University of Basel
Department of Environmental Sciences, Section of Conservation Biology

# Lack of reproductive isolation between the two subspecies of the Green Whip Snake *Hierophis viridiflavus* (Squamata, Colubridae)

## Master thesis

Handed in by
**Noah Meier**

Supervised by
**Prof. Dr. Bruno Baur**
**Dr. Sylvain Ursenbacher**

Submitted on
21.06.2022

# Lack of reproductive isolation between the two subspecies of the Green Whip Snake *Hierophis viridiflavus* (Squamata, Colubridae)

Noah Meier[1,2], Kay Lucek[1], Marco Zuffi[3], Federico Storniolo[4], Marcello Mezzasalma[5], Stefano Scali[6], Roberto Sacchi[4], Philippe Geniez[7], Sylvain Ursenbacher[1,8]

[1] University of Basel, Department of Environmental Sciences, CH-4056 Basel, Switzerland

[2] Naturhistorisches Museum Basel, Augustinergasse 2, CH-4001 Basel, Switzerland

[3] Museum of Natural History, University of Pisa, Via Roma 79, 56011 Calci (Pisa), Italy

[4] University of Pavia, Dipartimento di Scienze della Terra e dell'Ambiente, Viale Taramelli 24, 27100 Pavia, Italy

[5] University of Naples, Federico II, Corso Umberto I, 40, 80138 Napoli, Italy

[6] Natural History Museum of Milan, Corso Venezia, 55, 20121 Milano, Italy

[7] Laboratoire de Biogéographie et Ecologie des Vertébrés, EPHE, Univ. Montpellier II, F-34095 Montpellier Cedex 5, France

[8] info fauna CSCF & karch, Avenue de Bellevaux 51, CH-2000 Neuchâtel, Switzerland.

Corresponding author: Noah Meier, Naturhistorisches Museum Basel, Augustinergasse 2, CH-4001 Basel, Switzerland, noah.meier@bs.ch

**Abstract**

Speciation reflects the intrinsic power of nature to diversify. Naturally, the steps of speciation, such as reproductive isolation or ecological niche separation, can take place in various orders and time scales. Thereby, species delimitation is largely dependent on the applied species concept which emphasize different steps of speciation. The two subspecies of the Green Whip Snake *Hierophis viridiflavus viridiflavus* and *H. v. carbonarius* represent an interesting model system to study speciation since they likely evolved isolated by distance during glacial periods. So far little is known whether these two subspecies are reproductively isolated from each other. Furthermore, it is unknown how the complete or partial melanism of the subspecies *carbonarius* is regulated and why it occurs only in this eastern subspecies. Therefore, we evaluated the amount of gene flow within two transects across the contact zone of the subspecies with a genomic approach using double-digest restriction site-associated DNA (dd-RAD). Our molecular sample included 133 individuals that were genotyped on 24,817 single nucleotide polymorphisms (SNP). Admixture analysis supports the existence of two subspecies as well-defined clades. However, large gene flow within a cline of 300 km was detected. We further provide insight into the associations between phenotypic characters and genomic markers, as we quantified the proportion of yellow pigmentation and used geometric morphometrics for a sample of about 50 highly admixed individuals. We conclude that the two subspecies represent evolutionary significant units (ESU). Due to the amount of gene flow in a very large area, it seems that post-zygotic reproductive barriers have not evolved. It remains unclear whether these reproductive barriers will evolve in future or if the contact zone will further expand and the two subspecies will undergo a species collapse. On a taxonomic basis, we suggest continuing treating the two ESU as subspecies.

**Keywords:**

contact zone, dd-RAD, geometric morphometrics, melanism, species collapse

## Introduction

No matter how similar the term, species and speciation are two very different concepts. While speciation is a continuous evolutionary process, species - the ultimate outcome - are treated as distinct anthropogenic units. This discrepancy is central to the still ongoing debate about suitable species concepts (Coyne & Orr, 2004; De Queiroz, 1998; Mayden, 1997). During speciation distinct intraspecific lineages may acquire various properties that allows to delimit them into species. These properties, such as reproductive barriers, distinct ecological niches or morphological and genomic differentiation, can be acquired in various orders and at different stages of the speciation process (De Queiroz, 1998, 2005, 2007; Kulmuni et al., 2020). However, many of these species properties can be reversible and do not result in complete reproductive isolation (Nosil et al., 2009). Postzygotic reproductive barriers are often less common between in clades that evolved in geographic isolation, e.g., in separate glacial refugia (Szymura & Barton, 1986) and may only evolve upon secondary contact, also called reinforcement. Species that lay somewhere on this gradient between freely interbreeding and being completely reproductively isolated are difficult to define taxonomically. Even though they are often subject to disagreements between systematists who refer to different species concepts, these species are excellent models to study speciation in progress. Furthermore, taxonomy and species delimitation can have tremendous implications on conservation approaches, such as the management of introduced species or the distribution of resources in the protection of endangered species (Ryder, 1986).

In the past, varieties of species that lack reproductive isolation and show a parapatric distribution pattern were commonly described as subspecies. Subspecies among each other show an even less consistent level of differentiation than species due to the lack of an unifying concept (Braby et al., 2012). As consequence, subspecies have become neglected in the past decades to variable extent depending on the taxonomic group. This shortcoming of the rank of subspecies might have led to a significant underestimation of genetic (and species) diversity, impressively shown by the estimation that 36% of the global avian subspecies are phylogenetically distinct (Phillimore & Owens, 2006). In the herpetological community, subspecies are even less considered than in the avifauna, supported by molecular studies rejecting subspecies that were exclusively defined based on morphology and / or restricted distribution (Burbrink et al., 2000). However, it was shown that especially species with large distribution areas are likely to harbour species complexes and their subspecies deserve more attention (Reiserer et al., 2013). Only recently it was shown that three former subspecies of

the Common Grass Snake *Natrix natrix s.l.* represent well-defined genetic lineages with only little hybridization within a narrow contact zone, which thus were elevated to the rank of species (Kindler et al., 2017; Pokrant et al., 2016). Several other European snake species with distinct intraspecific clusters await further phylogenetic examination as they might represent species complexes, such as the Smooth Snake *Coronella austriaca* or the Green Whip Snake *Hierophis viridiflavus* (Jablonski et al., 2019; Mezzasalma et al., 2015).

In fact, *H. viridiflavus* was subject to various studies in the past in regard of its ecology, but also evolutionary history and systematics. Especially the specific status of the parapatric subspecies *carbonarius* (Bonaparte, 1833), which differs from the nominate subspecies by complete melanism (lack of yellow dots) has become a challenge regarding the current taxonomy. Bruno (1980) suggested to elevate the subspecies *carbonarius* to species level due to its locally sympatric but otherwise mostly parapatric occurrence with *viridiflavus* and pholidotic differences, i.e. less ventral scales. Schätti and Vanni (1986) showed that melanotic individuals of the subspecies *carbonarius* from southern and northeastern Italy have fewer ventral scales than non-melanotic individuals of the subspecies *viridiflavus*. However, they stated that the variation within these two subspecies outnumbers the differentiation between the subspecies and especially the occurrence of intermediate phenotypes in the contact zones appeared to be more complex. Therefore, they declared *H. viridiflavus* as single species and dismissed any subspecies (Schätti & Vanni, 1986).

The first molecular analyses of *H. viridiflavus* revealed two clearly differentiated clades based on a mitochondrial sequence and few anonymous nuclear markers tested on 22 individuals primarily from Italy and some from southeastern France (Nagy et al., 2002). Since these clades corresponded to the exclusive distribution ranges of the two subspecies, they declared *H. viridiflavus* as not monotypic and revalidated *H. v. carbonarius* as subspecies (Nagy et al. 2002). The subspecies also show indications for different life histories, where clutch sizes in individuals from southern Italy were smaller compared to intermediate and larger clutch sizes of individuals from Tyrrhenian islands and France (Zuffi et al., 2007). It remains unanswered, whether these differences could be just plastic in relation to additional ecological factors, such as climate and food sources, or rather reflect genetic differences between the subspecies. Covering most of the distribution area of *H. viridiflavus,* a more recent phylogeographic study reported an average genetic divergence of 4% between the western *viridiflavus* and the eastern *carbonarius* subspecies (Rato et al., 2009). However, the same study also found evidence for

incomplete lineage sorting with a single nuclear gene sequenced and an inconsistency between the molecular clade assignment and the occurrence of melanotic individuals, concluding that the clades are not separate species (Rato et al., 2009). This discordance was also found in another study with more mitochondrial genes, where the mitochondrial markers showed a clear differentiation between the western and the eastern clade but not the single nuclear marker that was analysed (Mezzasalma et al., 2015). Further evidence for lineage separation between *viridiflavus* and *carbonarius* was found, though, from geometric morphometrics of the head pholidosis, i.e. shape and size of head scales, and differences in the karyotype of the female's W chromosome, being submetacentric in the eastern clade and telocentric in the western clade (Mezzasalma et al. 2015). Concludingly the authors suggested the split of *Hierophis carbonarius* from the nominal species (Mezzasalma et al. 2015). However, the aforementioned study only used a sparse sampling in the contact zone between the two clades along the Apennine mountains and had very limited power to detect gene flow between the clades given the markers used. Consequently *carbonarius* has been considered as a subspecies by taxonomists as long as no better resolution of the situation in the contact zone would be available (Speybroeck et al., 2020). So far, investigations on the contact zone between the western *viridiflavus* and eastern *carbonarius* subspecies were only done by very few candidate genes that are thought to be involved in melanotic pattern (Sencuk et al. 2021). While these markers indicated some introgression in the contact zone, no correlation between the melanotic pattern with candidate genes was found.

