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Reproductive isolation between oviparous and viviparous lineages of the Eurasian common lizard *Zootoca vivipara* in a contact zone

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Contact zones between two evolutionary lineages are often useful for understanding the process of speciation because the observed genetic pattern reflects the history of differentiation. The Eurasian lacertid lizard *Zootoca vivipara* is a potentially interesting model for studying the role of reproductive mode in the speciation of squamate reptiles because it has both oviparous (*Zootoca vivipara carniolica*) and viviparous (*Zootoca vivipara vivipara*) populations that have recently been shown to be genetically distinct. We studied a newly-discovered syntopic area of these two *Zootoca* subspecies in the central Italian Alps using genetic markers to investigate the level of introgression between them. Patterns of genetic differentiation in a fragment of the mitochondrial DNA *cytb* gene and a set of nuclear microsatellites show that the speciation process is complete in this area, with no evidence of recent introgression. Phylogenetic and genotypic divergence suggests that the two subspecies have experienced long independent evolutionary histories, during which genetic and phenotypic differences evolved. The possible roles of biogeography, reproductive mode, and cytogenetic differentiation in this speciation process are discussed. *Biological Journal of the Linnean Society*, 2015, ••, ••–••.

ADDITIONAL KEYWORDS: gene flow – hybridization – microsatellites – speciation.

INTRODUCTION

The Eurasian lacertid lizard *Zootoca vivipara* is a potentially unique model for studying the role of reproductive mode in the speciation of squamate reptiles. Despite its scientific name, this species shows both viviparous and oviparous reproduction (Surget-Groba *et al.*, 2001). Although there are two other species of squamate lizards with both modes of reproduction (the Australian scincid lizards *Lerista bougainvillii* and *Saiphos equalis*; Qualls & Shine, 1998; Smith, Austin & Shine, 2001) only

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Z. vivipara is known to have potentially hybridizing egg-bearing and live-bearing natural populations (Surget-Groba *et al*., 2002; Lindtke, Mayer & Böhme, 2010).

Insight into the process of differentiation and speciation has often been obtained by the study of contact (or hybrid) zones between two lineages of the same species. For example, unimodal hybrid zones, where hybrid genotypes predominate, and bimodal zones, where hybrids are rare and parental genotypes prevail, reflect different stages of the speciation process. However, in contact zones where hybrids are lacking (i.e. speciation is complete), either genetic and phenotypic differentiation in allopatry has precluded hybridization upon secondary contact, or pre-zygotic or post-zygotic barriers have reinforced partial reproductive isolation (Jiggins & Mallet, 2000; Coyne & Orr, 2004). In the case of *Z. vivipara*, the subspecies *Zootoca vivipara vivipara* (viviparous) is found in many wet meadows in central western Europe, whereas oviparous populations of *Z. vivipara* occupy two allopatric areas in southern Europe: one in the Pyrenees (*Zootoca vivipara louislantzi*; Arribas, 2009) and the other in the central eastern Alps (*Zootoca vivipara carniolica*; Surget-Groba *et al*., 2002). The distributions of *Z. v. louislantzi* and *Z. v. vivipara* do not overlap, whereas *Z. v. carniolica* and *Z. v. vivipara* are parapatric in the Alpine chain. Phylogenetic studies have demonstrated that the latter two lineages show profound genetic divergence at the mitochondrial and nuclear genes (Surget-Groba *et al*., 2006; Cornetti *et al*., 2014), whereas morphometric analyses have concluded that they are morphologically indistinguishable (Guillaume *et al*., 2006). In the Alps, syntopic locations of *Z. v. carniolica* and *Z. v. vivipara* are rare, probably as a result of specific ecological differences (Voituron, Heulin & Surget-Groba, 2004), and previous surveys have only identified one area with potentially hybridizing oviparous and viviparous populations (Carinthia, Austria; Lindtke *et al*., 2010). In this contact zone, Lindtke *et al*. (2010) reported several putative hybrids with apparently intermediate developmental traits.

