A new vertebrate for Europe: The discovery of a range-restricted relict viper in the western Italian Alps

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Abstract

We describe *Vipera walser*, a new viper species from the northwestern Italian Alps. Despite an overall morphological resemblance with *Vipera berus*, the new species is remarkably distinct genetically both from *V. berus* and other vipers occurring in Western Europe, and shows closer affinities to species occurring only in the Caucasus. Morphologically, the new species appear to be more similar to *V. berus* than to its closest relatives occurring in the Caucasus, but can be readily distinguished in most cases by a combination of meristic features as confirmed by discriminant analysis. The extant population shows a very low genetic variability measured with mitochondrial markers,

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26 suggesting that the taxon has suffered a serious population reduction/bottleneck in the

27 past. The species is extremely range-restricted (less than 500 km^2), and occurs only in

28 two disjunct sites within the high rainfall valleys of the Alps north of Biella. This new

29 species should be classified as globally 'Endangered' due to its small and fragmented

30 range, and an inferred population decline. The main near-future threats to the species are

31 habitat changes associated with reduced grazing, along with persecution and collecting.

32

33 **Keywords** Vipers, *Vipera berus*, *Vipera walser*, Reptile conservation, New species,

34 bPTP species delimitation model, Alps, Biogeography, Climate change

35

Introduction

37 The identification of species is an essential building block in biodiversity valuations both

38 at global and local scales, and incomplete knowledge of an area's diversity may result in

39 sub-optimal conservation practices (Bickford et al., 2007). The full faunas and floras of

40 sites are often not known, meaning that conservation decisions have to be made based on

41 studies of just a subset of taxonomic groups (Howard et al., 1998), use of higher

42 taxonomies (Balmford et al., 1996), or incomplete species lists (Polasky et al., 2000).

43 Recently, the use of genetic markers has allowed the identification of morphologically

44 similar species with strong genetic differentiation, generally considered as 'cryptic

45 species' (Bickford et al., 2007), an approach that allows identification of divergent

46 evolutionary lineages despite subtle, or even no morphological differences. The addition

47 of these cryptic species to site lists has, in some cases, changed quite radically our

48 assessments of site importance or species-specific conservation priorities (Bickford et al.,

49 2007).

50   The Adder *Vipera berus* (Linnaeus, 1758) has a wider distribution than any other

51   terrestrial snake, occurring between France and the Pacific coast of Russia (Saint Girons,

52   1980). Perhaps surprisingly for a species with such a large range, just three subspecies

53   have so far been recognised: *V. b. berus* in the bulk of its distribution; *V. b. bosniensis*

54   Boettger, 1889; and *V. b. sachanlinesis* Zarevsky, 1917. Studies have confirmed genetic

55   differentiation between *V. b. bosniensis* and *V. berus*, and strongly suggest that

56   morphological differentiation of *V. b. sachalinensis* is related to recent local adaptation

57   (Kalyabina-Hauf et al., 2004, Ursenbacher et al., 2006). Additionally, the same studies

58   demonstrated the occurrence of one genetic clade in Northeastern Italy, Slovenia and

59   Southern Austria, based on samples collected east of the city of Milan (Kalyabina-Hauf

60   et al., 2004, Ursenbacher et al., 2006). In this clade, large genetic differentiation was

61   observed suggesting the possibility of several glacial refugia (Ursenbacher et al., 2006).

62   Moreover, genetic analyses also considered *V. barani* Böhme & Joger, 1983, and *V.*

63   *nikolskii* (Vedmederya, et al., 1986) as directly belonging to the *V. berus*-complex

64   (Kalyabina-Hauf et al., 2004, Zinenko et al., 2010).