Based on the history of systematic research on *H. viridiflavus* described above, the first goal of this study was to evaluate to which degree genome-wide gene flow between the two subspecies occurs, especially along the putative contact zones. In this context, we assessed whether the phenotypic differentiation between the two subspecies, such as melanism, showed a broad genomic footprint. As second goal, we investigated the genetic diversity within the whole distribution area of the species to determine the putative origin(s) of allochthonous populations in Switzerland (Meier et al., 2022). For that, we conducted an extensive sampling in the contact zones of the two clades, emphasizing the northern contact zone where most of the inconsistency between morphology and genetics has previously been found (Rato et al. 2009). To overcome the aforementioned issues associated with sampling only a few genes, we generated a genome wide dataset using double-digest restriction-site associated DNA sequencing (ddRADseq; Peterson et al. 2012). We expected to find a lot of gene flow between the two subspecies ($H_0$), since various intermediate phenotypes in terms of melanism are

prevalent in large areas (Jagar, 2011; Rato et al., 2009; M. Zuffi, 2008). On the other hand, strong genetic differentiation of tested mitochondrial genes suggests considerable lack of gene flow - at least in the past - and likely could indicate the existence of reproductively isolated clades ($H_1$).

## Materials and Methods

### *Collecting samples*

Based on the occurrence of the two subspecies *viridiflavus* and *carbonarius* (Mezzasalma et al. 2015), we defined two transects across the known contact zones: 1) the Italian states Piedmont–Lombardy–Emilia Romagna; 2) Umbria–Marche (Fig. 2C–D). Sampling took place between March and June 2021. The sampling localities were as evenly distributed along the contact zone as possible. We chose spots to search that matched with the habitat preference of the species (Di Nicola et al., 2019; Heimes, 1993; Speybroeck et al., 2016), especially warm, dry, south-exposed, and structurally rich habitats (Scali et al., 2008). Snakes were searched by sight and caught by hand. Whenever available, likely hiding spots such as corrugated metal plates were checked as well, especially in structurally poor habitats, such as the plains in the Po valley, which are intensively used for agriculture.

We took a blood sample of 100 µl and 50 µl for adults and juveniles, respectively, from the caudal vein (Bush & Smeller, 1978) and stored at room temperature in 0.9 ml Queen's lysis buffer (Seutin et al. 1991). In addition, two ventral scales were taken with scissors and stored in 1 ml of pure ethanol. For each caught individuals we collected standard metrics such as total length (TL), snout-vent length (SVL) and the weight. We determined the sex of the adult individuals based on the shape of the tail basis and the head. We took standardized pictures of each individual using a colour reference plate with an integrated ruler: 1) head dorsal view; 2) head ventral view; 3) body mid length dorsal view; 4) body mid length ventral view; 5) whole individual in dorsal view.

In addition, tissue samples from individuals found dead on the road were taken and stored in 1 ml pure ethanol. If these individuals were still in good condition, pictures were taken as outlined above and the whole specimen was stored in pure ethanol. Sloughs found in the field were also collected and stored dry at -20 °C. In total 80 samples (57 blood, 12 tissue, 11 sloughs) and eight samples *(*eight tissue) were collected in the northern contact zone and the eastern contact zone, respectively (Supplementary File S1). Beside these fresh collected

samples from the contact zone, 146 tissue samples from reference material that was collected from all over the distribution area was obtained from various museal collections (Supplementary File S1). We further obtained samples of seven specimens of the closely related Balkan Whip Snake *Hierophis gemonensis* as outgroup (Supplementary File S1). For all samples (N=241), DNA was extracted using the Qiagen DNeasy kit (Qiagen, Zug, Switzerland) following the manufacturer's protocol.

*Geometric morphometrics*

Standardized images of head dorsal scales were used to conduct a geometric morphometrical analysis in 56 individuals. All the pictures were combined to a TPS file using TPSUTIL (v. 1.8.1; Rohlf 2015). We set the identical 21 landmarks (Supplementary File S2 and Fig. 1) that were used in previous studies on the *H. viridiflavus* (Mezzasalma et al. 2015) with TPSDIG (v. 2.3.1; Rohlf 2015). The landmarks were then aligned by a Generalized Procrustes analysis (function "gpagen") and the shape similarity between specimens was investigated with a principal component analysis (function "gm.prcomp") in R (v.4.0.4; R Core Team, 2021) using the package GEOMOPRH (Adams & Otárola-Castillo, 2013). We fitted a linear regression between the principal component analysis axis 1 (PC1) of the geometric morphometric data and the TL and extracted the residuals from this model to correct for allometric shape differences.

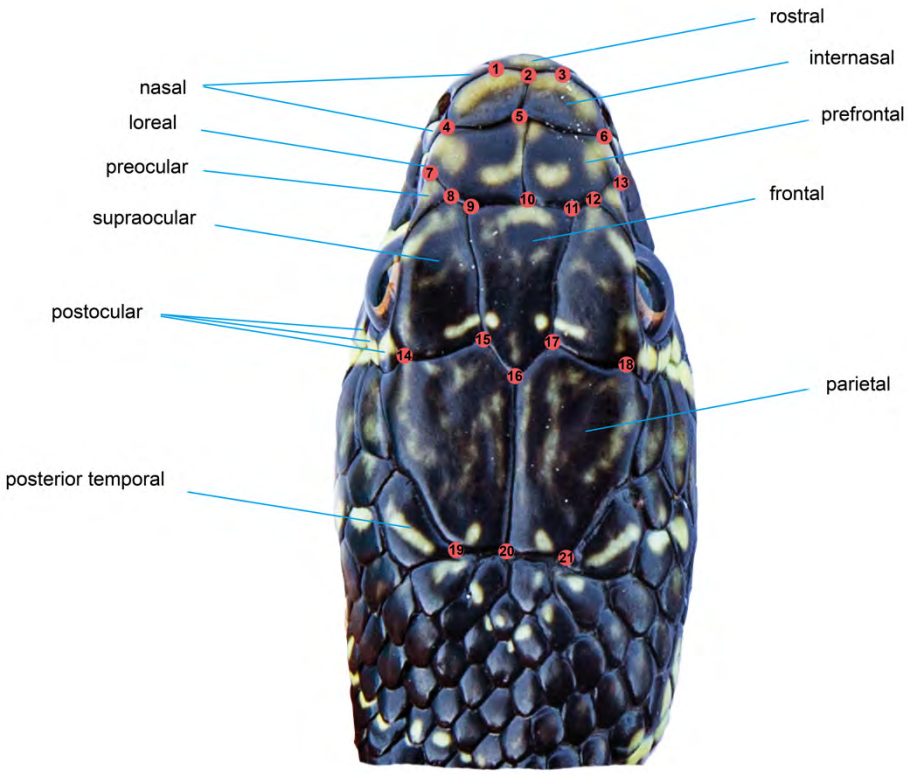


**Figure 1.** Position of the landmarks (n=21), for geometric morphometric analysis of the head pholidosis.

*Quantification of melanism*

We used standardized images of dorsal scales at mid body length to quantify the extent of yellow pigmentation in 46 individuals. First, the pictures were processed in Adobe Lightroom (v.11.0; Adobe, 2021) by correcting the white balance and colour setting using a colour checker Pantone system and producing camera profiles for each image. Then the pictures were cropped to a window with a width of 10 dorsal scales in the midline and the full body width in the height of the image. Contrast and lightning were manually corrected to optimize the visibility of the yellow pigmentation. The images were then split into colour clusters (Supplementary File S3) in R (R Core Team, 2021) using the function "getKMeanColors" form the package COLORDISTANCE (Weller & Westneat, 2019). The proportion of yellow pigmentation was calculated by dividing the sum of the size of all colour clusters with RGB values above R=0.3, G=0.35, B=0.35 by the sum of the size of all colour clusters.

*Sequencing & genotyping*

Samples with too low DNA concentrations or strong degradation on agarose gels were omitted, resulting in a total of 148 retained for genotyping out of 241 samples. All remaining individuals were genotyped using a double digest restriction-site associated DNA (ddRAD)

approach with the restriction enzymes EcoRI and Nla III. Library preparation and sequencing was outsourced (CD-Genomics; New York, USA). Sequencing was performed on an Illumina Novaseq 6000 with 150 bp paired-end reads. To obtain a sequencing depth of 10 M reads per sample, some samples were sequenced two to three times. All raw data is available through NCBI (BioProj PLACEHOLDER).

*SNP calling*

Using FASTP (v. 0.23.2; Chen et al. 2018), we removed poly-G tails and dropped reads with more than 40% of the bases below a Phred quality score of 30. We mapped all retained reads against the reference genome of the Corn Snake *Pantherophis guttatus* (Linnaeus, 1766) (Ullate-Agote et al. 2014; NCBI: GCA_001185365.2) with BWA (v. 0.7.17; Li and Durbin 2009) following duplicate removal with SAMTOOLS (v. 1.14; Li et al. 2009). We then called single nucleotide polymorphisms (SNPs) with BCFTOOLS (v. 1.12; Li 2011). Subsequently, we filtered SNPs to be biallelic with a minimal depth of 6 per sample, a minimal GQ of 20 and applying a minor allele frequency filter of 0.03 with VCFtools (v. 0.1.16; Danecek et al. 2011). Samples with more than 65% missing data and SNPs that were missing in more than 50% of the samples were removed. This approach resulted in 24,817 SNPs for 134 individuals. One individual was excluded in further analysis due to unreliable sampling location.

*Molecular analyses*

In order to search for subspecific clusters and evaluate their gene flow, we applied several downstream analyses on the obtained molecular data set. We used ADMIXTURE (v. 1.3.0; Alexander and Lange 2011) assuming one to ten genetic clusters (K = 1-10). We chose the best model based on the lowest cross-validation value. In order to estimate the level of genetic differentiation, we calculated $F_{ST}$ values between non-admixed individuals of the obtained clusters using GENODIVE (v. 3.0.5; Meirmans 2020) with 1000 bootstrap replicates. We further reconstructed a neighbour joining network using SPLITSTREE4 (v. 4.18.1; Huson and Bryant 2006) for all retained individuals in order to understand the individual relationship within the clusters. Furthermore, to characterize the contact zone we fitted simple sigmoid cline models across all individuals in our data set (N=133). We used clade assignment as dependent variable and calculated the distance from the putative contact zone (estimated based on results from admixture) as independent variable in QGIS (QGIS.org, 2022). We gave individuals from the western side of the contact zone negative indices in order to separate them from those from the eastern side. Alternatively, we tested the distance from the

westernmost individual independent variable. We calculated clines based on a maximum-likelihood approach with the package HZAR (Derryberry et al., 2014) in R (R Core Team, 2021). We selected the best-supported model using the Akaike information criterion. Lastly, we tested linear regression models to find associations between the phenotypic data, i.e., melanism and head morphometrics, and the assigned genetic cluster (clade assignment). To identify SNPs that are directly or indirectly associated with the intensity of melanism, we employed a genome-wide association study (GWAS) approach for the 40 genotyped individuals for which we had complete phenotypic data. We used the proportion of yellow pigmentation as phenotype and sex as covariate and built a covariate linear mixed model implemented in GEMMA (v. 0.98.1; Zhou and Stephens 2012). Putative outlier SNPs were identified with the package QQMAN (Turner, 2018) in R (R Core Team, 2021).

