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Relics of the Europe's warm past: Phylogeography of the Aesculapian snake

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ABSTRACT

Understanding how species responded to past climate change can provide information about how they may respond to the current global warming. Here we show how a European reptile species responded to the last natural global warming event at the Pleistocene–Holocene transition that led to the Holocene climatic optimum approximately 5000–8000 years ago. The Aesculapian snake, *Zamenis longissimus*, is a thermophilous species whose present-day distribution in the southern half of Europe is a remnant of much wider range during the Holocene climatic optimum when populations occurred as far north as Denmark. These northern populations went extinct as the climate cooled, and presently the species is extinct from all central Europe, except few relic populations in locally suitable microhabitats in Germany and the Czech Republic. Our phylogenetic and demographic analyses identified two major clades that expanded from their respective western and eastern refugia after the last glacial maximum (18,000–23,000 years ago) and contributed approximately equally to the present range. Snakes from the relic northern populations carried the Eastern clade, showing that it was primarily the snakes from the eastern, probably Balkan, refugium that occupied the central and northern Europe during the Holocene climatic optimum. Two small, deep-branching clades were identified in near the Black Sea and in Greece. These clades provide evidence for two additional refugia, which did not successfully contribute to the colonization of Europe. If, as our results suggest, some populations responded to the mid-Holocene global warming by shifting their ranges further north than other populations of the same species, knowing what populations were able to expand in different species may provide information about what populations will be important for the species' ability to cope with the current global warming.

1. Introduction

The relationship between geographical distributions of species and climate implies that many species distributions will shift northward as the global climate warms (Parmesan, 2006), and this poses a major challenge to conservation policy and practice (Hoegh-Guldberg et al., 2008; Keith et al., 2009). Climatic conditions profoundly different from the present occurred in the past (Hewitt, 2000), and understanding the impact of past climates can improve predictions of the future effects (Blois et al., 2010). The most significant climatic processes in the recent past have been the glacial and interglacial cycles of the Quaternary (Davis and Shaw, 2001). During the glacials, temperate species were restricted to refugia, typically at low latitudes, which served as sources for the colonization of the higher latitudes as the climate warmed (Hewitt, 2000; Bennett and Provan, 2008). Over recent years, molecular phylogeography has been used to infer species distributions during the last glacial maximum (LGM; 18,000–23,000 years ago) by comparing the genetic composition of current populations in the post-glacially colonized regions with that of putative refugial populations (Hewitt, 2000; Schmitt, 2007). The focus on species' distributions during the LGM may, however, be of limited value to study the process by which species will respond to the current warming trend, which is expected to cause further expansions of temperate-adapted species, particularly at northern edge boundaries of their present-day ranges (Parmesan, 2006). From this perspective, it should be informative to study species whose northern distributions were larger during a recent period of warmer climate than today (e.g. Enghoff et al., 2007), and to identify what populations within the present range expanded to the northern areas where the species went extinct as the climate cooled after that warm period ended.

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In the mid-Holocene, approximately 5000–8000 years ago, the climate was warmer than today, particularly in the northern hemisphere, with annual mean temperatures 2–2.5 °C higher than today. During that period – the Holocene climatic optimum – some thermophilous European species now inhabiting more southern latitudes were distributed further north than they are today (Ljungar, 1995; Sommer et al., 2007). Perhaps the best-documented case is that of the European pond terrapin *Emys orbicularis*. Numerous finds of subfossil specimens showed that the distribution of this species, which is now absent from large parts of Europe north of the Alps (Sommer et al., 2007), reached its maximum extent during the Holocene climatic optimum, when it occurred as far north as Britain, Denmark and Sweden (Sommer et al., 2007). In a recent phylogeographic study of this species, the extant and extinct Holocene populations across all central Europe between France and the Balkans revealed little diversity of mitochondrial DNA (mtDNA) haplotypes, pointing to the post-glacial colonization of central and western Europe by a single lineage probably originating from Balkan refugia (Lenk et al., 1999; Fritz et al., 2007; Sommer et al., 2009).