65   The Adder was first recorded in the western Italian Alps in 1879 by the

66   herpetologist Michele Lessona at 'Monasterolo', west of the city of Torino, a site

67   separated from the main range of *Vipera berus* by a gap of almost 120 km (Lessona,

68   1879). During the 1930s, the herpetologist Felice Capra outlined more precisely the

69   distribution of this *Vipera berus* population, discovering two populations in the Alps

70   north of the town of Biella. He found remarkable differences in head lepidosis, especially

71   in individuals from the Biella Prealps, from those of *Vipera berus* from central and

72   northern Europe (Capra, 1954). In 2006, Ghielmi and colleagues published the first record

73   for a new Piemonte locality, at Valle Strona in Verbania Province, representing a disjunct

74 population of the species and an expansion of the known range in Piemonte region

75 (Ghielmi et al., 2006).

76   In 2005, Ghielmi noticed that while *V. berus* is associated geographically with

77 one of its main prey items *Zootoca vivipara vivipara* (Lichtenstein, 1823) across almost

78 all of its European range, the entire known range of *Vipera berus* in western Alps

79 coincided with *Z. carniolica* Mayer, Böhme, Tiedemann & Bischoff, 2000, formerly

80 considered a subspecies of the common lizard, but now treated as a separate species, and

81 the ancestral oviparous sister species of the widespread *Z. vivipara* (Surget-Groba et al.,

82 2002, 2006, Cornetti et al., 2015). This raised the possibility that small and disjunct

83 populations of *V. berus* in the western Alps might be differentiated from other *V. berus*

84 populations.

85   The aims of this paper are to investigate the genetic and morphological diversity

86 within the adders from Piemonte, evaluate the phylogenetic relationships with other

87 adders and European vipers, formalise the description of this new taxon, and to make

88 recommendations for further research and management aimed at its conservation.

89

90 **Material and Methods**

91 Between 2011 and 2014, 31 individuals of a viper attributed to *Vipera berus* were found

92 in Piemonte (hereafter called the adder from Piemonte), in the western Italian Alps.

93 Morphological analyses were carried out on an additional 16 specimens currently

94 deposited in the Natural History Museum of Genova (14 specimens), in the Natural

95 History Museum of Torino (1 specimen) and in the Insubric Civic Museum of Natural

96 History at Clivio and Induno Olona (1 specimen). For each individual captured,

97 geographic coordinates and altitude were recorded with a GPS. Photographs were taken

98 in order to document body and head lepidosis. For most individuals, non-invasive DNA

4

99   samples were taken by cutting the distal margin of a ventral scale with a sterile scalpel

100   blade or by using buccal swabs (as detailed in Beebee, 2008). All sampled individuals

101   were immediately released at the exact place of capture. Three additional genetic samples

102   were collected in 2002 and 2003 during a previous survey (Ghielmi et. al., 2006).

103   Ventral, subcaudal and midbody scale rows were counted using standard

104   techniques (Dowling, 1951, Saint Girons, 1978). Total length, snout-vent length (SVL)

105   and tail length were recorded to the nearest millimetre. The following scales were counted

106   (scales located on head sides were counted on both sides): rostral; loreals (defined as the

107   scales between nasal, upper labials and perioculars); perioculars; apicals; frontal (when

108   fragmented, the number of fragments was recorded); parietals (when fragmented, the

109   number of fragments was recorded) and subocular (following the method proposed by

110   Vacher & Geniez, 2010). Comparative scale counts and character states were based on a

111   sample of 48 individuals of the adder population from Piemonte and 135 *Vipera berus*

112   from the alpine region and belonging to the Italian clade (see Ursenbacher et al., 2006).

113   This represents the geographically closest population, and the one showing some

114   convergent features in terms of lepidosis, compared to the more northerly *V. berus*

115   populations. Statistical comparisons were conducted using Student's *t*-test (if sample

116   variances equal), Welch's *t*-test (unequal variances), and Wilcoxon's test. Multivariate

117   comparisons of selected morphological characters between populations of *V. berus* and

118   the adder from Piemonte were conducted using a permutational multivariate analysis of

119   variance (PerMANOVA; Anderson, 2001) based on the Bray–Curtis dissimilarity

120   measure with 1,000 random permutations. To represent graphically the differences

121   between the two species, non-metric multidimensional scaling (nMDS) was used.

