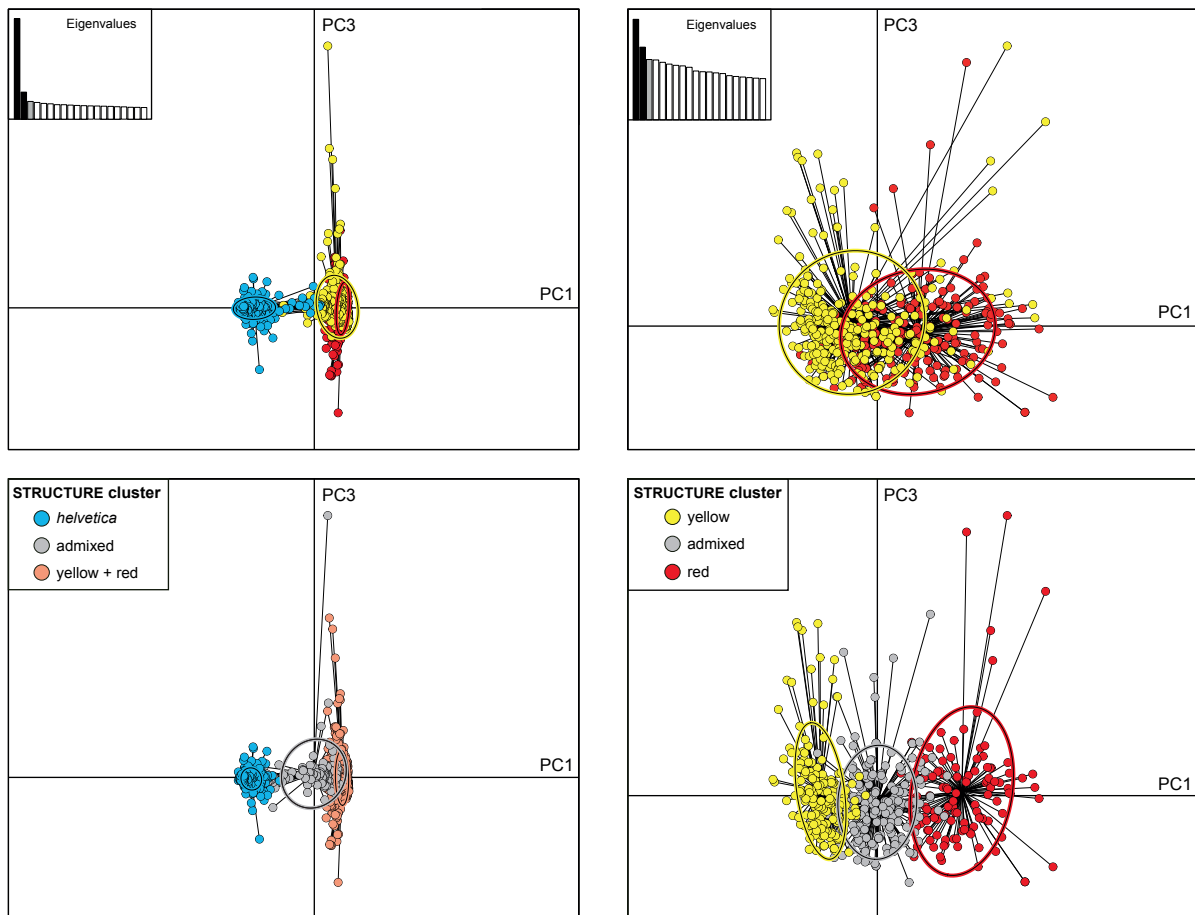


Figure S4. PCA axes 1–3 for microsatellite data of both contact zones. Samples are coloured according to mtDNA lineages (top) and STRUCTURE clusters (bottom). Admixed individuals were categorized according to HYBRIDLAB results. PCAs for the yellow and red lineages correspond to the samples from Figure 3c. Non-native samples were excluded. The oval outlines represent 95% confidential intervals. For *helvetica* and the eastern lineages (left), the x axis explains 16.6% and the y axis 2.9% of variation. For the eastern lineages (right), the x axis explains 3.8% and the y axis 2.4% of variation.

