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1 **Phylogeny and diversification of mountain vipers (*Montivipera*, Nilson et al. 2001)**
2 **triggered by multiple Plio-Pleistocene refugia and high-mountain topography in the Near**
3 **and Middle East**

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14
15 **Abstract**

16 The Near and Middle East is a hotspot of biodiversity, but the region remains underexplored at the
17 level of genetic biodiversity. Here, we present an extensive molecular phylogeny of the viperid snake
18 genus *Montivipera*, including all known taxa. Based on nuclear and mitochondrial data, we present
19 novel insights into the phylogeny of the genus and review the status of its constituent species.
20 Maximum likelihood methods revealed a montane origin of *Montivipera* at 12.3 Mya. We then
21 analyzed factors of mountain viper diversity. Our data support substantial changes in effective
22 population size through Plio-Pleistocene periods. We conclude that climatic oscillations were drivers
23 of allopatric speciation, and that mountain systems of the Near and Middle East have strongly
24 influenced the evolution and survival of taxa, because climatic and topographical heterogeneities
25 induced by mountains have played a crucial role as filters for dispersal and as multiple refugia. The
26 wide diversity of montane microhabitats enabled mountain vipers to retain their ecological niche
27 during climatic pessima. In consequence the varied geological and topographical conditions between
28 refugia favoured genetic isolation and created patterns of species richness resulting in the formation
29 of neoendemic taxa. Our data support high concordance between geographic distributions of
30 *Montivipera* haplotypes with putative plant refugia.

31
32 **Keywords**

33 *Montivipera*, Near East and Middle East, phylogeny, divergence times, phylogeography, allopatric
34 speciation

35

36 **1. Introduction**

37 In recent decades, biota of the Mediterranean Basin have been studied extensively to understand the
38 determinants of present-day biodiversity. The patterns revealed by multiple authors indicate that
39 biodiversity of the Mediterranean region has had a turbulent history triggered by climatic changes. In
40 particular, the deterioration of warm, moist Tertiary climates during the Plio-Pleistocene appears to
41 have had profound effects on faunal and floral compositions.

42 There is ample evidence for Plio-Pleistocene climatic oscillations as drivers for glacial refugia,
43 hotspots of genetic diversity, postglacial re-colonization routes and so on (e.g. Hewitt, 1996; 2000;
44 2004a; 2011). Climatic oscillations during the Plio-Pleistocene caused expansion or regressive
45 fragmentation of fauna and flora (e.g. Hewitt, 2000; 2004a; Varga and Schmitt, 2008) at both global
46 (e.g. Bennett, 1997) and regional (Svenning and Skov, 2007) scales. Owing to regional differences in
47 landforms, different species respond differentially to climatic changes. In central and northern
48 Europe, biota underwent latitudinal shifts over long distances, changing organismal abundances and
49 species compositions dramatically, including through extinction of the Pleistocene megafauna (e.g.,
50 Hofreiter and Stewart, 2009). However, Pleistocene climatic cycles also profoundly affected the
51 distribution and composition of Mediterranean biota (Taberlet et al., 1998; Weiss and Ferrand,
52 2007). In particular, for thermophilic animals, southern regions of the Mediterranean acted as
53 refugia, by providing suitable habitats during adverse climate periods (e.g. Ursenbacher et al.,
54 2006a,b Joger et al. 2007): refugial areas accumulated populations of species through both range
55 expansions and contractions resulting in latitudinal clines in species richness (e.g. Hewitt, 2004b). As
56 a result, the European peninsulas in the Mediterranean (Iberia, Italy and the Balkans) are rich in
57 endemic reptiles (see Cheylan and Poitevin, 1994), because refugia appear to have reduced
58 extinction rates and, through their isolation, favoured the emergence of new evolutionary lineages
59 (e.g. Hungerer and Kadereit, 1998). This is even more applicable to the circum-Mediterranean region
60 as a whole, which constitutes one of the world's major biodiversity hotspots (Médail and Myers,
61 2004).

62 One of the goals of phylogeographic examinations is to infer the historical and contemporary forces
63 that have shaped the genetic architecture of populations and closely related species (Avise, 2009)
64 through the use of gene genealogies. Numerous studies have shown that dramatic changes of
65 environmental conditions have left still-detectable traces in the genome of current biota. These
66 genetic consequences of climatic oscillations have been studied for many organisms in the European
67 part of the Mediterranean Basin. In contrast, although the ecosystems of the Near and Middle East
68 harbor a similarly rich biological diversity, a much more substantial proportion remains partially
69 undocumented (Ansell et al., 2012), especially at the level of genetic diversity (Krupp et al., 2009),

70 and phylogeographic studies remain rare, impeding our understanding of the processes that have
71 shaped the biodiversity of the region.

72 The Near and Middle East have been described as either a center of origin with active speciation (e.g.
73 [Hungerer and Kadereit, 1998](#)), or as Plio-Pleistocene refugia for relict biota ([Médail and Diadema, 2009](#)). Extremely relevant to evolutionary biogeography is the high topographic relief of the region,
75 creating a variety of heterogeneous Mediterranean oro-biomes, which allowed biota to retain their
76 ecological niches during climatic pessima by altitudinal range shifts. Thus, geomorphological settings
77 conserve regional genetic diversity as refugia and initiate vicariant allopatric speciation, because of
78 distributional dissection. These effects have been shown to be relevant for European biota in high
79 mountain systems (e.g. [Schmitt, 2009](#)). Generally, geographic vicariance is considered to be the most
80 common mode for speciation (e.g. [Futuyma, 1998](#); [Barraclough and Vogler, 2000](#); [Turelli et al., 2001](#)).
81 However, allopatric speciation driven by vicariance is not simply a geographic event ([Wiens, 2004](#)).
82 Wiens illustrates how niche conservatism drives allopatric lineage splitting in mountain systems.
83 Intrinsic physiological factors constrain species to their ecological niches over time and reduce their
84 fitness outside of the niche ([Holt and Gaines, 1992](#); [Holt, 1996](#)). The process impedes gene flow
85 ([Wiens, 2004](#)) and creates phylogenetic pattern in ecological data ([Wiens et al., 2010](#)).

86 Many groups of reptiles make ideal model organisms for the study of the impact of past climatic
87 changes on patterns of species diversity and distribution, due to their low vagility and often narrow
88 ecological niches. Moreover, reptiles are often important or even dominant components of the fauna
89 of Mediterranean and semi-arid ecosystems. The Near and Middle East contain a high diversity of
90 reptile taxa, including a number of endemic lineages. Among other groups, the Near and Middle East
91 are notable for harboring the highest diversity of true vipers within Eurasia. Eurasian vipers have
92 been the subjects of intensive surveys of phylogeny (e.g. [Lenk et al., 2001](#); [Wüster et al., 2008](#);
93 [Ursenbacher et al., 2008](#)) and phylogeography (e.g. [Ursenbacher et al., 2006a,b](#); [Barbanera et al., 2009](#);
94 [Ferchaud et al., 2012](#); [Zinenko et al., 2015](#)). However, while the overwhelming majority of
95 papers focus on the genus *Vipera*, only scant data are available about the Near and Middle Eastern
96 endemic genus *Montivipera*.

97 Mountain vipers (*Montivipera*) are excellent model organisms to study the impact of past climatic
98 oscillations for allopatric speciation in the mountain systems of the Near and Middle East. These
99 snakes are endemic to the Near and Middle East, from the Aegean coast of Anatolia and neighboring
100 islands to the highlands of central Iran. Most taxa have a montane distribution above an elevation of
101 1400 m a.s.l. and are mainly confined to oro-Mediterranean habitats, which expanded and retracted
102 with climatic cycles since late Miocene. As sit-and-wait predators with a short and stout
103 physiognomy, mountain vipers have a low vagility/movement capability (e.g. [Mebert et al. 2015](#)) that
104 increases the effects of physical vicariance and isolation by distance.