## Results

*Population structure and admixture*

The admixture analyses revealed that the model with two clusters was the best supported (Supplementary File S4). With two clusters, all individuals sampled in the northern contact zone were genetically admixed with a continuous gradient from the western *carbonarius* clade towards the eastern *viridiflavus* clade (Fig. 2A, C). Furthermore, individuals from Corsica, southern Vaud and Ticino in Switzerland were genetically admixed between these clades. Non-admixed individuals were only found further west in France and northern Vaud in Switzerland for the western clade and in north-eastern and southern-most Italy and Croatia for the eastern clade (Fig. 2A). The $F_{ST}$ value between non-admixed individuals from the eastern and western clade is 0.190.

*Network analysis*

The NeighborNet network fell into four broad groups: the non-admixed individuals from the western *viridiflavus* clade, the non-admixed individuals from the eastern *carbonarius* clade, the admixed hybrid *viridiflavus* x *carbonarius* individuals and the outgroup *H. gemonensis.* The admixed hybrids are shown in the network as gradient between the two subspecies with many additional splits (parallel lines connecting different branches) indicating ambiguities in the network, which can be explained by hybridization and homoplasy (Fig. 3).

For individuals of the introduced range from Vaud in Switzerland (Figs 2B, 3), the network analysis indicated that individuals from allochthonous populations in the south of Vaud (e.g., 46431 and 46528) are clustering together with the individuals from Ticino (e.g., HIVI005 and HIVI006), suggesting that Ticino could be the origin of this introduction. Both the populations from Ticino and southern Vaud are genetically admixed *viridiflavus* x *carbonarius*. In contrary, individuals from populations in the north of Vaud (e.g., 41159 and 41162) are clustering together with individuals from France (e.g., BEV_11025 and BEV_9093) and Geneva (2744_72), corresponding to the non-admixed *viridiflavus* subspecies.

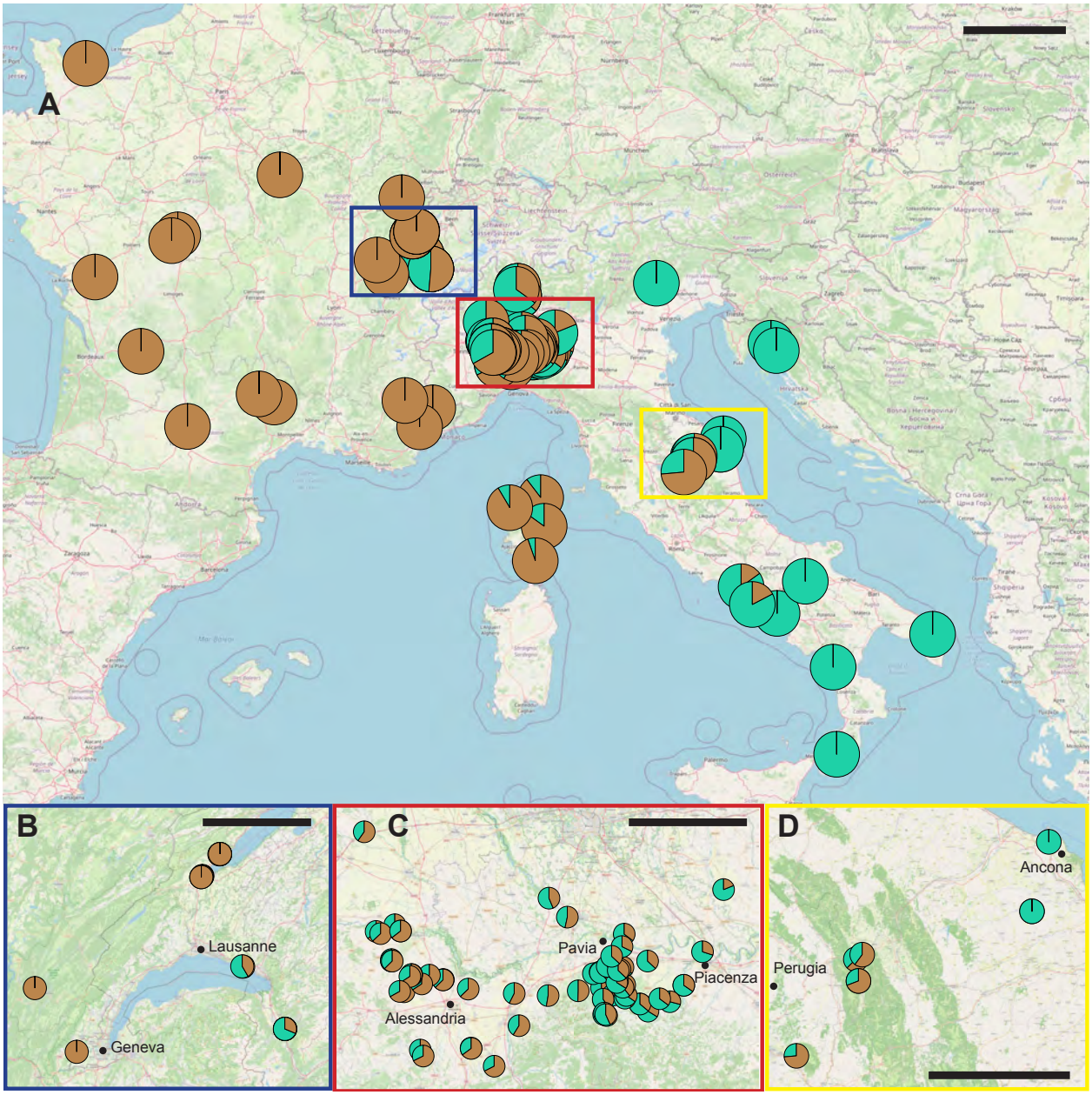


**Figure 2.** Admixture results (K=2) based on 24,817 SNPs for 133 individuals. proportion of clade assignment shown as pie charts (cyan = eastern clade, i.e., H. v. carbonarius; brown = western clade, i.e., H. v. viridiflavus). A. whole data set. B. *c*lose-up to western Switzerland. C. *c*lose-up to northern transect between Alessandria and Piacenza. D. *c*lose-up to eastern transect between Perugia and Ancona. colored frames indicate the position of the close-ups in panel A. bars indicate 200 km in A, and 40 km in B–D.

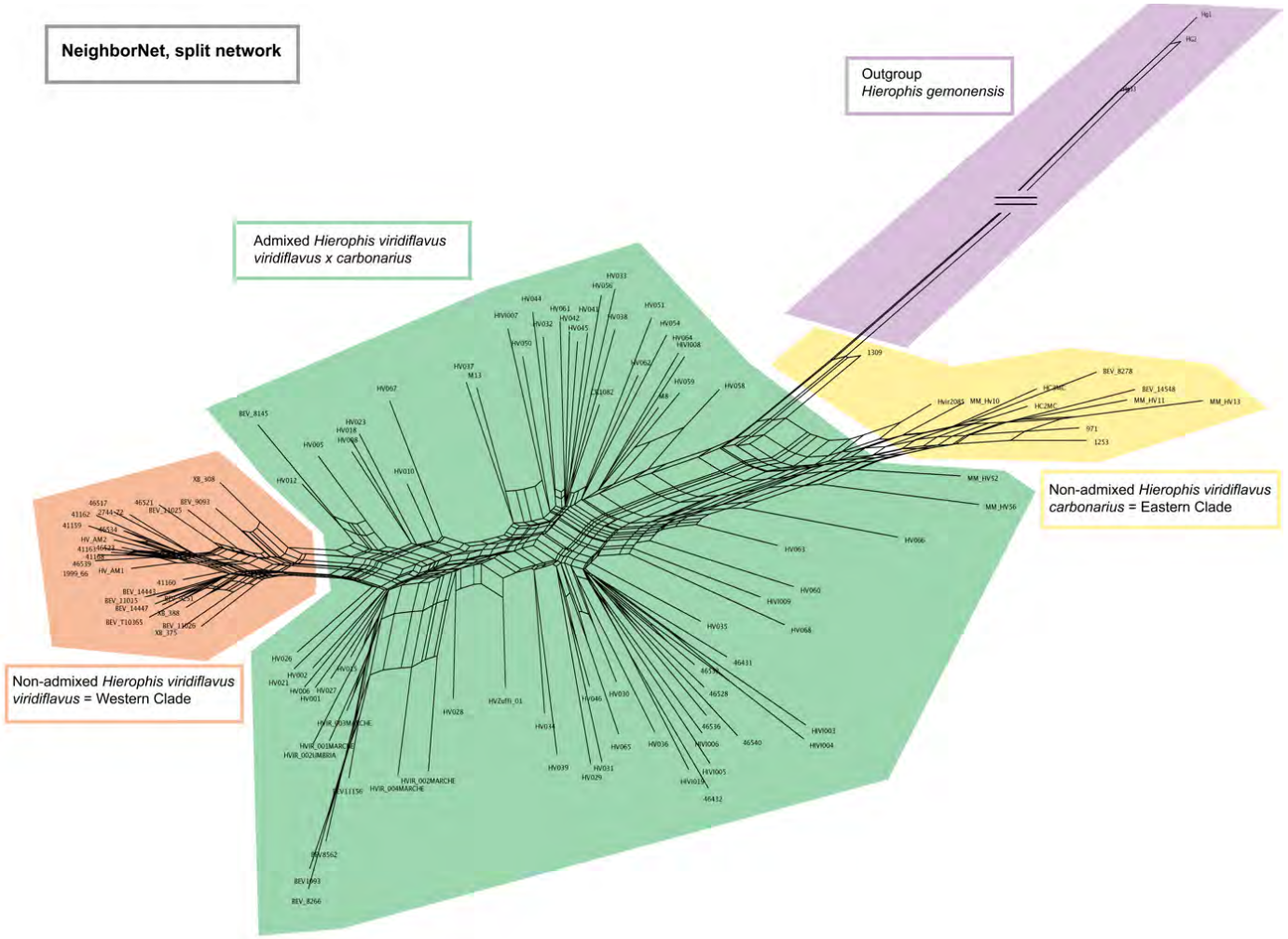


**Figure 3.** NeighborNet split network with 274 splits (parallel lines connecting the branches). Orange = non-admixed individuals from the western Clade (= *H. v. viridiflavus*). Yellow = non-admixed individuals from the eastern (= *H. v. carbonarius*). Green = admixed individuals between western and eastern clade (*H. v. viridiflavus* x *H. v.* carbonarius). Purple = outgroup individuals (= *H. gemonensis*), branch was shortened to improve the visibility of the remaining network. samples that clustered very close in the network were omitted in order to improve clarity of the network. Splits / ambiguities in the network are shown as parallel lines connecting different branches in the network.

