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Research Article

Association between Genetic Admixture and Morphological Patterns in a Hybrid Zone between the Two Iberian Vipers, *Vipera aspis* and *V. latastei*

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Understanding how hybridization influences the morphology and fitness of hybrids is essential for studying adaptive evolution and ecological speciation. Secondary contact zones, where separately evolving populations meet and hybridize, offer valuable insights into the evolutionary processes driving speciation and provide an excellent system to address these questions. In this study, we investigate patterns of morphological and genetic variation of two congeneric viper species, *Vipera aspis* and *V. latastei*, across a contact zone in northern Spain (Oja-Tirón), where vipers with mixed morphology are often detected, but genetic studies addressing hybridization and relating patterns of genetic and morphological admixture are lacking. Using nine morphological traits (scalation and colouration) and 18 microsatellite markers, we (1) estimated the extent of hybridization, (2) morphologically characterized parental species and hybrids, and (3) evaluated the correlation between patterns of genetic and morphological admixture. Analyses revealed a bimodal hybrid zone with high rate of hybridization (22%) and prevalence of late-generation hybrids (F2 and backcrosses). Morphological analyses differentiated the two parental species, and a positive correlation ($r = 0.95$) was found between morphological and genetic patterns. The hybrid group displayed on average an intermediate morphology between the parentals, yet morphologically intermediate hybrids were rare in our dataset. Instead, most hybrids resembled the parental species with whom they share most of the genetic background. Notably, the hybrid group exhibited greater morphological variation than the parental groups. Traits with adaptative value, such as ventral scales and dorsal marks, showed significant differences between hybrids and the two parental species. Introgression of these traits may confer ecological advantages to hybrids, enhancing local adaptation. Overall, this study reveals a positive correlation between patterns of morphological and genetic variation across a hybrid zone and provides insights into the phenotypic consequences of hybridization on these viper species.

1. Introduction

Secondary contact zones, where separately evolving populations come into contact and hybridize, serve as natural laboratories for studying the evolutionary processes and mechanisms underlying speciation [1]. The extent to which species hybridize in these areas is primarily determined by the degree of reproductive isolation between species. Generally, hybridization is more common among closely related species, as distantly related species had more time to accumulate incompatibilities and

develop stronger barriers to gene flow [2]. These barriers can manifest as pre-zygotic mechanisms, which prevent mating or impede fertilization, such as ecological segregation (spatial and/or temporal), sexual selection, and incompatible reproductive organs and gametes, or they can be post-zygotic, occurring after fertilization and reducing the viability, fertility, and fitness of hybrid offspring, thereby limiting gene flow between species [2, 3].

A critical factor influencing the extent of hybridization and the stability of hybrid zones is the fertility and fitness of the hybrids [3, 4]. If hybrids display lower fitness than the parentals, hybridization will be limited, and parental genotypes and phenotypes will be the most abundant in the hybrid zone (see [3] for a review on bimodal hybrid zones). Genetic markers are widely employed in the study of hybrid zones for hybrid identification and to estimate the frequency of the different hybrid classes (F1, F2, and backcrosses). Additionally, the use of phenotypic traits can provide important insights into the direct consequences of hybridization on the development, performance, and overall fitness of the organism (e.g., Darwin's finches [5, 6]). Morphology, in particular, plays an essential role in the survival and successful reproduction of organisms, as certain traits have important known biological functions, such as reproductive behaviour, feeding, or habitat use [7, 8]. Therefore, they are particularly informative in the study of hybrid zones and the fitness of hybrids (e.g., [5, 9]). First-generation hybrids typically exhibit "mixed" or intermediate morphologies and in some instances manifest atypical or transgressive traits relative to the parental taxa (e.g., [10, 11, 12]). In contrast, late-generation hybrids often resemble one of the parental species, requiring molecular methods for accurate detection [2]. Understanding how hybridization influences the morphology and overall fitness of hybrid populations is an important topic in hybridization research, particularly valuable in the context of research on adaptive evolution and ecological speciation.

European vipers (family Viperidae, genus *Vipera*) constitute a clade of venomous snakes that inhabit diverse environments and exhibit high morphological variation within and between species [13, 14, 15]. They display several secondary contact zones (hybrid zones) along their parapatric distributions where morphologically intermediate individuals (based on scalation and colouration traits) have been detected and confirmed as hybrids with genetic markers (e.g., [16, 17, 18, 19, 20]). Hybridization, however, is usually rare (only few hybrids detected), likely as a consequence of strong reproductive barriers between generally highly divergent species, either pre-zygotic (ecological divergence and niche segregation) or post-zygotic (due to selection against hybrids [20]). Notably, *Vipera aspis* and *Vipera latastei* stand out as an exception among other hybridizing European vipers. Unlike other pairs, these two species frequently present individuals with intermediate or mixed phenotypes at hybrid zones (e.g., [21, 22, 23]), suggesting the occurrence of substantial hybridization. They showcase a rather old evolutionary history (divergence around 9.1 Mya based on mtDNA inferences [13, 24]) marked by past hybridization events and mitochondrial transfer between their ancestors, which rendered deep discrepancies between nuclear and mitochondrial phylogenetic inferences [25]. Interestingly, hybridization events continue to occur in the secondary contact zones between these species. In the hybrid zone of the High Ebro (northern Spain), approximately 20% of the captured specimens displayed intermediate morphology, most of which were confirmed as hybrids using molecular methods [19]. Sympatry between these species is known to occur in other areas of the Iberian Peninsula, four areas in the southern slopes of the Pyrenees, and one in the north-western slopes of the Iberian System [22, 26]. In the later contact zone, named Oja-Tiron, 46 specimens with intermediate traits were found among a total of 549 specimens, suggesting the occurrence of hybridization between *V. aspis* and *V. latastei* [23]. A previous ecological study found high niche overlap between both species in this area and highlighted the role of anthropogenic landscape change in increasing habitat overlap and possibly species interactions, i.e., competition and hybridization [27]. However, a combination of genetic and morphological data is still required to assess the extent of hybridization and explore the relationship between morphological patterns and hybridization across the contact zone. This multidisciplinary approach will allow a deeper understanding of the evolutionary processes and mechanisms maintaining this contact zone and shed light on the phenotypic and adaptative consequences of hybridization between these species.

