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Rare genetic admixture and unidirectional gene flow between *Vipera aspis* and *Vipera berus* at their contact zone in western France

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Abstract. Asp vipers (*Vipera aspis*) and adders (*Vipera berus*) coexist in the Loire-Atlantique department in France where the two species reach their respective range limits. This contact zone is of special interest since hybridization has been recently discovered there. We carried out extensive sampling to further investigate the hybrid status of morphologically typical individuals and to evaluate the proportion of genetically admixed individuals in this area. Using microsatellite markers, no hybrids with typical morphological traits of either asp vipers or adders were detected. All recently investigated individuals with intermediate morphological traits were shown to be hybrids. A rather low proportion of genetically admixed individuals (1.5-3%) and a very small number of supposed second-generation hybrids suggest reduced fertility of first-generation hybrids or low viability of their progeny. The investigation of mtDNA of newly sampled hybrids support the finding that hybridization only occurs between female *V. aspis* and male *V. berus*. Several possible explanations for the unidirectional hybridization are discussed and consequent future studies suggested.

Keywords: contact zone, Eurasian vipers, interspecific hybridization, Viperidae.

Introduction

Studying contact zones is essential for understanding hybridization processes (Tarroso et al., 2014). Natural hybridization can be observed in about 10% of species in major faunal groups, while it is even more frequent in plants (Mallet, 2008). While hybridization is mainly detected in closely related taxa due to the lack of (or limited) reproduction barriers, it can sometimes also be observed in well-defined species (Mallet, 2008). Reproductive barriers can be either pre- or postzygotic (Rundle and Nosil, 2005). Prezygotic barriers to hybridization include spatial and temporal isolation, natural selection against immigrants, sexual isolation and postmating, prezygotic isolation (Rundle and Nosil, 2005). In the absence of prezygotic reproductive barriers, postzygotic barriers can still impede hybridization as F1 hybrids are often sterile, while F2 hybrids are often not viable or exhibit reduced fitness (Stebbins, 1958). These latter barriers can be related to either intrinsic or ecologically-induced postzygotic isolation, while sexual selection against hybrids may represent another postzygotic barrier (Rundle and Nosil, 2005). If reproductive barriers do not fully isolate two species, introgression can result in reticulated phylogenies, adaptations and speciation (Mallet, Besansky and Hahn, 2016). Introgression does not always affect both species equally and asymmetric introgression is frequent (Currat et al., 2008).

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In snakes, it has been shown that some valid species are hybridizing in the wild (e.g., Mebert, 2008; Mebert et al., 2011), but it was mostly observed for phylogenetically closely related ones. However, introgressive hybridization is still a rarely observed phenomenon in snakes (e.g., Sanders, Rasmussen and Guinea, 2014; Kindler et al., 2017; Schultze et al., 2019). In the genus *Vipera*, the speciation of most taxa is quite recent (about 17 Myr ago for the oldest one, but less than 2 Myr for the youngest; Freitas et al., 2020), leading to several contact zones with F1 and later-generation hybrids, with variable levels of introgression, mainly dependent on the time since the species have been separated. For instance, in the contact zone between *Vipera aspis*, *V. latastei* and *V. seoanei* in Spain, frequent hybridization between *V. aspis* and *V. latastei* occurrs (two closely related species), whereas hybrids between *V. latastei* and *V. seoanei* (belonging to two different subgenera) are only rarely present (Tarroso et al., 2014). Natural hybrids of viper species are also found e.g., between *V. berus* and *V. ammodytes* (Mihalca et al., 2003; Czirják et al., 2015), *V. aspis* and *V. ammodytes* (Lapini, 1988; Bagnoli, Capula and Luiselli, 2014) and between *V. berus* and *V. renardi* (Pavlov et al., 2011). Additionally, hybrids of viper species were repeatedly bred in captivity (Mertens, 1964; Saint Girons, 1977; Faoro, 1986; Saint Girons, 1990; Schweiger, 2009). In the *Vipera* genus, many species show parapatric distributions (Chamorro et al., 2020) and frequently a lack of total genetic isolation (e.g., Czirják et al., 2015) with evidences for incomplete reproductive barriers (Pavlov et al., 2011; Tarroso et al., 2014). However, not all contact zones result in hybridization between the parapatric species (e.g., Luiselli, Filippi and Lena, 2009).

The para-Mediterranean *Vipera aspis* (asp viper, subgenus *Vipera*) and the Euro-Siberian *Vipera berus* (adder, subgenus *Pelias*) diverged about 10 to 15 Mya (Szyndlar and Rage, 2002; Freitas et al., 2020). While the warm-adapted *V. aspis* occurs throughout northeastern Spain, the southern half of France, Italy, northwestern Slovenia and the Black Forest, the cold-adapted *V. berus* is distributed throughout a large Eurosiberian range (Saint Girons, 1980a). Both species can easily be distinguished by their morphology, including dorsal pattern, head scalation and the presence (*V. aspis*) or absence (*V. berus*) of an upturned snout (Saint Girons, 1980a). While *V. aspis* has 42 chromosomes, *V. berus* has only 36 (Saint Girons, 1977). The two species usually occupy contrasted climatic niches and habitats (Saint Girons, 1975; Scali et al., 2011) as they differ in physiology and water requirements (Guillon et al., 2013). Still, contact zones exist in Switzerland (Monney, 1996), Italy (Scali et al., 2011), Slovenia (Mebert et al., 2015) and France (Saint Girons, 1975; Naulleau, 1986). Even if the ecological niches are different between both species, there is always some slight overlap at the margin of both niches, where the species usually occupy contrasted microhabitats (Guillon et al., 2013), and where supposed hybrids were found in different parts of France: one individual with mixed morphological traits was photographed in Auvergne (Geniez, 2015), a few individuals were found and morphologically identified as hybrids by Viaud-Grand-Marais (1869) in the Deux-Sèvres department (*V. berus* population currently extinct) and by Saint Girons (1975) in the Loire-Atlantique department. These supposed hybrids showed intermediate morphological traits, specifically head scalation, dorsal pattern, or eye colour, not typical for either *V. aspis* or *V. berus*. The existence of hybrids in the Loire-Atlantique department was later genetically confirmed by Guiller, Lourdais and Ursenbacher (2017).

