

Phylogeography and conservation of the populations of *Zootoca vivipara carniolica*

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Abstract

The lizard *Zootoca vivipara* has both oviparous and viviparous populations which belong to distinct clades. This study aims to elucidate the geographic distribution, phylogeography and conservation priority of the oviparous subspecies, *Zootoca vivipara carniolica*, that has recently been identified in Slovenia. We studied seven Slovenian populations, two northeastern Italian populations and five northwestern Italian populations. The seven cytochrome b haplotypes that we identified from the Slovenian and Italian oviparous populations form a monophyletic basal clade. The high homogeneity of the Slovenian and northeastern Italian populations (nucleotide diversity $\pi=0.06\%$) is a striking contrast with the pattern observed in northwest Italy where each of the five populations studied presents a private haplotype ($\pi=1.03\%$). The results obtained suggest that all the extant lineages of *Z. v. carniolica* have originated in an Italian refuge. The reproductive and phylogenetic distinctiveness of *Z. v. carniolica* clearly justifies conservation of its populations, especially in Italy where the highest genetic diversity and the most ancestral haplotype were observed. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The lizard *Zootoca vivipara* (formerly *Lacerta vivipara*) is an Eurasian lacertid that has both oviparous and viviparous populations. Viviparous populations are widely distributed from central France and the British Isles north to Scandinavia and northeastern Asia (Sakhalin and Hokkaido Islands; Arnold et al., 1978; Heulin and Guillaume, 1989; Takenaka, 1991). Oviparous populations have only been observed in the southernmost part of the geographic range. A cluster of oviparous populations, geographically separated from the viviparous populations, in the extreme southwestern portion of the species' range from the Cantabrian Mountains (northern Spain) to the Pyrénées Mountains

and Aquitaine lowlands (southwestern France) have been studied previously (Lantz, 1927; Braña and Bea, 1987; Heulin and Guillaume, 1989). More recent investigations revealed that the populations from Slovenia and an adjacent population from the Carnian Alps in southern Austria are also oviparous (Böhme et al., 1999; Heulin et al., 2000; Mayer et al., 2000).

Our analyses of the mtDNA cytochrome b gene sequences revealed the existence of four major clades in *Z. vivipara*. The Slovenian oviparous populations form a basal clade, whereas the Spanish-French oviparous clade and two viviparous clades occupy upper branches of this phylogenetic tree (Fig. 1; Surget-Groba et al., 2001). The study of other mtDNA segments (12s and 16s rRNA sequences), which also revealed the basal position of the oviparous populations from Slovenia and from the Austrian Carnian Alps, led taxonomists to propose a subspecific status—*Zootoca vivipara carniolica*—for this oviparous clade (Mayer et al., 2000).

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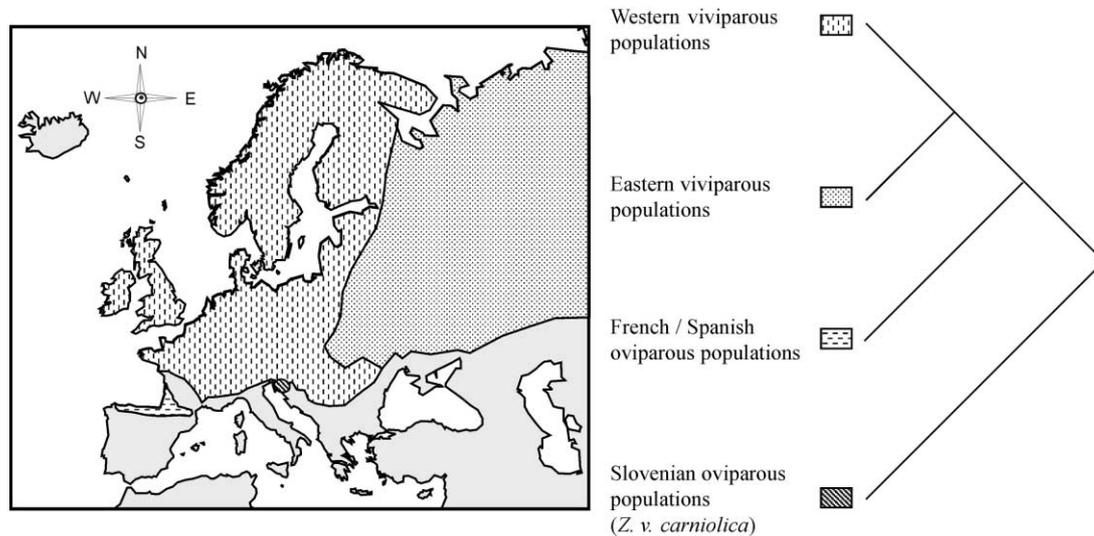