105 Mountain vipers have a comparatively recent history of discovery: seven out of ten taxa have been
106 described since the 1960s, and new taxa might be remain to be discovered (Rajabizadeh et al., 2011).
107 Based on analyses of immunological distances (Herrmann et al., 1992) and DNA-DNA hybridization
108 experiments (Nilson et al., 1999), *Montivipera* was initially separated from *Vipera* as a subgenus, and
109 later raised to full genus rank (Joger, 2005). *Montivipera* consists of two allopatrically distributed
110 species complexes (Nilson and Andrén, 1986). The *xanthina*-complex includes the monotypic species
111 *xanthina* Gray, 1849, *bornmuelleri* Werner, 1898, *wagneri* Nilson & Andrén, 1984, *bulgardaghica*
112 Nilson & Andrén, 1985 and *albizona* Nilson, Andrén & Flärdh 1990, and is restricted to Anatolia and
113 the Levant. As the eastern counterpart, species of the *raddei*-complex are known from Armenia,
114 Azerbaijan, Iran and Turkey. Four species with two subspecies have been described: *raddei* Boettger,
115 1890 with the nominate subspecies and *raddei kurdistanica* Nilson & Andrén 1986, *latifii* Mertens,
116 Darevsky & Klemmer, 1967, *albicornuta* Nilson & Andrén, 1985 and recently *kuhrangica* Rajabizadeh,
117 Nilson & Kami, 2011.

118 The history of the taxonomy and systematics of the genus *Montivipera* was marked by a heated
119 scientific debate about species concepts and phenotype diversity (see Schätti et al., 1991; 1992; Nilson
120 and Andrén, 1992). In the absence of conclusive data, this controversy created much confusion and a
121 persistent lack of consensus on the systematic situation of the group. As a result, different
122 herpetologists adopted different classifications on the sole basis of personal preference (e.g. David
123 and Vogel, 2010; Phelps, 2010). Legitimately, Wüster et al. (1997, p.335) stated that "Until a full
124 analysis of the population phylogeny of these forms is carried out, using large samples and preferably
125 a wide range of characters, and in particular molecular markers, the situation is likely to remain
126 confused." We here follow Wüster et al. (1997) and provide the first molecular phylogenetic and
127 phylogeographic analysis of the genus *Montivipera*, based on extensive sampling through most of its
128 range. In addition to the academic interest of the group, mountain vipers are of considerable medical
129 importance due to their wide distribution in the Middle East, causing many envenomations in rural
130 regions with morbidity and mortality in Turkey (Ozay et al., 2005). However, *Montivipera* venoms
131 and the level of compositional variation in them (Chippaux et al., 1991) have not been thoroughly
132 characterized pharmacologically, and specific or evidence based polyvalent antivenoms for these
133 taxa are only available for members of the *raddei*-complex (Razi Institute Teheran). Understanding
134 the phylogeny and species diversity within the genus represents an essential underpinning for
135 rigorous studies of venom variation and antivenom effectiveness.

136 The first goal of this study is thus to review the state of *Montivipera* systematics. Using a dense
137 sampling embracing all known taxa and a multilocus mitochondrial and nuclear dataset to overcome
138 the limitations of studies based solely on mitochondrial DNA (Galtier et al., 2009), we reconstruct the
139 phylogenetic history of the genus. Based on a multilocus analysis with four nuclear and three

140 mitochondrial genes we test the monophyly of the *xanthina*- and-*raddei*-complexes, determine its
141 constituent taxa and test, if the taxa *bornmuelleri*, *wagneri*, *bulgardaghica* and *albizona* are
142 monophyletic.

143 The second objective is to ascertain the determinants of present-day lineage diversity in time and
144 space. We analyze, whether *Montivipera* has a montane origin, and if Plio-Pleistocene climatic
145 oscillations have left a spatially arranged genetic imprint on the genome of mountain vipers, and,
146 more specifically, whether population size changes over their demographic history are still
147 detectable in the current genome. Finally, we discuss, whether the observed genetic diversification is
148 the result of isolation in Quaternary glacial refugia.

149 Our study illustrates the importance of Near and Middle Eastern mountain systems for allopatric
150 speciation, and recovers for the first time the phylogenetic history within the genus *Montivipera* on
151 the basis of nuclear and mitochondrial genes.

152 **2. Material and Methods**

153 **2.1. Specimen acquisition and molecular protocols**

154 A total of 115 viper samples were gathered from colleagues, zoological institutions, or were donated
155 with permission from museum collections ([Tab. A.1](#)).

156 Genomic DNA was extracted from muscle, scale clips or exuvia using DNeasy Blood & Tissue Kit
157 (Qiagen) according to the manufacturer's instructions. We amplified three protein-coding
158 mitochondrial (mt) genes (CYTB, COX1, ND5) from 115 viper samples with 2489 alignment positions
159 totall. As nuclear markers, we amplified four nuclear (nc) genes (RAG1, BACH1, MKL1, MC1R) with
160 5013 alignment positions totall. All of them have been previously used for multilocus species
161 delimitation in Squamates (e.g. [Vidal and Hedges, 2005](#); [Lynch and Wagner, 2010](#); [McVay and](#)
162 [Carstens, 2013](#); [Tolley et al. 2013](#)) and show polymorphism within and between closely related taxa.

163 We designed specific PCR primers for most loci amplified in this study ([Tab. A.2](#)).

164 For the amplification of target genes the TaKaRa Ex Taq™ PCR reaction system was used, containing
165 2.5 µl 10XBuffer, 2 µl dNTP Mix, 2.5 U enzyme, 1 µl of 10 pmol primer each, 1 µl genomic DNA, filled
166 up with dH₂O to 25 µl volume in total. Polymerase chain reaction was carried out, using the
167 automated Eppendorf Mastercycler®gradient. Conditions for PCR reaction were specific for each
168 gene and are given in [Table A.3](#). After PCR products were cooled down and stored until use at 8 °C.

169 Dye terminator cycle sequencing was set up according to suppliers' instructions (DTCS Quick Start Kit,
170 Beckman Coulter) in a two step thermal reaction with 30 cycles of 96°C 20 s, 60°C 4 min. For Dye-
171 terminator removal we used the Agencourt CleanSEQ system (SPRI-technology), and ran the samples
172 on a Beckman Coulter CEQ 8000 sequencing apparatus. All new DNA sequences generated for this
173 study were submitted to GenBank ([FJxx–FJxx](#)).

174 **2.2. Sequence alignment and mtDNA phylogenetic analyses**

175 Mitochondrial and nuclear sequences were edited and assembled using SEQUENCHER (Gene Codes).
176 Gene fragments were aligned separately using ClustalW ([Thompson et al., 1994](#)) implemented in
177 Bioedit 7.0.9 ([Hall, 1999](#)).

178 Heterozygous sequences were identified visually by checking for double peaks (point mutations) in
179 the electropherograms. Alleles were reconstructed for each specimen, using the software PHASE v.
180 2.1.1 ([Stephens et al., 2001; Stephens and Scheet, 2005](#)) by conducting two independent runs under
181 the default settings. Then the most likely haplotype pairs for each individual were chosen.

182 The program PartitionFinder ([Lanfear et al., 2012](#)) was used to determine the best partitioning
183 strategy and substitution models for the analysis. However, to identify the most appropriate models
184 of sequence evolution for each gene and dataset, we tested also other partitioning strategies using
185 MrModeltest 2.3 ([Nylander, 2004](#)).

186 The phylogenetic history of mt-genes was reconstructed, using Bayesian inference (BI) and Maximum
187 likelihood (ML). For Bayesian inference (BI) we used MrBayes 3.1.2 ([Ronquist and Huelsenbeck, 2003](#))
188 and partitioned the analysis by genes and codon positions. We ran the analyses with one cold and
189 three heated chains (MC^3) for 50 million generations sampling every 1000th generation and
190 discarding the first 25% of the trees as burn-in. Convergence was estimated in Tracer v1.5 ([Rambaut
191 and Drummond, 2007](#)) and observed with the convergence diagnostic parameters implemented in
192 MrBayes.