*Cline models*

In order to characterize the genetic gradient in the contact zone of the two genetic clades, we fitted simple sigmoid cline models. Firstly, we fitted a cline model to the genomic data from the admixture analysis against the distance gradient calculated from the westernmost individual. The model was supported with an AIC of 36.4, the centre of the cline was estimated at 788.9 km (95% CI = 708.3 – 862.9 km) and the width was estimated as 972.8 km (95% CI = 679.3 – 1323.1). However, admixture analyses (Fig. 2) revealed that the contact zone does not strictly follow a west-east gradient but rather runs along the Apennine mountains. Hence, we ran a second cline analysis using the closest distance to the putative contact zone (Fig. 8). This model was much better supported with an AIC of 15.65. The centre of the cline was estimated to be 21.1 km further westwards (95% CI = -43.4 km – 2.9 km). The width of the cline was estimated as 303.0 km (95% CI = 195.1 km – 579.4 km).

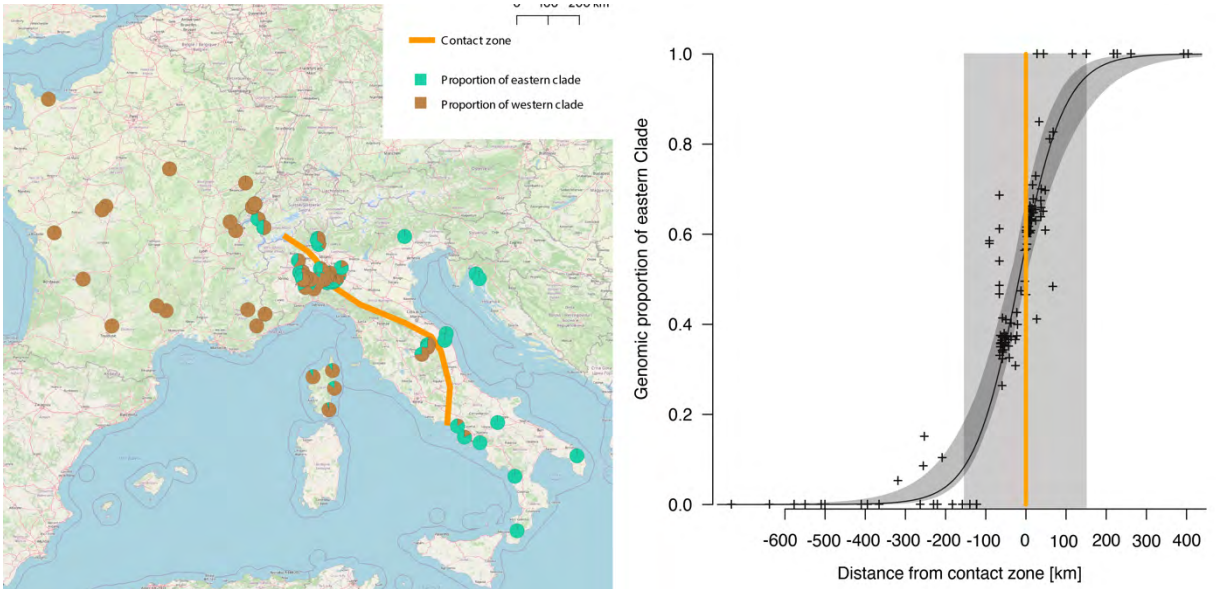


**Figure 8.** A. Position of putative contact zone (orange line), drawn in QGIS based on admixture results. B. Cline analysis with genomic admixture data and the distance from the putative contact zone (orange vertical line). Individuals from the western side of the contact zone were assigned with negative distances. The centre of the cline was estimated 21.1 km further westwards (95% CI = -43.4 km – 2.9 km). The width of the cline (light grey rectangle) was estimated as 303.0 km (95% CI = 195.1 km – 579.4 km).

*Geometric morphometrics colour analysis*

We used 21 landmarks to analyse the pholidosis of the head in 56 individuals. Even though the landmarks were aligned with a Generalized Procrustes analysis (Fig. 4B), the linear regression between the PC1 of the Procrustes coordinates and TL of the snakes (Fig. 4A) was highly significant (estimate = -635.98, SE = 88.54, $P < 0.0001$) and explained 50.8% of the

variation due to allometric shape differences. We observed qualitatively that smaller individuals had relatively shorter prefrontal and internasal scales, but relatively longer frontal and parietal scales (Fig. 6B).

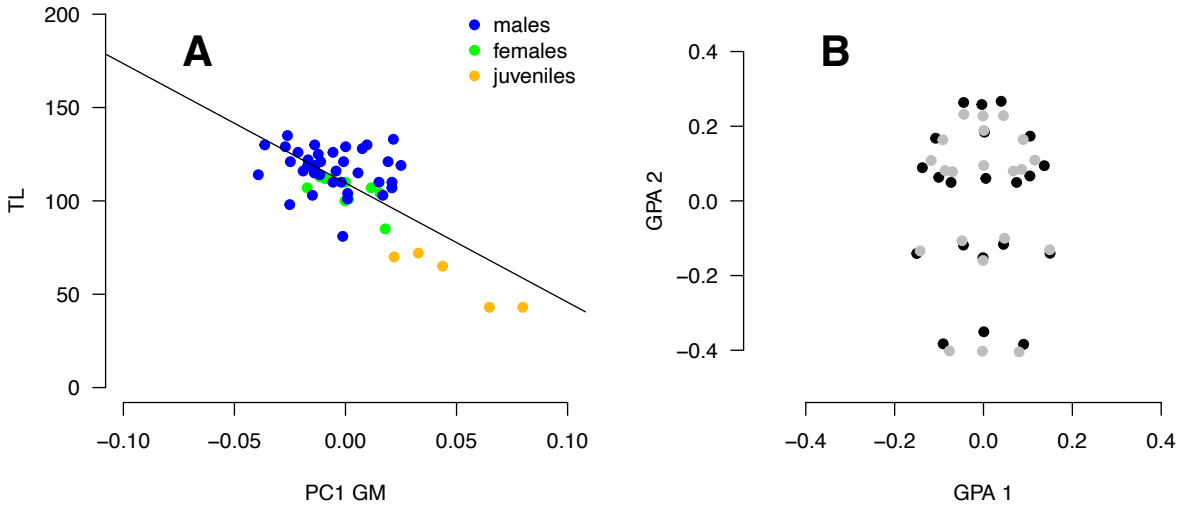


**Figure 4.** A. Principal component analysis axis 1 of Procrustes values (PC1 GM) based on 21 landmarks on head pholidosis plotted against the total length (TL) of the individuals. A linear model found a strong correlation between the total length and PC1 (estimate = -639.0, SE = 87.85, P < 0.0001, $R^2 = 0.508$). B. The generalised Procrustes values for the individual with the maximum (grey) and minimum (black) PC1 value, indicating the maximal shape difference in head pholidosis.

We calculated the proportion of yellow pigmentation in a similar section of the individual for 46 adult individuals as approximation for the melanism. Most individuals ranged between 0 – 22% with a median of 7.1% (Fig. 7). Interestingly, we found two individuals with of an aberrant colour morph, recognized by a much higher proportion of yellow pigmentation, 53.8% (Fig. 5A) and 48.4% (Fig. 5B). These individuals were excluded in all the linear regression models due to their leverage as outliers (Figs 6–7). A linear regression between the proportion of yellow pigmentation and the clade assignment was significant (estimate = -0.93, SE = 0.40, $p = 0.025$). This model explained 12.5% ($R^2$) of the variation. (Fig. 6B). Given that only 1/8 of the variation in melanism is explained by clade assignment, it is less surprising that we also found individuals at the same location with similar genotypes but very different phenotypes (Fig. 5C).

To determine whether the two genetic clades have distinct head pholidosis, we further tested linear regression models between PC1 of the geometric morphometrics of the head and the clade assignment (estimate = -0.87, SE = 1.40, $p = 0.54$, $R^2 = 0.01$; Fig. 6A), between the

residuals of the linear regression of the PC1 of the geometric morphometrics of the head and the TL and the clade assignment (estimate = -9.10, SE = 14.55, p = 0.54, $R^2$ = 0.01; Fig. 6C) and between the proportion of the yellow pigmentation and the residuals of the linear regression of the PC1 of the geometric morphometrics of the head and the TL (estimate = 33.32, SE = 38.27, p = 0.39, $R^2$= 0.019; Fig. 6D). However, the tested models (Fig. 6A, C–D) were not statistically significant and explained less than 2% of the observed variation.


**Figure 5.** Colour variation in four individuals from the contact zone. A. individual HV016, proportion of yellow pigmentation = 48.41%, clade assignment = 64% western clade. B. individual HV036, proportion of yellow pigmentation = 53.84%, clade assignment = 38% western clade. C. Two individuals from the exact same location showing similar genetics but different morphology. Left. individual HV054, proportion of yellow pigmentation = 2.17%, clade assignment = 35.6% western clade. Right. individual HV053, proportion of yellow pigmentation = 18.7%, clade assignment = 36.0% western clade.