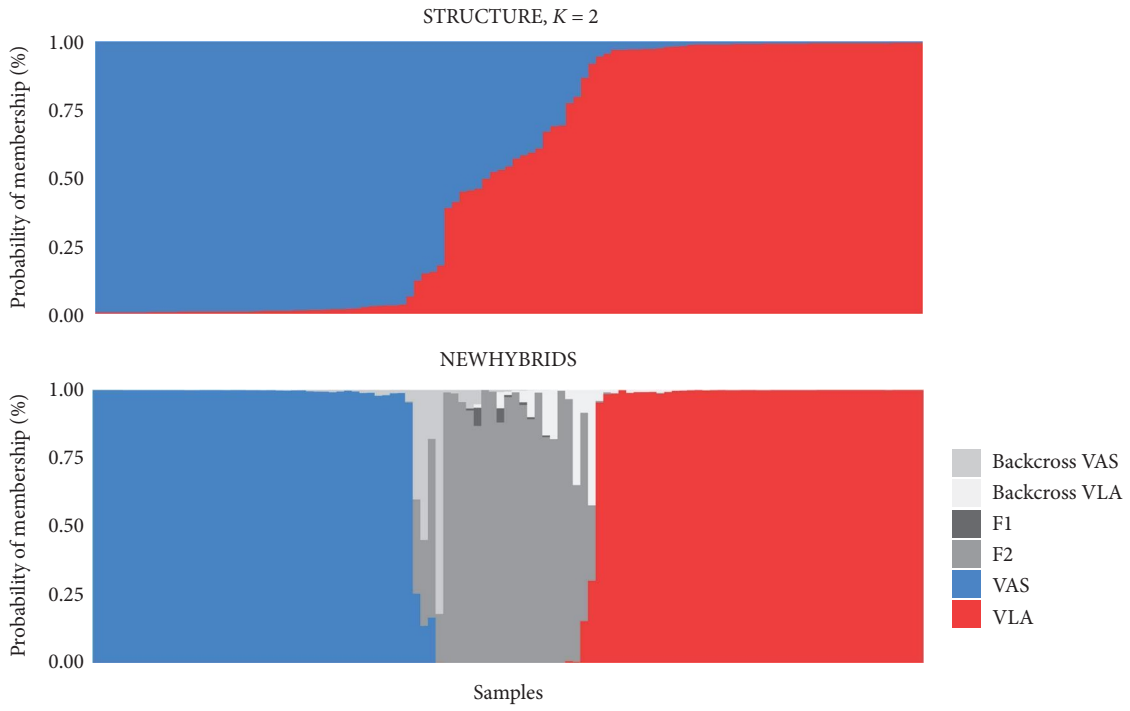
In the present study, we used a novel set of microsatellite markers developed specifically for the two species [28] and morphological traits recognized as diagnostic for species classification [22, 23] to (i) assess the extent of genetic admixture between *V. aspis* and *V. latastei* in the contact zone of Oja-Tirón and identify their hybrids; (ii) morphologically characterize parental species and the hybrids;and finally (iii) evaluate the consistency between the proportion of genetic admixture and morphological classification.

2. Material and Methods

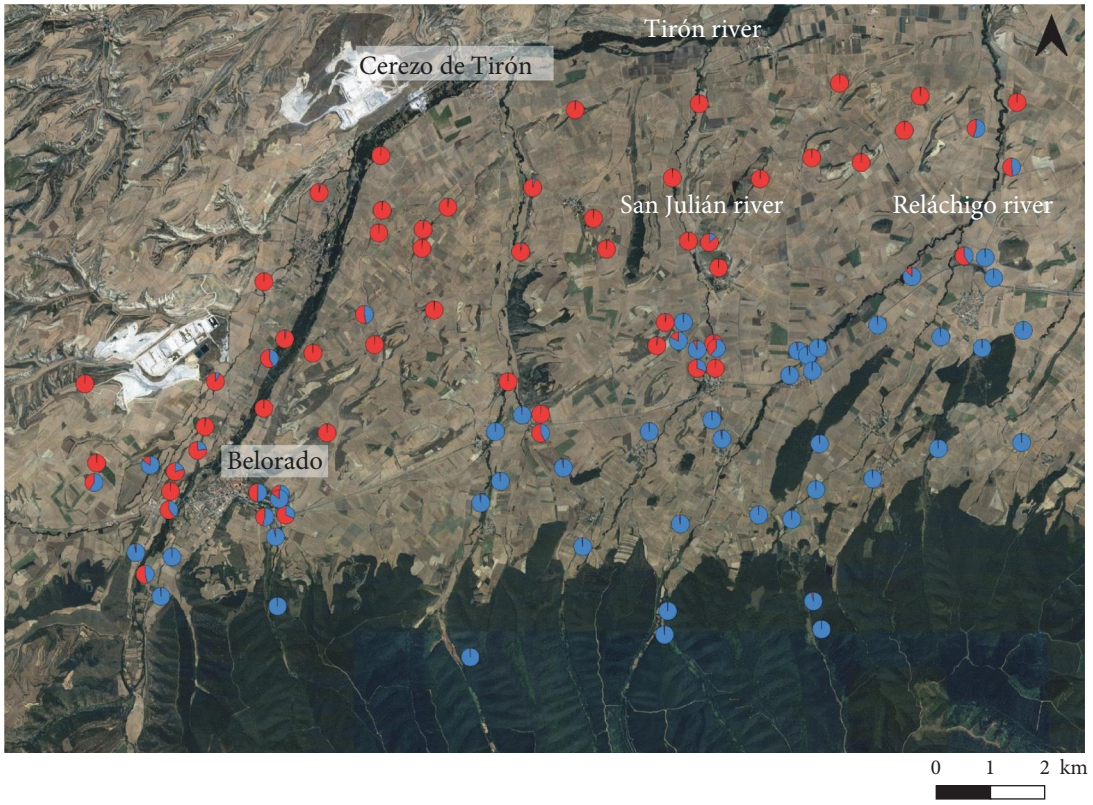
2.1. Data Collection. A total of 109 vipers from the contact zone of Oja-Tirón (Burgos, La Rioja, Spain) were analysed in this study (Table S1). Individuals were selected from an area of 30 km^2 (centroid: 30T 495000; 4695000; ETRS1989 datum; Figure 1) covering the local contact between species in Tiron, San Julian, and Relachigo river valleys and adjacent areas [23].