193 For maximum likelihood we used the software PhyML version 3.0 ([Guindon et al., 2010](#)), under the
194 GTR model with four substitution rate categories and 1000 non-parametric bootstrap replicates.
195 We specified *Macrovipera* as the outgroup for all analyses, as they are likely to be the sistergroup of
196 *Montivipera* ([Lenk et al., 2001; Wüster et al., 2008, Stümpel and Joger, 2009](#)).

197 **2.3. Species tree reconstruction and molecular dating**

198 We used a coalescent-based method to estimate a time calibrated species tree from four nuclear
199 (RAG1, BACH1, MKL1, MC1R) and three mitochondrial (CYTB, COX1, ND5) genes, using a Bayesian
200 framework implemented in the computer software *BEAST v. 1.8.0 ([Drummond et al., 2012](#)). Unlike
201 concatenated analyses, which shoehorn all loci into a single tree topology, this approach enables
202 multiple independent loci to be analysed simultaneously within a framework that accounts for gene
203 tree incongruence resulting from incomplete lineage sorting. This approach is preferable to
204 concatenation, which can lead to poor performance of standard phylogenetic estimates ([Kubatko
205 and Degnan 2007](#)).

206 Species tree approaches assume OTUs to be reproductively isolated, so that shared haplotypes are
207 the result of retention of ancestral haplotypes rather than ongoing gene flow. It follows that any

208 group that has an independent evolutionary history, can be designated as ‘species’ for the analysis.
209 Here, *Montivipera xanthina* has a substantial, well supported phylogeographic mt-DNA structure that
210 coincides with differences in the phenotypic appearance and ecological adaptations between the
211 populations of geographical regions (see below). We argue that this suggests the presence of a
212 taxonomically unrecognized diversity and consequently treated the populations of *Montivipera*
213 *xanthina* suggested by the mtDNA gene tree as independent evolutionary entities.

214 Molecular dating is critically affected by the quality of calibration points. Calibrations at internal
215 nodes are usually based on the fossil record, which is largely incomplete and biased ([Lieberman, 2002](#);
216 [Hedges and Kumar, 2004](#)). Its use and interpretation is often problematic. According to
217 [Gandolfo et al. \(2008\)](#) fossil calibration errors may be caused mainly by five factors: (1) fossil
218 preservation, (2) taxonomic assignment of the fossil, (3) identification of fossil homologies, (4)
219 sampling effort, and (5) fossil age determination. Especially in terrestrial environments, in which the
220 fossil record is poorer, identifications at the species level are difficult ([Padian et al., 1994](#)).
221 Consequently the availability of reliable calibration dates is traditionally restricted to few model
222 organisms ([Benton and Donoghue, 2007](#)). Most viper fossils are isolated vertebrae, so that their
223 taxonomic identification is problematic and relationships between extant and extinct species are in
224 many cases unclear ([Szyndlar and Rage, 1999](#)). [Head \(2005\)](#) pointed out that ontogenetic variation in
225 snake vertebrae is not well understood. In fact, the size of vertebrae is a character for taxonomic
226 assignment of fossil vipers ([Szyndlar and Rage, 1999](#)). Consequently, the fossil record of Eurasian
227 vipers does not provide enough verified evidence to date their cladogenesis.

228 For all of these reasons, we have used secondary calibrations of robust divergence time calculations
229 to improve the precision and accuracy of time estimates. Any node of a robust primary divergence
230 time calculation can be used as a secondary calibration point in a separate analysis, if there are no
231 known biases ([Hedges and Kumar, 2004](#)). [Stümpel \(2012\)](#) computed a chronogram based on 50
232 amino acid sequences of complete mt-genomes, representing the full diversity of amniotes. In order
233 to avoid fossil calibration errors inside viperids Stümpel dated the pedigree with 10 prominent
234 tetrapod calibration points of [Szyndlar and Rage \(1990\)](#), [Rage et al. \(1992\)](#), [Evans \(2003\)](#), [Müller and](#)
235 [Reisz, \(2005\)](#) and [Benton and Donoghue \(2007\)](#), using relaxed clock models. Based on these
236 calculations, extant species from *Montivipera* and *Macrovipera* shared their last common ancestor
237 (MRCA) at 15.3 Mya. Following lithological-palaeogeographic maps of [Popov et al. \(2004\)](#) the
238 divergence time correlates with a long standing isolation of “Asia Minor” during the Langhian,
239 between 16 and 15 Mya. The second calibration point we used, is the branching point between
240 extant species of the *Montivipera xanthina*- and *Montivipera raddei*-complexes. Calculations of
241 [Stümpel \(2012\)](#) date the timing of divergence at 10.7 Mya. However, the tectonic event that fits the
242 palaeobiogeographical reconstruction of oriental vipers, and which may have acted as vicariant

243 event, was the opening of a marine seaway along the Bitlis and Eastern Anatolian Fault zones in the
244 middle Serravallian (13-12.2 Mya) ([Stümpel, 2012](#)). Consequently we used both tectonic events as
245 calibration points to date the cladogenesis of the species tree. The initial divergence between
246 *Montivipera* and *Macrovipera* was modeled with a normal distribution with a mean of 15.5 Mya and
247 a standard deviation of 0.5 Mya, providing a 95% confidence interval of 14.68 and 16.32 Mya. For the
248 split between species of *Montivipera xanthina*- and *Montivipera raddei*-complexes we used a normal
249 distribution with a mean of 12.6 Mya and a standard deviation of 1.2 Mya, giving a 95% CI of 10.63–
250 14.57 Mya.

251 The analysis was run for 600 million generations sampling every 3000th generation, of which the first
252 25% were discarded as burn-in. To test the most appropriate partitioning strategy and substitution
253 models for the analysis, we used the program PartitionFinder ([Lanfear et al., 2012](#)), applying
254 partitions to the first/second and third codon for every gene.

255 To account for lineage-specific rate heterogeneity we used a Log-normal relaxed clock model and
256 specified a birth-death process for modeling the dynamical process of speciation and extinction.
257 Convergence statistics were monitored by effective samples sizes (ESS), analyzing the run in Tracer
258 version v1.5 ([Rambaut and Drummond, 2007](#)). A consensus tree with divergence times was obtained
259 from the 150,000 trees after discarding the first 25% as burn-in.

260 **2.4. Mitochondrial phylogeography**

261 For estimating the phylogeographic history we used statistical methods, implemented in the
262 software PhyloMapper 1b1 ([Lemmon and Lemmon, 2008](#)), which allows testing of a priori
263 hypotheses. We first tested the phylogeographic association between phylogenetic and geographic
264 distance for the mt-data matrix (CYTB, COX1, ND5) within each group after optimizing all parameters
265 and then generating the null distribution by performing 10,000 randomizations. Significance of the
266 test statistics rejects the null hypothesis that no association exists between geographic proximity and
267 genealogical proximity within the clade. We then tested, if the individuals of each species complex
268 tend to migrate in a non-random direction, using the overall directionality test as described by
269 [Lemmon and Lemmon \(2008\)](#). To estimate the geographic location of the ancestors of the *raddei*-
270 and *xanthina*-complex we calculated the locations of ancestors and estimated likelihood surfaces.
271 We initially performed the estimates for a wide geographic range, using a low resolution factor and
272 subsequently constrained the geographic grid for the final analyses. For the species complex of
273 *Montivipera xanthina* we constrained the latitude from 35.0 to 40.5 and the longitude from 32.0 to
274 40.0, and for the species complex of *Montivipera raddei* we applied a latitude from 35.0 to 40.0 and a
275 longitude from 44.0 to 52.0, using a resolution of 0.3 in each group. All analyses were optimized by
276 10,000 replications.

