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Short Notes

Museum specimens indicate genetic erosion in an endangered lizard

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Abstract. Genetic variability, one of the main factors that guarantees species persistence, and species' conservation status are generally evaluated with indices calculated at the present time. Natural history collections might help compare historical and current genetic diversity so to identify major trends. Here we analysed museum specimens of the lizard *Zootoca vivipara carniolica*, with a specific and stringent protocol for degraded DNA, in order to contrast its past and current genetic variability, using fragments of one mitochondrial DNA gene. Part of the distributional range of *Z. v. carniolica* (Po Plain, Italy), heavily impacted by human activities, was investigated. We found two previously unknown haplotypes in populations that are extinct today, suggesting the loss of these haplotypes and thus an overall shrinking of genetic variability. We argue that these results, together with the increasing threats posed by climate and land use changes, suggest that specific conservation measures for the persistence of *Z. v. carniolica* in Northern Italian lowlands have to be considered.

Keywords: ancient DNA, anthropization, genetic variability, habitat loss, mtDNA, viviparous lizard.

Introduction

Maintenance of genetic diversity through time is among the main tenets of conservation biology, since genetic variation is one of the key factors that guarantees adaptive potential to populations and therefore species' persistence (Frankham, Ballou and Briscoe, 2004; Spielman, Brook and Frankham, 2004). Genetic variability is usually measured at the present time and implications on the conservation status of wild species are based on standard genetic indices that are then used to infer species adaptive potential (Frankham, 2005).

Several powerful methods (e.g. Approximate Bayesian Computation; Bertorelle, Benazzo and Mona, 2010), given the summary statistics calculated from current samples, can evaluate the demographic dynamic of populations over time. From a conservation perspective, estimating the fluctuation of effective population size and consequently if and how genetic diversity changes through time, might help implement management strategies in the face of on-going and anticipated global environmental changes. However, a more precise prediction of loss or gain of genetic variability may be achieved analysing samples from the past. In this case, direct estimation of an increase or reduction of genetic variation can be determined (Ramakrishnan and Hadly, 2009).

Obtaining genetic information from historical specimens is very challenging (Lounsberry et al., 2014; Røed et al., 2014; Tison et al., 2015), despite significant advances in genomic techniques over the last decade (Orlando and Cooper, 2014). The major limitation for the analyses of historical samples is that DNA

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is generally degraded and fragmented to sequences of no more than a few hundred base pairs in length. For this reason, genetic analyses of museum specimens are traditionally targeted to the mitochondrial genome for most of the organisms (Wandeler et al., 2007), including reptiles (Stuart et al., 2006; Stuart and Fritz, 2008; but see Ruane and Austin, 2017). In fact, mitochondria are present in multiple copies per cell providing a higher probability of retrieving DNA molecules.

Here we analysed a set of museum specimens of the lacertid lizard *Zootoca vivipara* focusing on the lineage *Z. v. carniolica* (Mayer et al., 2000) with the aim of comparing its current and past mitochondrial genetic variation. *Zootoca v. carniolica*, possibly considered a separate species (Cornetti et al., 2015a, 2017), is of conservation concern and, according to mitochondrial and nuclear DNA variation, it has been considered an Evolutionary Significant Unit (ESU, Surget-Groba et al., 2002; Cornetti et al., 2014).

According to karyological studies and its reproductive oviparous mode, *Z. v. carniolica* can be viewed as the ancestral form of the species, in contrast to the viviparous *Z. vivipara vivipara* (Odierna et al., 2001; Surget-Groba et al., 2001). Phylogeographical analyses suggested that *Z. v. carniolica* populations have had a refuge in the southern margin of the Alpine chain (Northern Italy and Slovenia) and Po plain during Quaternary glacial phases (Surget-Groba et al., 2001, 2006). The oviparous reproductive mode, that made this lineage well-adapted to the warmer climates, likely prevented a northward expansion of *Z. v. carniolica*, determining its current relatively small distributional range (Surget-Groba et al., 2002). Additionally, since it tends to live at low-middle altitudes and even in few relict spots in the Po plain, this lineage is particularly vulnerable to anthropogenic modifications. In fact, the most suitable habitats for *Z. v. carniolica* (marshes or peatbogs) are threatened by human activities, in particular by recent drying of moist areas in the Po Plain and transformation of lowland biotopes into agricultural fields (Surget-Groba et al., 2002). Additionally, changing climatic conditions have been documented to exert a negative impact on the survival of such habitats even in mountainous regions (Bragazza, 2008). Together, these factors are progressively increasing fragmentation of *Z. v. carniolica* populations, as demonstrated by population genetic analyses (Cornetti et al., 2015b).