Vipers were captured during fieldwork expeditions conducted in the study area from 2015 to 2022. They were measured and photographed, and buccal swabs were collected and preserved in ethanol for DNA extraction. Morphological data were collected through direct observation of the specimens in the field and/or by the examination of specimens' photos. The final dataset was mostly comprised by adult specimens (81%) and by a similar proportion of males and females (Table S1). Data were missing for less than 3% of the sample, in a maximum of two morphological traits per individual. Missing data were replaced by the group average (i.e., *V. aspis*, *V. latastei*, or hybrid, considering the genetic classification).

2.2. Laboratory Procedures. Genomic DNA extractions were performed from buccal swabs, following a standard saline



(a)



(b)

Figure 1: (a) Bar plots showing STRUCTURE Bayesian assignment to two groups ($K = 2$) and NEWHYBRIDS Bayesian assignment to the parental species and four hybrid classes (F1, F2, and backcrosses). Each bar represents a single individual (in the same order in both plots), and colours represent proportional membership coefficients. (b) Map showing the geographic distribution of the 109 individuals assigned to two genetic clusters by STRUCTURE analyses (Q1 in blue represents *V. aspis*, and Q2 in red represents *V. latastei*). Google satellite was used as a background layer. The main topographic features and urban areas are highlighted in the map.

DNA extraction method. Samples were genotyped for a set of 18 novel microsatellite loci, recently developed for *V. aspis*, *V. latastei*, and *V. seoanei* [28]. We specifically selected these loci from the larger pool of 48 microsatellites developed in the aforementioned study, based on their proven reliability in amplification and their high intra- and interspecific polymorphism across our studied species. The codes for the selected loci are as follows: Vasp16, Vasp3, Vasp4, Vasp7, Vasp8, VLa1, VLa20, VLa21, Vasp10, Vse10, Vse11, Vse15, Vse19, Vse2, Vse21, Vse24, Vse25, and Vse9 (for detailed information on each locus, see [28]). The 18 loci were multiplexed in two reactions of 8 and 10 markers each; forward primers were labelled with fluorescent dye markers (FAM, NED, VIC, and PET; [29]). PCRs were performed in a total volume of 10 μL with 1–2 μL template DNA, 100–150 nM of each primer, and 5 μL Multiplex MasterMix Kit (Qiagen). Touchdown PCR conditions started with an initial denaturation step of 15 min at 95°C; first round (nine cycles) of 30 s at 95°C, 2 min for annealing (decreasing 0.5°C/cycle) at 64°C, and 1 min at 72°C; and second round (36 cycles) of 30 s at 95°C, 1 min at 58°C, 30 s at 72°C, and a final extension of 30 min at 60°C. Amplifications were performed in Biorad T100 Thermal Cyclers, and the PCR products were separated by capillary electrophoresis on an automatic sequencer *ABI35000xl Genetic Analyzer* (AB Applied Biosystems). Fragments were scored and manually checked using GENEMAPPER 5.1 (Applied Biosystems).

2.3. Genetic Admixture Analyses. The existence of null alleles, large allele dropout, and stuttering was verified using MICRO-CHECKER v2.2.3 [30]. No genotyping errors were detected in our dataset; therefore, all loci were retained in the following steps. The number of alleles (N_A) and expected heterozygosity (H_E) was estimated for each locus using ARLEQUIN v3.5 [31].

Principal component analysis (PCA) was first used as an exploratory method to visualize patterns of multilocus genotypic differentiation among individuals without a priori grouping, using ADEGENET 2.0 package [32]. Then, we applied a Bayesian method implemented in STRUCTURE 2.3.4 [33] to detect admixture between *V. aspis* and *V. latastei* in the contact zone and identify parental species and hybrids. For this purpose, a $K = 2$ was defined, and an admixture model with correlated allele frequencies was employed. Ten independent Markov chain Monte Carlo (MCMC) runs of 1 million iterations were performed, following a burn-in period of 100,000 steps. The output of the 10 runs was averaged with CLUMPAK [34].

In order to define the appropriate threshold for hybrid identification, a simulated hybrid dataset based on a filtered dataset of non-admixed/pure specimens was generated using the *hybridize* function of ADEGENET 2.0 [32]. As non-admixed, we considered all individuals with estimated posterior probabilities above 0.99 in STRUCTURE analyses. A total of 60 vipers (29 *V. aspis* and 31 *V. latastei*) met the criteria and were used as input to simulate 30 individuals of each of the four hybrid classes (F1, F2, and backcrosses). The simulated dataset was run in STRUCTURE under the same settings. The results of STRUCTURE based on the empirical and simulated data were finally compared to set a posterior probability threshold for hybrid identification. A threshold of 0.94 was chosen considering the mean and standard deviation of the assignment of simulated genotypes (i.e., specimens are classified as pure when $q > 0.94$ and as hybrids when $q < 0.94$).