• Table S1 continued

Voucher	Locality	mtDNA data			Microsatellite data			Reference
		mtDNA clade	ND4 haplotype	cyt b haplotype	First STRUCTURE run	Second STRUCTURE run	Third STRUCTURE run	
OREB2	Switzerland: St. Gallen: Oberuzwil	3	y1	y1	0.07	-	-	This study
OREB3	Switzerland: St. Gallen: Oberuzwil	3	y42	y36	0.08	-	-	This study
OREB4	Switzerland: St. Gallen: Oberuzwil	3	y42	y36	0.05	0.98	0.04	This study
OREB5	Switzerland: St. Gallen: Oberuzwil	3	y1	y1	0.01	0.99	0.25	This study
OREB6	Switzerland: St. Gallen: Oberuzwil	3	y1	y1	0.01	0.99	0.24	This study
OREB7	Switzerland: St. Gallen: Oberuzwil	3	y42	y36	0.04	0.98	0.06	This study
QUB1	Switzerland: St. Gallen: Quinzen	E	h1	h12	1.00	-	-	This study
SMN01	Switzerland: St. Gallen: Schmerikon	E	h1	h13	1.00	-	-	This study
SMN02	Switzerland: St. Gallen: Schmerikon	E	h1	h13	0.99	-	-	This study
SMN03	Switzerland: St. Gallen: Schmerikon	E	h1	h3	0.91	-	-	This study
SEW01	Switzerland: St. Gallen: Senwald	E	h1	h9	1.00	-	-	This study
NEB01	Switzerland: St. Gallen: Thal	3	y40	y1	0.28	-	-	This study
NEB02	Switzerland: St. Gallen: Thal	E	h1	h9	0.24	-	-	This study
NEB03	Switzerland: St. Gallen: Thal	3	y1	y1	0.32	-	-	This study
NEB04	Switzerland: St. Gallen: Thal	3	y1	y1	0.27	-	-	This study
NEB05	Switzerland: St. Gallen: Thal	E	h1	h9	0.31	-	-	This study
THA01	Switzerland: St. Gallen: Thal	3	y1	y1	0.22	-	-	This study
THA02	Switzerland: St. Gallen: Thal	3	y40	y36	0.36	-	-	This study
THA03	Switzerland: St. Gallen: Thal	3	y1	y1	0.21	-	-	This study
WIL01	Switzerland: St. Gallen: Will	3	y1	y36	0.16	-	-	This study
AAD01	Switzerland: Thurgau: Aadorf	3	y37	y1	0.28	-	-	This study
AAD02	Switzerland: Thurgau: Aadorf	3	y37	y1	0.45	-	-	This study
AAD03	Switzerland: Thurgau: Aadorf	3	y37	y1	0.23	-	-	This study
JUV01	Switzerland: Thurgau: Alterswilen	3	y28	y1	0.01	0.99	0.12	This study
JUV02	Switzerland: Thurgau: Alterswilen	3	y29	y1	0.12	-	-	This study
JUV03	Switzerland: Thurgau: Alterswilen	3	y27	y1	0.11	-	-	This study
JUV04	Switzerland: Thurgau: Alterswilen	3	y1	y1	0.36	-	-	This study
JUV05	Switzerland: Thurgau: Alterswilen	3	y27	y1	0.11	-	-	This study
JUV06	Switzerland: Thurgau: Alterswilen	3	y27	y1	0.23	-	-	This study
NWN01	Switzerland: Thurgau: Alterswilen	3	y1	y1	0.19	-	-	This study
NWN02	Switzerland: Thurgau: Alterswilen	3	y1	y1	0.16	-	-	This study
BUR01	Switzerland: Thurgau: Bürglen	3	y1	y1	0.17	-	-	This study
BUR02	Switzerland: Thurgau: Bürglen	3	y1	y1	0.16	-	-	This study
FRA01	Switzerland: Thurgau: Frauenfeld	3	y1	y1	0.23	-	-	This study
FRA02	Switzerland: Thurgau: Frauenfeld	3	y37	y1	0.09	-	-	This study
KRE01	Switzerland: Thurgau: Kreuzlingen	3	y1	y1	0.09	-	-	This study
KRE02	Switzerland: Thurgau: Kreuzlingen	3	y1	y1	0.29	-	-	This study
KRE03	Switzerland: Thurgau: Kreuzlingen	3	y1	y1	0.19	-	-	This study
MUE01	Switzerland: Uri: Erstfeld	E	h1	h1	1.00	-	-	This study
GEN01	Switzerland: Vaud: Begnins	E	h1	h1	1.00	-	-	This study