Methods

Thirty-nine historical specimens, preserved in ethanol or formaldehyde, were collected from two Natural History Museums (table 1, supplementary table S1). The list included specimens collected in the Po Plain and the eastern Italian Alps, between 1865 and 1981. Since *Z. v. vivipara* and *Z. v. carniolica*, the two lineages that are parapatric in Northern Italy, are difficult to distinguish morphologically (Rodriguez-Prieto et al., 2017) and the latter was described only recently (Mayer et al., 2000), a priori we could not perform an indisputable taxonomic identification. However, given the phylogeographic history of the genus *Zootoca*, specimens collected in the Po Plain are likely to belong to *Z. v. carniolica*. A few milligrams of skin tissue were taken from each specimen and stored in 95% ethanol before molecular analyses were performed. Genomic DNA was extracted using a phenol-chloroform protocol (Sambrook and Russell, 2006).

Because of the predicted high degree of DNA degradation, we designed new pairs of primers (supplementary fig. S1 and table S2) allowing for the amplification of short fragments of the mitochondrial gene Cytochrome b (*cytb*). PCR amplifications were carried out in a 50 μl reaction mix containing: 2 μl of template DNA, 5 μl of AmpliTaq Gold 360 Buffer 10X, 4 μl of 25 mM Magnesium Chloride, 5 μl of 360 GC Enhancer Buffer (Applied Biosystem), 40 mM dNTPs, 10 pM of each primer and 1 unit of AmpliTaq Gold® 360 DNA Polymerase (Applied

Table 1. List of locations and specimens analysed. Area, refers to fig. 1a-b; *N*, number of extracted samples per location; Amplified, number of samples successfully amplified for at least one *cytb* fragment (* indicates sequenced individuals ascribable to *Z. v. vivipara* lineage according to *cytb* haplotype); Haplotypes, observed haplotypes in the historical specimens successfully amplified for the 311-bp *cytb* fragment (in brackets, the number of samples carrying the haplotype); Preservation, method of preservation.

Museum	Location	GPS coordinates		Altitude (m)	Year	Area	*N*	Amplified	Haplotypes	Preservation
		North	East							
Torino	Padola di Cadore	46°36′06″	12°28′46″	1216	1908	eastern Alps	8	5 (1*)	HS1 (1), OS1/OS2/OS7 (1)	Ethanol
	S. Stefano di Cadore	46°33′28″	12°32′58″	918	1908	eastern Alps	2	2	-	Ethanol
	Vanchiglia	45°04′09″	7°42′10″	227	1879	western Po Plain	2	2	HS2 (1), OS3/OL12 (1)	Ethanol
	Casalgrasso	44°49′05″	7°37′35″	240	1885	western Po Plain	1	1	HS3 (1)	Ethanol
	Cerea	45°11′43″	11°12′41″	17	1877	central Po Plain	1	1	-	Ethanol
Verona	Risaie del Busolo	45°24′38″	11°06′54″	42	1865	central Po Plain	2	0	-	Formaldehyde
	Casaleone	45°10′23″	11°11′53″	15	1874	central Po Plain	13	4	-	Formaldehyde
	Legnago	45°11′37″	11°18′11″	18	1862	central Po Plain	3	2	-	Formaldehyde
	Cerea	45°11′43″	11°12′41″	17	1868	central Po Plain	4	0	-	Formaldehyde
	Palude del Busatello	45°03′57″	11°06′51″	12	1981	central Po Plain	3	3	OS2/OS7 (1)	Ethanol