In the study of Guiller, Lourdais and Ursenbacher (2017), ten individuals from the contact zone showing mixed morphological traits were genetically analysed and compared to reference individuals of each parental species ($N = 2 \times$

20) sampled about 10 km away from the contact zone. No individuals with typical morphological traits of *V. aspis* and *V. berus* from the contact zone itself were analysed in this former study. Therefore, an evaluation of the proportion of genetically admixed individuals as well as an assessment of a potential hybrid status of individuals with typical morphological traits is not available. Herein we performed an expanded sampling to answer the following questions:

(i) What is the hybrid status of morphologically intermediate individuals? We predict that mainly F1 and only rarely post-F1 lineages occur.

(ii) Are there hybrids with typical morphological traits of one of the parental species? Since we predicted a rare presence of post-F1 lineages, we expect at most a few hybrids with typical morphological traits.

(iii) What is the proportion of genetically admixed individuals in the contact zone? By analysing randomly sampled individuals using microsatellite markers we expect a low proportion of genetically admixed individuals.

(iv) Is gene flow directional? By investigating the mtDNA as a marker of the maternal lineage, we expect a unidirectional hybridization with *V. aspis* as the maternal parent as it was the case for all hybrids analysed in the study of Guiller, Lourdais and Ursenbacher (2017).

Materials and methods

Species ecology in the study area

The Loire-Atlantique department constitutes the northern limit of the asp viper distribution, while it represents the southern limit of the adder distribution (Naulleau, 1986). In this area, *V. berus* is growing faster, reach sexual maturity earlier and give birth to a higher number of viable offspring than *V. aspis* (Saint Girons, 1975; Guiller, 2012; Guiller, Legentilhomme and Lourdais, 2012). The main mating season of both species is in spring, but *V. aspis* can also occasionally mate in autumn (Saint Girons, 1975). The spermiogenesis of *V. berus* takes place in spring and a pre-mating ecdysis is required before the onset of reproduction. In *V. aspis*, spermiogenesis mainly occurs in summer and males are ready to mate soon after their emergence from hibernation (Saint Girons, 1975). While male *V. aspis* are ready to mate earlier than male *V. berus*, in contrary, the ovulation of female *V. aspis* takes place a bit later than this of female *V. berus* (Saint Girons, 1975).

Sampling

A total of 170 *V. aspis*, 212 *V. berus* and 11 individuals with intermediate morphological traits were sampled randomly since 2010 in one particular location of the contact zone (municipality of Blain in the Loire-Atlantique department). More details about the extent (1.35 km^2) and structure of the study area can be found in Guiller et al. (2018). Of those 11 morphologically intermediate individuals, seven were specifically chosen to investigate their hybrid status in Guiller, Lourdais and Ursenbacher (2017). The other four individuals with intermediate morphological traits were newly sampled following the previous study and included in the present one. To assess the proportion of genetically admixed individuals of *V. aspis* and *V. berus* in the study site and to detect possible hybrids lacking intermediate morphological traits, 112 of the remaining 386 non-analysed individuals were chosen randomly for genetic analyses (selecting the first 112 samples collected, checking for a similar proportion of both species and both sexes: 30 male and 28 female *V. aspis*, 24 male and 28 female *V. berus* and two females with intermediate morphological traits).

Furthermore, the two morphologically intermediate individuals which were not included in the random sample as well as three additional morphologically intermediate individuals newly sampled in other parts of the contact zone (in municipalities of Le Gâvre and Marsac sur Don) were also investigated. These last three samples were only considered to assess if individuals with intermediate morphological traits are hybrids and if hybridization is directional.

Since in the previous study (Guiller, Lourdais and Ursenbacher, 2017), microsatellite primers, which could only be amplified in one of the two focal species, were also included, and as Structure and NewHybrids (see methods below) could not deal with a large number of missing data, it made sense to re-analyse the samples using only microsatellite markers working for both species. Therefore, six of the seven samples collected after 2010 (mentioned above) as well as two of three additional samples with intermediate morphological traits collected during a non-random sampling before 2010 were re-analysed with the same microsatellite markers as in the present study (see below). One of these latter two re-analysed individuals was showing intermediate morphological traits but was genetically identified as pure *V. aspis* by Guiller, Lourdais and Ursenbacher (2017). Two samples could not be re-analysed due to a lack of extant DNA. For the previous study, reference samples of individuals showing typical morphological traits (20 *V. aspis* and 20 *V. berus*) were collected about 10 km away from the contact zone and were also re-analysed. Consequently, the complete dataset for the genetic analysis regrouped a total of 165 samples (120 collected after 2010 in the contact zone of which 112 randomly chosen to assess proportion of genetically mixed individuals; 43 from different locations).