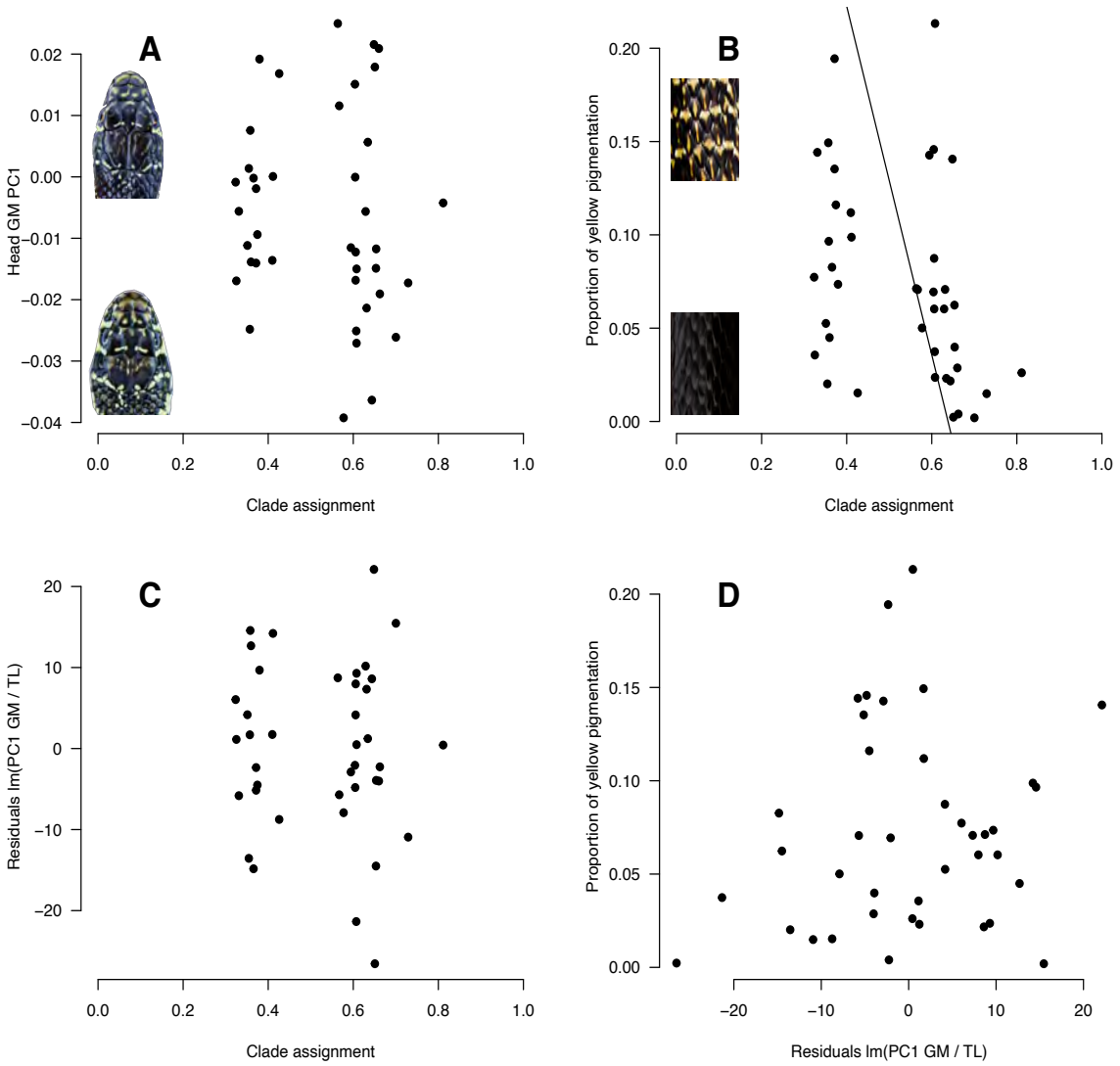


**Figure 6.** Linear regression models between the clade assignment, the geometric morphometrics (GM) of the head pholidosis and the proportion of yellow pigmentation. A. PC1 of the GM plotted against the clade assignment based on admixture (K=2) results (estimate = -0.87, SE = 1.40, $p = 0.54$, $R^2 = 0.010$). B. Proportion of the yellow pigmentation plotted against the clade assignment based on admixture (K=2) results (estimate = -0.93, SE = 0.40, $p = 0.025$, $R^2 = 0.125$). C. Residuals of the linear regression between the PC1 of the GM and the total length plotted against the clade assignment based on admixture (K=2) results (estimate = -9.10, SE = 14.55, $p = 0.54$, $R^2 = 0.010$). D. Proportion of the yellow pigmentation plotted against the residuals of the linear regression between the PC1 of the GM and the total length (estimate = 33.32, SE = 38.27, $p = 0.39$, $R^2 = 0.019$). Only significant regression models are shown as lines in the graphs. Images present the most extreme phenotypes for geometric morphometric of the head pholidosis (A) and the proportion of the yellow pigmentation (B). Juveniles (N=5) and individuals with a proportion of yellow pigmentation above 0.4 (N=2) were omitted in all plots and models.

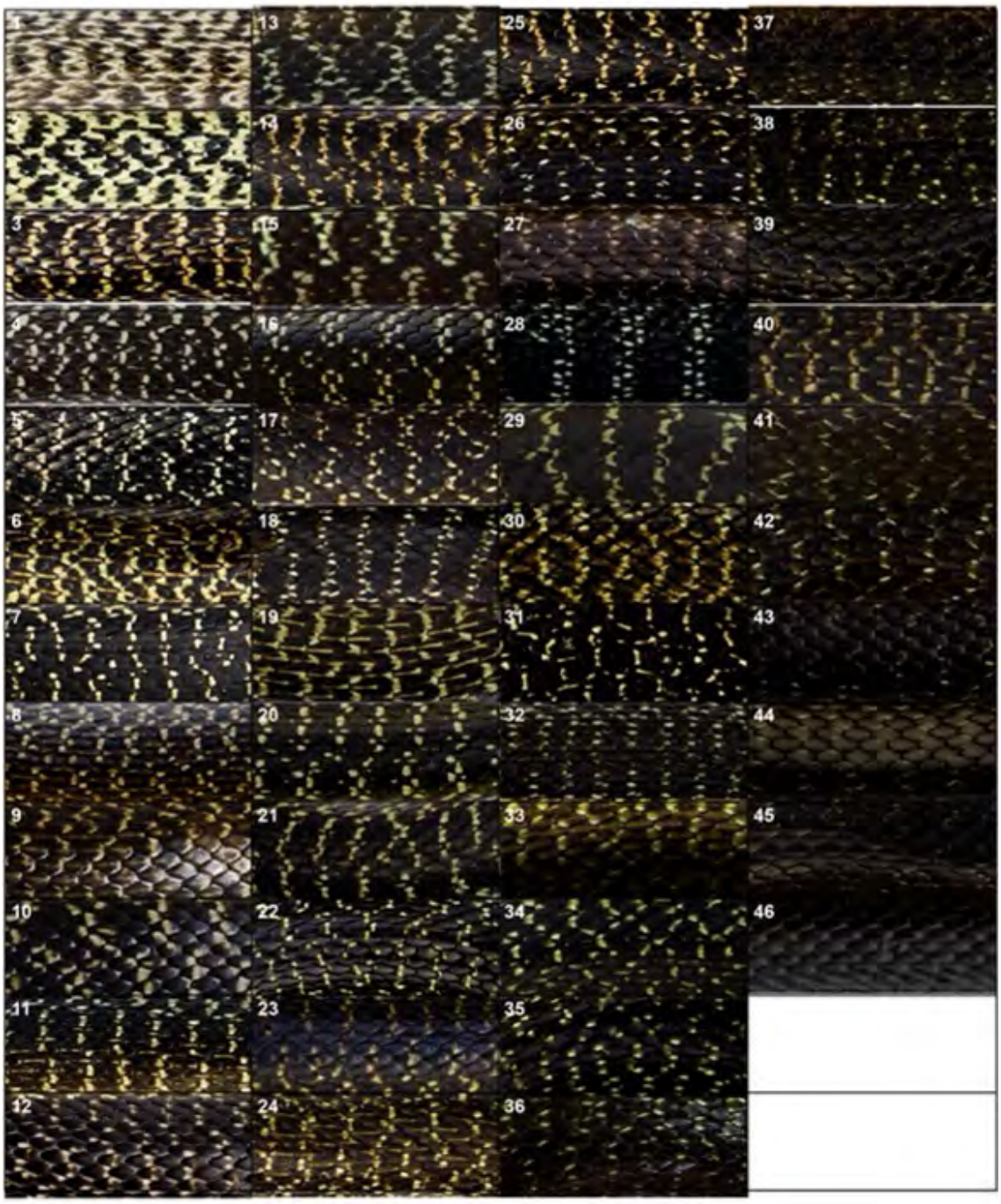

**Figure 7.** Images of dorsal scale pigmentation after preparation for quantification of melanism. Individuals displayed on image 1–46 with decreasing proportion of yellow pigmentation: 1. HV036, 53.84%; 2. HV016, 48.41%; 3. HV037, 21.33%; 4. HV011, 19.44%; 5. HV053, 18.71%; 6. HV052, 18.02%; 7. HV007, 14.94%; 8. HV031, 14.58%; 9. HV010, 14.43%; 10. HV012, 14.42%; 11. HV035, 14.27%; 12. HV056, 14.06%; 13. HV018, 13.53%; 14. HV008, 11.61%; 15. HV013, 11.19%; 16. HV028, 9.88%; 17. HV009, 9.66%; 18. HV041, 8.74%; 19. HV020, 8.27%; 20. HV026, 7.73%; 21. HV023, 7.35%; 22. HV062, 7.12%; 23. HV033, 7.08%; 24. HV039, 7.07%; 25. HV038, 6.94%; 26. HV051, 6.24%; 27. HV029, 6.03%; 28. HV045, 6.03%; 29. HV019, 5.26%; 30. HV034, 5.01%; 31. HV014, 4.5%; 32. HV030, 3.99%; 33. HV065, 3.74%; 34. HV021, 3.56%; 35. HV049, 2.87%; 36. HV066, 2.61%; 37. HV043, 2.36%; 38. HV044, 2.31%; 39. HV054, 2.17%; 40. HV022, 2.02%; 41. HV067, 1.53%; 42. HV061, 1.49%; 43. HV055, 0.4%; 44. HV048, 0.23%; 45. HV063, 0.2%; 46. HV058, 0%

*Genome wide association study*

We evaluated the Wald p-value from the univariate mixed linear model analysis from GEMMA for outlier SNPs that were associated the prevalence of yellow pigmentation (Figure 8). We found that 14 SNPs were strongly associated with Wald p-values lower than $10^{-5}$ (Supplementary File S6). The gene annotations of these SNPs were extracted from the reference genome of *P. guttatus* (GCA_001185365.2). However, we did not find any genes known to be associated with pattern regulation or the melanin production, such as POMC or MSH (Senczuk et al., 2021).