In the present study, we analyze in detail a second potential contact zone between *Z. v. carniolica* and *Z. v. vivipara* (Clade E; *sensu* Surget-Groba *et al*., 2006) identified during recent field surveys (L. Cornetti, G. F. Ficetola, S. Hoban, C. Vernesi, unpublished data) using a set of highly variable genetic markers, with the aim of obtaining insight into the speciation process. These results have important implications for the taxonomy of the genus and, consequently, for the conservation status of relatively rare *Z. v. carniolica* populations.

MATERIAL AND METHODS

During recent alpine-wide field surveys, a relatively small area (0.72 km^2) of potential overlap was identified between *Z. v. vivipara* and *Z. v. carniolica* in the alpine valley Valmora (central northern Italy, 46°02′15″ to 46°02′36″N; 9°37′09″ to 9°38′01″E; 1400–1600 m above sea level; Fig. 1). Subsequently, during the summers of 2012 and 2013, 60 lizards were captured by hand within this area over 27 nonconsecutive days by one to four surveyors. To confirm that there was adequate sampling coverage, mixture models for open populations were used to estimate the local abundance of lizards for four sites of the study area (Kéry *et al*., 2009) (Fig. 1; see also Supporting information, Table S1). The analyses suggested that the 60 captured individuals represented at least 50% of the resident lizard population.

Three-millimetre tail tips were collected and stored at room temperature in 95% ethanol until DNA extraction. Genomic DNA was extracted using the Qiagen DNeasy Tissue Kit (Qiagen Inc.). For each sample, a 385-bp fragment of the mitochondrial gene cytochrome b (*cytb*) was amplified and sequenced as described previously (Cornetti *et al*., 2014). *cytb* is the most extensively sequenced marker for the *Zootoca* genus and therefore is useful for comparing our results with previous studies, as well as to confirm subspecies identification, because no morphological traits unequivocally distinguish the two forms. Thirteen microsatellite (short tandem repeat; STR) markers (Lv-4-alpha, Lv-2-145, Lv-4-X, and Lv-4-115: Boudjemadi *et al*., 1999; B114: Remon *et al*., 2008; Lacviv04, Lacviv06, Lacviv26, Lacviv07, Lacviv27, Lacviv30, Lacviv05, and Lacviv17: Agata *et al*., 2011) were also amplified in seven multiplexed runs under the conditions described in the Supporting information (Table S2).

Sequence fragments were edited with FINCHTV, version 1.4.0 (Geospiza, Inc.; http://www.geospiza.com), assembled using SEQUENCHER, version 4.7 (Gene Codes Corporation), and aligned using CLUSTAL X (Thompson *et al*., 1997). These and all publicly available haplotypes found across the Alpine chain were collapsed into a median-joining network using NETWORK, version 4.6.1.1 (http://www.fluxus-engineering.com/sharenet_rn.htm), so that the subspecies of each of our samples could be identified.

The STR data were tested for deviations from Hardy–Weinberg equilibrium and linkage disequilibrium using GENEPOP, version 4.0 (Rousset, 2008). Possible genotyping errors (the presence of null alleles, large allele dropout, and stuttering) were assessed with MICRO-CHECKER, version 2.2.3 (Van Oosterhout *et al*., 2004). Because some markers had null alleles (see Results), we used FREENA (Chapuis