DNA extraction and microsatellite amplification

DNA of 165 individuals was extracted from clipped ventral scales using the DNeasy Blood and Tissue Kit (Qiagen, Hombrechtikon, Switzerland). Polymerase chain reactions (PCR) of microsatellite markers were initially conducted using 21 fluorescent-labelled primers developed for *V. aspis* (Geser et al., 2013) and *V. berus* (Ursenbacher, Monney and Fumagalli, 2009) in five different multiplex PCR. Multiplex PCRs were performed in 10 μl reactions with the Type-it Microsatellite PCR Kit (Qiagen) following manufacturer's instructions (32 cycles using 2 μl template DNA and primers with a concentration between 0.1 and 0.3 μM after preliminary tests) in an Eppendorf Mastercycler Gradient (Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). PCR products were visualized with an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) and lengths of the alleles were assessed using Peak Scanner™ Software v2.0 (Applied Biosystems). Due to poor performance of the multiplex PCR, a lack of cross-species amplification or a lack of polymorphism, 14 primers were removed after first tests. Finally, only seven microsatellite markers (Va-P8, Va-P20, Va-P25, Va-P26, Va-P29, Va-P51 and Vb-A8) could be amplified and were polymorphic in both species and were kept for further analyses.

Assessment of population structure and hybridization level

To detect possible hybrids and to see if the results with seven microsatellites are consistent with the previous results, a Bayesian cluster analysis was performed using Structure 2.3.4 (Pritchard, Stephens and Donnelly, 2000). Structure was run three times with different settings. First, correlated allele frequencies were used and no prior information was entered into the software (Falush, Stephens and Pritchard, 2003). Second, the same analysis was performed using independent allele frequencies (as the involved species diverged $>$ 10 Mya ago). Finally, a third analysis was performed using prior population information to test for hybrids (reference samples were denoted as pure using USEPOPINFO $=$ 1 with POPFLAG $=$ 1 for *V. aspis* and POPFLAG $=$ 2 for *V. berus*, and USEPOPINFO $=$ 0 for individuals of the contact zone; Hubisz et al., 2009). All the three analyses were performed using the admixture model which can "deal with hybrid zones in a natural way" as mentioned in Pritchard (2009). Ten replicates per K ranging from 1 to 10 were ran, each with 400 000 MCMC iterations after a burn-in of 200 000 iterations. As the results were similar with all the three methods, only the outcome of the first analysis is shown hereinafter.

The most probable number of clusters K was assessed using the mean log-likelihood L(K) as well as the ΔK method (Evanno, Regnaut and Goudet, 2005), both implemented in StructureHarvester Web 0.6.94 (Earl and vonHoldt, 2012). The ten runs corresponding to the most probable number K were pooled using the pipeline CLUMPAK (Kopelman et al., 2015).

To assess the level of hybridization of individuals, NewHybrids 1.1 beta (Anderson and Thompson, 2002) was used allowing to calculate the probability of an individual being assigned to one of the following genotypes: pure *V. aspis* (PVa), pure *V. berus* (PVb), F1, F2, first-generation backcross with *V. aspis* (F1 $\times$ *V. aspis*; BcVa) and first-generation backcross with *V. berus* (F1 $\times$ *V. berus*; BcVb). The analysis was performed twice with a burn-in of 100 000 followed by 1 000 000 sweeps, once using "Jeffreys" priors and once using "Uniform" priors, both without placing priors on mixing proportions and allele frequencies. As the two methods yielded similar results and as assignments of the simulated data were more efficient and accurate using Jeffreys priors, only results of the analysis with Jeffreys priors are shown below. The 20 *V. aspis* and 20 *V. berus* reference individuals from Guiller, Lourdais and Ursenbacher (2017) were included in the analysis denoted as pure individuals collected from another location (using the z and s option of the program). The three additional individuals with intermediate morphological traits were also denoted as sampled in another location (using the s option of the program).

To assess the statistical power of Structure and NewHybrids for correctly identifying individuals as purebreds or hybrids, 500 individuals of each of the following levels were simulated with HybridLab 1.0 (Nielsen, Bach and Kotlicki, 2006; Vähä and Primmer, 2006): pure *V. aspis* (PVa), pure *V. berus* (PVb), F1, F2, first-generation backcross with *V. aspis* (F1 $\times$ *V. aspis*; BcVa) and first-generation backcross with *V. berus* (F1 $\times$ *V. berus*; BcVb). To simulate the 500 individuals of each parental species, only individuals which were assigned $\geqslant$ 99% to one of the species with Structure ($=$ 110 individuals in total) were used as input. Using those simulated 1000 individuals as input, 500 F1 and subsequently, 500 F2 and 1000 backcrosses (500 BcVa and 500 BcVb) were simulated. With those simulated 3000 individuals, the analyses with Structure and NewHybrids were repeated with the same settings stated above.

A Principal Coordinates Analysis (PCoA) was performed on a matrix of genetic distances using GenAlEx 6.51b2 (Peakall and Smouse, 2006; Peakall and Smouse, 2012) to visualize the genetic differences between the species and to assess a supposed intermediate status of hybrids.

The proportion of genetically admixed individuals was calculated in two different ways. First, only the 112 randomly chosen individuals were taken into account for the calculation. Second, all the individuals sampled from 2010 onwards were included in the calculation: on one hand, all morphologically intermediate individuals were genetically analysed (sampled after 2010: $N = 11$, newly analysed and re-analysed: $N = 10$) to ensure their hybrid status; on the other hand, the proportion of hybrids in the randomly chosen morphologically typical individuals ($\times$/110 as 2 of 112 were morphologically intermediate) was applied to the total number of morphologically typical individuals sampled since 2010 ($N = 382$). The sum of hybrids (number of hybrids among morphologically intermediate individuals + proportion of hybrids in randomly sampled morphologically typical individuals * total number of morphologically typical individuals) was finally divided by the total number of sampled individuals to get a second measurement of the proportion of genetically admixed individuals. For this evaluation, the three additional individuals with intermediate morphological traits sampled in other parts of the contact zone were excluded.