Fig. 1. Phylogenetic relationships and geographical distribution of the different clades of *Zootoca vivipara* previously identified (Surget-Groba et al., 2001).

However there is no reason to suppose a priori that this clade is restricted to the “carniolian” region,¹ nor that it has originated from this region as speculated by Mayer et al. (2000). Indeed, we very recently discovered oviparous populations of *Z. vivipara* not only in the Italian region (Frioule) adjacent to Slovenia, but also far away in the Piemonte-Lombardia region in northwest Italy (Ghielmi et al., 2000, 2001). Hence, it is necessary to examine whether these Italian oviparous populations are phylogenetically related to the Slovenian populations of *Z. v. carniolica*.

The aim of this study is to examine the phylogenetic position of the Italian oviparous populations and also to evaluate conservation priorities. Indeed, the southernmost populations of *Z. vivipara* are generally restricted either to relatively rainy mountain areas or, discontinuous, wet biotopes (in particular peatbogs and fens) in the lowlands (Heulin, 1989; Heulin and Guillaume, 1989; Guillaume et al., 1997). The populations are most vulnerable in the latter case, because human activities such as drainage and turf digging may increase the fragmentation of the favourable habitat for the species in lowlands. This conservation problem seems of particular importance in the Po basin, which marks the southernmost extent of the distribution in Italy, and which contains a few endangered relict lowland populations. Historical records and museum specimens indicate that the species was distributed throughout this region prior to 1940, whereas recent field investigations (subsequent to 1970) confirmed the survival of only about 10 small, isolated populations in this region (De Betta, 1857, 1863; Massolongo, 1859; Lessona, 1878; Camerano, 1885; Vandoni, 1914; Tortonese, 1942;

Lugaro, 1957; Lapini, 1983; Giovine, 1989; Salmaso and Osella, 1989; Richard and Semenzato, 1992; Baratelli and Ghielmi, 1994; Semenzato et al., 1996; Fig. 2). The five oviparous populations recently identified by us in Piemonte-Lombardia (including two lowland populations: see Varese and Busatello in Fig. 2) are therefore of obvious conservation interest.

Molecular markers, which are irreplaceable tools for investigating phylogenetic and phylogeographic questions (Avice et al., 1987), are also increasingly employed to estimate parameters (among-population gene flow; genetic distinctiveness of taxonomic units) useful to guide conservation actions (Moritz, 1994a,b, 1995; Avice, 1995). In this study, we perform a phylogenetic analysis of the mtDNA cytochrome b sequences, including new samples from Italian and Slovenian oviparous populations and representative of the major clades previously identified by Surget-Groba et al. (2001). We also compare the nucleotide diversity of Italian and Slovenian oviparous populations in order to assess their genetic distinctiveness and, hence, to determine possible conservation priorities. We discuss to what extent the genetic diversity of these populations could be related to their phylogeographic history and/or to habitat fragmentation.

2. Material and methods

2.1. Sampling

We studied the mtDNA characteristics of 124 individuals from 14 populations: five populations from northwestern Italy, two populations from northeastern Italy, and seven populations from Slovenia (population codes and locality names in Table 1; Map in Fig. 2). The

¹ Historically: up to 1918 a southern Austrian region that is now in Slovenia.