The present study examines the phylogeography of another thermophilous reptile species whose present-day distribution in Europe is a remnant of a much wider distribution during the Holocene climatic optimum, the Aesculapian snake *Zamenis longissimus* (previously known as *Elaphe longissima*). Like the pond terrapin, the Aesculapian snake become extinct over much of the northern Holocene distribution, although there are several extant relic populations in the area north of the Alps (Musilová et al., 2007). The present-day distribution of *Z. longissimus* is restricted to the southern parts of Europe west and east of the Alps, roughly between the north of Spain and large part of France in the west to the Carpathian Mountains and Black Sea coast in the east, and including much of Italy and the Balkans (Fig. 1A). A large distribution gap north of the Alps separates the French populations in the west from the Austrian and Slovakian populations in the east (Fig. 1A). However, the numerous subfossil finds from the Atlantic and Sub-boreal periods (Fig. 1A), in particular from Germany (Peters, 1977; Böhme, 1991, 1994, 2000; Gomille, 2002) and Poland (Szyndlar, 1984), provide evidence that during the warmest period of the Holocene the Aesculapian snakes were widely distributed in central Europe up to Denmark (Ljungar, 1995; Richter and Noe-Nygaard, 2003). The northern populations become extinct during the Holocene, likely due to the climatic deterioration that followed the warm period (Joger et al., 2010). However, some remnant populations in this northern distribution survived into the recent past, such as on the island of Sjæland in Denmark where the Aesculapian snakes were recorded until 1863 (Hvass, 1942; Ljungar, 1995), and in Germany and in the Czech Republic where extant populations of *Z. longissimus* still exist (Hecht, 1928; Mertens, 1948; Böhme, 1993; Mikátová and Zavadil, 2001). Four relic populations of the Aesculapian snake are known in the area north of the Alps (Musilová et al., 2007). Two of them are in western Germany (near Schlangenbad in the Taunus Mountains and Hirschhorn in the Neckar River valley), one in the southeast of Germany (near Burghausen in the Salzach River valley), and one in the northwest of the Czech Republic (near Karlovy Vary in the Eger River valley) (Fig. 1A). These relic populations, restricted to favorable warm and humid microhabitats along wooden slopes of river valleys, are considered highly vulnerable to extinction due to the restricted area they occupy and anthropogenic habitat deterioration (Heimes, 1988; Waitzmann, 1993; Musilová et al., 2007).

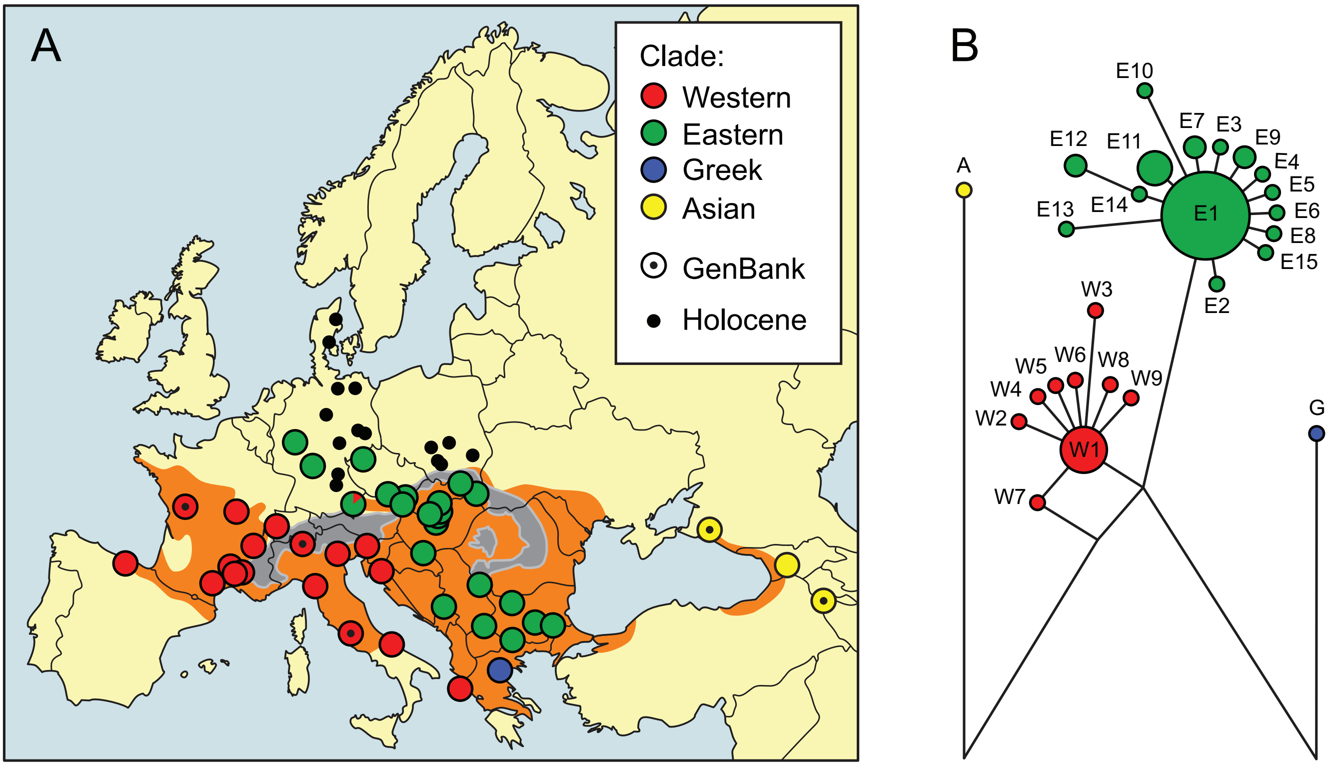

Fig. 1. Phylogeography of *Zamenis longissimus*. (A) The collection sites and the distribution of clades within the present range (ochre), including data from the five sequences retrieved from GenBank (Table 1). Holocene subfossil records in Europe north of the present range are shown. Approximate distributions of major mountain regions of the Alps and of the Carpathians are indicated by grey shading. See text (Sections 1 and 2.2.) for references to published data. (B) Unrooted median-joining network of the haplotypes. Haplotypes in the four clades identified in the maximum-likelihood analysis (Fig. 2) are represented in different colored circles with the colors corresponding to those used in (A). The size of the circles is proportional to the haplotype frequency.