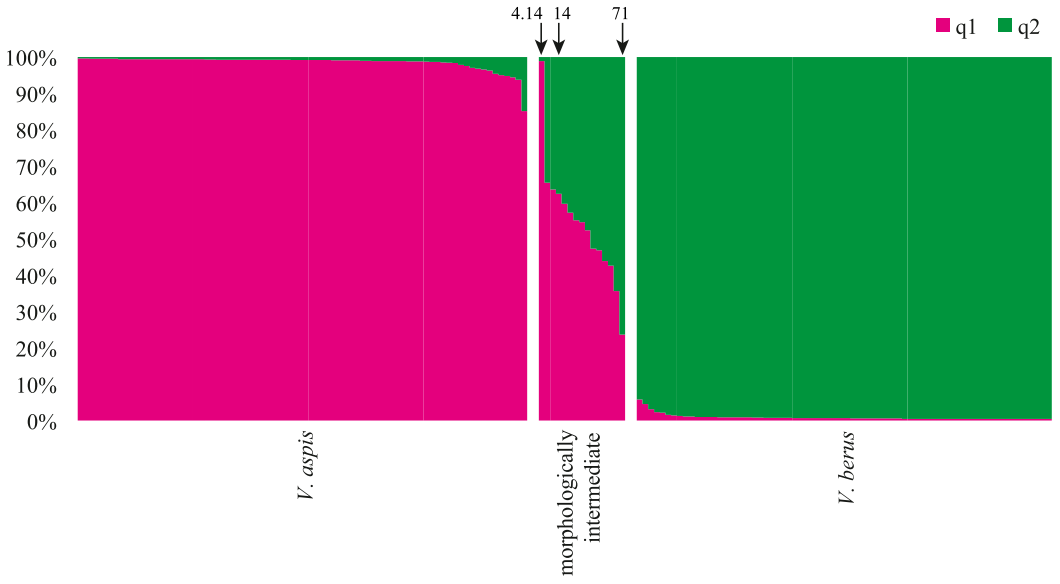

Figure 1. Structure analysis (K = 2) of *V. aspis* and *V. berus* samples collected in the Loire Atlantique department (France). Morphologically intermediate individuals include individual 4.14 which was genetically assigned to *V. aspis*. The hybridization level of individuals 14 and 71 could not be clearly assigned.

Assessment of the maternal lineage

To assign the maternal lineage, a portion of the cytochrome *b* of the putative hybrids was amplified following the protocol mentioned in Ursenbacher et al. (2006), or, when only short DNA fragments could be replicated, a small part of the cytochrome *b* (but sufficient to discriminate between *V. aspis* and *V. berus*) was amplified using the new primers L15192Vb (5′-CACACATTGCACGAGGC-3′) and H15301Vb (5′-AACATAGCCGAAGAAGGC-3′) and similar PCR conditions were applied. The sequences were later compared to already published sequences of *V. berus* and *V. aspis* with Nucleotide BLAST in order to determine the maternal lineage.

Results

Assignment analyses

Among the 165 genetically analysed individuals, 14 could be identified as hybrids, all of them having intermediate traits. One morphologically intermediate individual (4.14) was genetically identified as pure *V. aspis*, as in Guiller, Lourdais and Ursenbacher (2017). All the morphologically typical individuals were identified as purebred (78 individuals as pure *V. aspis* and 72 individuals as pure *V. berus*).

StructureHarvester identified the most probable number of clusters K = 2 (fig. 1) using the mean log-likelihood L(K) (supplementary fig. S1) as well as the ΔK method (supplementary fig. S2). Structure assigned all the 14 morphologically intermediate individuals (except for 4.14) on average 50.8% to *V. aspis* (max value: 76.3%, individual 71; table 1), while the morphologically typical individuals were genetically assigned to their according species by at least 85.1% to *V. aspis* and 94.2% to *V. berus* and on average 98.6% to *V. aspis* and 99.1% to *V. berus* (tables 1 and 2).

As NewHybrids performed better in identifying later generation hybrids using Jeffreys priors, only the output of this analysis is shown (tables 1 and 2 and fig. 2). NewHybrids confirmed the result of Structure and none of the morphologically intermediate individuals (except for 4.14) could be assigned to either one of the parental forms with a probability of more than 22.7%. Twelve of the 14 morphologically intermediate individuals (except for 4.14) were considered to be F1 with a probability of at least 81.3%, while one (71) was assigned as F1 with a

Table 1. Genetic assignment of morphological intermediate individuals (putative hybrids between *Vipera aspis* and *V. berus*) in the Loire-Atlantique department (France) using Structure (K = 2) and NewHybrids. Probabilities of classification using NewHybrids > 0.6 are highlighted in bold. The individuals 70, 71 and 106 are not from exactly the same location but were found about 1 km apart. Individuals in *italics* were non-randomly sampled before 2010, while the other individuals were randomly sampled after 2010. PVa = pure *Vipera aspis*; PVb = pure *Vipera berus*; BcVa = F1 × *Vipera aspis*; BcVb = F1 × *Vipera berus*.