A Bayesian analyses implemented in NEWHYBRIDS 1.1 was used to identify and classify admixed individuals as F1s, F2s, or backcrosses [35]. NEWHYBRIDS uses parental populations to estimate allele frequencies and then calculates the posterior probability of belonging to one of six classes: parent A, parent B, F1, F2, backcross to parent A, and backcross to parent B. The z option was applied to identify pure individuals ($q > 0.94$ threshold in STRUCTURE). Ten independent MCMC runs with 1 million steps were performed, following a burn-in period of 100,000 iterations. Analyses were run twice using "Jeffreys" and "Uniform" priors for allele frequency and admixture estimations. Both priors yielded similar results, but identification of later generation hybrids was more efficient using Jeffreys; therefore, only the results of this analysis are presented. Since hybrid classes are usually identified with lower statistical power in NEWHYBRIDS, a second threshold for the assignment probabilities of hybrid classes of 0.6 was adopted, as used in other studies [20, 36]. Due to these low probabilities and because the validity of the assumed distribution of priors cannot be assessed statistically in Bayesian analyses [37], the same set of simulated genotypes was run in NEWHYBRIDS to evaluate its performance in identifying different classes of hybrids between *V. aspis* and *V. latastei*.

Additionally, for each individual, we calculated a maximum likelihood estimate of the hybrid index, i.e., the proportion of alleles that were inherited from one of the two hybridizing parental species, and the average interspecific heterozygosity, using Introgress 1.2.3 package [38]. Introgress works with co-dominant, dominant, and haploid marker data and does not require fixed allelic differences between parental populations to estimate the interspecific heterozygosity and hybrid index of each individual. If parental populations exhibit fixed differences at all loci, these metrics simply account for the number of alleles inherited from each of two parental populations at each locus. If the parental populations do not exhibit fixed differences at all loci scored, as in this study, the counts are of alleles from the allelic class associated with a given parental population and the frequency of allelic classes in the parental species can be used to account for uncertainty in the ancestry of particular alleles [38]. Both variables were then plotted against each other using the triangle.plot function implemented in the same package to provide a more complete picture of the genetic structure of hybrid populations. F1 hybrids are expected to have a hybrid index of 0.5 and heterozygosity of 1.0 across all loci. F2 hybrids can have variable but mostly lower heterozygosity. Unlike F2 hybrids, backcrosses are expected to be restricted to the sides of the triangle [39].

2.4. Morphological Analyses. To characterize the morphology of parental and hybrid individuals and examine concordance between morphological and genetic traits, a set of one colouration and eight pholidotic traits were considered for

statistical analyses. Morphological traits analysed included the number of marks from the dorsal design between the head and the vent (DMARK) and the number of ventral (VENT), subcaudal (SUBC), apical (APIC), intercanthal plus intrasupraocular (INTER), loreal (LOR), infralabial (INFRA), supralabial (SUPRA), and periocular (PERI) scales. Four of these traits (i.e., APIC, INTER, VENT, and DMARK) are recognized as diagnostic for species identification [20, 22], while the remaining are frequently considered in studies addressing morphological variation in vipers (e.g., [15, 40]). For bilateral traits (i.e., LOR, INFRA, SUPRA, and PERI), the mean between both sides was considered. Prior to analyses, all traits were log transformed to meet the assumptions of most statistical tests.

Exploratory ANOVAs were performed for each morphological trait to test for sexual dimorphism and for differences between parental species and hybrids (from now on treated as "groups"). For this purpose, ANOVA comparisons were done considering sex, groups, and their interaction as fixed factors. ANOVA procedures were evaluated for significance using residual randomization permutation procedures (1,000 permutations) as implemented in the R package RRPP [41]. Post hoc pairwise comparisons were performed when significant effects were found.

Sexual dimorphism was detected in VENT and SUBC. The first trait showed significant differences between groups, but since sex × group interactions were not significant, further analyses were conducted on all specimens together (Text S1). Only traits showing statistically significant differences between groups were considered in the following steps.

A PCA was conducted to examine the multivariate morphological structure of the dataset. The two first axes that explained most of the morphological variability were plotted. Points were coloured according to their genetic assignment as parental or hybrids. A linear discriminant analysis (LDA) was then performed to predict, for each individual, the probability of belonging to each of the parental clusters. For this purpose, the model was fit using only the non-admixed individuals, and predictions of group membership were performed for the entire dataset. Finally, a Pearson correlation coefficient was computed to assess the linear relationship between the posterior probabilities of genetic (i.e., obtained from STRUCTURE) and morphological (i.e., from the LDA) classification, using the full dataset (i.e., parental and admixed individuals) and a reduced dataset comprising only hybrid specimens. In addition, we compared the morphological disparities (morphological diversity) of the hybrids and the two parental species, using the R package geomorph (function morphol.disparity). Morphological disparity was estimated as the variance of Procrustes distances and statistically compared using a residual randomization permutation procedure (1,000 permutations [42]). All analyses were performed using R language for statistical programming (R version 4.0.3).

3. Results

3.1. Identification of Purebred and Hybrid Individuals. Missing data among the analysed loci ranged from 0% (in Vasp4, VLA20, VLA21, Vasp10, Vse24, and Vse9) to 17% (in Vasp3). With the exception of Vasp3, all loci had less than 10% of missing data. The number of alleles per locus ranged from 7 (Vas16 and Vse21) to 29 (Vse25), and values of expected heterozygosity ranged from 0.65 in locus VLA1 to 0.95 in locus Vse25.