Because of their outlying geographical position and the association with thermal spa regions (i.e. Schlangenbad and Karlovy Vary), the origin of these populations has been debated. Some theories suggested that they may be derived from individuals released by the ancient Romans or later (see Böhme, 1993; Mikátová and Zavadil, 2001; Gomille, 2002), and it was only with the discovery of subfossil localities in central Europe that it has become clear that the populations still existing there must be considered as relics from the earlier continuous distribution (Gomille, 2002; Musilová et al., 2007). However, no study has addressed in detail the phylogeographic relationships between the isolated populations and those from the main distribution area (Joger et al., 2010), and the Czech Karlovy Vary population has not yet been studied genetically. An earlier protein electrophoretic comparison revealed only slight geographic structuring among populations of *Z. longissimus*, including German populations, based on frequency differences of common transferrin variants (Lenk and Joger, 1994). Recently, Joger et al. (2006) in a coarse-scale analysis of a portion of the mtDNA cytochrome *b* gene distinguished an Eastern haplotype group, found along the Black and Aegean seas, and a Western haplotype group, comprising remaining European sites. However, the limited coverage and low haplotype resolution within the groups did not permit testing of specific phylogeographic hypotheses (Joger et al., 2006, 2010).

Here we explore the sequence variation at two mtDNA-encoded genes (cytochrome *c* oxidase subunit I and cytochrome *b*) in 92 *Z. longissimus* from 38 localities over most of the geographical range to describe its phylogeographic history. In particular, we address whether there are distinct genetic clades in Europe reflecting different LGM refugia and the Holocene migration routes. Special focus is given to the relationships among the relic central European populations and those from the main distribution area in western and eastern Europe, and we evaluate the relative contribution of different populations to the Holocene expansion in the areas north of the Alps where the species is now largely extinct.

2. Materials and methods

2.1. Sample collection

We used various types of source material for DNA extraction, including ethanol-preserved tissue of road-killed specimens, ventral scale clips, and buccal swabs (Beebee, 2008). Altogether 92 individual samples of Aesculapian snakes were gathered, which originated at 38 localities from across the distribution range (Table 1, Fig 1A). In addition, one individual of the southern Italian *Zamenis lineatus* and one Persian Ratsnake *Zamenis persicus* were included as outgroup, which are rat snake species distinct from but related to the Aesculapian snake (see Utiger et al. 2002).

2.2. Data collection

Total genomic DNA was extracted using DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). Two mtDNA genes were amplified with polymerase chain reaction (PCR). A part (536 bp) of the cytochrome *c* oxidase subunit I (*coxI*) was amplified using the primers COI(+)b and COI(−)bdeg (Utiger et al., 2002), and a part (1102 bp) of the cytochrome *b* (*cytb*) gene with the primers L14910 and H16064 (Burbrink et al., 2000). The amplifications were carried out in 50 μl reactions and the cycling conditions were initial denaturation at 94 °C for 2 min, followed by 34 cycles of denaturation for 30 s at 94 °C, 40 s at annealing temperature (50 °C for *coxI* and 45 °C for *cytb*), and 1 min elongation at 72 °C, and the thermocycling program ended with a final elongation step at 72 °C for 10 min. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and both strands were directly sequenced with the same primers as those used for the amplification. The sequence analysis was carried out on a 3730xl DNA analyzer (Applied Biosystems). Sequences were aligned manually and the *coxI* sequence for each individual was combined with the *cytb* sequence for the same individual, and the redundant haplotypes were removed using Collapse version 1.2 (Posada, 2006). Nucleotide sequences of each unique haplotype identified in this study have been deposited in the GenBank database (accession nos: HQ392534–HQ392568). A total of five shorter *Z. longissimus* sequences were retrieved from GenBank and compared with the homologous parts of the new sequences to increase the geographical coverage (Table 1, Fig 1A). Only the new sequences were subjected to the analyses, however. The GenBank sequences included were three partial (513 bp) *coxI* sequences from one site in Russia (Krasnodar area), one site in Italy (Rome) and one site in Switzerland (Olivone), available from the study of Utiger et al. (2002), and two partial (597 bp) *cytb* sequences from one site in western France (Villiers en Bois) and one site in eastern Turkey (Ararat), available from the study of Lenk et al. (2001).