Biosystem). The thermocycling conditions consisted of incubation at 95°C for 10 min, followed by 50 cycles of 95°C for 45 s, variable annealing temperatures (see supplementary table S2) for 45 s, and 72°C for 1 min, with a final extension of 72°C for 7 min. For all extractions and amplifications, contamination was rigorously checked by means of negative controls. In particular, we added one or more negative controls in every set of twelve samples subjected to DNA extraction and subsequent PCR. The DNA extraction negative controls were amplified with the same set of primers for further confirming absence of contamination. Personnel wore a set of disposable lab coat, facemask and gloves for DNA extraction and another set for PCR. All the procedures took place in a laboratory exclusively devoted to ancient DNA analyses placed in a building that never hosted modern common lizard samples, DNA or PCR products. To further reduce contamination, every surface was cleaned with 10% bleach before and after each extraction and amplification. PCRs were set up in a workstation equipped with UV light and HEPA-filtered airflow. Amplicons, after ExoSAP-IT (USB Corporation, Cleveland, OH) purification, were sequenced with Big Dye Terminator cycle sequencing (Applied Biosystems) in both directions and then were run on an ABI 3130xl Genetic Analyzer (Applied Biosystems) sequencer.

In order to detect potential contaminations and/or misincorporations we cloned PCR products of one amplicon (cytb_1-R/MVZ04) from three samples and all the four amplicons for one additional specimens (see details in table S1). After running PCR products on a 2% agarose gel, the bands of the adequate size were excised and then purified with a commercial kit (QIAquick gel kit, Qiagen). The TOPOTA Cloning Kit (Invitrogen) was used under the manufacturer's protocol for cloning the amplicons in chemically competent *E. coli* cells. For each amplicon, about 15 positive colonies containing the insert of the expected size were subjected to sequencing in both directions with Big Dye

Terminator cycle sequencing (Applied Biosystems) using the universal primers M13F and M13R. We sequenced an average of about 8 clones for each PCR product. The cloned fragments proved to be 100% identical to those obtained by direct sequencing. Thus, it appeared that our mtDNA sequences were not affected by bias due to post-mortem DNA damage (and consequently deamination of cytosine to uracil) nor by major contamination.

Raw sequences, after the removal of primer sequences, were edited with FinchTV and assembled in contigs using Sequencer 4.7 (Gene Codes. Corporation, USA). Alignment of the resulting contigs together with publicly available *cytb* sequences of *Z. v. carniolica* (AF444037-42; AF248005; AY714929; KF886541-46) was performed with ClustalX (Larkin et al., 2007) and checked by eye. We constructed a median joining network with TCS 1.21 (Clement et al., 2000) including the six specimens for which the entire target sequence was obtained (see Results and discussion) and the publicly available sequences of *Z. v. carniolica*. The network was designed using tcsBU (Múrias Dos Santos et al., 2015).

Results and discussion

We obtained positive amplification in 48 out of 156 PCRs (39 samples × 4 fragments, see details in supplementary table S1) corresponding to about 31% of the reactions. As expected, the high level of DNA degradation prevented the amplification for the great majority of the sample set. More specifically, all the four fragments were obtained for six specimens; for two and six specimens, we obtained three and two amplicons, respectively, while only one amplicon was amplified in six specimens. For about half of the specimens (19) no fragments were obtained. We found a significant difference (Fisher's exact test, $P < 0.0001$) in the success of amplification based on the preservation method; for 14 (82%) specimens preserved in ethanol at least one fragment was successfully amplified, while for only six (27%) specimens preserved in formaldehyde at least one fragment was successfully amplified. Preservation in ethanol guaranteed a positive amplification in about 57% of the reactions (39/68), instead we achieved reliable DNA amplification in only about 10% of the reactions (9/88) concerning specimens preserved in formaldehyde, demonstrating that the extent of DNA damage is determined by the type of fixation (Zimmermann et al., 2008).