The first two components of PCA revealed two-well separated clusters, with intermediate genotypes (potential hybrids) positioned between them. No genetic substructure was observed in the dataset (Figure S1). Among the 109 genotyped individuals, STRUCTURE analyses identified 85 as pure ($q > 0.94$), 43 of which were assigned to *V. latastei* and 42 to *V. aspis* clusters, and 24 individuals as hybrids ($q < 0.94$) (Table S2 and Figure 1). Most individuals assigned to parental clusters showed probabilities of membership above 0.99 (mean = 0.99, SD = 0.01; Table S2 and Figure 1). By comparing the results of the observed and simulated dataset, we confirmed that STRUCTURE analyses performed well in identifying simulated hybrids and parental individuals, and a threshold of 0.94 was set for hybrid classification, as all simulated hybrid individuals showed probabilities below this value, and non-admixed individuals used to generate the simulated dataset ($q > 0.99$) were above it (Table S3).

All hybrid specimens (based on structure; $q < 0.94$) were identified as hybrids by NEWHYBRIDS with probabilities of membership above 0.70 (obtained by summing the four hybrid classes). Among the 24 hybrids, 20 were identified as F2 ($q > 0.65$, mean = 0.90, $SD = 0.11$) and two as backcrosses with *V. aspis* ($q > 0.55$). No F1 hybrids were detected in our dataset. Two hybrids were not attributed to any class due to low posterior probabilities ($q < 0.40$; Table S2 and Figure 1). Tests with the simulated dataset demonstrated an overall good performance of this method for the identification of specific hybrid categories. NEWHYBRIDS was able to correctly classify 93% of simulated F1 hybrids, 67% of simulated F2 hybrids, 83% of simulated backcrosses with *V. aspis*, and 73% of simulated backcrosses with *V. latastei* (Table S3). The triangle plot showing the relationship between interspecific heterozygosity across loci and ancestry of individual samples showed the absence of F1 hybrids but presence of at least two backcrosses and 22 F2s in this hybrid zone, corroborating the results of NEWHYBRIDS (Figure 2).

3.2. Morphological Characterization of Purebred and Hybrids. Among the nine morphological traits analysed, DMARK, VENT, and APIC showed statistically significant differences ($p < 0.05$) between the two species and the hybrids (Text S1). *V. aspis* specimens have more DMARK (mean = 58.4; SD = 5.6; range = 49–71) and VENT (mean = 146.8; SD = 3.9; range = 139–154) compared to *V. latastei* (DMARK: mean = 42.2, SD = 4.3, and range = 31–53; VENT: mean = 140, SD = 3.9, and range = 131–148). The hybrids show intermediate values between the two parental species for these traits, between 36 and 69 DMARK (mean = 47.4, SD = 7.2) and 133–155 VENT (mean = 143.6, SD = 4.6). *V. latastei* specimens have from 2 to 7 APIC that contrasts with the 2 or 3 APIC typically found in *V. aspis*, and the hybrids have between 2 and 5 APIC (Figure 3 and Table S4).

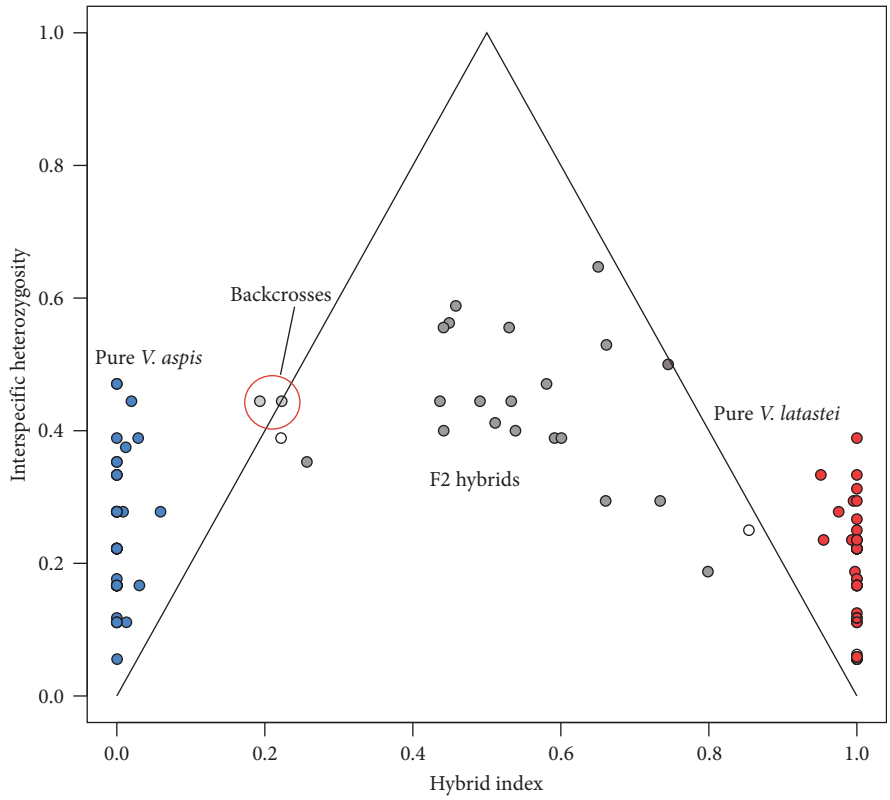


FIGURE 2: Triangle plot representing the hybrid index vs. the interspecific heterozygosity across the hybrid zone between *V. aspis* and *V. latastei*, based on 18 microsatellite loci. The hybrid index ranges from 0 (pure *V. aspis*) to 1 (pure *V. latastei*). The interspecific heterozygosity ranges from 0 (complete homozygosity) to 1 (complete heterozygosity). Individuals are coloured according to NEWHYBRIDS classification (Figure 1). The two genotypes that were not assigned to any hybrid class due to low posterior probabilities ($q < 0.4$) are not coloured.