2.3. Data analyses

Summary measures of sequence polymorphism were calculated using DnaSP version 4.90.1 (Rozas et al., 2003). The phylogenetic relationships among the sequences were reconstructed by using the maximum likelihood (ML) optimality criterion. The program jModelTest 0.1.1 (Posada, 2008) was used to identify the best-fit model of sequence evolution for our data using the Akaike information criterion (AIC). The phylogenetic analyses were performed by the nearest neighbour interchanges approach implemented in PhyML version 3.0.1 (Guindon and Gascuel, 2003) and using the TIM + G evolutionary model (where TIM stands for Transitional Model and G is for the gamma-distributed rates across sites) (Posada, 2003). Branch support for the phylogenetic partitioning of the sequences was quantified by the approximate likelihood ratio test (aLRT) (Anisimova and Gascuel, 2006) using the nonparametric Shimodaira-Hasegawa-like (SH-like) procedure implemented in PhyML (Guindon and Gascuel, 2003), which provides a fast but accurate alternative to the bootstrap method (Anisimova and Gascuel, 2006). The aLRT test assesses whether the branch being studied provides a significant likelihood gain, in comparison with the null hypothesis that involves collapsing that branch, but leaving the rest of the tree topology identical.

A median-joining haplotype network was constructed using the program Network version 4.5.1.6 (Bandelt et al., 1999).

We used several different approaches to examine signatures of past population size change in mtDNA clades. We note that the present-day distributions of clades do not necessarily equate to currently physically separated populations; they are instead taken as markers of past expansions of the populations that carried them (see Atkinson et al., 2009). First, we calculated three commonly used summary statistics *D* (Tajima, 1989), *Fs* (Fu, 1997) and R_2 (Ramos-Onsins and Rozas, 2002) with DnaSP (Rozas et al., 2003). The significance of the *D*, *Fs* and R_2 statistics was tested by generating random samples under constant population size using a coalescent simulation conditioned on the number of polymorphic sites (Ramírez-Soriano et al., 2008). For neutral markers, significant low *D*, *Fs* and R_2 values can be expected in cases of population expansion (Tajima, 1989; Fu, 1997; Ramos-Onsins and Rozas, 2002).

As another way of assessing signatures of population size change, we considered the distribution of the number of pairwise nucleotide differences (mismatch distribution) by contrasting observed distributions with those expected from models of population size change. We tested whether the data fitted the sudden demographic expansion model (Rogers and Harpending, 1992) or

Table 1
Sample location and size (n) and the haplotypes found with frequencies in parentheses. Five GenBank sequences are listed with their accession numbers and our matching haplotype in parentheses.

Locality	Country	Latitude/longitude	n	Clade	Haplotypes
Asenovgrad	Bulgaria	42.02 N/24.87 E	2	E	E1 (1), E6 (1)
Harmanli	Bulgaria	41.88 N/25.85 E	1	E	E1 (1)
Loveč	Bulgaria	43.12 N/24.32 E	1	E	E1 (1)
Sandanski	Bulgaria	41.57 N/23.45 E	1	E	E15 (1)
Island of Krk	Croatia	45.22 N/14.53 E	2	W	W1 (2)
Karlovy Vary	Czech Republic	50.33 N/13.05 E	9	E	E1 (9)
Podyjí National Park	Czech Republic	48.81 N/15.97 E	8	E	E1 (8)
Alzon	France	43.97 N/3.52 E	1	W	W1 (1)
Loire valley	France	46.64 N/3.74 E	1	W	W2 (1)
Olargues	France	43.53 N/2.92 E	1	W	W8 (1)
Sassenage	France	45.20 N/5.66 E	1	W	W1 (1)
St. Césaire	France	44.00 N/4.00 E	1	W	W7 (1)
St. Laurent le Minier	France	43.92 N/3.63 E	1	W	W5 (1)
Kutaisi/Sataplias	Georgia	42.26 N/42.71 E	1	A	A (1)
Burghausen	Germany	48.16 N/12.83 E	7	E, W	E1 (6), W1 (1)
Hirschhorn	Germany	49.47 N/8.92 E	5	E	E11 (5)
Schlangenbad	Germany	50.08 N/8.10 E	7	E	E1 (7)
Corfu	Greece	39.66 N/20.75 E	1	W	W1 (1)
Stomio, Mt Ossa	Greece	39.89 N/22.62 E	1	G	G (1)
Esztergom	Hungary	47.80 N/18.83 E	5	E	E1 (5)
Harkany	Hungary	45.85 N/18.24 E	1	E	E13 (1)
Letkés	Hungary	47.87 N/18.82 E	1	E	E12 (1)
Szob	Hungary	47.85 N/18.82 E	1	E	E1 (1)
La Spezia	Italy	44.12 N/9.85 E	1	W	W9 (1)
Aviano	Italy	46.07 N/12.58 E	1	W	W1 (1)
Foresta Umbra	Italy	41.49 N/15.58 E	2	W	W1 (1), W4 (1)
Kobarid	Italy/Slovenia	46.23 N/13.57 E	1	W	W3 (1)
Mt Durmitor	Montenegro	43.13 N/19.02 E	1	E	E5 (1)
Beljanica Mts	Serbia	44.08 N/21.66 E	1	E	E1 (1)
Pčinja	Serbia	42.35 N/21.90 E	1	E	E1 (1)
Bratislava	Slovakia	48.07 N/17.12 E	2	E	E1 (1), E3 (1)
Čabraď	Slovakia	48.33 N/19.00 E	1	E	E1 (1)
Hankovce	Slovakia	49.20 N/21.41 E	1	E	E9 (1)
Kováčov Hills	Slovakia	47.85 N/18.75 E	6	E	E1 (43), E12 (1), E14 (1)
Little Carpathians Mts	Slovakia	48.19 N/17.12 E	5	E	E1 (2), E2 (1), E4 (1), E10 (1)
Vihorlat Mts	Slovakia	48.88 N/22.20 E	7	E	E1 (3), E7 (2), E8 (1), E9 (1)
Mendexa	Spain	43.35 N/3.50 W	1	W	W6 (1)
South Jura Mts	Switzerland	46.63 N/6.43 E	1	W	W1 (1)
GenBank sequences					
Villiers en Bois	France	46.15 N/0.40 W	1	W	AJ277671 (W1)
Ararat	Turkey	39.70 N/44.30 E	1	A	AJ277672 (A)
Krasnodar Area	Russia	45.03 N/38.98 E	1	A	AY122699 (A)
Rome	Italy	41.90 N/12.48 E	1	W	AY122696 (W1)
Olivone	Switzerland	46.53 N/8.95 E	1	W	AY122697 (–)