Individual	Sex	Structure		NewHybrids Jeffreys						mtDNA
		q1	q2	PVa	PVb	F1	F2	BcVa	BcVb	
1.32	Female	0.655	0.345	0.002	0.000	**0.880**	0.039	0.077	0.001	*V. aspis*
4.28	Male	0.469	0.531	0.000	0.000	**0.813**	0.115	0.051	0.021	*V. aspis*
4.7	Female	0.473	0.527	0.000	0.000	**0.986**	0.006	0.006	0.002	*V. aspis*
4.8	Female	0.596	0.404	0.000	0.000	**0.973**	0.008	0.019	0.001	*V. aspis*
6.16	Female	0.439	0.561	0.000	0.000	**0.973**	0.014	0.008	0.005	*V. aspis*
10.16	Male	0.551	0.449	0.000	0.000	**0.971**	0.013	0.015	0.001	*V. aspis*
14	Male	0.624	0.376	0.024	0.000	0.055	0.442	0.479	0.000	*V. aspis*
59	Female	0.524	0.476	0.000	0.000	**0.972**	0.011	0.014	0.002	*V. aspis*
62	Female	0.573	0.428	0.000	0.000	**0.978**	0.007	0.014	0.001	*V. aspis*
76	Male	0.357	0.643	0.000	0.000	**0.953**	0.024	0.008	0.015	*V. aspis*
82	Male	0.545	0.455	0.000	0.000	**0.978**	0.008	0.013	0.001	*V. aspis*
70	Female	0.426	0.574	0.000	0.001	**0.895**	0.066	0.019	0.019	*V. aspis*
71	Female	0.237	0.763	0.000	0.227	0.453	0.193	0.007	0.120	*V. aspis*
106	Male	0.636	0.364	0.001	0.000	**0.927**	0.023	0.046	0.002	*V. aspis*
*4.14**	Male	0.989	0.011	**0.995**	0.000	0.000	0.000	0.005	0.000	*V. aspis*

*Genetically identified as pure *V. aspis* despite its intermediate morphological traits.

Table 2. Assignment and classification of the individuals of *V. aspis* (PVa) and *V. berus* (PVb) analysed in the Loire Atlantique department (France). On the left, results of Structure (K = 2) are shown followed by the percentage of correctly identified individuals using two different thresholds (0.9 and 0.95, respectively). On the right, the percentage of correctly identified individuals using two different thresholds (0.9 and 0.6, respectively) in NewHybrids.

Category	STRUCTURE (CLUMPAK-results)				NEWHYBRIDS – Jeffreys		
	Average proportion of assignment (range)		Correct assignment		Average probability (range)	Correct classification	
	q1	q2	0.9	0.95	Q	0.9	0.6
PVa	0.986 (0.851-0.996)	0.014 (0.004-0.149)	98.72%	94.87%	0.991 (0.857-1.000)	98.72%	100%
PVb	0.009 (0.005-0.058)	0.991 (0.942-0.995)	100%	98.61%	0.999 (0.968-1.000)	100%	100%
Morph. intermediate	0.507 (0.237-0.655)	0.4963 (0.345-0.763)	–	–	–	–	–
4.14*	0.989	0.011	–	–	0.995	–	–

*Genetically identified as pure *V. aspis* despite its intermediate morphological traits.

probability of 45.3% and the last individual (14) as a BcVa (47.9%) or less likely as F2 (44.2%).

Evaluation of statistical power

The different methods performed relatively well in assigning and classifying simulated pure individuals of both species (table 3). Structure was able to correctly identify 90.4% of the simulated pure *V. aspis* and 97.8% of the simulated pure *V. berus* when using a threshold of 0.9. However, using a threshold of 0.95, only 18.6% of the simulated pure *V. aspis* and 31.4% of the simulated pure *V. berus* could be correctly identified. Similarly, NewHybrids was able to correctly assign 76.2% of simulated pure *V. aspis* and 96.6% of simulated pure *V. berus* using a threshold of 0.9. Structure as well as NewHybrids performed better in identifying pure *V. berus* than pure *V. aspis*. As hybrids are usually

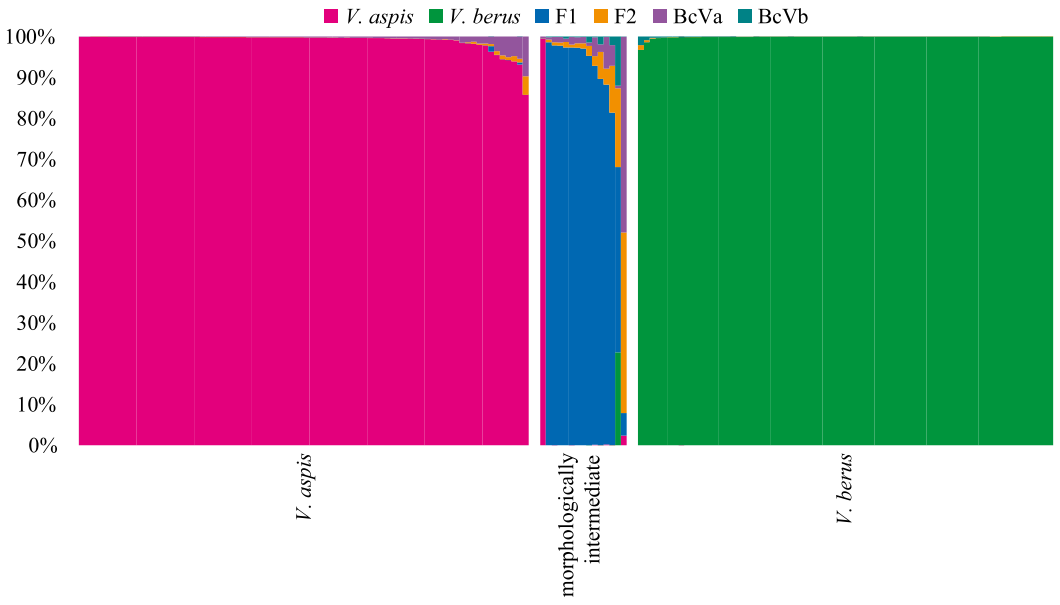

Figure 2. NewHybrids results using Jeffreys priors of *V. aspis* and *V. berus* samples collected in the Loire Atlantique department (France). Morphologically intermediate individuals include individual 4.14 which was genetically assigned to *V. aspis*. The hybridization level of individuals 14 and 71 could not be clearly assigned. BcVa = F1 × *Vipera aspis*; BcVb = F1 × *Vipera berus*.