277 **2.5. Neutrality tests and demographic analyses**

278 In order to detect a population's departures from equilibrium conditions, which may result from
279 changes in population size, selection or gene flow, we used mt-DNA (CYTB, COX1, ND5) to calculate
280 nucleotide diversity for each clade in addition to Tajima's D ([Tajima, 1989](#)) and Fu's Fs ([Fu, 1997](#)),
281 under the neutral model. For historically stable populations, both D and Fs would be expected to be
282 close to zero. Negative values of both D and Fs would be indicative of recent population expansion,
283 whereas positive values would be expect from a recent population bottleneck or from negative
284 selection ([Slatkin and Hudson, 1991](#); [Rogers and Harpending, 1992](#); [Charlesworth et al., 1995](#)).
285 Significance was assessed for both statistics by comparison with data simulated under a constant
286 population size model, with significant P values indicating rejection of the hypothesis of constant
287 population size.

288 Population expansions have also been shown to leave particular signatures in the distribution of
289 pairwise sequence differences. Unimodal and smooth mismatch distributions indicate panmictic
290 populations, which undergo sudden range expansions ([Slatkin and Hudson, 1991](#); [Rogers and](#)
291 [Harpending, 1992](#)). In contrast, multimodal mismatch distributions suggest structured or diminishing
292 population and ragged distributions indicate a stable and widespread population ([Excoffier et al.,](#)
293 [1992](#); [Rogers and Harpending, 1992](#); [Rogers et al., 1996](#); [Excoffier and Schneider, 1999](#)). Statistically
294 significant differences between observed and simulated expected distributions were evaluated with
295 the sum of the square deviations (SSD) and Harpending's raggedness index (RI), with significant P
296 values indicating rejection of the recent expansion hypothesis ([Slatkin and Hudson, 1991](#); [Rogers and](#)
297 [Harpending, 1992](#)). All analyses were performed using Arlequin v.3.1 ([Excoffier et al., 2005](#)).

298 To visualize changes in effective population size through time, we also inferred the demographic
299 history of mountain vipers, using the extended Bayesian skyline plot (EBSP), as implemented in the
300 Bayesian phylogenetic software BEAST ([Drummond and Rambaut, 2007](#)). The coalescent-based
301 approach permits the analysis of multiple unlinked loci, enabling the rate and pattern of the
302 evolutionary process to vary among loci. For both phylo-groups we performed two independent runs
303 with 500 million generations for the *bornmuelleri*-clade (sampling every 3000 iterations) and 800
304 million for the *xanthina*- and *raddei*-clade (sampling every 3500 iterations). Results of each run were
305 visualized using Tracer v1.5 ([Rambaut and Drummond, 2007](#)) to ensure stationarity and convergence
306 had been reached, and that effective sample sizes (ESS) were higher than 200.

307 3. Results

308 3.1. DNA sequence characteristics and phylogenetic results

309 The concatenated mt-DNA matrix with 111 individual *Montivipera* DNA sequences and 2489 aligned
310 positions (825 bp COX1, 1062 bp CYTB, 602 bp ND5) is characterized by 424 invariable, 178
311 polymorphic and 150 parsimony informative sites with 58 unique haplotypes totally.

312 The nuclear data set embraces 270 *Montivipera* sequences, with 5013 alignment positions (2481 bp
313 RAG1, 1105 bp BACH1, 777 bp MKL1, 650 bp MC1R) 43 polymorphic sites and 59 unique sequences.
314 Bayesian inference (BI) and Maximum Likelihood (ML) analysis of mt-DNA data produced concordant
315 trees with considerable phylogenetic structure with distinct geographic associations (Fig. 2). Within
316 the genus *Montivipera* the BI and ML genealogies support a sister-group relationship between the *M.*
317 *raddei*- and the *M. xanthina*-complexes, with maximal statistical robustness and an uncorrected p-
318 distance of 0.107. Haplotypes of the *M. xanthina*-complex segregate into two evolutionary clades
319 with considerable divergences (p-distance = 0.069). The eastern *bornmuelleri*-clade embraces the
320 nominal taxa *M. bornmuelleri*, *M. wagneri*, *M. bulgardaghica*, *M. albizona* and a new taxon from
321 Syria, which is separated by a p-distance of 0.028 from its sister taxa *bulgardaghica* and *albizona*. The
322 *bornmuelleri*-clade has a monophyletic origin and its evolutionary lineages are separated by a
323 maximum p-distance of 0.040. The Anatolian *xanthina*-clade displays unexpectedly deep
324 phylogeographic structure, suggesting long standing evolutionary isolation between groups, with
325 higher p-distances (up to 0.056) than between the species of the *bornmuelleri*-clade. The common
326 ancestry of the Anatolian populations is not well supported, suggesting the possibility of alternative
327 genealogical relations (Fig. 2). However, each evolutionary lineage within the Anatolian clade is
328 supported by maximal posterior probabilities and bootstrap values, with specimens from western
329 Taurus in basal position, which are the sister-group of populations from Lycia and those from the
330 Aegean coast.

331 In contrast to the high genetic structure of the *xanthina*-complex, we found only 16 unique mt-gene
332 sequences among the *raddei*-complex, with a maximum genetic distance of p=0.029, indicating a
333 historically young radiation. Haplotypes of the nominal taxon *M. raddei kurdistanica* are paraphyletic
334 and also a common ancestry of *M. albicornuta* is statistically not well supported. *Montivipera*
335 *kuhrangica* represents a separate evolutionary lineage, having a common ancestry with *M. raddei*.
336 As expected the nuclear data set of *Montivipera* has a low variability with a maximal genetic distance
337 of p=0.0203. Measures of the nuclear genetic distances confirm a more recent origin of the *raddei*-
338 complex and an older divergence of the *xanthina*-complex. The genetic distance of the *Montivipera*
339 *xanthina*-complex (p-distance = 0.0201) is 6-fold higher than within the *raddei*-complex (p-distance =
340 p=0.0032).

341 **3.2. Species tree and molecular dating**

342 Post run diagnosis parameters of Tracer observed high effective sample sizes (ESS) and indicated that
343 runs of the *Beast analyses converged.
344 The topology of the time calibrated multilocus species tree (Fig. 3) from the combined data set of
345 nuclear and mitochondrial genes is congruent with the mt-genealogies obtained with MrBayes and
346 PHYML and strongly supports a sister relationship between the *Montivipera xanthina* and the *M.*

347 *raddei*-complex. Species of the *xanthina*-complex segregate into two clades with high Posterior
 348 Probabilities for a monophyletic origin of the *bornmuelleri*-clade. The relatively low support (PP 0.83)
 349 for the *xanthina*-clade suggests contradictory topologies with a possible paraphyletic origin of *M.*
 350 *xanthina* (Fig. 3). Divergence times support a late Miocene diversification of the *M. xanthina*-
 351 complex. Populations of *M. xanthina* from the Taurus Mountains were identified as the oldest
 352 evolutionary lineage, which split off from its sister-group 5.2 Mya ago, and are thereby older than
 353 basal lineages within the *bornmuelleri*-clade. In contrast, divergence time estimates derived from
 354 sampled *raddei* populations were considerably closer to the present and have a Pliocene origin.

355 **3.3. Mitochondrial Phylogeography**

356 We found significant evidence for phylogeographic association in the *M. xanthina*- and the *M. raddei*-
 357 complex at the $\alpha = 0.001$ level (Tab. 2) and in the analyzed clades individuals tend to migrate in an
 358 non-random direction (overall directionality test: $p < 0.001$).

359 We then estimated the geographic locations for the ancestors of the *M. xanthina*- and *M. raddei*-
 360 complexes (Fig. 4). According to the analysis the mountain vipers of the *xanthina*-complex had their
 361 origin in the Anatolian Taurus Mountains (latitude 37.94, longitude 34.78, lnL -381.33). Present
 362 haplotype distributions suggest a colonization of early ancestors mainly in east-west directions.
 363 *Montivipera bornmuelleri* from the Levant is the only recent population that indicates an ancestral
 364 colonization advance also to southern territories. Despite their spatial proximity, *xanthina*
 365 populations from Greek and Turkish Thrace go back to different dispersal events and do not share a
 366 common ancestor (Fig. 1).