Nevertheless, when dealing with historical specimens, obtaining short DNA sequences can provide valuable information. In fact, by retrieving short fragments of *cytb* we could perform a reliable taxonomic identification of the specimens, given that *Z. v. carniolica* and *Z. v. vivipara* show about 5% of sequence divergence at this gene (Cornetti et al., 2014). For 20 specimens (about 50% of the sample set) we could recover between one to three amplicons (see details in supplementary table S1) allowing us to show that all but one of the 20 specimens belonged to *Z. v. carniolica*. One specimen (Zovi_06_mT, table S1) from the eastern Alps was, in fact, classified as *Z. v. vivipara* (table 1) after its nucleotide sequence was aligned with both *Z. v. carniolica* and *Z. v. vivipara* modern haplotypes. This finding was not unexpected since the two lineages were found in syntopy in an area (Carinthia, Austria) not far from Padola di Cadore, eastern Alps (Lindtke, Mayer and Böhme, 2010). We did not find previously unknown haplotypes when we analyzed the 14 specimens that yielded one to three short fragments. However, more precise conclusions can be obtained by comparing longer sequences of modern and historical samples.

For six museum specimens we successfully assembled a *cytb* fragment of 311 bp (table 1), meaning that we achieved good quality chromatograms for all the four amplicons simultaneously. In the 311-bp long sequences, some short sequence gaps, due to non-overlapping amplicons, occurred (a 7 bp gap between

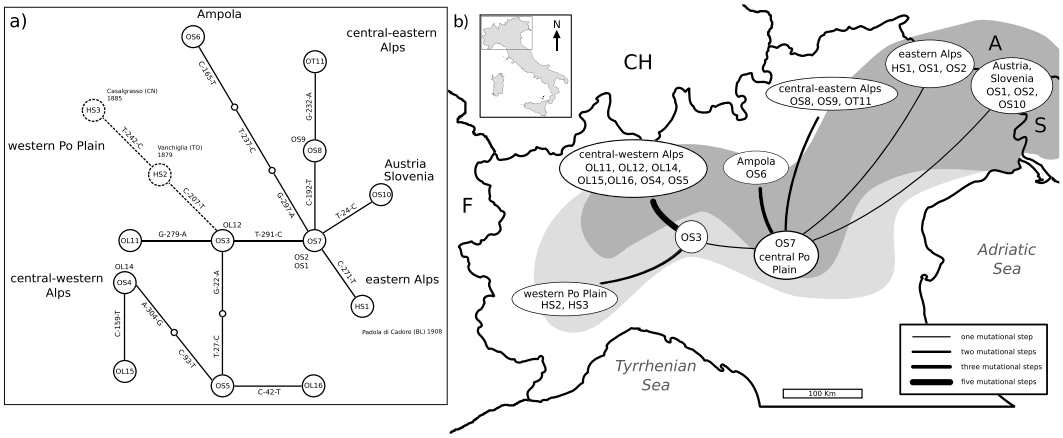

Figure 1. a) Cytb haplotype network including all the *Z. v. carniolica* haplotypes publicly available (OS, OL, OT) and the sequences obtained in this study (HS, historical sample). Haplotype labels beside big circles indicate sequences identical (due to the fact that a shorter fragment, in comparison with the original haplotype definition, was analysed in this study) to the haplotype showed inside the circle. The haplotypes depicted in the figure are not completely confined to single geographic populations. Small empty circles represent mutational steps between haplotypes; numbers beside branches indicate the position of the substitution and the type of nucleotide change; dashed lines represent connections to haplotypes coming from locations where *Z. v. carniolica* is considered extinct. b) Map of Northern Italy with a simplified representation of cytb haplotype network. The haplotypes depicted in the figure are not completely confined to single geographic populations. Thickness of network connections is proportional to the maximum number of mutations between two nodes. Grey areas represent the approximate current (dark gray) and historical (light grey) distribution of *Z. v. carniolica* according to the literature.

the fragments amplified by MVZ05/cytb4F and cytb3F/cytb3R and a 14 bp gap between the fragments amplified by cytb3F/cytb3R and cytb2F/cytb2R) and were filled with Ns. Three out of the six historical *Z. v. carniolica* haplotypes found in this study (all deposited in GenBank database under Accession Nos. MH049454-MH049459, table S3) have never been observed before. The haplotype network, which does not include details about haplotype frequency since information was missing in some of the original papers, highlighted, in fact, the occurrence of new sequences and connections with nowadays existing haplotypes, suggesting a reduction in genetic variation especially in the Po Plain (fig. 1a).