Table 3. Assignment and classification of the simulated individuals with different hybridization levels based on *V. aspis* and *V. berus* samples collected in the Loire Atlantique department (France). On the left, the percentage of correctly identified simulated pure individuals using two different thresholds (0.9 and 0.95, respectively) in the Structure analysis (K = 2). On the right, the percentage of correctly identified simulated individuals of the different hybridization levels using two different thresholds (0.9 and 0.6, respectively) in NewHybrids. PVa = pure *Vipera aspis*; PVb = pure *Vipera berus*; BcVa = F1 × *Vipera aspis*; BcVb = F1 × *Vipera berus*.

Category	STRUCTURE (CLUMPAK-results)				NEWHYBRIDS – Jeffreys		
	Average proportion of assignment (range)		Correct assignment		Average probability (range)	Correct classification	
	q1	q2	0.9	0.95	Q	0.9	0.6
PVa	0.931 (0.779-0.958)	0.069 (0.042-0.221)	90.40%	18.60%	0.915 (0.192-0.992)	76.20%	96.40%
PVb	0.056 (0.045-0.142)	0.944 (0.858-0.955)	97.80%	31.40%	0.966 (0.609-0.989)	96.60%	100%
F1	0.504 (0.295-0.651)	0.496 (0.349-0.706)	–	–	0.817 (0.061-0.963)	34.20%	90.20%
F2	0.511 (0.130-0.905)	0.489 (0.096-0.870)	–	–	0.581 (0.008-1.000)	34.60%	51.20%
BcVa	0.733 (0.308-0.954)	0.267 (0.046-0.692)	–	–	0.677 (0.004-0.967)	24.40%	72.20%
BcVb	0.268 (0.046-0.553)	0.732 (0.447-0.954)	–	–	0.695 (0.007-0.951)	18%	77.20%

identified with lower statistical power, a second threshold for the assignment probabilities of NewHybrids was set to 0.6 as it was also done by do Prado et al. (2017). Using this threshold of 0.6, NewHybrids was able to correctly classify 90.2% of simulated F1, 51.2% of simulated F2, 72.2% of simulated BcVa (F1 × *Vipera aspis*) and 77.2% of simulated BcVb (F1 × *Vipera berus*). NewHybrids performed badly in identifying hybrids using a threshold of 0.9 and could only classify 18-34.6% of the individuals correctly. The application of the different thresholds on our real data demonstrates the reliability of our results: using a threshold of 0.9, Structure and NewHybrids yielded exactly the same outcome and could correctly assign 98.72% of

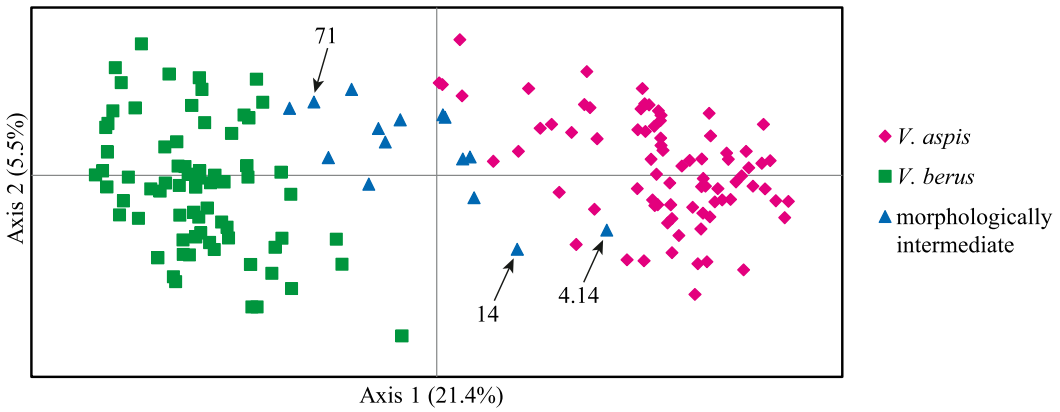

Figure 3. Principal Coordinates Analysis (PCoA) based on 10 microsatellite markers showing the distribution of morphologically intermediate individuals in between the pure *V. aspis* and pure *V. berus* individuals in the Loire Atlantique department (France). Individual 4.14 was morphologically intermediate but genetically assigned to pure *V. aspis*.

the pure *V. aspis* and 100% of the pure *V. berus* (table 2). Among the 15 individuals with intermediate morphological traits 12 could be classified as F1 with NewHybrids using a threshold of 0.6 (lowest value 0.813 for individual 4.28, table 1). Two individuals with intermediate morphological traits (14 and 71) could not be clearly assigned to any hybrid category with NewHybrids using a threshold of 0.6 (highest values 0.479 and 0.453, respectively, table 1). Only one individual was assigned to one of the species with Structure using a threshold of 0.9 (highest value 0.763 for individual 71, table 1).