367 The origin with the maximum likelihood estimate for the basal ancestor of the *raddei*-complex is
 368 located in the Persian Alborz Mountains (lat 35.06, long 49.18, lnL -145.99).

369 **3.4. Population genetic analyses and historical demography**

370 Extended Bayesian skyline plots (EBSP) of the *bornmuelleri*- and *xanthina*-clade indicate a substantial
 371 population size change over their demographic history. Both clades had maintained high population
 372 size during Pleistocene glaciations of Northern hemisphere (Fig. 6). The EBSP of the *xanthina*-clade is
 373 bimodal with a broad peak between 2.8 and 1.6 Mya during late Piacenzian and Gelasian and a
 374 second peak in current times. In contrast, the *bornmuelleri*-clade reached its highest population
 375 between 1.2 and 0.4 Mya, when the *xanthina* population decreased to its all-time low. Since the
 376 Middle Pleistocene, the EBSP reveals a rapid decrease of the *bornmuelleri*-clade. In recent times the
 377 population started to increase slightly to current size. Populations of the *M. raddei*-complex (Fig. 7)
 378 were constant over long time periods and decreased around 116.000 years ago with End of Eemian
 379 warm phase and beginning of Tarantian stage of upper Pleistocene. The negative population trend
 380 turned 8.000 years ago and started to increase to the present day.

381 Mismatch frequencies were calculated separately for the *xanthina*-, *bornmuelleri*- and the *raddei*-
 382 clade (Fig. 5). The shapes of the observed distributions deviate from a smooth unimodal pattern
 383 simulated under a sudden expansion model. Mismatch distributions have multimodal characteristics
 384 for all groups. In the *raddei*-complex the mismatch distribution has a high frequency of sequence
 385 pairs with low mismatch counts, indicating a shrinking or declining N_e . Thus the associated
 386 Raggedness-Index is high for the bimodal distribution in *raddei* and much smaller for the multimodal
 387 distributions of *xanthina* and *bornmuelleri*. The variances (SSD) and Harpending raggedness index (RI)
 388 indicate that the observed distributions differ significantly from the distributions expected under the
 389 model of population expansion in all groups for SSD. For the raggedness index significance was only
 390 assessed for *bornmuelleri*. Fu's *Fs* and Tajima's *D* are positive and differ from zero except for *raddei*.
 391 In concordance with the EBSP, high values for *Fs* and *D* suggest a recent population bottleneck or
 392 negative selection in *bornmuelleri* and *xanthina*. However, test statistics for Tajima's *D* and Fu's *Fs*
 393 cannot reject the null hypothesis (H_0) that the sample of DNA sequences were taken from a
 394 population with constant effective population size (Tab. 1).

395 4. Discussion

396 4.1. Mitochondrial genealogy uncovers hidden genetic diversity within *Montivipera*

397 Our mitochondrial based phylogeny is a continuation of Stümpel et al. (2009) and represents the first
 398 study that includes all known taxa. The results provide significant new insights into the evolutionary
 399 history of mountain vipers. Previous mt-genealogies of Lenk et al. (2001) considered only three OTU's
 400 of mountain vipers and revealed a paraphyly of the *Montivipera xanthina*-complex, with *M. raddei*
 401 being closer to *wagneri* than *xanthina*. A CYTB based Maximum-Parsimony tree of Garrigues et al.
 402 (2005) with six OTUs of mountain vipers revealed the species of the *xanthina*-complex as a
 403 monophyletic assemblage, but without resolving their relations, because of low statistical support
 404 and a basal polytomy.

405 Our BI and ML genealogies support a sister relation between the *raddei*-complex and the *xanthina*-
 406 complex with maximal statistical robustness, previously suggested by Nilson and Andrén (1986)
 407 based on morphological data and revealed with mt-marker by Garrigues et al. (2005). The monophyly
 408 of both complexes coincides with considerably differences in scalation, of which the circum-ocular
 409 ring, separating the supraocular from the eye, is most conspicuous (Nilson and Andrén, 1986).

410 Our phylogenetic inference showed that the *xanthina*-complex consists of two monophyletic groups,
 411 which correspond to east Anatolia (*bornmuelleri*-clade) and west Anatolia (*xanthina*-clade). Within
 412 the east Anatolian clade, we found *bornmuelleri* to be the most basal taxon. After the description of
 413 *Vipera bornmuelleri* Werner, 1898 as full species, it was synonymized with *Vipera lebetina xanthina*
 414 by Schwarz (1936), until Mertens (1967) resurrected the mountain viper from Lebanon as valid

415 species under the assumption that *Daboia palaestinae* belongs to the “Rassenkreis” (species
416 complex) of *xanthina*. This taxonomic position remained largely undisputed and most herpetologists
417 (e.g. Joger, 1984; Brodmann, 1987; Golay et al., 1993) followed Mertens (1967). Only Schätti et al.
418 (1991) doubted its validity and synonymized the populations from the Levant with *xanthina*, without
419 granting them any taxonomic status. Golay et al. (1993) treated *bornmuelleri* as a subspecies of
420 *xanthina*. Our mitochondrial genealogy strongly supports *bornmuelleri* as an independent
421 evolutionary lineage, which belongs to the east Anatolian clade and has no common ancestry with
422 *xanthina*, as suggested by Schätti et al. (1991).

423 *Montivipera bornmuelleri* is the sister taxon of the nominal taxa *wagneri*, *bulgardaghica* and
424 *albizona*, which have a common ancestry. *Montivipera wagneri* was collected in 1846 by Moritz
425 Wagner in the vicinity of Lake Urmia. Until its rediscovery by Teynié (1987) only the single female
426 holotype was known (Nilson and Andrén, 1984). Today Wagner’s mountain viper is only known from
427 a small isolated enclave around the Aras river catchment in east Anatolia (Joger et al. 1988). Recently
428 Göçmen et al. (2014) reported new localities of mountain vipers extending their range in Anatolia.
429 However, in combination with the distinct morphology (Joger et al., 1988) and considerable
430 differences in blood protein analyses (Herrmann et al., 1987), our results validate the populations
431 from the Aras region as full species *M. wagneri*.

432 The sister-group of *wagneri* embraces the two nominal species *bulgardaghica* and *albizona*. The
433 discourse about their systematics is discussed page by page in Schätti et al. (1991). Both taxa are
434 restricted to the Taurus Mountains, but their distribution is only known from few individual localities
435 and detailed sampling locations from the few caught specimens have never been published.
436 However, the possibility of a parapatric contact zone between *bulgardaghica* and *albizona* exists and
437 was discussed by Schätti et al. (1991). Our analyses show that haplotypes of *M. bulgardaghica* are
438 nested within *albizona* and do not support the species status of *albizona*. The nearest populations of
439 *M. albizona* are known from Tahtalı Dağları (Teynié, 1991) and Dibek Dağları (own observations), less
440 than 200 km from Bolkar Mountains. Interestingly Schätti et al. (1991) mention a specimen caught in
441 Kar Boğaz, which displayed a coloration that could be a morphological indicator for genetic contact
442 between both taxa. Our data confirm this hypothesis. One specimen from the type territory, which
443 was morphologically identified as *bulgardaghica*, shares an *albizona* haplotype.

444 Due to homoplasies in color pattern Bettex (1993) supposes *albizona* to be synonymous with
445 *wagneri*, and Phelps (2010) treats *bulgardaghica* as conspecific with *bornmuelleri*. A specimen from
446 the Syrian coastal Mountains near Slanfah (المنفج), formerly identified as *M. xanthina* by Sindaco et
447 al. (2006), represents a new taxon basal to *bulgardaghica*.