To the best of our knowledge, all lowland populations in western Po Plain are extinct at the present time (Ghielmi et al., 2006). The occurrence of the *Z. v. carniolica* in Casalgrasso (Piedmont, western Po Plain) was reported by Camerano (1885), while more recently this population, as well as the one of Vanchiglia (TO, Piedmont, western Po Plain), was considered extinct by Andreone and Sindaco (1998) due to habitat destruction. This is remarkable and suggests that genetic variation has been eroded due to habitat loss and consequent population extinction in the western Po Plain.

Similarly, suitable habitats in the central Po Plain have been decreasing dramatically during the last decades (Semenzato, Richard and Amato, 1996) and, with the disappearance of moist areas, *Z. v. carniolica* populations have also likely been shrinking. In fact, nowadays a tiny proportion (roughly 1.5%) of the original surface of the marshes "Valli Grandi Veronesi" (about 200 km^2, during the 19th century) persists in the surrounding of Casaleone, Legnago or Cerea, namely "Palude Brusà-Vallette" (148 ha) and "Palude di Ostiglia-Busatello" (123 ha), mainly due to land conversion for agricultural purposes (Salmaso and Osella, 1989). Remarkably, the last reported observation of *Z.*

v. carniolica in the moist area of "Palude Brusà-Vallette" dates back to 1998 (Pollo, 1999). The site of Legnago presents similar conditions; in this area, lands have been almost completely altered by human activities (e.g. agriculture, industry, building). However, no firm conclusion can be drawn for a possible loss of genetic diversity in the central Po Plain, since the long *cytb* fragment was obtained for only one specimen, which carried haplotype OS1, OS2 or OS7. The occurrence of OS7 haplotype in the central Po Plain is in agreement with what Surget-Groba et al. (2002) found by analysing several individuals of *Z. v. carniolica* in the Busatello marsh (the same location where the museum specimen was collected from), one of the main wet areas of the central Po Plain. All those individuals, in fact, carried the OS7 haplotype, which was considered the oldest haplotype for its central position in the network. Despite the addition of new sequences, the OS7 haplotype remained the node with the highest number of connections (5) in our network comprising ancient haplotypes (fig. 1a). This seems to confirm the scenario where the extant lineages of *Z. v. carniolica* likely originated from a refugium in the central Po Plain, thus corroborating the evolutionary significance of the Busatello population. Moreover, the inclusion of historical samples did not alter the mtDNA phylogeographical pattern where variation appears to be geographically partitioned. Specimens coming from Piedmont and Lombardy belonged to the same group of closely-related haplotypes (central-western Alps, fig. 1b) that also includes haplotypes from historical samples collected in the western Po Plain. The ancestral haplotype OS7 occurred in the central Po Plain. The central-eastern Alps group was formed of three haplotypes, whereas a historical specimen (HS1) and two additional haplotypes are included in the eastern Alps cluster. Haplotypes occurring in Austria and Slovenia were just one mutational step off OS7, while more than 20 specimens from Ampola lake and surroundings (Trentino, central Pre-Alps; Surget-Groba et al., 2002; Cornetti et al., 2014) shared the same haplotype, being three steps off OS7 (fig. 1a-b). Genetic drift appears the most likely driver of this local differentiation.

Over a time period of less than two centuries, at least two haplotypes from historical samples have likely disappeared, suggesting that populations from northern Italian lowland experienced a reduction in mtDNA diversity. These populations used to inhabit moist habitats that have been progressively eroded by human activities and climate change. As a result most, if not even all, of lizard western Po Plain populations have disappeared (Ghielmi et al., 2006). Since the remnant lowland (viz. Busatello) and mid-altitude populations (from western to eastern Alps) are increasingly facing the threats posed by climate and land use changes, we argue that our results provide new evidence in favour of specific conservation measures for *Zootoca v. carniolica* populations.

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