Principal coordinate analysis (PCoA)

The PCoA showed an intermediate position of the hybrids between the two parental species (fig. 3). The first coordinate axis of the PCoA explained 21.4% of the variation, the second coordinate axis 5.5%, respectively. The genetically pure *V. aspis* with intermediate morphological traits (4.14) was situated in between other genetically pure *V. aspis* even though it was placed at an edge position. The morphologically intermediate individual 71, which was assigned with a probability of 76.3% to belong to *V. berus*, was situated among the other putative hybrids yet nearer to pure *V. berus* samples than to pure *V. aspis* samples. Similarly, the morphologically intermediate individual 14, which was assigned to *V. aspis* with a probability of 62.4% in Structure and which could not be clearly assigned with NewHybrids, was situated in between the pure *V. aspis* and the hybrids.

Proportion of genetically admixed individuals and direction of gene flow

Two of the 112 newly analysed and randomly selected individuals were identified as hybrids what results in a proportion of genetically admixed individuals of 1.79%. Using the second evaluation method, we estimated a proportion of genetically admixed individuals of 2.80% ($[11 + (0/110) * 382]/393$), which is very close to the evaluation based on randomly selected samples only. Consequently, the proportion of genetically admixed individuals can be assumed to be between 1.5% and 3% in this particular contact zone. The analysis of the cytochrome *b* revealed that the mtDNA of all the individuals with intermediate morphological traits can be assigned to the haplotype of *V. aspis* from the reference population (GenBank No: KX781250, table 1).

Discussion

Our study demonstrates that hybridization between the two species occurs but is rare

and we confirm unidirectional crossing. All analysed individuals showing typical morphological traits of one species could be properly assigned to their supposed species suggesting that hybrids can be already identified based on their intermediate morphology. We discuss our findings below.

Hybridization mainly to F1 level

In contrast to the previous study by Guiller, Lourdais and Ursenbacher (2017), most of the hybrids (except of individuals 14 and 71) could be assigned to F1. The differences can be explained by the use of markers that amplified well in both species and likely provide more reliable results.

Only one individual (14) could be most likely assigned as a backcross (F1 × parental) or F2. We can assume that F1 hybrids (and rare F2 or backcrosses) have a low fitness. Even if F1 hybrids seem to have the same survival rate as their parental species (several F1 individuals from this study were observed in the field over several years by GG), a similar fecundity would result in a higher proportion of F2 and backcrosses. Low viability of second-generation hybrids was observed in litters of wild F1 females that gave birth in captivity. These litters ($N = 6$) produced only three viable second-generation hybrids (born by two different females) and these juveniles were never found after release. Additionally, the different litters of these six F1 females produced three stillborn, one undeveloped embryo and 14 undeveloped ova (GG, unpubl. data). Likewise, Schweiger (2009) tried to cross hybrids between *V. aspis* and *V. ammodytes* with each other, but F2 hybrids or backcrosses were either not viable at all or lived at most 1.5 years. Similarly, Faoro (1986) was able to breed a generation of F2 hybrids of *V. aspis* with *V. ammodytes*, but only one offspring was born healthy. Although *V. aspis* is more closely related to *V. berus* than to *V. ammodytes* (Freitas et al., 2020), it can be expected that viable F2 hybrids of *V. aspis* and *V. berus* exist only exceptionally. Consequently, the proportion of hybrids in the contact zone is possibly quite stable and virtually no introgression occurs.

Low proportion of genetically admixed individuals

Interspecific hybridization rates in natural populations are usually 0.1% or less per generation (Mallet, Besansky and Hahn, 2016). Species are alleged to be able to hybridize more often if their divergence time is less than 15 Mya (Bolnick and Near, 2005). This is supported by our study, where the occurrence of hybridization is confirmed with a divergence time of 13-14 Mya between the two focal species (Freitas et al., 2020). In the Loire-Atlantique department, the proportion of genetically admixed individuals is still quite low (between 1.5 and 3%) and could be considered comparable to the contact zone occurring in Slovenia (one single individual with some signs of ancient hybridization among 28 genetically tested *V. berus* and *V. aspis* individuals; Mebert et al., 2015) or in northern Spain, where hybrids between *V. aspis* and *V. seoanei* (two species separated by the same time span as *V. aspis* and *V. berus*; Freitas et al., 2020) were not detected (but the habitat is not considered optimal for *V. seoanei*) and only one between *V. latastei* and *V. seoanei* was found (Tarroso et al., 2014). Even if *V. seoanei* possesses a similar reproductive cycle as the asp viper, with an additional mating period in autumn (in opposite to *V. berus*; Saint Girons 1980b), the differentiation between these species belonging to two different subgenera (*Pelias* and *Vipera* 1) seems to be sufficient to imply a limited gene flow. Similar observations were conducted between *Vipera* 1 and *Vipera* 2 groups (following Nilson and Andrén, 1997) as no hybrids were detected in Slovenia between *V. aspis* and *V. ammodytes* (Mebert et al., 2015). In contrast, hybridization within the same subgenus (e.g., within *Vipera 1* or *Pelias*) is more common, as locally observed between *V. latastei* and *V. aspis* (Tarroso et al., 2014) or related to old gene flow

between *V. kazankovi* and *V. renardi* (Zinenko et al., 2016) or *V. renardi* and *V. berus* (Pavlov et al., 2011). The old split between the 3 subgenera (about 10 Myr) is perhaps sufficient to strongly reduce reproductive compatibility to the formation of only a few F1 and backcross individuals, whereas the more recent speciation within the subgenus (< 10 Mya; see Freitas et al., 2020) permits more important gene exchanges, which is still possible to detect long time after introgression (Zinenko et al., 2016; Pavlov et al., 2011).