MTD T 10079	Switzerland: Vaud: Vinzel	E	h1	-	1.00	-	-	Kindler et al. (2013)
DAT01	Switzerland: Zürich: Därwil	3	y37	y1	0.16	-	-	This study
DAT02	Switzerland: Zürich: Därwil	3	y37	y1	0.20	-	-	This study
DUB01	Switzerland: Zürich: Dübendorf	3	y1	y1	0.98	-	-	This study
DUB02	Switzerland: Zürich: Dübendorf	3	y1	y1	0.99	-	-	This study
DUB03	Switzerland: Zürich: Dübendorf	3	y1	y1	0.98	-	-	This study
DUB04	Switzerland: Zürich: Dübendorf	3	y1	y1	0.99	-	-	This study
FIS01	Switzerland: Zürich: Fischenthal	E	h1	h1	0.86	-	-	This study
FIS02	Switzerland: Zürich: Fischenthal	E	h1	h9	1.00	-	-	This study
FIS03	Switzerland: Zürich: Fischenthal	E	h1	h1	1.00	-	-	This study
FCH01	Switzerland: Zürich: Flaach	3	y37	y1	0.02	0.96	0.09	This study
FCH02	Switzerland: Zürich: Flaach	3	y1	y1	0.12	-	-	This study
FCH03	Switzerland: Zürich: Flaach	3	y37	y1	0.17	-	-	This study
FCH04	Switzerland: Zürich: Flaach	3	y1	y1	0.14	-	-	This study
FCH05	Switzerland: Zürich: Flaach	unknown	-	-	0.17	-	-	This study
FCH06	Switzerland: Zürich: Flaach	3	y1	y1	0.20	-	-	This study
GAT01	Switzerland: Zürich: Gattikon	E	h11	h23	0.98	-	-	This study
GAT02	Switzerland: Zürich: Gattikon	E	h1	h1	0.98	-	-	This study
PPA01	Switzerland: Zürich: Lake Pfäffikon	E	h1	h26	0.97	-	-	This study
MUE07	Switzerland: Zürich: Langnau am Albis	E	h1	h1	0.99	-	-	This study
MAR01	Switzerland: Zürich: Marthalen	3	y38	y42	0.08	-	-	This study
MAR02	Switzerland: Zürich: Marthalen	3	y1	y35	0.25	-	-	This study
MAR03	Switzerland: Zürich: Marthalen	3	y38	y42	0.04	0.97	0.09	This study
NEE01	Switzerland: Zürich: Neerach	3	y1	y1	0.31	-	-	This study
NEE02	Switzerland: Zürich: Neerach	3	y1	y1	0.16	-	-	This study
NEE03	Switzerland: Zürich: Neerach	3	y1	y1	0.23	-	-	This study
NEE04	Switzerland: Zürich: Neerach	3	y1	y32	0.05	0.99	0.13	This study
OBF01	Switzerland: Zürich: Obfelden	E	h1	h1	1.00	-	-	This study
OBF02	Switzerland: Zürich: Obfelden	E	h1	h10	1.00	-	-	This study
PFU01	Switzerland: Zürich: Pfungen	3	y33	y1	0.46	-	-	This study
PFU02	Switzerland: Zürich: Pfungen	3	y1	y1	0.31	-	-	This study
PFU03	Switzerland: Zürich: Pfungen	3	y1	y1	0.28	-	-	This study
STA01	Switzerland: Zürich: Stäfa	E	h1	h1	0.91	-	-	This study
STA02	Switzerland: Zürich: Stäfa	E	h1	h1	0.96	-	-	This study
STA03	Switzerland: Zürich: Stäfa	E	h1	h1	0.88	-	-	This study
STA04	Switzerland: Zürich: Stäfa	E	h1	h1	0.97	-	-	This study
MUE02	Switzerland: Zürich: Stallikon	unknown	-	-	0.96	-	-	This study
TAT01	Switzerland: Zürich: Thalheim an der Thur	3	y1	y1	0.12	-	-	This study
GRF01	Switzerland: Zürich: Uster	E	h1	h1	0.78	-	-	This study
WE01	Switzerland: Zürich: Weiach	3	y1	y1	0.16	-	-	This study
WE02	Switzerland: Zürich: Weiach	3	y1	y36	0.21	-	-	This study
WE03	Switzerland: Zürich: Weiach	3	y33	y1	0.02	0.96	0.06	This study
MUE03	Switzerland: Zürich: Wettswil am Albis	unknown	-	-	0.99	-	-	This study