122   Discriminant analysis was used for visually confirming or rejecting the hypothesis that

123   the two species are morphologically distinct. Analyses were carried out separately on

5

124   males and females. For the nMDS, the following variables were considered: subcaudals,

125   crownscales; apicals; perioculars; parietals; and loreals (only on the right side because the

126   adder from Piemonte showed a much higher degree of asymmetry on loreal scales count,

127   compared to other *V. berus*). The analysis was carried out on the adder from Piemonte

128   and three groups of *V. berus* from the Italian clade having the same number of samples,

129   in order to evaluate intraspecific variability. Analyses and tests were computed with R

130   v3.1.2 (R Core Team, 2014) and PAST3 (Hammer et al., 2001).

131   DNA was extracted using a QIAamp DNA Mini Kit (Qiagen) and several portions

132   of the mitochondrial DNA were amplified. A portion of the Cytochrome *b* (cytb; 927 bp)

133   was amplified for all samples following Ursenbacher et al., (2006), whereas portions of

134   the 16S (473 bp), the ND4 (796 bp), and the Control Region (CR; 1058 bp) were

135   amplified following respectively Lenk et al. (2001), Arevalo et al. (1994) and

136   Ursenbacher et al. (2006) for each cytb haplotype and each region (six individuals). PCRs

137   were conducted in 25 µl volumes with 2µl of DNA template, 1xPCR buffer (Qiagen),

138   2mg ml^{-1} of Q solution (Qiagen), 2mM of MgCl2, 0.2mM dNTPs, 0.5µM of each primer

139   and 0.5 units of Taq polymerase (Qiagen). Successfully amplified fragments were

140   sequenced by Macrogen Inc (Seoul, South Korea). Sequences were deposited in GenBank

141   (no XXXX - to be completed on publication). Additionally, sequences of the different

142   *Vipera berus* genetic clades (individuals It1, Se2 and Ch2 in Ursenbacher et al., 2006), as

143   well as several other viper species (*V. ammodytes ammodytes* (Linnaeus, 1758), *V. aspis*

144   *francisciredi* Laurenti, 1768*, V. ursinii ursinii* (Bonaparte, 1835) – all from Italy; *V.*

145   *darevskii* Vedmederja, Orlov & Tuniyev, 1986 – Turkey; *V. eriwanensis* Reuss, 1933 –

146   Iran; *V. kaznakovi* Nikolsky, 1909 – Georgia, Genbank: FR727103, FR727034; *V.*

147   *kaznakovi* Nikolsky, 1909 – Russia, GenBank: KC176736; *V. dinniki* Nikolsky, 1913 –

148   Georgia, GenBank: KC176731, AJ275773; *V. u. graeca* Nilson & Andrén, 1988 –

149   Greece, GenBank: FR727087, FR727018; *V. anatolica* REF – Turkey, GenBank:

150   KC316113; *V. seoanei* REF – Spain, GenBank: AJ275782, DQ186030, FR727035,

151   DQ185984; DQ185938; and *Macrovipera lebetina* (Linnaeus, 1758) – Turkey), most

152   obtained from GenBank, were used as outgroups. Sequences were aligned by eye. The

153   appropriate model of sequence evolution was determined using the program JModelTest

154   v2.1.5 (Darriba et al., 2012). The chosen model was applied to the data matrix in order to

155   produce maximum likelihood (ML) estimates using PHYML v3.0 (Guindon & Gascuel,

156   2003). Maximum parsimony (MP) and Neighbour Joining (NJ) analyses were performed

157   using MEGA v6.06 (Tamura et al., 2007) with the model suggested by JModelTest.

158   Robustness of the trees was assessed through bootstrap resampling with 1,000 repetitions.

159   In addition, Bayesian inference analysis was done with the software MrBayes v3.12

160   (Huelsenbeck & Ronquist 2001) using the GTR+I+G model of substitution. The analysis

161   was run with four chains of 5x 10^6 generations, and sampling was performed every 100

162   generations. The first 10% of trees was discarded (burn-in) after the control that the runs

163   were stable using Tracer v1.6 (Rambaut et al., 2014), and the analyses done four times to

164   avoid local optima (see Huelsenbeck & Imennov 2002). The genetic p-distances between

165   the different species were generated with MEGA.