448 Our mitochondrial genealogy confirms the statement of Nilson and Andrén (1986) and of Nilson et al.
449 (1990) that *M. xanthina* is the closest relative of the four east Anatolian mountain vipers, which

450 represents a divergent evolutionary lineage. Following [Nilson and Andrén \(1986\)](#), *M. xanthina* is also
 451 characterized by autapomorphies, such as ten supralabials and high number of subcaudals. However,
 452 the monophyly of *xanthina* (s.str.) is statistically significant in the MrBayes analysis, but is less
 453 robustly supported by the species tree of *BEAST.

454 Within *M. xanthina* our mt-genealogy recovers unexpected high levels of genetic diversity with a well
 455 supported phylogenetic structure. This is unexpected, as *M. xanthina* displays comparatively low
 456 variability in external morphology within its rather large range, as [Nilson and Andrén \(1986\)](#) note.
 457 Phenotypes do not display eye-catching differences in coloration or pattern, like *M. wagneri* and *M.*
 458 *albizona*, or a distinctive dorsal pattern like *M. bornmuelleri*. However, the unexpected cryptic
 459 genetic diversity revealed here suggests the presence of unrecognized taxa ([Stümpel and Joger, 2009](#)).
 460 [Nilson and Andrén \(1986\)](#) performed a hierarchical cluster analysis based on morphometric
 461 characters for species of the genus *Montivipera* and found considerable intraspecific variation within
 462 *M. xanthina*, but the results were not consistent for both sexes, and the authors distinguished,
 463 without drawing taxonomic conclusions, two subgroups (a northern and a southern *xanthina*) below
 464 the subspecies level. Their findings partially support the substantial genetic structure within
 465 *xanthina*.

466 In our data set the *M. raddei*-complex is a genetically relatively homogenous lineage, with the lowest
 467 haplotype diversity within the mountain vipers, possibly indicating their historically young radiation.
 468 The recently described species *M. kuhrangica* ([Rajabizadeh et al., 2011](#)) is the sister taxon of *M.*
 469 *raddei* and reflects the very incomplete knowledge of the distribution area, especially in the southern
 470 Zagros Mountains.

471 Given the poor exploration and the large geographic distance to the next populations of *raddei* (s. l.)
 472 it seems possible that unknown haplotypes may have been overlooked. [Obst \(1982\)](#) treats the taxa
 473 *latifii* and *raddei* as diverging populations of the same species, and [Schätti et al. \(1991\)](#) added also
 474 *albicornuta* to the synonyms of *raddei*. [Nilson and Andrén \(1986\)](#) hypothesized *albicornuta* and *latifii*
 475 to have a common ancestor. Our phylogenetic inference revealed *latifii* as a separate evolutionary
 476 lineage, which is confirmed by its distinctive ecological adaptation to alpine habitats in the Alborz
 477 Mountains ([Mertens et al., 1967; Andrén and Nilson, 1979](#)). Samples assigned to the subspecies
 478 *raddei kurdistanica* are scattered throughout the *raddei*-complex.

479 **4.2. Speciation and divergence times**

480 A key aim of this study was the molecular dating of important nodes for the reconstruction of
 481 biogeographical histories. For estimating rates of molecular evolution in a tree, nodes must be fixed
 482 to a time scale. Key means of clock calibration are fossil data, providing minimum constraints on the
 483 timing of lineage divergence events ([Benton and Ayala, 2003; Benton and Donoghue, 2007](#)). It is
 484 obvious that the quality of the fossil record has a large impact on the inferred divergence times of

485 the pedigree. Eurasian vipers have a very poor fossil record and the taxonomic identification of fossils
486 is often problematic. However, according to [Antunes and Rage \(1974\)](#) and [Szyndlar and Rage \(1999\)](#)
487 oriental vipers of the genera *Macrovipera* or *Montivipera* appeared in the European fossil record for
488 the first time in the lower Miocene (MN 3, 22.1 – 17 Mya). But the single vertebra from Lisboa, which
489 is the evidence for the first appearance, could not be assigned with absolute taxonomic confidence.
490 For the following Mammal period of the Neogene (MN 4, 17 – 16 Mya) [Szyndlar and Rage \(1999\)](#)
491 claim that oriental vipers were already widespread in Europe and remained so until the Pleistocene,
492 embracing a time period of at least 15 Mya. However, molecular divergence times do not confirm an
493 early Miocene origin of *Macrovipera* and *Montivipera*. To date divergences among Colubroidea,
494 [Wüster et al. \(2008\)](#) used a mitochondrial data matrix, mainly calibrated with fossil snake calibration
495 points. According to the authors' analysis, *Macrovipera* was separated from *Montivipera* about 11
496 Mya ago, considerably younger than our results. The taxonomic affinity and/or stratigraphic age of
497 calibration points used by [Wüster et al. \(2008\)](#) were doubted by [Lukoschek et al. \(2012\)](#), who
498 demonstrated that the use of mitochondrial-only data by [Wüster et al. \(2008\)](#) may have inflated the
499 ages of distal nodes relative to basal ones due to the saturated third codon position of mtDNA loci.
500 Consequently [Lukoschek et al. \(2012\)](#) corrected the split between *Montivipera* vs. *Macrovipera* of
501 [Wüster et al. \(2008\)](#) to younger ages – even less compatible with the estimates presented here.
502 [Szyndlar and Rage \(1999\)](#) note that a distinction between fossil species of *Macrovipera* and
503 *Montivipera* is hardly possible. Given the uncertainty of taxonomic identification it seems likely that
504 extinct lineages and members of the stem-group were pooled by [Szyndlar and Rage \(1999\)](#) and may
505 thus bias biogeographic hypotheses and systematic assignments.
506 To be free from circularity derived from the biased fossil record of snakes, we used secondary
507 calibration points of [Stümpel \(2012\)](#), which were calculated using protein sequences of complete mt-
508 genomes and are in concordance with vicariant events in the Tethyan realm and confirm the
509 divergence times for the MRCA of Viperidae and Viperinae published by [Wüster et al. \(2008\)](#).
510 The topology of the multilocus *BEAST chronogram ([Fig. 4](#)) is congruent with the mitochondrial
511 genealogy resulting from the MrBayes run. Nodes of the combined analysis of mtDNA and nuclear
512 loci suggest a late Miocene (12.3 Mya) origin for the MRCA of *Montivipera*. The time frame correlates
513 with a prominent tectonic event in the Middle East, the uplift of the Turkish-Iranian plateau to an
514 elevation of 1.5-2 km a.s.l. ([Şapaş and Boztepe-Güney, 2009](#)). Along with the increase of elevation,
515 climatic, spatial, biotic and evolutionary factors changed. The most obvious is the generally linear
516 decrease in temperature, which decreases by an average of approximately 0.68 °C for each 100 m
517 increase in elevation ([Barry, 2008](#)), so that the Turkish-Iranian plateau cooled down by approximately
518 10.2 to 13.6 °C due to the uplift. Other abiotic factors like air pressure, solar radiation and humidity
519 change predictably along the montane gradients. These determinants are well known to impact

520 species richness ([McCain and Grytnes, 2010](#)) and are thus likely to have strongly influenced
521 organismal communities and habitats in the Near and Middle East. Flora and fauna respond to these
522 changes in their speciation and extinction rates. We propose this scenario as a driver for the
523 *Montivipera* stem-group to adapt to mountainous conditions. The diversification of the *xanthina*-
524 clade began in the early Pliocene at 4.7 Mya, as already hypothesized by [Nilson and Andrén \(1986\)](#). It
525 is worth reiterating that this group was considered as monotypic until recently. The relatively old
526 origin suggests extensive cryptic diversity. Recent species of the *bornmuelleri*-clade are of
527 considerable younger age and have their origin in the late Pliocene. Based on immunological
528 distances [Herrmann et al. \(1987\)](#) determined an age of less than 5 Mya for the MRCA of the
529 *bornmuelleri*-clade. [Wüster et al. \(2008\)](#) estimated the taxa *Montivipera xanthina* having separated
530 from *Montivipera albizona* approximately 4 Mya ago.