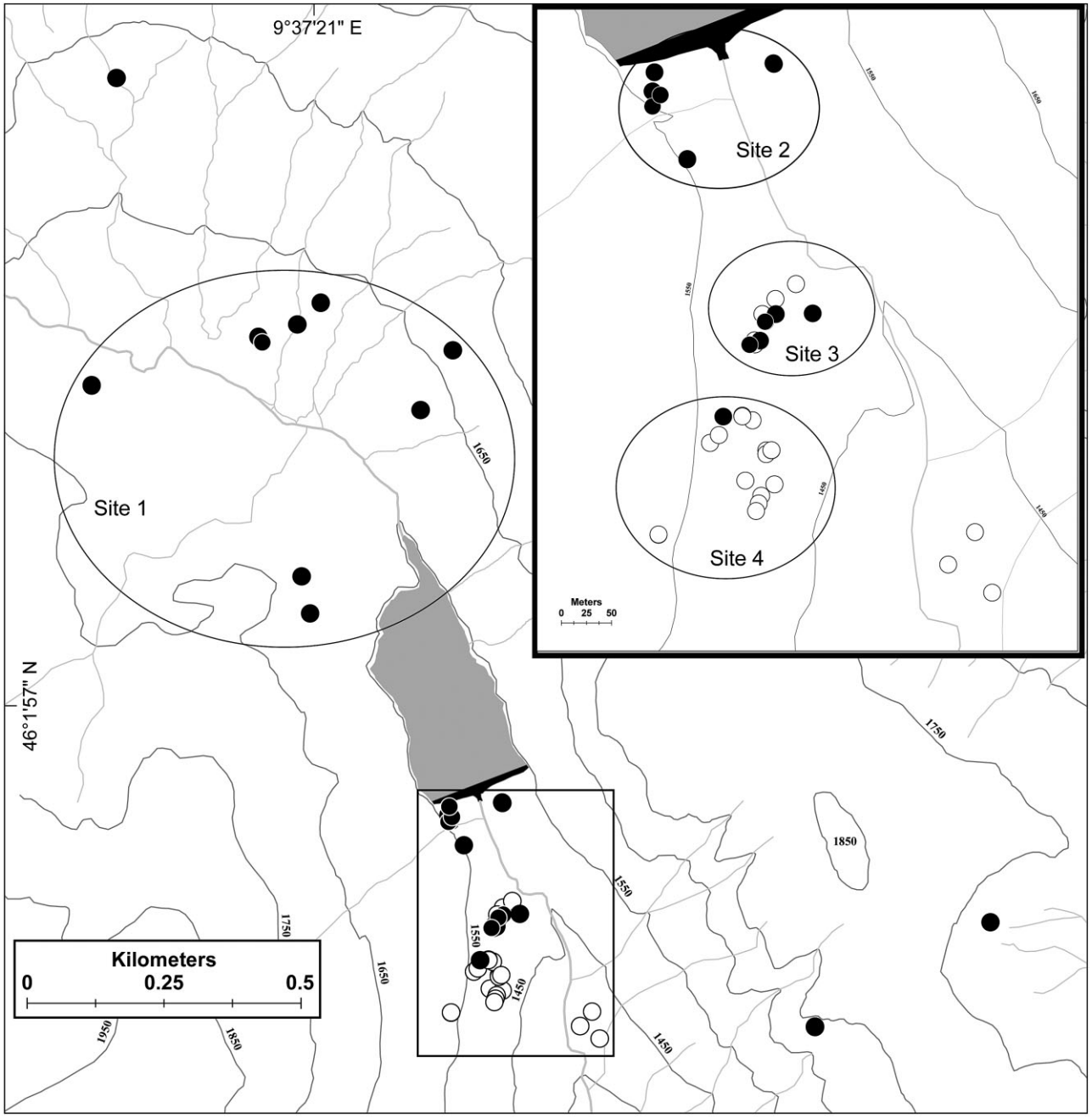

Figure 1. Detailed map of sampling area. Closed circles represent the capture sites of *Zootoca vivipara vivipara* and open circles represent the capture sites of *Zootoca vivipara carniolica*, identified to 'subspecies' according to *cytb* haplotype. The sites used for the estimation of lizard abundance are numbered 1–4.

& Estoup, 2007) to calculate whether such null alleles induced a positive bias in the estimates of F_{ST}. Genetic variation at STRs and subspecies differentiation were investigated using the R package DIVERSITY (Keenan *et al*., 2013); number of alleles (N_A), allelic richness (A_R), and observed and expected heterozygosity (H_O and H_E, respectively) were calculated for each subspecies, whereas F_{ST} and G'_{ST} were calculated between subspecies. Factorial correspondence analysis (FCA) implemented in GENETIX (Belkhir *et al*., 2004) was used to visualize the distribution of genetic variation across individuals.

NEWHYBRIDS, version 1.1 beta (Anderson & Thompson, 2002) and STRUCTURE, version 2.3.4 (Pritchard, Stephens & Donnelly, 2000) were used for inferring hybridization between subspecies, with indi-

vidual lizards being categorized as belonging to either parental subspecies (pure *vivipara*, pure *carniolica*) or one of the hybrid categories (F_1, F_2, or backcross) using a Bayesian algorithm and Markov chain Monte Carlo sampling. We ran 10 independent analyses using uniform priors, and a burn-in of 2.5×10^5 followed by 10^6 iterations. To detect possible hybrids, we also ran 10 independent analyses of STRUCTURE using $K = 2$ clusters, representing the two potentially hybridizing subspecies (burn-in of 2.5×10^5 followed by 10^6 iterations).