the instantaneous range expansion model (Excoffier, 2004), using Arlequin version 3.1 (Excoffier et al., 2005). The models were fitted to the data by a generalized non-linear least-square approach, which allowed estimation of the parameter $\tau = 2\mu t$, the time since the onset of the population expansion scaled by the mutation rate (Schneider and Excoffier, 1999). A parametric bootstrapping approach (Schneider and Excoffier, 1999) was used to obtain the probability that the observed data conform to the model using the sum of square deviations (SSD) between the observed and expected mismatch distribution as a test statistic, and to estimate the confidence intervals (CI) of τ.

To further investigate the demography of the clades, we used the Bayesian skyline plot, a piecewise-linear function of population size against time, which provides continuous indications of trends in demographic history (Drummond et al., 2005). The Bayesian skyline plots were estimated using BEAST version 1.5.3 (Drummond and Rambaut, 2007), allowing three steps in $Ne \times \mu$ (the effective population size scaled by the mutation rate) through time. The distribution of skyline plots was sampled using Markov chain Monte Carlo, producing an estimate and 95% highest posterior density (HPD) intervals of the population size through time that incorporate uncertainty in the tree and substitution model parameters (Drummond et al., 2005). The analysis was carried out with several independent runs for each clade. Each run consisted of 10 million (Western clade) or 20 million iterations (Eastern clade), with the first 10% removed as burn-in, and trees and parameters were sampled every 1000 iterations. Convergence upon the stationary distribution was assessed by estimating the effective sample size (ESS) for the parameters and the analysis was considered to have converged if repeated runs generated similar results, with a minimum ESS of 200 for each parameter (Kuhner and Smith, 2007).

3. Results

The *coxI* and *cytb* sequences (1638 bp) were obtained for each of the 92 Aesculapian snakes. The genetic diversity represented by these sequences is summarised in Table 2. Maximum likelihood analysis with the outgroup revealed the existence of four distinct clades separated with an average corrected sequence divergence of 0.4–1.2% and supported by modest to high bootstrap and aLRT values (Fig. 2). The same four groups of haplotypes were discerned also in the median-joining network (Fig. 1B). The clades had almost non-overlapping geographic distributions (Fig. 1A). Two clades, Western and Eastern, accounted for the majority of haplotypes and represented the entire sampling distribution in Europe (except

Table 2
Summary statistics in different phylogenetic subsets (*coxI* and *cytb* combined).

	n	*k*	*S*	$\pi \pm$ SD (%)	*h* ± SD	R_2	*D*	*Fs*	τ (95% CI)
Whole species	92	26	48	0.17 ± 0.03	0.672 ± 0.055	0.0338^{**}	-2.228^{***}	-14.264^{**}	–
Eastern clade	73	15	16	0.04 ± 0.01	0.490 ± 0.072	0.0291^{***}	-2.324^{***}	-16.226^{***}	0.72 (0.00–1.81)
Western clade	17	9	9	0.07 ± 0.02	0.735 ± 0.117	0.0673^{***}	-2.171^{**}	-7.105^{***}	1.21 (0.00–2.28)

n, sample size; *k*, number of haplotypes; *S*, number of polymorphic sites; π, nucleotide diversity; *h*, haplotype diversity; τ, expansion time estimated from mismatch distribution under the sudden demographic expansion model with percentile confidence intervals based on 1000 simulated samples.
Statistical significance for *D*, *Fs* and R_2 statistics.
** $P < 0.01$.
*** $P < 0.001$.