MUE04	Switzerland: Zürich: Wettswil am Albis	unknown	-	-	0.99	-	-	This study
WIN01	Switzerland: Zürich: Winterthur: Sennhof	E	h1	h1	0.44	-	-	This study
WIN02	Switzerland: Zürich: Winterthur: Sennhof	E	h1	h1	0.21	-	-	This study
WIN03	Switzerland: Zürich: Winterthur: Sennhof	E	h1	h1	0.41	-	-	This study
WIN04	Switzerland: Zürich: Winterthur: Sennhof	E	h1	h1	0.31	-	-	This study
ZEL01	Switzerland: Zürich: Zeli	E	h1	h1	0.38	-	-	This study
ZEL02	Switzerland: Zürich: Zeli	E	h1	h1	0.31	-	-	This study
ZEL03	Switzerland: Zürich: Zeli	E	h1	h1	0.25	-	-	This study
ZEL04	Switzerland: Zürich: Zeli	E	h1	h1	0.44	-	-	This study
ZEL05	Switzerland: Zürich: Zeli	E	h1	h1	0.58	-	-	This study
ZEL06	Switzerland: Zürich: Zeli	E	h1	h1	0.38	-	-	This study
ZEL07	Switzerland: Zürich: Zeli	E	h1	h1	0.39	-	-	This study
ZHC01	Switzerland: Zürich: Zürich	E	h1	h1	0.96	-	-	This study
ZHC02	Switzerland: Zürich: Zürich	E	h1	h1	1.00	-	-	This study
ZHN01	Switzerland: Zürich: Zürich: Affoltern	E	h1	h12	1.00	-	-	This study
ZHN02	Switzerland: Zürich: Zürich: Affoltern	E	h1	h3	0.83	-	-	This study
KL001	Switzerland: Zürich: Zürich: Airport	3	y1	y1	0.31	-	-	This study
KL002	Switzerland: Zürich: Zürich: Airport	3	y1	y1	0.25	-	-	This study
KL003	Switzerland: Zürich: Zürich: Airport	3	y1	y1	0.41	-	-	This study
KL004	Switzerland: Zürich: Zürich: Airport	E	h1	h12	0.35	-	-	This study
KL005	Switzerland: Zürich: Zürich: Airport	3	y1	y1	0.22	-	-	This study
KL006	Switzerland: Zürich: Zürich: Airport	3	y1	y1	0.34	-	-	This study
KL007	Switzerland: Zürich: Zürich: Airport	E	h1	h12	0.26	-	-	This study
KL008	Switzerland: Zürich: Zürich: Airport	3	y1	y1	0.44	-	-	This study
KL009	Switzerland: Zürich: Zürich: Airport	3	y1	y1	0.26	-	-	This study
KL010	Switzerland: Zürich: Zürich: Airport	3	y1	y1	0.33	-	-	This study
ZFMK 47034	Turkey: Aegean Region: between Söke and Doğanbey	7	-	gy12	0.02	0.03	-	Kindler et al. (2013)
ZFMK 82946	Turkey: Aegean Region: Dalyan	7	HF679889	gy13	0.01	0.03	-	Kindler et al. (2013)
MTD D 25227	Turkey: Aegean Region: Selçuk	7	gy11	HF680188	0.02	0.01	-	Kindler et al. (2013)
MTD D 25229	Turkey: Aegean Region: Selçuk	7	gy8	-	0.15	-	-	Kindler et al. (2013)
MTD D 25230	Turkey: Aegean Region: Selçuk	7	gy8	HF680189	0.03	0.02	-	Kindler et al. (2013)
NHSMW 38053	Turkey: Black Sea Region: 10 km E Gerdec	8	gn14	gn16	0.02	0.02	-	This study
ZFMK 71143	Turkey: Black Sea Region: between Hopa and Arhavi	8	-	gn15	0.00	0.02	-	Kindler et al. (2013)
ZFMK 71144	Turkey: Black Sea Region: between Hopa and Arhavi	8	-	gn14	0.00	0.01	-	Kindler et al. (2013)
ZFMK 71145	Turkey: Black Sea Region: Buzluca	8	gn13	gn18	0.00	0.02	-	Kindler et al. (2013)
REV 37392	Turkey: Marmara Region: Beşevler	7	gy7	gy8	0.00	0.02	-	This study
MTD D 42725	Ukraine: Crimean: Lachytse	8	gn1	gn8	0.00	0.03	-	Kindler et al. (2013)
MTD D 42724	Ukraine: Oblast Herson: Holsi Prystan'; Herson'ske	8	gn6	gn12	0.00	0.04	-	Kindler et al. (2013)