Three additional traits, INTER, LOR, and INFRA, show statistically significant differences between the two parental species only (Text S1). *V. latastei* presents in average higher number of INTER (mean = 31.9, SD = 5.9), LOR (mean = 8, SD = 1.5), and INFRA (mean = 12.1, SD = 0.8) than *V. aspis* (Table S4).

The first two principal components of the PCA, which account for 67.6% of the total variation in the dataset, result to the emergence of two distinct morphological groups corresponding to each parental species and one intermediate group that overlaps with both, comprising the hybrid specimens. PC1 was mostly influenced by APIC, INTER, and DMARK and PC2 by LOR, INFRA, and VENT (Figure 3 and Text S1). Consistently, LDA based on the six informative morphological traits was able to correctly classify all purebred individuals, with high probabilities ($p = 0.95$ for *V. latastei* and $p = 0.85$ for *V. aspis*). DMARK, VENT, and APIC were the traits that contributed the most to the discriminant function (Text S1).

A positive correlation was found between the posterior probabilities of classification obtained from STRUCTURE and LDA when considering the full dataset ($r\ (107) = 0.95$, $p < 0.05$) and the reduced dataset including only hybrid specimens ($r\ (22) = 0.71$, $p < 0.05$). Morphological and genetic probabilities of classification however were not consistent for several hybrids which have mixed ancestry (q closer to 0.50) but show high morphological probabilities of classification to one of the parental species ($p \sim 1.0$).

Morphological classifications were generally consistent with the individuals' ancestry. From the 24 hybrids, 10 were classified as *V. aspis* and 14 as *V. latastei* based on their morphology ($p > 0.5$). Backcrosses with *V. aspis* were classified as *V. aspis*. Moreover, from the 10 hybrids classified as *V. aspis*, seven exhibit higher genetic proportion of the species ($q > 0.54$ in STRUCTURE analyses). Similarly, 11 out of the 14 morphologically classified *V. latastei* are genetically closer to *V. latastei* ($q > 0.52$). Notably, almost all exceptions presented similar genetic proportions between parental species ($0.45 < q < 0.55$) (Figures 4, S2 and Table S1).

Morphological disparity analyses showed significant differences ($p = 0.035$) between hybrids and *V. latastei* and marginally significant differences between hybrids and *V. aspis* ($p = 0.052$), with hybrids exhibiting greater variance in morphology (0.26 vs. 0.19 in the two species).

4. Discussion

In this study, we explored the association between genetic and morphological patterns in a hybrid zone between two Western Mediterranean vipers. We found a relatively high rate of hybridization (22%) and prevalence of late-generation hybrids (F2 and backcrosses) between *V. aspis* and *V. latastei*