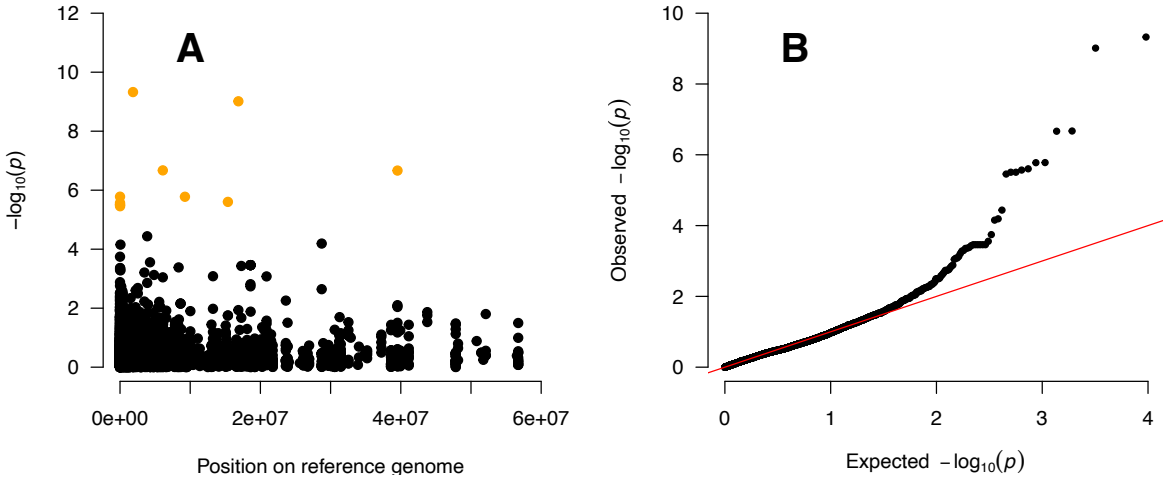


**Figure 8.** A. Manhattan plot showing SNPs that are associated with the characters state of melansim, calculated with a univariate mixed linear model in GEMMA. The y-axis shows -log10 transformed Wald p-values. SNPs with a p-value < $10^{-5}$ are marked in orange. B. A quantile-quantile plot based on the p-values, indicating that some SNPs have a far lower p-value than it would be expected in a normal distribution.

## Discussion

*Evolutionary history*

Our molecular analysis underlines the existence of two well defined lineages with substantial genetic differentiation between their allopatric occurrences ($F_{ST}$ =0.19). This observation is consistent with the 4% genetic divergence reported from mitochondrial genes, which has been translated to a level of differentiation that is similarly observed between closely and taxonomically well separated snake species in Europe (Rato et al., 2009, Mezzasalma et al., 2015). However, our admixture analyses revealed tremendous gene flow along a rather broad (>100 km in northern Italy) contact zone between the two subspecies. The large admixture between the genetic lineages suggests limited or no prezygotic barriers and the lack of selection against hybrids and intermediate morphotypes and post-zygotic reproductive barriers. The lack of complete reproductive barriers is common in young lineages that differentiated in allopatry during the last glacial cycles, such as species of the Fire-bellied oads *Bombina* (Nürnberger et al., 2016; Szymura & Barton, 1986) or the Spadefoot Toads *Pelobates* (Dufresnes et al., 2019). In such cases, reproductive isolation may have evolved upon secondary contact, through reinforcement (see also Hoskin et al., 2005). For example, there is strong selection against hybrids limiting the gene flow between the two well defined sibling species *Bombina variegata* and *B. bombina* (Szymura and Barton, 1986). Similar to theses well-studied amphibian species, it appears most likely that the subspecies of *H. viridiflavus* are currently experiencing secondary contact after they were isolated by distance due to different glacial refugia, as suggested before by Mezzasalma et al. (2018). But given the high mobility of the species and the already widely extended contact zone it is questionable whether *viridiflavus* and *carbonarius* will reach further differentiation in future. Under the assumption that there are no selective pressures against hybrids and little niche differentiation for the two subspecies, the contact zone could further expand in future in large parts of the post-glacially recolonized area. Given the extensive distribution range of the species, a complete fusion of the gene pool appears doubtful, more likely non-admixed individuals will prevail within the former glacial refugia. The scenario of species collapse has been observed, though, in species without reproductive isolation with more limited distribution ranges, such as postglacial clades in the Three-spined Stickleback *Gasterosteus aculeatus* species complex at lake Enox (Taylor et al., 2005)

A potential scenario for the outcome of extensive admixture is displayed in Ticino, Switzerland. *H. viridiflavus* in Ticino is only connected southwards to its conspecifics in Italy

and usually displays an intermediate phenotype, darkish but not completely black (Hofer, 2001). Genetically, we found that they are strongly admixed between the two subspecies (Fig. 2). However, due to sequencing of the mitochondrial gene *Cyt-b* (Meier et al., 2022), we know that the mitochondrial gene pool exclusively corresponds to *carbonarius*. Hence, the most likely scenario is that primarily males from the subspecies *viridiflavus* have entered the Ticino in the past and interbred with females of the subspecies *carbonarius*. Furthermore, expansion of the contact zone can be accelerated if individuals are translocated by humans. This has been shown in Vaud (Hofer, 2001), where introduced populations of non-admixed *viridiflavus* in the North and admixed *viridiflavus* x *carbonarius* in the South are present, even though the Alps were never traversable for the species towards the Italian populations and the *carbonarius* gene pool.

*Association of genetics and morphology*

In the context of evolutionary history, we were also interested to understand the colour polymorphism in *H. viridiflavus*, ranging from the nominate black with yellow dots and stripes to various intermediate forms, such as the brown and abundistic phenotype, to the completely black, melanotic form (Rato et al., 2009; Senczuk et al., 2021; M. Zuffi, 2008). The occurrence of melanism in European vipers is evidently linked to environmental factors, such as solar radiation, temperature and latitude (Martínez-Freiría et al., 2020). However, the phenotypes of *H. viridiflavus* are segregated between the two subspecies, respectively occur within their contact zone. Hence, there is neither a latitudinal gradient, nor meteorological differences which could explain the occurrence of melanism (Mezzasalma et al., 2018).
On a molecular basis, studies on more distantly related garter snakes *Thamnophis sirtalis* have shown that melanism is inherited as a simple Mendelian recessive trait (King, 2003). But since there are so many intermediate phenotypes in *H. viridiflavus*, a single-locus recessive Mendelian inheritance can hardly explain the observed diversity. Indeed, we found few SNPs that showed a more significant association with the melanotic character state. These SNPs are partly positioned in annotated genes in the reference genome of *P. guttatus* and occur throughout the genome. However, none of these annotations were directly linked to genes involved in melanogenesis. This is little surprising, since annotations of genes are far from complete, especially in genomes with lots of contigs such as *P. guttatus* (119,215 contigs) and annotated genes sometimes are only distantly linked to specific SNP position. In addition, RADseq only samples a small fraction of the genome. Hence, some of the outlier SNPs might only be indirectly associated with melanism. While this approach seems to be seeking for the needle in the hay stack, also studies that focused specifically on candidate genes for melanism

have difficulties to show clear associations (Cox et al., 2013; Senczuk et al., 2021). Moreover, it is unclear how the aberrant "high yellow" colour morphs (Fig. 7a–b) are genetically regulated and if they might be connected to similar mechanisms as melanism (see also Di Nicola et al. 2021).We conclude that a more dense genomic sampling or breeding experiments, such as QTL mapping, would be necessary to understand the mechanisms underlying the colour variation in *H. viridiflavus*.

*Taxonomic treatment*

Given the long history of taxonomic revalidations of *H .viridiflavus* (Mezzasalma et al., 2015; Nagy et al., 2004; Rato et al., 2009; Schätti & Vanni, 1986; Speybroeck et al., 2020), we aimed to answer the question, whether the subspecies *carbonarius* should be treated as sibling species to the nominate subspecies *viridiflavus*. Admixture analysis implicates that gene flow occurs regularly between the two subspecies within a large contact zone. Followingly, reproductive barriers are likely weak or inexistent and hybrids between the two subspecies seem to not be negatively affected by natural selection. We conclude that it is appropriate to treat *viridiflavus* and *carbonarius* as subspecies given their parapatric distribution area and the prevalence of hybridization in the wide contact zone. However, despite of the gene flow in the contact zone, we find good evidence that the two subspecies represent distinct evolutionarily significant units (ESU) due to the strong genetic differentiation of the non-admixed individuals. While taxonomist usually expect species to be reproductively isolated, conservationists should protect the whole biodiversity, including the intraspecific genetic diversity (Casacci et al., 2014). ESU represent an important tool in order to translate this intraspecific diversity into conservation approaches (Borges et al., 2018). Treating the subspecies of *H. viridiflavus* as ESUs not only allows to evaluate needs for conservation programs separately, but also emphasizes the need to analyse the genetic identity of specimens at introduced locations. In future, it would be interesting to monitor shifts in the contact zone, as this could represent an example of "species collapse" in progress. We further want to encourage herpetologists to acknowledge subspecies, as they represent a significant contribution to biodiversity.