Museum acronyms of vouchers for Table S1:

BEV – Laboratoire de Biogéographie et Ecologie des Vertébrés, Centre d’Ecologie
Fonctionnelle & Evolutive, Montpellier

LMNM – Landesmuseum Natur und Mensch, Oldenburg

MTD D – Museum of Zoology, Senckenberg Dresden (Herpetological Collection)

MTD T – Museum of Zoology, Senckenberg Dresden (Tissue Collection)

MWLK – Museum der Westlausitz, Kamenz

NHMW – Naturhistorisches Museum Wien

NME – Naturkundemuseum Erfurt

SMF – Forschungsinstitut und Naturmuseum Senckenberg, Frankfurt a.M.

SMNS – Staatliches Museum für Naturkunde Stuttgart

SMNK – Staatliches Museum für Naturkunde Karlsruhe

ZFMK – Zoologisches Forschungsmuseum Alexander Koenig, Bonn

ZMB – Museum für Naturkunde Berlin

ZMH – Zoologisches Museum Hamburg

ZMUO – Zoologisk museum, Universitetet i Oslo

ZSM – Zoologische Staatssammlung München

ZSUM – Zoologische Sammlung der Philipps-Universität Marburg

Table S2. Akaike Information Criterion (AIC) scores for fitted clines under different models using the R package HZAR (Derryberry *et al.* 2014). Bold values in blue indicate the best-fit model from the 15-model comparison.

	Contact zone I		Contact zone II	
	Microsatellites	mtDNA	Microsatellites	mtDNA
Null Model	152.846	184.261	68.427	254.247
Model 1	38.966	17.566	11.670	74.262
Model 2	22.132	n/a	11.366	n/a
Model 3	17.449	21.839	15.249	78.504
Model 4	18.120	25.038	18.909	n/a
Model 5	17.692	n/a	18.865	82.518
Model 6	23.491	30.390	22.930	86.930
Model 7	29.795	21.169	15.795	78.360
Model 8	13.501	n/a	15.365	n/a
Model 9	19.169	26.013	18.782	82.610
Model 10	36.967	21.694	15.573	78.360
Model 11	26.258	n/a	15.353	n/a
Model 12	21.600	26.034	19.168	82.724
Model 13	15.214	21.694	15.111	78.360
Model 14	15.295	n/a	15.119	n/a
Model 15	20.469	25.999	19.306	82.733

Table S3. European Nucleotide Archive (ENA) accession numbers of ND4 and *cyt b* haplotypes.