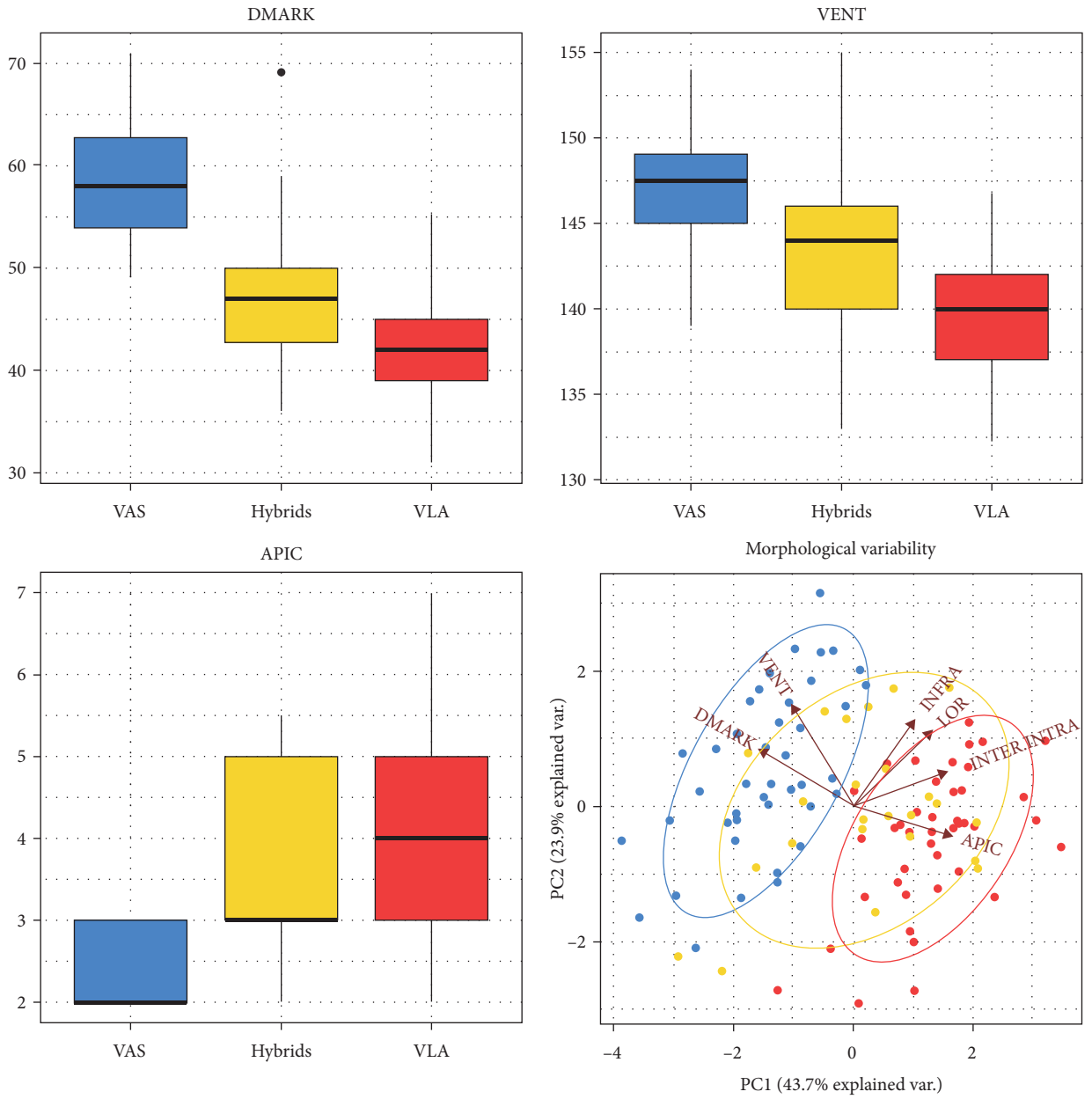


Figure 3: Boxplots of the three morphological traits that exhibited statistically significant differences between the two species and their hybrids: DMARK, VENT, and APIC. PCA summarizing the morphological variability of the morphological traits that exhibited statistically significant differences between the species and the hybrids (DMARK, VENT, and APIC) and the two parental species only (INTER, LOR, and INFRA). Samples were coloured according to their genetic assignation. Ellipses represent 95% confidence intervals for each group.

and a high correlation between morphological and genetic assignments ($r = 0.95$), with hybrids displaying a similar morphology to the closer parental species.

4.1. High Hybridization Rate between Western Mediterranean Vipers. Our study points to frequent and ongoing hybridization occurring between *V. aspis* and *V. latastei* in the hybrid zone of Oja-Tirón. Among the 109 specimens analysed, 22% were identified as late-generation hybrids. Notably, first-generation hybrids (F1s) were not detected in our sample. A similar pattern of common hybridization and the absence of F1 hybrids between these species were reported in the hybrid zone of the High Ebro (hybridization rate of 21% [19]) and other studies across hybrid zones (e.g., amphibians [43], fishes [44], and plants [45]). This trend contrasts to findings of other contact zones involving distinct pairs of *Vipera* species, where hybridization is punctual and does not go beyond F1 filial (*V. renardi*/*V. berus* [18]; *V. ammodytes*/*V. aspis* [16]; *V. seoanei*/*V. aspis* and *V. seoanei*/*V. latastei* [19]; and *V. aspis*/*V. berus* [17, 20]). High genetic differentiation between these pairs of species likely imposes strong pre- and post-zygotic isolating barriers to gene flow, such as high ecological differentiation

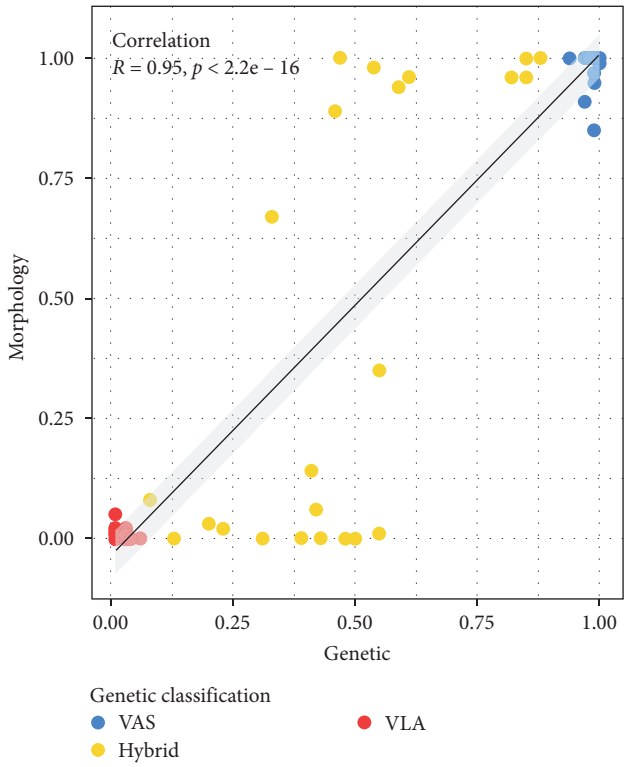


Figure 4: Correlation between the genetic and morphological probabilities of classification to each of the parental species. The *X* and *Y* axes represent the membership probability belonging to the *V. aspis* cluster, based on genetic data (obtained from STRUCTURE; *X* axis) and based on morphological data (obtained from DA; *Y* axis).