531 Despite the late Miocene origin of the *raddei* and *xanthina* stem-group, the most extant evolutionary
532 lineages emerged not before the Pleistocene, except of the Lycian and Taurus lineages, which are of
533 considerable older age. The absence of old lineages within the *raddei*-complex suggests a massive
534 loss of lineage diversity through time. The global climate system experienced drastic changes from
535 the middle Eocene to the present with global cooling and an overall increase of seasonality
536 ([Mosbrugger et al., 2005](#)), which resulted in numerous shifts in the distribution and abundances of
537 species ([Hewitt, 2004a](#)). However, [Avise et al. \(1998\)](#) calculated that 57% of the recent herpetofauna
538 goes back to Pleistocene speciation events. The same time frame is mentioned by [Veith et al. \(2003\)](#)
539 and [Plötner et al. \(2010\)](#) as relevant for the speciation of Anatolian anurans. Besides the climatic
540 effects we could identify geological settings in Anatolia that are likely to have been relevant for
541 lineage differentiation of *Montivipera* populations. The river Göksu Nehri, breaking the Taurus
542 Mountains between the cities Mut and Silifke, is a barrier for montane biota. The valley bottom, with
543 an elevation of less than 250 m a.s.l., is a barrier for dispersal of montane organisms, dividing
544 *Montivipera* populations into an eastern (*bornmuelleri*-) and a western (*xanthina*)-clade. Beyond
545 that, the tectonic evolution of the Isparta Angle might have triggered the isolation of the basal
546 *xanthina* lineage from its sister-group. The Isparta Angle is a junction between the Aegean and
547 Cyprus arcs, with a long-term polyphase deformation history, which is characterized by a massive E-
548 W compression, resulting in the N-S orientation of main structural lines ([e.g. Van Hinsbergen et al.,](#)
549 [2010; Poisson et al., 2011 and references therein](#)). The inner Isparta Angle hosts several basins and
550 lakes, which might constitute barriers to the dispersal of montane *xanthina* populations. Evolutionary
551 lineages east of the Isparta Angle (Isparta, Geyik Dağı) are clearly separated from the West Anatolian
552 lineages (Lycia, Aegean).

553 **4.3. Phylogeography, population genetic analyses and historical demography**

554 Descriptive summary statistics and inferential methods of both mt and ncDNA are congruent and
 555 support substantial changes in effective population size of mountain vipers through time ([Fig. 6](#)).
 556 Based on our data, we argue that climatic oscillations during the Pleistocene, together with the high
 557 relief Near and Middle Eastern mountain systems, were key drivers of lineage diversity of mountain
 558 vipers.

559 Mountain vipers are spatially constrained to montane habitats, especially the taxa of the *raddei* and
 560 *bornmuelleri*-clades, which inhabit exclusively an elevational zone between 1400 and 2800 m a.s.l.
 561 We hypothesize the stem-group of *Montivipera* to have originated in oro-biomes, adapting to a
 562 seasonal climate and a diurnal lifestyle. The spatial hypothesis that *Montivipera* has a montane
 563 origin, is supported by the Likelihood estimates (center of origin), which reveal a montane origin of
 564 both the MRCA of *xanthina*-complex and the MRCA of the *raddei*-complex. In addition, the
 565 phylogenetic results ([Fig. 2](#)) show that the most basal and oldest lineages are invariably distributed in
 566 mountainous habitats, outnumbering lowland populations, which are of considerably younger age
 567 and nested deep within otherwise montane clades. Today species of the East Anatolian *bornmuelleri*-
 568 clade are ecologically confined to oro-Mediterranean habitats between 1400 and 2500 m a.s.l. In the
 569 Taurus Mountains the zone corresponds to the *Cedrus-Abies* forests (Querco-Cedretalia libani)
 570 outlined by the range of the Astragalo-Brometalia ([Parolly, 2004](#)). Evolutionary lineages of the
 571 *xanthina*-clade have a broader ecological amplitude distributed from sea level up to 2000 m a.s.l.
 572 The ultimate causes of why some lineages of the west Anatolian *xanthina*-clade display more
 573 plasticity remain unclear. However, we argue that the ancestral ecological trait is montane and
 574 adaption to lowland habitats occurred secondarily.

575 Our data indicate that the populations suffered substantial changes in effective population size over
 576 time. Growth and decline of populations can be associated with two relatively abrupt climate
 577 transitions, the onset of major northern hemisphere glaciations at approximately 2.7 Mya and the
 578 mid-Pleistocene transition (at approximately 900 ka), when the dominant periodicity of glacial
 579 response changes from 41 to 100 kyr ([Milankovitch, 1941; Paillard, 2001](#)). This historical pressure on
 580 natural systems could have shaped species ranges and been the driver for demographic processes.
 581 EBSP indicate that ancestors of the *xanthina* and the *bornmuelleri*-clades responded differently to
 582 the change of environmental conditions. The *xanthina*-clade expanded during the warm phase of the
 583 Pliocene ('Green House effect'), but reached maximum population size at the beginning of the
 584 Pleistocene. During the climatic reorganization and the end of the Pliocene warm period (5–3 Mya
 585 ago) ancestors of the *xanthina*-clade must have successfully adapted to the changing abiotic and
 586 biotic conditions. During the continuous cooling of earth climate and the switch in the frequency of
 587 the astronomical Milankovitch cycles, ancestors of the *xanthina*-clade responded with a negative

588 population growth rate, due to the deterioration of environmental conditions. Finally, the *xanthina*-
589 clade shows evidence of population growth at the end of the Pleistocene, presumably as a result of
590 the increase in available habitats for this warm, lowland-adapted species during the current
591 interglacial.

592 Unlike in the *xanthina*-clade, evidence from EBSP indicates that ancestors of the *bornmuelleri*-clade
593 increased population sizes during the Pleistocene, and had the adaptive capacity to use the
594 ecological opportunities arising from Pleistocene climate oscillations through adaptive responses
595 such as cold tolerance. Similarly, the *bornmuelleri*-clade shows no evidence of late Pleistocene
596 population expansion, which is to be expected for a species inhabiting cool, high altitude habitats,
597 which may have shrunk and become restricted to higher elevations as a result of late Quaternary
598 climatic warming. In contrast populations of the *raddei*-complex collapsed from 116,000 to 8,000
599 years ago, coinciding with the final glacial episode of the Pleistocene (Tarantian), supposedly because
600 of late Pleistocene hyperaridity. Pollen records from Lake Urmia in Iran give evidence that the lack of
601 moisture supply during last glaciation changed the herbaceous vegetation to a xerophytic *Artemisia*
602 and grass steppe ([Djamali et al. 2008](#)). In Iran the late glacial to early Holocene transition is marked
603 by the expansion of deciduous forests ([Djamali et al. 2008](#)), indicating the increase of
604 paleoenvironmental moisture supply and the extension of suitable habitats with an increasing
605 population size of *M. raddei* ancestors.

606 Mountains have a high richness of different climatic zones and microhabitats. On a larger scale, this
607 richness is primarily related to the change of abiotic factors along the altitudinal gradient and, on a
608 more local scale, by slopes facing different geographic directions. In a spatio-temporal scenario,
609 different microhabitats are very dynamic in terms of their distribution at different elevations at
610 different times, but stable in terms of their continued existence within the mountain system. They
611 thus enable species to retain their ecological niches during climatic changes by means of changes in
612 their elevational distribution. The local range or 'biogeographical stasis' is therefore linked to
613 capacity of the mountain range to provide the required microhabitat of the species despite changes
614 in overall climatic conditions ([Médail and Diadema, 2009](#)). As a result of the elevational shifts, the
615 habitats of montane species became restricted during global warming, because eco-zones shift to
616 higher elevations, resulting in loss of available surface area. Conversely, climatic cooling shifts the
617 range of acceptable ecological conditions back to lower altitudes and formerly isolated populations
618 probably became connected again. In mountainous regions, climatic oscillations are thus a driving
619 force of allopatric speciation: due to phylogenetic niche conservatism ([Wiens, 2004](#)), species tend to
620 retain similar ecological niches over time ([Ricklefs and Latham, 1992; Peterson et al., 1999, Webb et](#)
[al., 2002](#)), and their ranges are thus fragmented and reconnected repeatedly through climatic cycles.