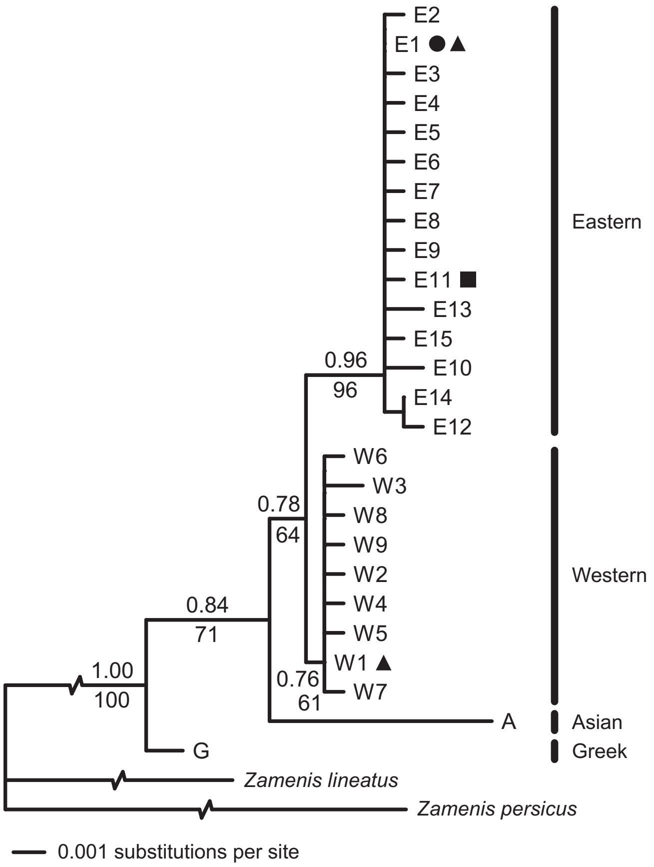

Fig. 2. Maximum likelihood phylogeny of *Zamenis longissimus* haplotypes. The statistical support for major bipartitions is expressed as the SH-like aLRT probabilities and as percentage bootstrap values. Haplotypes followed by a black symbol were found in the relic populations north of the Alps (●, Schlangenbad and Karlovy Vary; ■, Hirschorn; ▲, Burghausen). The tree has been rooted with sequences of *Zamenis lineatus* and *Zamenis persicus* representing outgroups to *Z. longissimus* sequences. For ease of presentation, three long branches were shortened to one tenth of their actual length.

central Greece), and two were minor clades of only one haplotype each (Figs. 1 and 2). The Western clade (haplotypes W1–W9) was found at all sites west and south of the Alps and along the Adriatic Sea between Apulia in the south of Italy and Corfu island off the west coast of Greece (Fig. 1A). The most frequent central haplotype in the Western clade (W1) accounted for 53% of all snakes sampled at the Western sites (Fig. 1B). The Eastern clade (haplotypes E1–15) was found at all sites north and east of the Alps, including the Carpathian basin and Balkans but excluding the Adriatic coast and Greece (Fig. 1A). The most frequent central haplotype in the Eastern clade (E1) accounted for 71% of all snakes sampled in this clade (Fig. 1B). The isolated populations near Schlangenbad and Hirschhorn in Germany and near Karlovy Vary in the Czech Republic all carried exclusively haplotypes of the Eastern clade (Fig. 1A). Only the most common Eastern haplotype (E1) was found at Schlangenbad and Karlovy Vary, but Hirschhorn had a unique haplotype not present anywhere else (E11), which is phylogenetically derived from E1. Of all the sampled localities, only near Burghausen in the southeast of Germany haplotypes of both the Eastern (E1) and Western (W1) clades were found in the same population (Fig. 1A). The Burghausen population is presently separated from the western edge of the main distribution in Austria but it is located very near to it and it thus marks the western edge of the range of the Eastern clade (Fig. 1A). The third, Asian clade was represented in the new sequences by a single individual from Georgia in the western Transcaucasia (haplotype A), and the Greek clade was carried only by a single individual from Mount Ossa (haplotype G) in central Greece.

Identical haplotypes were found among *Z. longissimus* samples represented by the shorter *coxI* and *cytb* sequences retrieved from GenBank (Table 1). After comparing the same length of sequence, the GenBank *coxI* sequence from Italy and *cytb* sequence from France were identical with the most common Western haplotype W1, and the GenBank *coxI* sequence from Russia and *cytb* sequence from Turkey were identical with the Asian haplotype A. The GenBank *coxI* sequence from Switzerland was separated by one mutation step from haplotype W1.

The 'star-shaped' genealogies of the Western and Eastern clades (Fig. 1B) produced significant low values of the *D*, *Fs* and R_2 statistics compared with the expectation under a constant population size (Table 2). There was also a good fit (P (simulated SSD $\geqslant$ observed SSD) > 0.1) between the observed and expected mismatch distributions for the sequences of both the Western as well as Eastern clade (Fig. S1), and the pure demographic expansion model provided essentially the same fit to the data as was obtained by using the range expansion model. This is the pattern expected if migration among neighbouring sub-populations (demes) during a large spatial expansion is high (Ray et al., 2003; Excoffier, 2004).