RESULTS

Detailed mapping of captures showed that *Z. v. vivipara* and *Z. v. carniolica* overlap considerably in Valmora, in particular in a wet meadow of approximately 1 ha of surface area, and should come into contact with one another because individuals of both lineages were captured on the same days and in close proximity (Fig. 1). All 60 samples were successfully sequenced for *cytb*. On the basis of haplotypes, our sample set consisted of 29 *Z. v. carniolica* (all with haplotype OS3, AF444038) and 31 *Z. v. vivipara* (23 of the VL_26, AF247976; eight of the VB1, KF898394, haplotype). The median-joining network, shown in Figure 2A, highlights the high level of divergence (19 mutations) between *Z. v. vivipara* and *Z. v. carniolica* haplotypes found in the contact zone under investigation (Fig. 1).

All thirteen STRs were successfully genotyped for all samples. The MICRO-CHECKER results, based on the grouped dataset (i.e. *Z. v. vivipara* and *Z. v. carniolica*), suggested the presence of null alleles for four markers; however, these were distributed evenly among subspecies (Lv115 and Lacviv04 in *Z. v. carniolica* and Lacviv07, Lacviv30 in *Z. v. vivipara*). In addition, three of these loci (all except Lacviv30), showed significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$), after correction for multiple testing using the false discovery rate (Benjamini & Hochberg, 1995). Only one out of 78 pairs of loci showed significant genotypic linkage ($P < 0.05$; Lv-4-X and Lacviv30). Because analyses with or without deviant loci led to very similar conclusions, we only report the results of analyses including all 13 STRs.

Visualization of the overall genotypic variation in STRs (Fig. 2B) suggests a marked genetic difference between individuals belonging to the two *cytb* clades, with no mitochondrial introgression. Genetic variability within the two populations, which can now be confidently referred to the viviparous and oviparous groups, was similar; although *Z. v. carniolica* had lower estimates for all indices, these differences were not significant (*t*-test, $P > 0.05$) (Table 1). The mean number of private alleles was 2.6 (56%) and 4.0 (66%) in *Z. v. carniolica* and *Z. v. vivipara*, respectively; the G'_{ST} value between the two groups was high and significant (0.891), as was the F_{ST} (0.381), which was very similar to the F_{ST} calculated, excluding null alleles, with FREENA (0.372).

Admixture analyses using NEWHYBRIDS clearly illustrated the lack of hybrid individuals in our sample set, and all samples were assigned to their pure parental subspecies with a probability above 99%. Similarly, STRUCTURE estimated a mean posterior probability of ranking *Z. v. carniolica* individuals to one cluster of 99.7% and *Z. v. vivipara* individuals to the other cluster of 99.5% (Fig. 2C).

DISCUSSION

In the studied area, speciation between the oviparous *Z. v. carniolica* and the viviparous *Z. v. vivipara* is complete because our multilocus analyses confirmed two highly distinct groups and the presence of hybrid individuals can be confidently excluded. Mitochondrial DNA sequences confirmed the deep haplotypic divergence between lineages (Fig. 2A), as previously suggested by Cornetti *et al*. (2014). We also reported profound genotypic differentiation (FCA) (Fig. 2B), corroborated by a high and significant G'_{ST} and F_{ST} values between subspecies and a high percentage of private alleles (56% and 66% in *Z. v. carniolica* and *Z. v. vivipara*, respectively). Thus, molecular analyses clearly illustrated the lack of gene flow between oviparous and viviparous lineages in this contact zone.