622 The mountains of the Middle East were not affected by glaciations to the same extent as northern
623 Europe, although at higher altitudes glacial erosive or depositional features have been found (e.g.
624 [Akçar & Schlüchter, 2005](#)). However, it is a fallacy to believe that the Near and Middle East did not
625 experience climatic fluctuations of large magnitude ([e.g. Joannin et al., 2010](#)). In mountains, the
626 upper vegetation zone is restricted by the snow line. Today, the habitats of *Montivipera* populations
627 have their upper elevational limit approximately 800-1500 m below the summer snow line. During
628 last glacial maximum (19-23 ka) the palaeo snowline was estimated to have been 1000 m below the
629 modern snow line ([Sarıkaya, 2011](#)), suggesting that the elevational range of mountain viper species
630 was similarly lowered, leading to range expansion and reconnection for populations of montane
631 species. This explains both, the pattern of allopatric speciation seen between montane forms in
632 separated mountain systems in the *bornmuelleri*-clade, but also the shallow divergences between
633 currently isolated populations of the *raddei*-complex.

634 This Plio-Pleistocene scenario has thus left distinctive marks on the genome ([Hewitt, 1996](#)) and
635 initiated vicariant allopatric speciation and dispersal. Allopatric populations, which experienced little
636 gene flow, can be isolated over long time periods, allowing them to acquire and retain unique and
637 high genetic variation ([Petit et al., 2003; Hampe and Petit, 2005](#)). During glacial-interglacial episodes
638 the Mediterranean mountains played a key role in speciation processes as refugia ([Médail and
639 Diadema, 2009](#)). This hypothesis is also supported by plant diversity-environment relationships in
640 southern Europe ([Svenning et al., 2009](#)).

641 Our results evidently imply restricted gene flow among the populations by the appearance of
642 physical vicariance. This phenomenon of decreasing chances of mating might have been caused by
643 the topographic relief and discontinuous habitats in the Near and Middle East.

644 Presumably, mountain viper populations survived glacial periods in allopatric refugial areas adjoining
645 mountain chains in the Near and Middle East, or in situ within valley systems of high mountains, with
646 each distinct regional clade having had its own refugium.

647 Comparable studies are rare for the Near and Middle East. For Asia Minor our results are in
648 concordance with other studies pointing out the impact of Anatolian Mountains for species diversity
649 ([e.g. Hewitt, 1999; 2000; Veith et al., 2003; Çiplak, 2003; 2004; Mutun, 2010; Bilgin, 2011](#)) and fit in
650 with the hypothesis of [Nilson et al. \(1990\)](#) that the Anatolian Diagonal is a key factor for
651 diversification of the *Montivipera xanthina*-complex and a hot spot for other biota ([Ekim and Güner,
652 1986; Duran et al., 2005](#)). It is worth mentioning that, despite their aquatic life history, eastern
653 Mediterranean water frogs ([Plötner et al., 2010](#)) show a highly congruent distribution of mt-
654 haplotypes with the Anatolian mountain vipers, supporting the broad relevance of vicariant
655 palaeogeological events for the evolution of Eastern Mediterranean biota. [Médail and Diadema
656 \(2009\)](#) identified multiple floral refugia in the Mediterranean mountains, which indicate continuous

657 divergence and speciation over many millions of years to the present. The high congruence between
658 the geographic distribution of *Montivipera* haplotypes and plant refugia ([Fig. 8](#)) is astonishing and
659 evidently indicates the importance of common historical events as drivers of speciation and
660 distribution across a broad swath of Near and Middle Eastern biota.

661 **4.4. Implications for mountain viper systematics and future work**

662 Translating the phylogeographic results obtained in this study into a formal taxonomic framework
663 remains challenging and subject to multiple different interpretations, depending on the species
664 delimitation criteria used ([de Queiroz, 2007](#)). Mountain vipers are allopatrically distributed and
665 inhabit isolated disjunct mountain areas and are therefore genetically and geographically isolated.
666 However, experimental hybridizations between *M. wagneri* and *M. xanthina* result in fertile offspring
667 (own observations). Further research may yet reveal natural hybrid zones, where populations share
668 haplotypes of different species. On the other hand, many isolated populations are clearly geographic
669 variants of one another, but display distinctive phenotypic features, so that no intermediates exist.
670 Speciation is a complex process culminating in the evolution of intrinsic isolation mechanisms, which
671 result in genetic isolation. During the preceding transition time, when populations diverge, it is
672 difficult to find objective criteria for species delimitation ([Hey, 2009](#)), because the lineage simply may
673 not yet have evolved distinctive properties. However, the presence of any unique property
674 constitutes evidence for lineage separation and the possession of several properties highly
675 corroborate the existence of different species ([De Queiroz, 2007](#)).

676 Proposals for taxonomic classification of the *Montivipera* taxa have been made in either the splitting
677 or lumping direction. [Nilson and Andrén \(1986\)](#), who described *wagneri*, *bulgardaghica* and *albizona*
678 as full species, stated that these taxa including *bornmuelleri* could also be treated as subspecies or
679 allospecies of one superspecies. In line with this argumentation, *Montivipera* would consist of three
680 species *xanthina*, *bornmuelleri* and *raddei*.

681 According to the molecular evidence presented in this paper, we suggest to treat each major
682 evolutionary lineage of *Montivipera* as valid species. Our molecular genealogy supports *bornmuelleri*,
683 *wagneri*, *bulgardaghica*, *albizona*, *raddei*, *latifii* and *kuhrangica* as valid taxa. They all represent
684 unique evolutionary lineages, separated by considerable genetic distances. The exception are
685 *albicornuta* and *kurdistanica*, where we found no evidence for monophyletic origins. Nominal
686 haplotypes of both taxa scatter through the tree and belong to the *raddei* haplo-group. In the
687 absence of unique morphological characters, *albicornuta* and *kurdistanica* should be collapsed into
688 *raddei* and considered geographic variants of the latter. *Montivipera albizona* has a unique nuclear
689 haplotype, which separates the taxon from the allopatric *bulgardaghica*, but the mtDNA genealogy
690 evidently suppose genetic contact between both taxa. Considering the allopatric distribution and
691 similar morphological and ecological synapomorphies, we prefer to treat *albizona* as a subspecies of

692 *bulgardaghica*. A single specimen from the Syrian Levant represents a new phyletic lineage in the
693 pedigree. However, without any further knowledge and additional specimens, further taxonomic
694 conclusions are hardly possible.

695 In the light of our genetic data, *M. xanthina* appears to constitute a cryptic species complex with
696 three or four new taxa. Each of them has a long standing history of isolation comparable to the
697 species of the *bornmuelleri*-clade. Prior to this genetic analysis, a phenotypic distinction between the
698 phyletic *xanthina* lineages was not possible, probably due to the lack of material. With the
699 phylogenetic background of this study, genetic information is available which can be included for
700 accurate species identification, and can guide the search for morphological characters that can help
701 differentiate these taxa. Taxonomic revisions have different connotations for further biological
702 analysis.

703 Together with *Macrovipera*, *Montivipera* is responsible for serious, often-lethal clinical problems in
704 the Near and Middle East (e.g. Chippaux 1998). Venom composition varies both interspecifically and
705 intraspecifically in many snakes, and this can have severe consequences for snakebite victims
706 (Casewell et al., 2013). Where victims of bites require antivenom, and in the absence of direct
707 evidence on venom composition of antivenom effectiveness, phylogenetic relatedness of lineages
708 could potentially inform antivenom choice. Moreover, the evolutionary tree for a group of species
709 can also inform conservation measures for these taxa.

710 Future morphological work including more samples is necessary to identify diagnostic characters for
711 species delimitation and to evaluate the species concept made here on the basis of molecular data
712 alone.

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