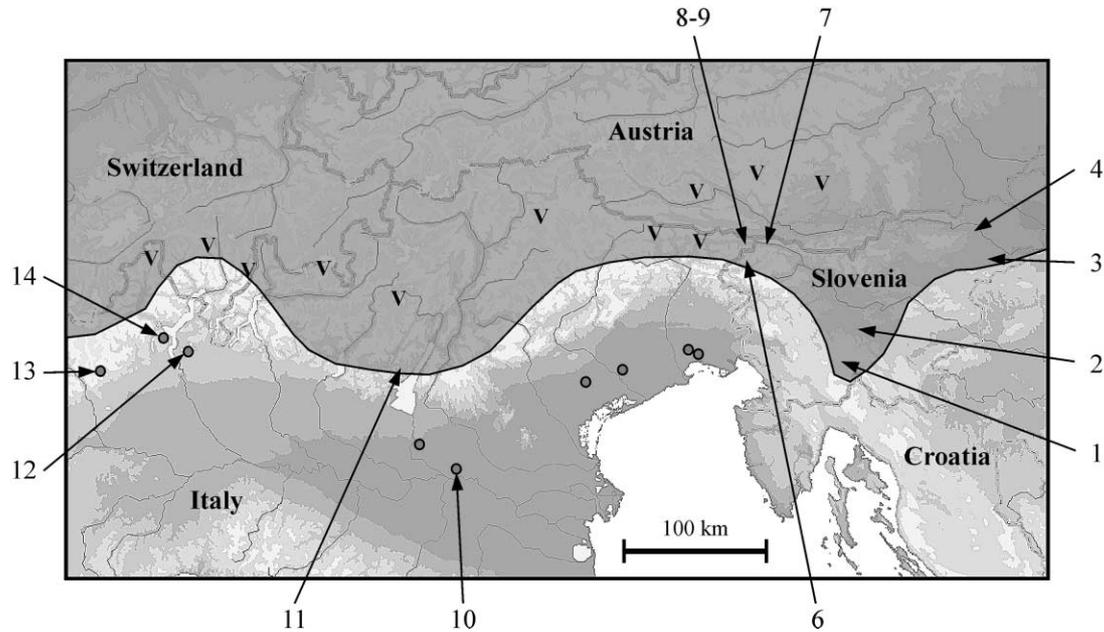


Fig. 2. Map of the sampled populations. The dark area corresponds to the main distribution range of the species, and the dots to disjunct populations of the Po basin. Nos. 1 to 14: oviparous populations sampled in this study (for locality names, see Table 1); V = localities where the viviparous form has been identified in the southern Alps (according to Ghielmi et al., 2001; Mayer et al., 2000; Odierna et al., 1998; Surget-Groba et al., 2001; and personal unpublished observations).

Table 1
Localities sampled and haplotypes identified in Slovenia and Italy^a

Country	Population code	Locality	Altitude	Latitude (° N)	Longitude (° E)	N	Haplotypes
Slovenia	1	Cernisko Jezero	480 m	45.772	14.398	12	OS1
	2	Ig	1000 m	45.958	14.536	2	OS1
	3	Medvedce	245 m	46.343	15.730	7	OS1
	4	Kot-Pohorje	1040 m	46.494	15.614	10	OS1
	5	Pavlicevo seldo	1200 m	46.425	14.589	1	OS1
	6	Mangart	1200 m	46.440	13.659	2	OS1 (1)/OS2(1)
	7	Zelenci	840 m	46.491	13.758	10	OS1
NE Italy	8	Fusine	875 m	46.492	13.653	12	OS1 (11)/OS2(1)
	9	Tarvisio	800 m	46.492	13.600	8	OS1
NW Italy	10	Busatello	15 m	45.101	11.093	7	OS7
	11	Bollone	830 m	45.736	10.586	11	OS6
	14	Lago Maggiore	850 m	45.877	8.488	10	OS5
	12	Varese	240 m	45.784	8.700	12	OS3
	13	Oropa	1180 m	45.629	7.974	19	OS4

^a Haplotypes as in Fig. 3; population codes as in Fig. 2; N sample size. When different haplotypes are present in one population, the number of individuals carrying each haplotype is bracketed.