The lack of hybrid genotypes of any category (F_1, F_2, and backcrosses) highlighted that interbreeding of the two subspecies of *Z. vivipara* in this hybrid zone is absent or extremely rare (Fig. 2C). Elsewhere, convincing evidence of natural hybridization between oviparous and viviparous *Z. vivipara* has also never been reported. Lindtke *et al*. (2010) claimed that hybridization occurs between wild populations of *Z. v. carniolica* and *Z. v. vivipara*, although the hybrid origin of these individuals could not be confirmed. However, hybridization in captivity has been noted previously (Arrayago, Bea & Heulin, 1996), with it being suggested that the geographically isolated *Z. v. louislantzi* and *Z. v. vivipara* can successfully hybridize, and that the fitness of F_1 hybrids was lower than that of parental forms. However, we expect a more reduced viability/fertility in a *carniolica* × *vivipara* F_1 hybrid than a *louislantzi* × *vivipara* cross. This is because *Z. v. vivipara* (mitochondrial *cytb* Clade E in Cornetti *et al*., 2014, with females having $2n = 35$ and a Z_1Z_2W sex chromosome system including W as a macrochromosome; Odierna *et al*., 2004) is more similar karyotypically and genetically to *Z. v.*

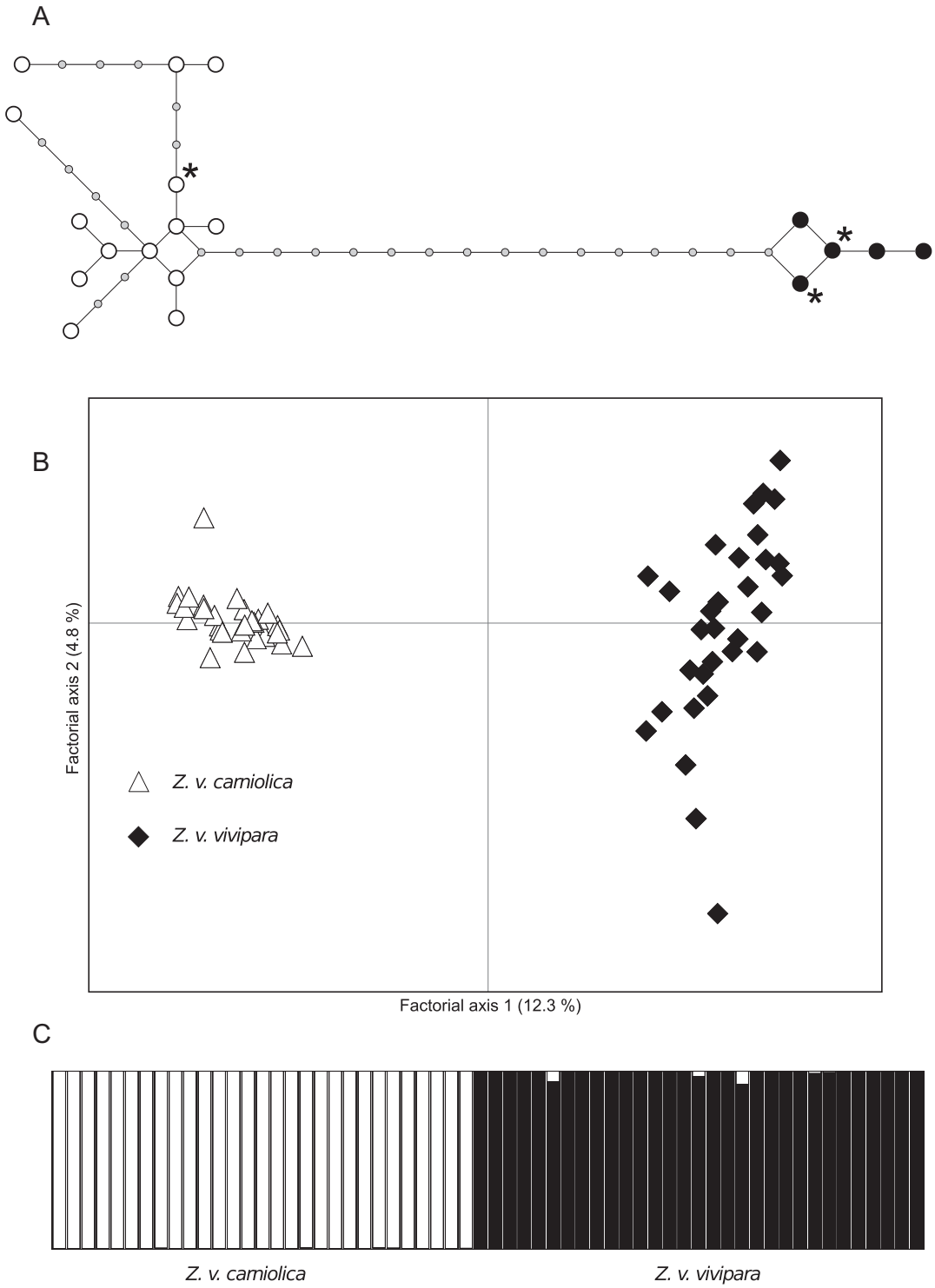

Figure 2. Analysis of mitochondrial and nuclear genetic variation of *Zootoca vivipara* in the Valmora contact zone. Network analysis including deposited sequences from Alpine distributions of common lizard subspecies: closed and open circles represent mitochondrial (mt)DNA haplotypes of *Zootoca vivipara vivipara* and *Zootoca vivipara carniolica*, respectively; circles indicated by asterisks correspond to haplotypes found in the present study; grey dots represent diverging mutations between observed haplotypes (A). FCA of genotypic variation between individuals divided according to *cytb* assignment (B). Plot representing the *Q*-value of individuals belonging to predefined mtDNA clusters as estimated by STRUCTURE (C).

Table 1. Genetic variation within the *Zootoca vivipara* subspecies

Subspecies	N	N_A	A_R	H_O	H_E
Zootoca vivipara carniolica	29	4.69	4.34	0.47	0.51
Zootoca vivipara vivipara	31	6.08	5.35	0.51	0.57

Number of alleles (N_A), allelic richness (A_R) observed and expected heterozygosity (H_O and H_E).