Because no hybrids have been found in the Alps (Monney, 1996; Scali et al., 2011), ecological factors could explain the occurrence of hybrids at lower altitudes like in the studied area. Even if habitat segregation was demonstrated in high altitudes (Monney, 1996; Scali et al., 2011; Mebert et al., 2015), as well as in our study area, where *V. berus* occupies basking sites with higher humidity and colder microconditions (Guillon et al., 2013), recent anthropogenic disturbance induced by the intensified agricultural farmland could be a possible explanation for an increased contact between the species and consequently the occurrence of hybrids in our study area (Grabenstein and Taylor, 2018). Furthermore, a higher homogeneity of habitats in the degraded farmland compared to the Alps could possibly reduce the effect of differentiated microhabitat selection by both species, leading to increased encounters between the two species and thus more frequent hybridization events. Additionally, differences in phenology probably pose the most likely explanation for the absence of hybrids in higher altitudes as different mating periods were observed in the Alps (Monney, 1996) while overlapping mating periods were demonstrated in our lowland study area (Guillon et al., 2013; Guiller et al., 2014).

Evidences for directional hybridization

In all hybrid individuals ($N = 14$), the mtDNA was always provided by *V. aspis*, meaning that the hybridization is unidirectional (male *V. berus* with female *V. aspis*). If the abundance of one species is exceeding the abundance of the other species in a contact zone, the probability of an interspecific hybridization would be enhanced (Rohde et al., 2015). As the abundance of both species is similar in the study site (Guiller et al., 2018), there is likely no impact on the hybridization in this regard. Several possible explanations for the unidirectional hybridization can be proposed:

(1) Prezygotic barriers: as *V. berus* is cold-adapted in opposite to *V. aspis*, one can hypothesise that males of *V. berus* are emerging earlier from hibernation and thus the likelihood for a female asp viper to encounter a male adder during the early mating season is bigger. However, in the study site, it has been shown that male *V. aspis* and male *V. berus* emerge at about the same time from hibernation (Guiller, Legentilhomme and Lourdais, 2014). Additionally, only male *V. berus* need to terminate their spermiogenesis (Prestt, 1971; Nilson, 1980) and undergo a pre-mating ecdysis after hibernation (Saint Girons, 1980b). This implies that male adders likely start their search for females later in the year than male *V. aspis*. Because of year-to-year variation in temperature and precipitation, the probability of an interspecific mating is likely fluctuating.

Moreover, both species use specific pheromones (Saint Girons, 1980a) and mating experiments conducted by Saint Girons (1975) demonstrated that single specimens of different viper species do not interbreed when placed in the same artificial enclosure. But when he formed an "artificial population" out of different species, interspecific copulations occurred. This observation could be explained by a blending of pheromones within the enclosure which impedes males from identifying their conspecific females. As those observations would explain interspecific copulation in a habitat where both species are abundant, an impact on the unidirectional hybridization remains questionable. But we can hypothesize that male asp vipers perform better in recognizing their

conspecific females in mixed populations than adders, what would prevent them from mating with female adders; or alternatively that adders are also attracted to asp viper pheromones. Differences in courtship behaviour could also explain unidirectional hybridization if *V. aspis* females respond to the courtship behaviour of *V. berus* males, but *V. berus* females not to that of *V. aspis* males.

Another possibility for a pre-zygotic reproductive barrier could be an incompatibility of *V. aspis* hemipenes with *V. berus* cloaca since hemipenes of *V. aspis* are longer with more basal spines than those of *V. berus* (Zuffi, 2002). Because copulation between a male asp viper and a female adder was observed by Saint Girons (1975), this explanation is not supported.

As multipaternity is known from both species (*V. aspis*: S. Nanni personal communication; *V. berus*: Ursenbacher, Erny and Fumagalli, 2009), it is likely that some females are mating with males of both species. Sperm competition could lead to the survival of *V. berus* sperms only what would explain the unidirectional hybridization. But if this would be the only reason, the lack of matings of female *V. berus* with only male *V. aspis* is improbable.

(2) Postzygotic barriers: copulation may be possible in both directions, but the genomes of the two species may form a viable zygote only in one direction resulting in asymmetric viability. As asp vipers have 42 chromosomes (2n), while adders have 36 chromosomes (2n), we can hypothesise that hybridization is only possible between females with 42 chromosomes and males with 36 chromosomes. In fishes, it has been shown that hybrids can survive if the number of maternal chromosomes is greater than or equal to the number of paternal chromosomes, whereas a hybridization hardly produces living progeny if the chromosomal number of the maternal fish is lower than that of the paternal fish (Liu, 2010). However, a crossing between a female *V. seoanei* ($2n = 36$ as for *V. berus*) and a male *V. aspis* ($2n = 42$) resulted in viable individuals with 39 chromosomes (2n; Saint Girons, 1977). But still, other genetic incompatibilities between *V. berus* females and *V. aspis* males are likely, e.g., due to cytoplasmic issues (Ferree and Barbash, 2009) or sex chromosomes (Matute and Gavin-Smyth, 2014).

Differential survival represents another postzygotic barrier which could explain the observed results. If there is embryonic survival, the hybrid neonates mothered by *V. berus* possibly do not survive in the wild. Considering the extensive sampling in the area by one of the authors (GG), it seems very unlikely that all hybrids between female *V. berus* and male *V. aspis* older than one year would have been overseen if there would be any.

The hypotheses mentioned before could be tested in captivity or semi-captivity. Monitoring of the hybrids in the field should give a better insight into the fitness, survival and fecundity of the hybrids. In fact, during the writing of this article three additional adult hybrids were detected and a female *V. aspis* produced a litter of four young, one typical *V. aspis* and three with intermediate morphological traits. By comparing the reproduction rates of the parental species as well as of the hybrids, a better understanding of the unidirectional hybridization could be achieved.

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