124 individuals studied include those (15 individuals from the Slovenian populations Nos. 1, 3, 4 and 7) previously studied by Surget-Groba et al. (2001), that all carried the same haplotype (OS1) of mtDNA cytochrome b.

Approximately 1 cm of autotomized-tail of each lizard was preserved in 95% ethanol, until processed for the mtDNA study. Most animals were then released at their collection site, except those (one to three per population) that were used to study reproductive mode (Ghielmi et al., 2000; Heulin et al., 2000).

2.2. Mitochondrial DNA analyses

Total DNA was chelex extracted according to the methods described in Estoup et al. (1996) from small amounts of tail. A 429 bp segment of mtDNA (408 bp from cytochrome b and 21 bp from the adjacent Glu-tRNA genes) was amplified using primers MVZ04 and MVZ05 (Smith and Patton, 1991).

The haplotype of at least one individual from each population was determined by sequencing, as previously

described in Surget-Groba et al. (2001). A single-strand conformation polymorphism analysis (SSCP: Orita et al., 1989) was used to infer the haplotypes of the other individuals by comparing the chromatogram of each individual to those of the individuals whose haplotypes had previously been sequenced. SSCP offers a sensitive and rapid method for determining which DNA samples in a set differ in sequence, so that only an informative subset needs to be sequenced. This method, which is widely used for clinical use, has been shown to be very useful for population genetics studies (Sunnucks et al., 2000). In this study, we used a capillary electrophoresis SSCP method (CE-SSCP, Inazuka et al., 1997) which is much more efficient than conventional SSCP technique (Ren, 2000). For the SSCP analysis, we used fluorescent-marked primers (MVZ04-hex and MVZ05-fam). PCR (0.5 μ l) was mixed to 0.5 μ l NaOH (0.1 N), 11 μ l deionised formamide and 0.5 μ l Genscan-1000 [ROX] size standard. This mix was run on an ABI 310 genetic analyser in a 3% Genscan polymer + 10% glycerol at 30 °C. Chromatograms were analysed with the Genscan software (Applied Biosystem). Any new variant of haplotype detected by SSCP, was sequenced .

2.3. Phylogenetic analyses and nucleotide diversity

We reconstruct a phylogenetic tree including all the new haplotypes identified in Slovenia and Italy and the haplotypes representative of the main clades previously described by us. For presentational convenience, the phylogenetic tree presented (Fig. 3) does not include all 29 haplotypes previously identified but only the most common haplotypes of each clade (genbank accession numbers: AF247976-AF248005): OS1 for Slovenian clade; OC1, OC2, OF1, OF2 for the Spanish-French oviparous clade ; VU1, VU2, VU3, VU4 for the eastern viviparous clade ; VB1, VB8, VB9, VB 10 for the western viviparous clade (Surget-Groba et al., 2001). We verified that this presentation simplification did not modify the topology of the tree. Sequence alignment was performed by eye and the phylogenetic relationships between haplotypes were inferred by maximum parsimony (branch and bound search) using PAUP version 4.0b3. Tree length and the consistency index (CI) were based on all characters. The reliability of the nodes was investigated by bootstrapping using 1000 replicates. All the sequences used in our study are deposited in the Genbank database under accession numbers AF444037-AF444042 for *Z. vivipara* and AF248006-AF248007 for *Lacerta bilineata* (formerly *Lacerta viridis*) and *Podarcis muralis* (two other lacertid lizards used as outgroup in the analysis).