that restricts co-occurrence and opportunity for cross-mating, and important genetic and chromosomal incompatibilities that result in low hybrid fertility and viability [17, 19, 20, 46]. In contrast, and despite the distant phylogenetic relationships observed at the nuclear level [25], reproductive isolation between *V. aspis* and *V. latastei* is not complete, resulting in substantial admixture in their contact zones and reproductively viable and fertile hybrids, as evidenced by the high number of late-generation hybrids detected in Oja-Tiron (this study) and in the High Ebro [19]. Yet, differences in ecological requirements and habitat selection patterns probably act as an important pre-zygotic barrier to gene flow, restricting hybridization to the sympatric areas located in the centre of the hybrid zone [19, 22, 23, 26, 27, 40]. Additionally, the lack of F1 hybrids and abundance of F2 and backcrosses in our sample may suggest the influence of post-zygotic extrinsic barriers, as ecological selection against intermediate hybrid genotypes. This phenomenon emerges as the primary mechanism explaining the low frequency/absence of F1s in analogous hybrid zones (e.g., plants [45, 47] and fishes[44, 48]). This can occur when F1 hybrids exhibit lower fitness, due to intermediate morphology and ecology, in relation to the parental species within their respective environments. Conversely, F2s and backcrosses demonstrate equivalent fitness to the parents, resulting in a skewed representation of observed frequencies towards F2 and backcrosses and low abundance of F1 hybrids, which can even pass undetected in sampling efforts. Consistently, ecological studies conducted in the High Ebro suggested that morphologically intermediate specimens showed lower fitness (in some traits) and were restricted to areas suboptimal for the parental species [19, 49], providing further support to this hypothesis. Together, these isolating mechanisms seem sufficient to maintain species boundaries and form a stable "bimodal" type hybrid zone in Oja-Tiron, where intermediate genotypes are rare and late-generation hybrids and parental species are more abundant ([3] and references herein).

4.2. Morphological Patterns in the Hybrid Zone and Their Association with Genetic Admixture. Our study showed a positive correlation between morphological and genetic patterns in this hybrid zone. PCA and discriminant analysis (DA) based on the six most informative traits demonstrated the morphological distinctiveness of pure *V. aspis* and *V. latastei*. The hybrids exhibit on average an intermediate morphology between the two parental species and displayed higher overall phenotypic variability compared to the more homogeneous parental groups. However, morphologically intermediate vipers were rare in our dataset, with most hybrids resembling the parental species with whom they share the highest genetic proportion. This lack of intermediate hybrid vipers can be attributed to the composition of the hybrid group, which consists exclusively of late-generation hybrids (F2 and backcrosses). Therefore, patterns of morphological variation seem to follow the same bimodal distribution of genetic patterns, with parental genotypes and phenotypes being the most common in this hybrid zone.

Despite this, mosaic morphologies, characterized by a mix of traits from both species, and intermediate states in some traits remain relatively common among F2 hybrids. Generally, when examining the morphology of hybrids, common assumptions include intermediate character states and character incoherence, with both typically serving as diagnostic indicators of introgressive populations (e.g., [10, 12, 50]). Traits under multigenic control tend to manifest intermediate states in first-generation hybrids, while those under simple genetic control may display parental or intermediate states, based on dominant vs. co-dominant allelic expression. This process results in structurally mosaic hybrids that represent a combination of both parental and intermediate traits [50, 51]. Consistently, all specimens displaying mixed morphologies were unequivocally identified as hybrids, establishing mixed morphology as a strong indicator of hybridization between the two viper species. Yet, the two backcrosses and some F2 hybrids displayed morphologies that were indistinguishable from that of one of the parentals. This implies that morphological traits, while generally reliable predictors of genetic admixture, may fail to identify late-generation hybrids. Morphological and genetic identification incongruences in highly hybridized populations have also been reported in other studies (e.g., [52, 53, 54]), highlighting the limitations associated with relying solely on morphological criteria to distinguish hybrids from pure individuals. A comprehensive approach,

integrating both morphological and genetic data, is needed in this context to achieve a thorough understanding of the dynamics of hybridization [52].

Our morphological analysis, encompassing eight scalation and one colouration trait, identified six traits as informative for species classification. Among these, the number of ventral scales and dorsal marks emerged as crucial indicators distinguishing hybrids from parental species. Macroevolutionary analyses on scalation traits in *Vipera* (and other related genera) revealed a strong phylogenetic signal and a lack of association with environmental variables, suggesting that these traits may have evolved primarily under neutral processes [15]. Interestingly, the number of ventral scales and dorsal marks deviate from this pattern, showing a significant association with several environmental variables [15, 40, 55]. In snakes, variation in the number of ventral scales was linked to water loss prevention and enhanced locomotion [40, 56, 57], while dorsal pattern and colouration serve thermoregulatory and antipredatory functions [55, 58, 59]. Therefore, the transference of these traits through hybridization may confer an ecological advantage to admixed populations and enhance adaptation to local environments [60, 61], but further studies are required to address the potential adaptative role of hybridization between *V. aspis* and *V. latastei* in this hybrid zone.

Data Availability

The datasets used and/or analysed during the current study are available upon request from the corresponding author.

Disclosure

Fieldwork was conducted with permits from the Spanish regional environmental authorities (for La Rioja, A/2015/013, A/2016/017, A/2017/021, A/2018/022, A/2019/028, A/2020/036; for Castilla y León, EP/BU/207/2015, EP/CyL/94/2016, EP/CyL/31/2017, EP/CyL/56/2018, EP/CyL/27/2019, AUES/CYL/192/2020).

Conflicts of Interest

The authors declare no conflicts of interest.

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Supplementary Materials

Table S1: samples from the Hybrid zone of Oja-Tirón analysed in this study. Table S2: probabilities of membership obtained from STRUCTURE, NEWHYBRIDS and LDA. Genetic classification of each individual based on the $Q > 0.94$ (STRUCTURE) and 0.6 (NEWHYBRIDS) thresholds for species and hybrid classification. Table S3: probabilities of membership obtained from STRUCTURE and NEWHYBRIDS using a dataset of non-admixed individuals and 30 simulated individuals for each hybrid class. Table S4: range, mean value and standard deviation of the nine morphological traits for the two parental species and the hybrids. Figure S1: genetic variation observed at 18 nuclear microsatellite loci over the two first axis of the PCA. Figure S2: correlation between genetic and morphological probabilities of classification to each of the parental species for the 24 hybrids. Text S1: details from univariate and multivariate statistics on morphological traits. (*Supplementary Materials*)

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