ND4 haplotype	Accession number	<i>cyt b</i> haplotype	Accession number
<i>helvetica</i>			
h1	LT839092	h1	LT839229
h2	LT839093	h2	LT839230
h3	LT839094	h3	LT839231
h4	LT839095	h4	LT839232
h5	LT839096	h5	LT839233
h6	LT839097	h6	LT839234
h7	LT839098	h7	LT839235
h8	LT839099	h8	LT839236
h9	LT839100	h9	LT839237
h10	LT839101	h10	LT839238
h11	LT839102	h11	LT839239
h12	LT839103	h12	LT839240
		h13	LT839241
		h14	LT839242
		h15	LT839243
		h16	LT839244
		h17	LT839245
		h18	LT839246
		h19	LT839247
		h20	LT839248
		h21	LT839249
		h22	LT839250
		h23	LT839251
		h24	LT839252
		h25	LT839253
		h26	LT839254
		h27	LT839255
		h28	LT839256
		h29	LT839257
Yellow lineage (lineage 3 of Kindler <i>et al.</i>, 2013)			
y1	LT839104	y1	LT839258
y2	LT839105	y2	LT839259
y3	LT839106	y3	LT839260
y4	LT839107	y4	LT839261
y5	LT839108	y5	LT839262
y6	LT839109	y6	LT839263
y7	LT839110	y7	LT839264
y8	LT839111	y8	LT839265
y9	LT839112	y9	LT839266
y10	LT839113	y10	LT839267
y11	LT839114	y11	LT839268
y12	LT839115	y12	LT839269
y13	LT839116	y13	LT839270
y14	LT839117	y14	LT839271
y15	LT839118	y15	LT839272
y16	LT839119	y16	LT839273
y17	LT839120	y17	LT839274
y18	LT839121	y18	LT839275
y19	LT839122	y19	LT839276
y20	LT839123	y20	LT839277
y21	LT839124	y21	LT839278

• Table S3 continued

Yellow lineage (lineage 3 of Kindler <i>et al.</i>, 2013)			
y22	LT839125	y22	LT839279
y23	LT839126	y23	LT839280
y24	LT839127	y24	LT839281
y25	LT839128	y25	LT839282
y26	LT839129	y26	LT839283
y27	LT839130	y27	LT839284
y28	LT839131	y28	LT839285
y29	LT839132	y29	LT839286
y30	LT839133	y30	LT839287
y31	LT839134	y31	LT839288
y32	LT839135	y32	LT839289
y33	LT839136	y33	LT839290
y34	LT839137	y34	LT839291
y35	LT839138	y35	LT839292
y36	LT839139	y36	LT839293
y37	LT839140	y37	LT839294
y38	LT839141	y38	LT839295
y39	LT839142	y39	LT839296
y40	LT839143	y40	LT839297
y41	LT839144	y41	LT839298
y42	LT839145	y42	LT839299
y43	LT839146		
Red lineage (lineage 4 of Kindler <i>et al.</i>, 2013)			
r1	LT839147	r1	LT839300
r2	LT839148	r2	LT839301
r3	LT839149	r3	LT839302
r4	LT839150	r4	LT839303
r5	LT839151	r5	LT839304
r6	LT839152	r6	LT839305
r7	LT839153	r7	LT839306
r8	LT839154	r8	LT839307
r9	LT839155	r9	LT839308
r10	LT839156	r10	LT839309
r11	LT839157	r11	LT839310
r12	LT839158	r12	LT839311
r13	LT839159	r13	LT839312
r14	LT839160	r14	LT839313
r15	LT839161	r15	LT839314
r16	LT839162	r16	LT839315
r17	LT839163	r17	LT839316
r18	LT839164	r18	LT839317
r19	LT839165	r19	LT839318
r20	LT839166	r20	LT839319
r21	LT839167	r21	LT839320
r22	LT839168	r22	LT839321
r23	LT839169	r23	LT839322
r24	LT839170	r24	LT839323
r25	LT839171	r25	LT839324
r26	LT839172	r26	LT839325
r27	LT839173	r27	LT839326
r28	LT839174	r28	LT839327
r29	LT839175	r29	LT839328
r30	LT839176	r30	LT839329
r31	LT839177	r31	LT839330