In addition to the classical phylogenetic methods (see earlier) that were used to identify the relative position of the main clades of the species, we also constructed a statistical parsimony network (Templeton, 1992) using

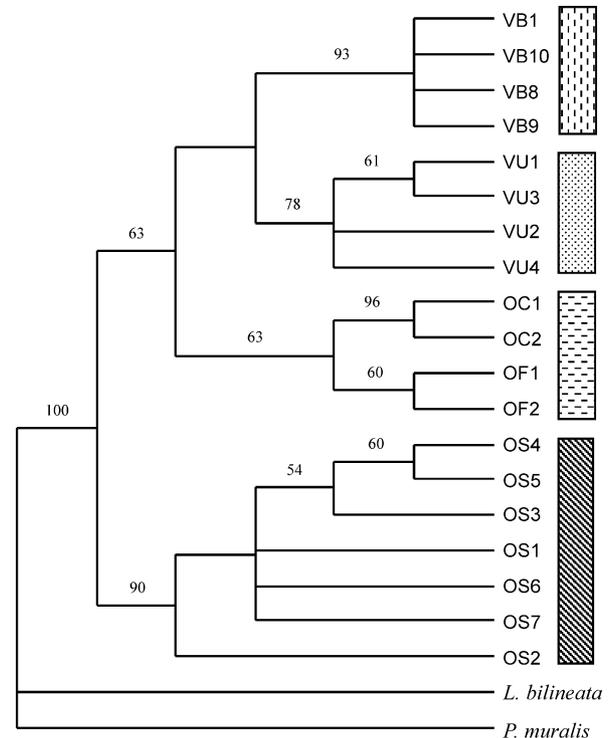


Fig. 3. Maximum parsimony consensus tree of the haplotypes of *Zootoca vivipara*. This phylogenetic tree includes representative haplotypes of the main clades previously identified by Surget-Groba et al. (2001), and the new haplotypes (OS2, OS3, OS4, OS5, OS6, OS7) identified in the present study. Bootstrap values indicated on the branches.

TCS version 1.12 in order to better understand the relationships between the “Italo-Slovenian” haplotypes. Indeed, network methods (Posada and Crandall, 2001) are better suited to analysing closely related haplotypes than classical phylogenetic methods. Nucleotide diversity (π) was calculated using DnaSP version 3.5.

3. Results

3.1. Phylogenetic relationships

Our study reveals that the OS1 haplotype previously identified in Slovenia (Surget-Groba et al., 2001), also exists in northeastern Italy (Fusine and Tarvisio). A new haplotype, OS2, was found in only two individuals: one coming from the Italian population of Fusine and the other coming from the neighbouring Slovenian population of Mangart. Five new haplotypes, each being characteristic of a single population, have also been identified in northwestern Italy: OS3 in the 14 individuals from Varese, OS4 in the 13 individuals from Lago Maggiore, OS5 in the 19 individuals from Oropa, OS6 in the 11 individuals from Bollone and OS7 in the seven individuals from Busatello (Table 1). The maximum parsimony search produced two trees of 176 steps

(consistency index $CI=0.80$, rescaled consistency index $RC=0.66$). The OS1 haplotype and the six new haplotypes (OS2 to OS7) identified branch together in the phylogenetic tree (bootstrap $P=90$) as shown in the strict consensus tree (Fig. 3). Hence the Slovenian and the Italian oviparous populations form a basal single clade, which corresponds to the subspecies *Z. v. carniolica* of Mayer et al. (2000).

The haplotype OS7 is the central haplotype of the network (Fig. 4). Four branches emerge from this haplotype. The first branch is composed of the three haplotypes from western Lombardia and Piemonte (OS3, OS4 and OS5), the second of the haplotype OS6 near the Lago di Garda, the third of the haplotype OS2 present in two populations of eastern Italy and western Slovenia and the fourth of the haplotype OS1 present in eastern Italy and in all Slovenian populations.

3.2. Population structure and diversity

The Slovenian and northeastern Italian populations form an homogeneous group, in which all but two individuals bear the OS1 haplotype. This is a striking contrast to the pattern observed in northwest Italy where each of the five populations have a specific and unique haplotype. As a consequence the nucleotide diversity of the northwest Italian group ($\pi=1.03\%$) is considerably higher than that of the Slovenian and northeastern Italian group ($\pi=0.06\%$). The clear-cut genetic differentiation of the five populations from northwest Italy (absence of shared haplotypes) indicates that, despite the geographic proximity of some of them (less than 30 km between Varese/Lago Maggiore), there is only very limited (if any) gene flow among these populations.