The inferred Bayesian skyline plots were similar for the Western and Eastern clade (Fig. S2). They both showed a slight increase in effective population size since an estimated time of the most recent ancestor, which itself appears younger in the Eastern than in the Western clade (Fig. S2), although the wide credibility intervals caution against over-interpretation.

4. Discussion

Current scenarios predict that global warming will make species shift their ranges northward as a result of colonization at the northern edge and extinction at the southern edge (Parmesan, 2006); some studies suggest that range shifts related to climate change are yet occurring (Thomas and Lennon, 1999; Brommer, 2004). Whether a species will be able to withstand the changing climate will depend on the capacity of its populations to sufficiently rapidly colonize newly suitable habitats and/or adapt to the novel climatic conditions (Davis and Shaw, 2001). In this study we have identified a specific clade that migrated to climatically suitable central and northern Europe during the mid-Holocene climatic warming and has left remainder populations during subsequent retreat that may be relevant for future range extension into the same area.

4.1. East–west subdivision of Europe

The western and eastern parts of *Z. longissimus* range are contiguous only along a narrow belt south of the Alps in the north of Italy and Slovenia (Fig. 1A). The phylogenetic and network analyses each supported the same conclusions of two well-differentiated east–west clades in Europe, separated by the Alps. All populations along and west of the Alps represented the Western clade that is occurring also in Italy and along the Adriatic coast as far as Greece. On the other hand, the populations east of the Alps and north of Greece represented the Eastern clade. This clade distribution is strongly reminiscent of phylogeographic discontinuities driven by refugial isolation during the LGM (Taberlet et al., 1998; Hewitt, 1999). Their genealogical distinctiveness and the level of sequence divergence (0.4%) support that these clades represent long separated and evolutionarily distinct lineages that were restricted to different LGM refugia (Avise et al., 1998). All methods of inferring demographic change were in agreement and suggested population expansion for both clades. It seems thus likely that, after the LGM, the Western clade dispersed from a western European refugium, whereas the Eastern clade originated from an easterly, probably Balkan refugium. This conclusion is in agreement with previous phylogeographic studies indicating that putative western refugia for widespread European reptiles were in the south of France (viperine snake *Natrix maura*: Guicking et al., 2006) and in the Apennine peninsula (European pond terrapin: Lenk et al., 1999; green lizard *Lacerta bilineata*: Böhme et al., 2007), and that there were multiple refugia for different species in the south of Balkans (European pond terrapin: Lenk et al., 1999; green lizard *L. viridis*: Böhme et al., 2007; slow worms *Anguis* spp.: Gvoždík et al., 2010; dice snake *Natrix tesselata*: Guicking et al., 2006).

4.2. Holocene colonization of central and northern Europe

The fact that the Western and Eastern clades contribute approximately equally to the present range (Fig. 1A) shows that both clades had the capacity to disperse from their southern refugia to higher latitudes following the LGM. The observed deep divergence of snakes from the Transcaucasia and eastern Turkey (Asian clade) and from central Greece (Greek clade) demonstrates the existence of evolutionarily long isolated populations in those areas that did not contribute to the post-glacial colonization of Europe (Fig. 1A).

Many animal and plant species show hybrid zones running down the center of Europe produced by the contact of different clades that colonized the area north of the Alps from the west and from the east (Taberlet et al., 1998; Hewitt, 1999). The Aesculapian snake become extinct in the north of the Alps during the Holocene due to cooling after the climatic optimum, but the phylogeographic relationships of the surviving relic populations provide information about the relative contribution of different refugia to that area. The pattern observed is most consistent with the hypothesis that the dispersal to central Europe north of the Alps occurred predominantly from the Balkan refugia and involved the Eastern clade, with negligible contribution from the western refugia. We were unable to study DNA of subfossil specimens, which in snakes are typically represented by single vertebrates (unlike in the pond terrapin where relatively large amounts of plastron or carapace tissue can be used; Sommer et al., 2009). Although we do not have the information about phylogeographic relationships of the extinct populations further north in Europe (e.g. in Denmark), our mtDNA results from the extant relic populations in Germany and Czech Republic strongly suggest that the snakes of the Eastern clade contributed most significantly to the Holocene expansion towards the north of Europe.

The Western and Eastern clade showed similar magnitudes of population growth (Fig. S2). The expansion in the western refugia even appears to have started earlier after the LGM than in the Balkan refugia (τ of 1.2 versus 0.7). The Western clade colonized a large part of western Europe where it is still present up to central France, but apparently it did not reach the northern central Europe as did the Eastern clade. It is thus possible that the post-glacial expansion from the Balkans was somewhat delayed due to a more continental climate with colder winters in eastern Europe at that time, but that the adaptations to more severe climatic conditions allowed the Eastern clade to colonize further north than the Western clade. A similar explanation was proposed for the European pond terrapin (Joger et al., 2010).