**Acknowledgements**

We are very grateful to everybody who contributed to this study in some way. We would like to thank the numerous curators from the natural history museums and further herpetologists, who provided tissue samples for molecular analyses: Olivier Glaizot from the Musée de Zoologie de Lausanne, Nicola Zambelli from the Museo Cantonale della Storia Naturale in Lugano, Andreas Schmitz from the Muséum d'histoire naturelle de la Ville de Genève, Edoardo Razzetti from the Natural History Museum of Pavia, Nicolas Vidal from the Natural History Museum of Paris, Alix Michon from the Ligue pour la protection des Oiseaux (LPO), Dimitri Laurent from the Group Herpétologique Rhône-Alpes (GHRA), Xavier Bonnet from the Centre d'Etudes Biologiques de Chizé and Luca Coppari. Furthermore, our gratitude belongs to all the people who supported the sample collection in the field, the lab work and bioinformatical analyses: Roger Meier, Hans-Peter Rusterholz, Daniel Croll and Christophe Praz. We further thank Bruno Baur for supervising the thesis project of Noah Meier and providing feedback on an earlier version of the manuscript.
The Italian ministry of Environment provided us with a permission to conduct the field work for this study. Bioinformatical analyses were run on the cluster Scicore at the University of Basel. This project was financially supported by the Swiss Federal Office for the Environment (FOEN).

**Data availability**

The raw sequences are uploaded to GenBank (NCBI Project PLACEHOLDER). The R script for the quantification of melanism, the geometric morphometrics analysis and the cline analysis is given in Supplementary File S5. Output data of the admixture analysis, the colour analysis and the geometric morphometrics are given in Supplementary File S7.

**Authors contributions**

NM and SU conceptualized the study, all co-authors made substantial contributions to the conception and design. NM, FS, MZ, PG and SU conducted field work or contributed to the preparation of museal samples. NM conducted the lab work. NM, KL and SU performed the bioinformatical analyses. NM wrote the manuscript with inputs from all co-authors. SU secured the funding for the study.

## References


Adams, D. C., & Otárola-Castillo, E. (2013). geomorph: An R package for the collection and analysis of geometric morphometric shape data. *Methods in Ecology and Evolution*, *4*(4), 393–399. https://doi.org/10.1111/2041-210X.12035

Alexander, D. H., & Lange, K. (2011). Enhancements to the ADMIXTURE algorithm for individual ancestry estimation. *BMC Bioinformatics*, *12*, 246. https://doi.org/10.1186/1471-2105-12-246

Borges, V. S., Santiago, P. C., Lima, N. G. S., Coutinho, M. E., Eterovick, P. C., & Carvalho, D. C. (2018). Evolutionary Significant Units within Populations of Neotropical Broad-Snouted Caimans (*Caiman latirostris*, Daudin, 1802). *Journal of Herpetology*, *52*(3), 282–288. https://doi.org/10.1670/17-074

Braby, M. F., Eastwood, R., & Murray, N. (2012). The subspecies concept in butterflies: Has its application in taxonomy and conservation biology outlived its usefulness?: the subspecies concept. *Biological Journal of the Linnean Society*, *106*(4), 699–716. https://doi.org/10.1111/j.1095-8312.2012.01909.x

Bruno, S. (1980). I serpenti del Veneto. I. Morfologia, tassonomia, geonemia. *Lavori Della Società Veneziana Di Scienze Naturali*, *5*(suppl.), 70.

Burbrink, F. T., Lawson, R., & Slowinski, J. B. (2000). Mitochondrial DNA phylogeography of the polytypic north amercian rat snake (*Elaphe obsoleta*): A critique of the subspecies concept. *Evolution*, *54*(6), 2107–2118.

Bush, M., & Smeller, J. (1978). Blood collection & injection techniques in snakes. *Veterinary Medicine*, 4.

Casacci, L. P., Barbero, F., & Balletto, E. (2014). The "Evolutionarily Significant Unit" concept and its applicability in biological conservation. *Italian Journal of Zoology*, *81*(2), 182–193. https://doi.org/10.1080/11250003.2013.870240

Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*, *34*(17), i884–i890. https://doi.org/10.1093/bioinformatics/bty560

Cox, C. L., Rabosky, A. R. D., & Chippindale, P. T. (2013). Sequence variation in the Mc1r gene for a group of polymorphic snakes. *Gene*, *513*(2), 282–286. https://doi.org/10.1016/j.gene.2012.10.065

Coyne, J. A., & Orr, H. A. (2004). *Speciation*. Sinauer Associates.

Danecek, P., Auton, A., Abecasis, G., Albers, C. A., Banks, E., DePristo, M. A., Handsaker, R. E., Lunter, G., Marth, G. T., Sherry, S. T., McVean, G., Durbin, R., & 1000 Genomes Project Analysis Group. (2011). The variant call format and VCFtools. *Bioinformatics*, *27*(15), 2156–2158. https://doi.org/10.1093/bioinformatics/btr330

De Queiroz, K. (1998). The General Lineage Concept of Species, Species Criteria, and the Process of Speciation. In *Endless Forms: Species and Speciation* (p. 19).

De Queiroz, K. (2005). A Unified Concept of Species and Its Consequences for the Future of

Taxonomy. *Proceeding of the California Academy of Sciences*, 21.

De Queiroz, K. (2007). Species Concepts and Species Delimitation. *Systematic Biology*, *56*(6), 879–886. https://doi.org/10.1080/10635150701701083

Derryberry, E. P., Derryberry, G. E., Maley, J. M., & Brumfield, R. T. (2014). HZAR: Hybrid zone analysis using an R software package. *Molecular Ecology Resources*, *14*(3), 652–663. https://doi.org/10.1111/1755-0998.12209

Di Nicola, M. R., Cavigioli, L., Luiselli, L., & Andreone, F. (2019). *Anfibi & Rettili d'Italia*. Edizioni Belvedere.

Di Nicola, M. R., Di Rosa, D., Mariacher, A., & Zabbia, T. (2021). *Hierophis viridiflavus carbonarius* (Western Whip Snake). Coloration. *Herpetological Review*, *52*(4), 873–874.

Dufresnes, C., Strachinis, I., Suriadna, N., Mykytynets, G., Székely, P., Vukov, T., Arntzen, J. W., Wielstra, B., Geffen, E., Gafny, S., Kumlutaş, Y., Ilgaz, Ç., Mizsei, E., Szabolcs, M., Kolenda, K., Smirnov, N., Géniez, P., Lukanov, S., Crochet, P.-A., … Denoël, M. (2019). Phylogeography of a cryptic speciation continuum in Eurasian spadefoot toads ( *Pelobates* ). *Molecular Ecology*, *28*, 3257–3270. https://doi.org/DOI: 10.1111/mec.15133

Heimes, P. (1993). *Coluber viridiflavus* Lacepède, 1789—Gelbgrüne Zornnatter. In W. Böhme (Ed.), *Handbuch der Reptilien und Amphibien Europas Schlangen (Serpentes) I*. AULA-Verlag.

Hofer, U. (2001). *Coluber* (*Hierophis*) *viridiflavus*. In U. Hofer, J.-C. Monney, & G. Dusej, *Die Reptilien der Schweiz: Verbreitung, Lebensräume, Schutz*. Springer.

Hoskin, C. J., Higgie, M., McDonald, K. R., & Moritz, C. (2005). Reinforcement drives rapid allopatric speciation. *Nature*, *437*(7063), 1353–1356. https://doi.org/10.1038/nature04004

Huson, D. H., & Bryant, D. (2006). Application of Phylogenetic Networks in Evolutionary Studies. *Molecular Biology and Evolution*, *23*(2), 254–267. https://doi.org/10.1093/molbev/msj030

Jablonski, D., Nagy, Z. T., Avcı, A., Olgun, K., Kukushkin, O. V., Safaei-Mahroo, B., & Jandzik, D. (2019). Cryptic diversity in the smooth snake (*Coronella austriaca*). *Amphibia-Reptilia*, *40*(2), 179–192. https://doi.org/10.1163/15685381-20181025

Jagar, T. (2011). Occurrences of the brown colour form of Western Whip (*Hierophis viridiflavus carbonarius* (Bonaparte, 1833)) in Slovenia. *Natura Sloveniae*, *13*(2), 51–55.

Kindler, C., Chèvre, M., Ursenbacher, S., Böhme, W., Hille, A., Jablonski, D., Vamberger, M., & Fritz, U. (2017). Hybridization patterns in two contact zones of grass snakes reveal a new Central European snake species. *Scientific Reports*, *7*(1), 7378. https://doi.org/10.1038/s41598-017-07847-9

King, R. B. (2003). Mendelian inheritance of melanism in the Garter Snake *Thamnophis sirtalis*. *Herpetologica*, *59*(4), 484–489. https://doi.org/10.1655/02-93

Kulmuni, J., Butlin, R. K., Lucek, K., Savolainen, V., & Westram, A. M. (2020). Towards the completion of speciation: The evolution of reproductive isolation beyond the first barriers. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *375*(1806),

20190528. https://doi.org/10.1098/rstb.2019.0528

Li, H. (2011). A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics*, *27*(21), 2987–2993. https://doi.org/10.1093/bioinformatics/btr509

Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics (Oxford, England)*, *25*(14), 1754–1760. https://doi.org/10.1093/bioinformatics/btp324

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., & 1000 Genome Project Data Processing Subgroup. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics (Oxford, England)*, *25*(16), 2078–2079. https://doi.org/10.1093/bioinformatics/btp352

Martínez-Freiría, F., Toyama, K. S., Freitas, I., & Kaliontzopoulou, A. (2020). Thermal melanism explains macroevolutionary variation of dorsal pigmentation in Eurasian vipers. *Scientific Reports*, *10*(1), 16122. https://doi.org/10.1038/s41598-020-72871-1

Mayden, R. L. (1997). A hierarchy of species concept: The denouement in the saga of the species problem. In Chapman & Hall (Eds.), *Species: The Units of Biodiversity* (London).

Meier, N., Glaizot, O., Schmitz, A., Zambelli, N., & Ursenbacher, S. (2022). Where are you from? Origin determination of the introduced green whip snake, *Hierophis viridiflavus* (Squamata: Colubridae), in Switzerland. *Herpetology Notes*, *15*, 335–344.