4. Discussion

4.1. Biogeographic history

To what extent the geographic variation in the genetic diversity of *Z. v. carniolica* reflects the biogeographic history of its different populations? Our previous research led us to propose a biogeographic scenario positing that the glacial phases of the Pleistocene may have obliged the populations of *Z. vivipara* to retreat into several allopatric southern refuges, and hence may have facilitated the differentiation of distinct oviparous and viviparous clades within this species. The viviparous populations, better adapted to the coldest climatic conditions, may have rapidly re-colonized northern countries during warming periods; whereas the oviparous populations may have been restricted for longer periods in southern regions (for more details on the scenario, see: Heulin et al., 1993, 1999; Guillaume et al., 2000; Odierna et al., 2001; Surget-Groba et al., 2001).

The highest genetic diversity observed in the Italian populations of *Z. vivipara carniolica* may well correspond to the conservation of an ancestral polymorphism in a refuge area, whereas the existence of single haplotype in most Slovenian populations could reflect a founder effect correlated to a recent colonization of this region. Indeed, colonization processes are often associated with a loss of variability (Austerlitz et al., 1997; Huchon et al., 1999).

It is worth noting that the hypothesis of an Italian refuge for this subspecies is also strengthened by the structure of the haplotype network (Fig. 4). Indeed, it is expected that the age of an haplotype is proportionnal to the number of its connection with the other haplotypes

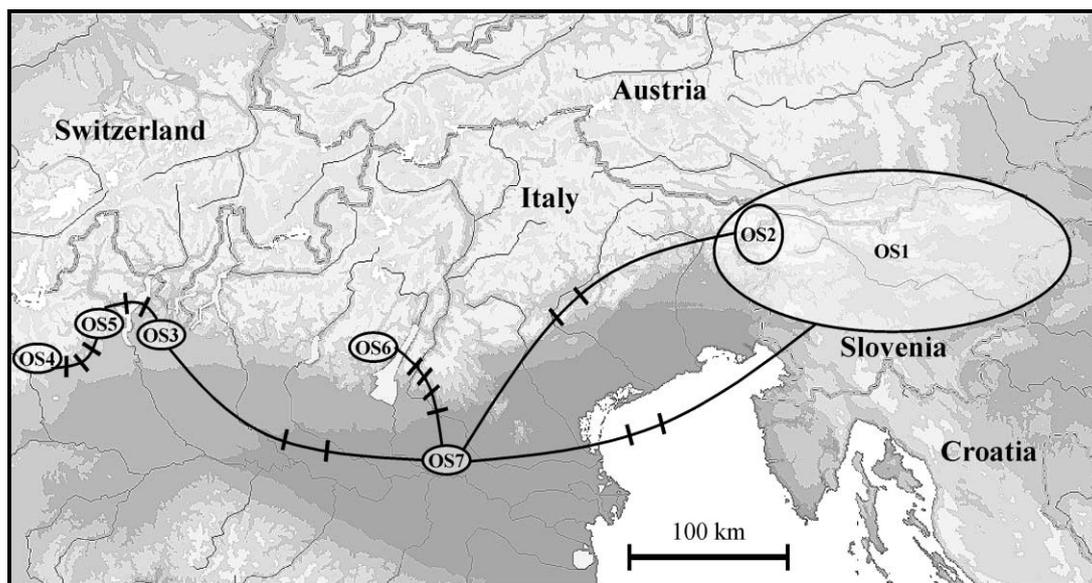


Fig. 4. Geographic repartition and network of haplotypes of *Z. v. carniolica*. The slashes represent the number of mutations between two haplotypes.

(Crandall and Templeton, 1993). The Italian haplotype OS7 which is at the center of the network and has four connections with other haplotypes is therefore probably the oldest haplotype. Hence the presence of the most ancestral haplotype in Italy tends to support the idea that all the extant lineages of *Z. v. carniolica* would have originated from this refuge area. On the contrary, eastern Italy and Slovenia present only tip haplotypes (with only one connection) which suggests that this region has been colonized recently by “young” haplotypes.