It may be that sample sizes in our study are not large enough to detect the Western clade at the northern sites if it was present in very low frequencies, or that it was lost from those sites by genetic drift. However, we consider parallel loss of the Western clade from all three sites as unlikely, unless it was at an adaptive disadvantage. In such case, its absence from central Europe could be the result of selection during the range contraction in addition to during the colonization.

As in the Aesculapian snake, the mtDNA clade from Balkan refugia (lineage II of Lenk et al., 1999) founded the (now extinct) Holocene populations of the pond terrapin (Sommer et al., 2009). However, western European populations of the pond terrapin in France also belonged to the Balkan lineage (Fritz et al., 2005), and not to a separate western lineage as in the Aesculapian snake (only pond terrapins from Italy and Adriatic coast formed distinct lineages from the Balkan lineage; Lenk et al., 1999). The phylogeography of the Aesculapian snake is thus more similar to that of the two sibling species of green lizards, where the western green lizard, *L. bilineata*, occurs in Europe west and south of the Alps and the eastern green lizard, *L. viridis*, occupies the Europe east of the Alps (Godinho et al., 2005; Böhme et al., 2006), resembling the distributions of the Western and Eastern clades of the Aesculapian snake. In the green lizards, however, isolated populations in the east of Germany (Brandenburg) and in the Czech Republic (Bohemia) belong to the eastern (*L. viridis*) mtDNA clade, but the isolated population in the west (Rhineland) and in the southeast of Germany (Baden-Württemberg) belong to the western (*L. bilineata*) clade (Böhme et al., 2007). Therefore, in contrast to the Aesculapian snake, both western and eastern populations of green lizards have clearly expanded to the area north of the Alps during the mid-Holocene warming (Böhme et al., 2006).

Burghausen is the westernmost edge population of the Eastern clade where most snakes carry Eastern haplotypes (Fig. 1A). The occurrence of a Western haplotype in this population might therefore represent a legacy of historical gene flow between the Western and Eastern populations at the time when Aesculapian snakes were still widespread north of the Alps. Alternatively, it can be a rare gene flow around or across the Alps from the south, where snakes of the Western clade occur in the north of Italy and in Slovenia (Fig. 1A). Our data also cannot exclude the possibility that the snake with the Western haplotype at Burghausen is the result of a recent introduction. Although infrequent, there is at least one case of introduction reported for Britain, where a population of the Aesculapian snake has become established in the grounds of a zoo in North Wales during the 1960s (see Musilová et al., 2007).

Before fossil evidence was available, it was suggested that also the extant populations in Germany and the Czech Republic may be have been introduced (Gomille, 2002; Musilová et al., 2007) and derived from individuals released by the ancient Romans (the German and Czech populations), or later by an Italian or French noble family or by a Greek merchant (the Czech population). Our genetic data support the argument that these populations are indeed mid-Holocene relics (Ljungar, 1995; Gomille, 2002; Joger et al., 2010): the snakes from Germany and from the Czech Republic are related to the other snakes of the Eastern clade, which includes snakes from the Carpathian basin and northern Balkans (Fig. 1A), and they are only distantly related to the snakes from Italy and France (Western clade) and from Greece (Western and Greek clades).

4.3. Relic peripheral populations and climate change

Our study provides an example of a species that responded to the last natural climate warming event by an expansion of a particular clade into the areas where the species is at present at the northern limit of its range, while the other clades made negligible or no contribution to the surviving northern populations. If, as appears to be the case, some species responded to the mid-Holocene warming by expansion of the northern edge that involved only, or primarily, some populations but not the others, identifying those populations that were able to advance could be of real importance in terms of our understanding of species' responses to future climate change. We thus suggest that one of the upcoming challenges in phylogeography is to infer what populations were able to expand further north during the Holocene climatic optimum, as those same populations may be relevant for species surviving current global warming. We believe that it is through the study of relic and peripheral northern edge populations that molecular data can be used to determine what populations were able to expand and colonize areas where the species presently are at the northern limit of their range. This needs to be investigated for different species with mtDNA and nuclear markers and, where possible, with DNA analysis of subfossil specimens. In species that only exist at their extreme northern margins by occupying locally favorable, i.e. microclimatically warm, habitat patches, increased temperatures will likely enable these populations to expand and colonize areas north of its present range (Cannon, 1998). Relic and peripheral northern populations may thus represent our best chance to conserve species threatened by global warming (Channell, 2004). These populations may carry adaptations that allow them to persist in more stressful environments than more southerly populations or populations at the centre of the species range, and that may be preadaptations to future changes in the environment (Channell, 2004). The loss of such populations thus could mean the loss of an adaptive genetic variation, which could be important to the survival of the species in the face of global warming (Nielsen et al., 2001).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.09.017.

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