• Table S3 continued

Red lineage (lineage 4 of Kindler <i>et al.</i>, 2013)			
r32	LT839178	r32	LT839331
r33	LT839179	r33	LT839332
		r34	LT839333
		r35	LT839334
		r36	LT839335
		r37	LT839336
		r38	LT839337
		r39	LT839338
		r40	LT839339
		r41	LT839340
		r42	LT839341
		r43	LT839342
		r44	LT839343
		r45	LT839344
		r46	LT839345
		r47	LT839346
		r48	LT839347
		r49	LT839348
		r50	LT839349
		r51	LT839350
		r52	LT839351
		r53	LT839352
		r54	LT839353
		r55	LT839354
		r56	LT839355
		r57	LT839356
		r58	LT839357
		r59	LT839358
		r60	LT839359
		r61	LT839360
		r62	LT839361
		r63	LT839362
		r64	LT839363
		r65	LT839364
		r66	LT839365
Lilac lineage (lineage 5 of Kindler <i>et al.</i>, 2013)			
11	LT839180	11	LT839366
12	LT839181	12	LT839367
13	LT839182	13	LT839368
14	LT839183	14	LT839369
15	LT839184	15	LT839370
16	LT839185	16	LT839371
17	LT839186	17	LT839372
18	LT839187	18	LT839373
19	LT839188	19	LT839374
110	LT839189	110	LT839375
111	LT839190	111	LT839376
112	LT839191	112	LT839377
113	LT839192	113	LT839378
114	LT839193	114	LT839379
115	LT839194	115	LT839380
116	LT839195	116	LT839381
117	LT839196	117	LT839382
118	LT839197	118	LT839383

• Table S3 continued

Lilac lineage (lineage 5 of Kindler <i>et al.</i>, 2013)			
l19	LT839198	l19	LT839384
l20	LT839199	l20	LT839385
		l21	LT839386
		l22	LT839387
Grey lineage (lineage 7 of Kindler <i>et al.</i>, 2013)			
gy1	LT839200	gy1	LT839388
gy2	LT839201	gy2	LT839389
gy3	LT839202	gy3	LT839390
gy4	LT839203	gy4	LT839391
gy5	LT839204	gy5	LT839392
gy6	LT839205	gy6	LT839393
gy7	LT839206	gy7	LT839394
gy8	LT839207	gy8	LT839395
gy9	LT839208	gy9	LT839396
gy10	LT839209	gy10	LT839397
gy11	LT839210	gy11	LT839398
		gy12	LT839399
		gy13	LT839400
Green lineage (lineage 8 of Kindler <i>et al.</i>, 2013)			
gn1	LT839211	gn1	LT839401
gn2	LT839212	gn2	LT839402
gn3	LT839213	gn3	LT839403
gn4	LT839214	gn4	LT839404
gn5	LT839215	gn5	LT839405
gn6	LT839216	gn6	LT839406
gn7	LT839217	gn7	LT839407
gn8	LT839218	gn8	LT839408
gn9	LT839219	gn9	LT839409
gn10	LT839220	gn10	LT839410
gn11	LT839221	gn11	LT839411
gn12	LT839222	gn12	LT839412
gn13	LT839223	gn13	LT839413
gn14	LT839224	gn14	LT839414
gn15	LT839225	gn15	LT839415
gn16	LT839226	gn16	LT839416
		gn17	LT839417
		gn18	LT839418
		gn19	LT839419
		gn20	LT839420
		gn21	LT839421
Lineage C of Kindler <i>et al.</i> (2013)			
c1	LT839227	c1	LT839422
		c2	LT839423
Lineage F of Kindler <i>et al.</i> (2013)			
f1	LT839228	f1	LT839424

Table S4. Used microsatellite loci. For primer sequences, annealing temperature and multiplex-sets, see Pokrant *et al.* (2016).

Locus	Repeat motif	Allele size range [bp]	Number of alleles	Original reference
Natnat09	(AC) ₂₂	80 - 144	25	Meister <i>et al.</i> (2009)
Natnat05	(GT) ₁₆	136 - 194	23	Meister <i>et al.</i> (2009)
μNt8new	(AC) ₁₅	75 - 123	20	Meister <i>et al.</i> (2009)
Nsμ3	(ATCT) ₁₄ ATC(CA) ₂₀	139 - 457	39	Prosser <i>et al.</i> (1999)
μNt3	(AC) ₁₆	111 - 163	24	Gautschi <i>et al.</i> (2000)
μNt7	(AC) ₁₇	164 - 212	25	Gautschi <i>et al.</i> (2000)
30	(CA) ₁₄	225 - 271	20	Burns & Houlden (1999)
Natnat11	(GA) ₁₃	102 - 228	25	Meister <i>et al.</i> (2009)
Natnat06	(GT) ₂₁	145 - 185	16	Meister <i>et al.</i> (2009)
Tbu A09	(AC) ₇	110 - 146	17	Sloss <i>et al.</i> (2012)
3TS	(GATA) ₁₉	186 - 270	20	Garner <i>et al.</i> (2002)
Eobμ1	(TG) ₂₁	120 - 142	12	Blouin-Demers & Gibbs (2003)
Eobμ13	(AC) ₂₀	118 - 162	19	Blouin-Demers & Gibbs (2003)