louislantzi (from sister Clade B, also with $2n = 35$ females and a Z_1Z_2W sex chromosome system and W as a macrochromsome) than to *Z. v. carniolica* (Clade A, with both males and females with $2n = 36$ with a ZW sex chromosome system and W as a microchromosome). However, even the W chromosomes of *Z. v. vivipara* and *Z. v. louislantzi* are not identical (Odierna *et al*., 2001); therefore, to confirm our hypothesis that *Z. v. vivipara* and *Z. v. carniolica* are separate species (i.e. they no longer interbreed), a specific captive study is needed to test hybridization success and the fertility of offspring among populations with the same reproductive modality and with same or different cytotypes, as well as among populations with the same cytotype but different reproductive modality. In addition, more detailed studies in the second hybrid zone should be carried out to confirm whether hybridization has also ceased in other parts of the *Z. v. vivipara* and *Z. v. carniolica* ranges.

The mitochondrial DNA and microsatellite results of the present study, together with previous phylogenetic studies, allow us to hypothesize about the timing of the transition from oviparity to viviparity in *Z. vivipara*. In reptiles, this switch is consistently associated with colonization of cold climates (Pincheira-Dinoso *et al*., 2013). Similarly, for *Z. vivipara*, oviparity is considered ancestral, and it has been demonstrated that the evolution and distribution of viviparous and oviparous populations were mainly shaped by Pliocene/Pleistocene climatic oscillations (Surget-Groba *et al*., 2001). It has been hypothesized that colder climatic conditions exerted a strong selective pressure on some populations pushed to southern-eastern areas of Europe during the Quaternary glacial phases, giving rise to viviparity. Viviparity then permitted the recolonization of northern Eurasia by these populations during interglacial periods. Surviving oviparous populations in the Italian peninsula, currently classified as *Z. v. carniolica*, presumably remained well-adapted to the warmer climate because their spatial and demographic re-expansion after glacial oscillations was limited to areas south of the Alps (Surget-Groba *et al*., 2002). The above hypothesis would indicate that the switch to viviparity in *Z. vivipara* occurred between 5.3 and 0.01 Mya, in the same range as mitochondrial phylogenetic analysis suggest that *vivipara* and *carniolica* began to differentiate (4.5 Mya, 95% confidence interval 6.1–2.6; Cornetti *et al*., 2014) and long before their secondary contact in Valmora [after the Last Glacial Maximum (LGM), approximately 10 000 years ago].