Meirmans, P. G. (2020). GENODIVE version 3.0: Easy-to-use software for the analysis of genetic data of diploids and polyploids. *Molecular Ecology Resources*, *20*(4), 1126–1131. https://doi.org/10.1111/1755-0998.13145

Mezzasalma, M., Dall'Asta, A., Loy, A., Cheylan, M., Lymberakis, P., Zuffi, M. A. L., Tomović, L., Odierna, G., & Guarino, F. M. (2015). A sisters' story: Comparative phylogeography and taxonomy of *Hierophis viridiflavus* and *H. gemonensis* (Serpentes, Colubridae). *Zoologica Scripta*, *44*(5), 495–508. https://doi.org/10.1111/zsc.12115

Mezzasalma, M., Di Febbraro, M., Guarino, F. M., Odierna, G., & Russo, D. (2018). Cold-blooded in the Ice Age: "Refugia within refugia", inter-and intraspecific biogeographic diversification of European whipsnakes (Squamata, Colubridae, *Hierophis*). *Zoology*, *127*, 84–94. https://doi.org/10.1016/j.zool.2018.01.005

Nagy, T. Z., Joger, U., Guicking, D., & WInk, M. (2002). Phylogeography of the European Whip Snake *Coluber* (*Hierophis*) *viridiflavus* as inferred from nucleotide sequences of the mitochondrial *cytochrome b* gene and ISSR genomic fingerprinting. *Biota*, *3*(1–2), 109–118.

Nagy, Z. T., Lawson, R., Joger, U., & Wink, M. (2004). Molecular systematics of racers, whipsnakes and relatives (Reptilia: Colubridae) using mitochondrial and nuclear markers. *Journal of Zoological Systematics and Evolutionary Research*, *42*(3), 223–233. https://doi.org/10.1111/j.1439-0469.2004.00249.x

Nosil, P., Harmon, L. J., & Seehausen, O. (2009). Ecological explanations for (incomplete) speciation. *Trends in Ecology & Evolution*, *24*(3), 145–156. https://doi.org/10.1016/j.tree.2008.10.011

Nürnberger, B., Lohse, K., Fijarczyk, A., Szymura, J. M., & Blaxter, M. L. (2016). Para-allopatry in hybridizing fire-bellied toads (*Bombina bombina* and *B. variegata*): Inference from transcriptome-wide coalescence analyses: Para-allopatry in *Bombina*. *Evolution*, *70*(8), 1803–1818. https://doi.org/10.1111/evo.12978

Peterson, B. K., Weber, J. N., Kay, E. H., Fisher, H. S., & Hoekstra, H. E. (2012). Double Digest RADseq: An Inexpensive Method for De Novo SNP Discovery and Genotyping in Model and Non-Model Species. *PLoS ONE*, *7*(5), e37135. https://doi.org/10.1371/journal.pone.0037135

Phillimore, A. B., & Owens, I. P. F. (2006). Are subspecies useful in evolutionary and conservation biology? *Proceedings of the Royal Society B: Biological Sciences*, *273*(1590), 1049–1053. https://doi.org/10.1098/rspb.2005.3425

Pokrant, F., Kindler, C., Ivanov, M., Cheylan, M., Geniez, P., Böhme, W., & Fritz, U. (2016). Integrative taxonomy provides evidence for the species status of the Ibero-Maghrebian grass snake *Natrix astreptophora*. *Biological Journal of the Linnean Society*, *118*(4), 873–888. https://doi.org/10.1111/bij.12782

QGIS.org. (2022). *QGIS Geographic Information System. QGIS Association.* http://www.qgis.org

Quiagen. (2020). *DNeasy® Blood & Tissue Handbook*.

R Core Team. (2021). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Rato, C., Zuffi, M., Corti, C., Fornasiero, S., Gentilli, A., Razzetti, E., Scali, S., Carretero, M., & Harris, D. J. (2009). Phylogeography of the European Whip Snake, *Hierophis viridiflavus* (Colubridae), using mtDNA and nuclear DNA sequences. *Amphibia-Reptilia*, *30*(2), 283–289. https://doi.org/10.1163/156853809788201126

Reiserer, R. S., Schuett, G. W., & Beck, D. D. (2013). Taxonomic reassessment and conservation status of the beaded lizard, *Heloderma horridum* (Squamata: Helodermatidae). *Amphibian and Reptile Conservation*, *7*(1), 23.

Rohlf, F. (2015). The tps series of software. *Hystrix, the Italian Journal of Mammalogy*, *26*(1). https://doi.org/10.4404/hystrix-26.1-11264

Ryder, O. A. (1986). Species Conservation and Systematics: The Dilemma of Subspecies. *Trends in Ecology & Evolution*, *1*(1), 9–10.

Scali, S., Mangiacotti, M., & Bonardi, A. (2008). Living on the edge: Habitat selection of *Hierophis viridiflavus*. *Acta Herpetologica*, *3*(2), 13.

Schätti, B., & Vanni, S. (1986). Intraspecific Variation in *Coluber viridiflavus* Lacépède, 1789, and validity of its subspecies (Reptilia, Serpentes, Colubridae). *Revue Suisse Zoologie*, 219–232.

Senczuk, G., Gramolini, L., Avella, I., Mori, E., Menchetti, M., Aloise, G., & Castiglia, R. (2021). No association between candidate genes for color determination and color phenotype in *Hierophis viridiflavus,* and characterization of a contact zone. *Journal of Zoological Systematics and Evolutionary Research*, *59*(3), 748–759. https://doi.org/10.1111/jzs.12451

Seutin, G., White, B. N., & Boag, P. T. (1991). Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal of Zoology*, *69*(1), 82–90. https://doi.org/10.1139/z91-013

Speybroeck, J., Beukema, W., Bok, B., Van Der Voort, J., & Velikov, I. (2016). *Field guide to the amphibians & Reptiles: Of Britain and Europe*. Bloomsbury.

Speybroeck, J., Beukema, W., Dufresnes, C., Fritz, U., Jablonski, D., Lymberakis, P., Martínez-Solano, I., Razzetti, E., Vamberger, M., Vences, M., Vörös, J., & Crochet, P.-A. (2020). Species list of the European herpetofauna – 2020 update by the Taxonomic Committee of the Societas Europaea Herpetologica. *Amphibia-Reptilia*, *41*(2), 139–189. https://doi.org/10.1163/15685381-bja10010

Szymura, J. M., & Barton, N. H. (1986). Genetic Analysis of a Hybrid Zone Between the Fire-Bellied Toads, *Bombina bombina* and *B. variegata*, Near Cracow in Southern Poland. *Evolution*, *40*(6), 1141. https://doi.org/10.2307/2408943

Taylor, E. B., Boughman, J. W., Groenenboom, M., Sniatynski, M., Schluter, D., & Gow, J. L. (2005). Speciation in reverse: Morphological and genetic evidence of the collapse of a three-spined stickleback (*Gasterosteus aculeatus*) species pair: Collapse of a stickleback pair. *Molecular Ecology*, *15*(2), 343–355. https://doi.org/10.1111/j.1365-294X.2005.02794.x

Turner, S. D. (2018). qqman: An R package for visualizing GWAS results using Q-Q and manhattan plots. *Journal of Open Source Software*, *3*(25), 731. https://doi.org/10.21105/joss.00731

Ullate-Agote, A., Milinkovitch, M. C., & Tzika, A. C. (2014). The genome sequence of the corn snake (*Pantherophis guttatus*), a valuable resource for EvoDevo studies in squamates. *The International Journal of Developmental Biology*, *58*(10-11–12), 881–888. https://doi.org/10.1387/ijdb.150060at

Weller, H. I., & Westneat, M. W. (2019). Quantitative color profiling of digital images with earth mover's distance using the R package colordistance. *PeerJ*, *7*, e6398. https://doi.org/10.7717/peerj.6398

Zhou, X., & Stephens, M. (2012). Genome-wide efficient mixed-model analysis for association studies. *Nature Genetics*, *44*(7), 821–824. https://doi.org/10.1038/ng.2310

Zuffi, M. (2008). Colour pattern variation in populations of the European Whip snake, *Hierophis viridiflavus*: Does geography explain everything? *Amphibia-Reptilia*, *29*(2), 229–233. https://doi.org/10.1163/156853808784124929

Zuffi, M. A. L., Fornasiero, S., & Bonnet, X. (2007). Geographic variation in reproductive output of female European whip snakes (*Hierophis viridiflavus*). *Herpetological Journal*, *17*, 219–224.

**List of supplementary files:**

Supplementary File S1: Collection data of samples

Supplementary File S2: Position of Landmarks

Supplementary File S3: Example output of colourdistance

Supplementary File S4: Cross-validation

Supplementary File S5: R script

Supplementary File S6: Filtered output data of GEMMA

Supplementary File S7: Output data of admixture, colour analysis and geometric morphometrics

University of Basel
Faculty of Science

**Declaration on Scientific Integrity**
(including a Declaration on Plagiarism and Fraud)
Translation from German original

Title of Thesis: Lack of reproductive isolation between the two subspecies of the Green Whip Snake Hierophis viridiflavus

Name Assessor: Prof. Dr. Bruno Baur

Name Student: Noah Meier

Matriculation No.: 17-054-610

With my signature I declare that this submission is my own work and that I have fully acknowledged the assistance received in completing this work and that it contains no material that has not been formally acknowledged. I have mentioned all source materials used and have cited these in accordance with recognised scientific rules.

Place, Date: Rheinfelden, 21.06.2022 Student: N. Meier

Will this work, or parts of it, be published?

○ No

● Yes. With my signature I confirm that I agree to a publication of the work (~~print~~/digital) ~~in the library, on the research database of the University of Basel and/or~~ on the document server of the department. ~~Likewise, I agree to the bibliographic reference in the catalog SLSP (Swiss Library Service Platform)~~. (cross out as applicable)

Publication as of: December 2022

Place, Date: 21.06.2022 Student: N. Meier

Place, Date: _____ Assessor: _____

*Please enclose a completed and signed copy of this declaration in your Bachelor's or Master's thesis*