Table S5. Genetic diversity of STRUCTURE clusters based on 13 microsatellite loci. n number of individuals, n_A number of alleles, $n_{\bar{A}}$ average number of alleles per locus, n_P number of private alleles, AR allelic richness, H_O average observed heterozygosity, H_E average expected heterozygosity. F_{IS} inbreeding coefficient, F_{ST} fixation index. Individuals with mixed ancestries not considered. All F_{IS} and F_{ST} values were statistically significant.

Microsatellites									
Cluster	n	n_A	$n_{\bar{A}}$	n_P	AR	H_O	H_E	F_{IS}	F_{ST}
First STRUCTURE run									
<i>helvetica</i>	350	156	12.000	45	4.505	0.404	0.548	0.265	0.40
Eastern lineages*	953	230	17.692	119	5.761	0.501	0.612	0.182	
Second STRUCTURE run									
Yellow + red lineage	502	121	9.308	19	7.185	0.453	0.510	0.112	0.18
Adjacent lineages**	100	186	14.308	84	14.006	0.510	0.712	0.285	
Third STRUCTURE run									
Yellow	175	83	6.385	12	6.025	0.423	0.467	0.095	0.11
Red	122	102	7.846	31	7.722	0.501	0.555	0.098	

*Yellow, red, lilac, grey and green lineages

**Lilac, grey and green lineages

Table S6. Simulated data. Twenty samples of each parental group were chosen as pure parental genotypes. Using this data, 20 genotypes of each hybrid class (F₁, F₂ and the two backcrosses) were modelled in HYBRIDLAB and analyzed with STRUCTURE. Individuals with Q values $\geq 92\%$ were reliably identified as pure *helvetica*, individuals with Q values $\geq 95\%$ as eastern grass snakes. Regarding the yellow and red lineages, the differentiation from backcrosses was more difficult. Although there was a misassignment rate of 5% for the pure yellow lineage, we decided to treat grass snakes with at least 80% cluster membership as pure yellow because otherwise the misidentification rate with backcrosses would be too high. Individuals with at least 83% were treated as pure red.

	Parental group 1	Parental group 2	F ₁ (referred to parental 1 cluster)	F ₁ (referred to parental 2 cluster)	F ₂ (referred to parental 1 cluster)	F ₂ (referred to parental 2 cluster)	Backcross parental 1	Backcross parental 2
	<i>helvetica</i>	Eastern lineages						
Average Q score	0.965	0.971	0.493	0.507	0.467	0.533	0.772	0.765
SD	0.013	0.007	0.034	0.034	0.095	0.095	0.078	0.066
Minimum Q	0.921	0.954	0.444	0.444	0.328	0.319	0.634	0.661
Maximum Q	0.997	0.979	0.556	0.556	0.681	0.672	0.968	0.912
Misassignment	if $Q < 92\%$: 0%	if $Q < 95\%$: 0%	if $Q < 92\%$: 0%	if $Q < 95\%$: 0%	if $Q < 92\%$: 0%	if $Q < 95\%$: 0%	if $Q < 92\%$: 5%	if $Q < 95\%$: 0%
	Yellow lineage	Red lineage						
Average Q score	0.880	0.890	0.480	0.520	0.580	0.420	0.740	0.720
SD	0.049	0.033	0.149	0.149	0.192	0.192	0.140	0.148
Minimum Q	0.770	0.825	0.191	0.310	0.206	0.150	0.442	0.353
Maximum Q	0.935	0.940	0.690	0.809	0.850	0.794	0.925	0.917
Misassignment	if $Q < 80\%$: 5%	if $Q < 83\%$: 0%	if $Q < 80\%$: 0%	if $Q < 83\%$: 0%	if $Q < 80\%$: 15%	if $Q < 83\%$: 0%	if $Q < 80\%$: 40%	if $Q < 83\%$: 30%

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