The evolutionary transition from oviparity to viviparity requires major structural, physiological, and therefore genetic changes (Murphy & Thompson, 2011). Nonetheless, and perhaps remarkably, this switch has been reported at least 115 times in squamate reptiles, out of a total of 140 switches for vertebrates (Sites, Reeder & Wiens, 2011). Thus, this change in reproductive mode in *Z. v. vivipara* may have determined the genetic differentiation between these subspecies. The ecological shift that coincided with the evolution of viviparity would have resulted in an allopatric distribution of the two subspecies and, in the Alps, in different altitudinal distributions [mean 1200 (range 450–1880) m asl and 1700 (1160–2160) m asl, for *Z. v. carniolica* and *Z. v. vivipara*, respectively; Cornetti *et al*., 2014], where genetic drift may have promoted further differentiation.

In addition to a switch in reproductive mode and drift, karyotypic divergence (as described above) may also have posed significant post-zygotic barriers upon secondary contact, such as hybrid subfertility, sterility or inviability (Coyne & Orr, 2004). F_1 hybrids between *carniolica* males × *vivipara* females are expected to show misalignment between the *carniolica* W microchromosome, the *vivipara* W macrochromosome, and a *carniolica* autosome during meiosis, and these three chromosomes may then fail to segregate regularly, causing germ cell death and/or resulting in inviable aneuploid gametes. Essentially, this type of chromosomal rearrangement may cause lowered hybrid fitness, potentially limiting gene flow between the two lineages (Faria & Navarro, 2010), playing an important role in the speciation process in many vertebrates, including lizards (Leache & Sites, 2009). On the basis of genotypic results reported in the present study and previous studies about karyotypic and phylogenetic divergence between *Z. v. vivipara* and *Z. v. carniolica*, we hypothesize that the speciation process between the two lineages was complete or almost complete before their secondary contact in the Alpine chain as a result of a switch in reproductive mode some time before the LGM. In this scenario, the role of reproductive mode may have made a strong contribution to genetic differentiation, although drift was almost certainly a contributing factor in allopatry.

In conclusion, *Z. v. vivipara* and *Z. v. carniolica* lineages and their contact zones provide excellent models for studying speciation and suitable subjects

for investigating the genomic basis of the oviparity/viviparity transition. Given the high level of genetic divergence and lack of gene flow between *Z. v. vivipara* and *Z. v. carniolica* reported in the present study, these two 'subspecies' should be considered as separate management units for conservation purposes. If the other contact zone also confirms these results, *Z. carniolica* should be recognized as a full species, distinct from *Z. vivipara*. Because the most suitable habitats for *Z. carniolica* are considered to be threatened by climate change and anthropization (Moore, 2002), conservation measures should be urgently re-evaluated because *Z. vivipara* is currently considered of least concern (IUCN, 2014).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Empirical Bayes estimation of lizard abundance in the four sites within the study area. Capture date, air temperature, precipitation, solar radiation, and number of surveyors were assumed to affect detection probability. We used the Akaike information criterion, corrected for small sample size, to identify the combination of predictors best explaining detection probability (Richards *et al*., 2011); we assumed a negative binomial error for the abundance component of models. Models were run using the package 'unmarked' in R (Fiske & Chandler, 2011). An empirical Bayes algorithm was used to estimate lizard abundance in the four sites and the 95% confidence interval.

Table S2. Thermocycling condition of microsatellites loci amplifications and genotyping. The 13 loci were amplified in seven multiplexes with an initial incubation at 94 °C for 10 min, followed by 30 cycles of 94 °C for 1 min, annealing temperature for 45 s, and 65 °C for 1 min, with a final extension of 65 °C for 10 min. Polymerase chain reaction (PCR) amplifications were optimized in a 20-μL reaction volume containing 1 μL of DNA, 2 μL HotMaster Taq Buffer 25 mM Mg_2 (Eppendorf), 100 μM dNTPs, a variable proportion of labelled forward and reverse primers, 1 U of HotMaster Taq Polymerase (Eppendorf), and double-distilled water. PCR products were run with an internal lane standard (LIZ) on an ABI 3130 (Applied Biosystems); alleles were scored using GENEMAPPER (Applied Biosystems).