Finally, one may also suspect that the warmer and dryer climate of the post pleistocene period has progressively restricted the favourable biotopes (in particular peatbog) available for *Z. vivipara* on the southern margin of its range, and that recent human activities (e.g. drying of numerous marshes of the Po basin, since 1940) have certainly further fragmented those relict biotopes in lowlands (Amadei, 1977; Cotta Ramusino, 1986; Giovine, 1989; Semenzato et al., 1996). The expected effects of such a habitat fragmentation, that is a marked genetic differentiation of the populations and a low genetic variability within each population (Wright, 1931; Cunningham and Moritz, 1998), corresponds to the genetic structure observed in the northwestern Italian populations of *Z. v. carniolica*.

4.2. Conservation implications

The populations of *Z. v. carniolica* from Slovenia and from northeastern and northwestern Italy are unique, not only because they are oviparous but, also, because they branch off at the base of the phylogenetic tree, before all other oviparous and viviparous clades of the species. Furthermore, we previously showed that females from the Slovenian oviparous populations exhibit a particular chromosomal formula ($2N=36$ chromosomes) different from those ($2N=35$, due to a fusion of two chromosomes from distinct pairs) observed in females from all other oviparous and viviparous clades of *Z. vivipara* (Odierna et al., 2001). A recent karyological investigation also revealed the existence of $2N=36$ chromosomes in oviparous females coming from the Italian population of Oropa, Bollone and Tarvisio (Odierna, unpublished data, personal communication). Given all these unique features, it seems worth protecting most of the populations of *Z. v. carniolica* that can be considered as an evolutionary significant unit (ESU: Moritz, 1994a,b).

Conservation actions should however take into account the fact that the genetic diversity is very unevenly distributed among and within the populations of *Z. v. carniolica*. We must first emphasize that despite the term “carniolica” refers to Slovenia, this subspecies has probably not originated from this region which only contains two of the haplotypes identified. On the

contrary the whole genetic diversity identified (seven haplotypes) is represented in Italy. Furthermore, the low genetic diversity of the Slovenian and northeastern Italian group of populations strikingly contrasts with the genetic differentiation observed among the five populations from northwest Italy (one particular haplotype in each of them). These five populations from northwest Italy, among which there is probably no gene flow, must for this reason be considered as true management units (MU: Moritz, 1994a,b) deserving specific—and urgent—conservation action. This is of particular importance for the lowland population of Busatello that represents a crucial element (see above paragraph and Fig. 4) in understanding the phylogeographic history of *Z. v. carniolica*.

4.3. Conclusions and prospects

This study clearly illustrates the reproductive and phylogenetic distinctiveness of *Z. v. carniolica* and allows us to define priorities in the conservation of its populations, especially in Italy where the highest genetic diversity was observed. Future research will aim, (1) at defining the reproductive and phylogenetic status of all the relict (hopefully not extinct) populations of *Z. vivipara* isolated in the Po basin, and (2) at assessing whether there are contact zones (and possibly interbreeding) between the oviparous *Z. v. carniolica* and the viviparous populations of *Z. vivipara* in the Alps (see also Ghielmi et al., 2001). Available data on the southernmost occurrences of the viviparous form (Fig. 2), actually suggest that such contacts might exist both in Italy, on the border Austria-northeast Italy (western part of the Carnic Alps) and near the Drave river in south Austria (Ghielmi et al., 2001; Mayer et al., 2000).

Indeed, we previously noted that, although it is possible to crossbreed Spanish-French oviparous lizards with the French viviparous lizards in the laboratory, there is an ecological barrier (absence of favorable wet habitat) which prevents any contact and interbreeding of these two groups of populations in southern France (Heulin et al., 1993; Arrayago et al., 1996). This situation has led us to consider whether the existence of such a barrier was a necessary condition to the survival of oviparous populations in *Z. vivipara*. The identification of a contact zone between the oviparous *Z. v. carniolica* and viviparous populations of *Z. vivipara*, would therefore be of obvious theoretical interest.

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