166   A Bayesian implementation of the Poisson Tree Processes (bPTP; Zhang et al.,

167   2013) model was accessed through the web interface available at http://species.h-its.

168   org/ptp/. PTP is a single-locus species delimitation method using only nucleotide

169   substitution information, implementing a model assuming gene tree branch lengths

170   generated by two independent Poisson process classes (within- and among-species

171   substitution events). The bPTP analysis was run using 100,000 MCMC generations, with

172   a thinning of 100 and burn-in of 0.1. The bPTP model can be used to delimit phylogenetic

173   species in a similar way to the popular and widely used General Mixed Yule Coalescent

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174 (GMYC) approach (Pons et al., 2006), but without the requirement for an ultrametric tree

175 (Zhang et al., 2013), eliminating the possible errors due to time calibration.

176   Additionally, two nuclear protein-coding loci (BACH1; Townsend et al., 2008

177 and RAG1; Townsend et al., 2004) were used to investigate the genetic variability within

178 the nuclear genome. PCRs and sequencing were conducted with the same protocol as for

179 the mtDNA, with, respectively, the primers F_Nik_Bach1 and R1_Bach1 (Stümpel,

180 2012), and the primers Rag1_F1 and Rag1_R1 (Stümpel, 2012), and the PCR

181 amplification cycles following Stümpel (2012). For this aspect, four individuals were

182 investigated: one individual from Piemonte, and one sample each of the following

183 species: *V. berus*, *V. a. francisciredi* and *V. a. ammodytes* (similar individuals as for the

184 mtDNA analysis). Sequences were deposited in GenBank (no XXXX - to be completed

185 on publication). The relationship between the different sequences were displayed using

186 TCS 1.21 (Clement et al., 2000) with a parsimony connection set up to 90% for BACH1

187 and to 95% for RAG1. Genetic differentiation was evaluated with p-distance using

188 MEGA.

189   Given the extinction risk of the taxon herein described, we have decided not to

190 collect any individuals from the wild, and to use the specimens deposited in the Natural

191 History Museum 'Giacomo Doria' of Genova as type specimens, while tissues samples

192 used for DNA analysis are deposited at the Muse, the Science Museum of Trento, Italy.

193

194 **Results**

195

196 Phylogeny

197 The best model selected by JModelTest was GTR+G (freq A = 0.3065 freq C = 0.2830;

198 freq G = 0.1187; freq T = 0.2919; R(a)=3.68; R(b)=47.2; R(c)=7.22; R(d) = 5.71;

8

199   R(e)=33.7; R(f) = 1.00; gamma shape = 0.210). All genetic reconstruction methods

200   demonstrated that the adder from Piemonte region belongs to a completely isolated

201   cluster, which is not related to any *V. berus* samples (Figure 1). The reconstruction for

202   each gene separately analysed also confirmed this conclusion (Supplementary material

203   Figure 1). Indeed, these snakes are genetically more closely related to the *V. ursinii*

204   complex than to the *V. berus* complex (Table 1). The adder from Piemonte appears more

205   closely related to the cluster regrouping *V. dinniki*, *V. kaznakovi* (from Georgia) and *V.*

206   *darevskii* even if the bootstrap support is limited. It is thus likely that the split between *V.*

207   *ursinii*, *V. darevskii-V. kaznakovi* and the adder from the Piemonte occurred during a

208   similar period. The p-distances between the adder from the Piemonte and the other vipers

209   used in this study are 3.97% with *V. eriwanensis*, and *V. ursinii ursinii*, 4.24% with *V.*

210   *darevskii*, 5.36% with the different clades of *V. berus,* and 14.8% with *V. ammodytes*

211   (Table 1). The Bayesian implementation of the Poisson Tree Processes (bPTP) species

212   delimitation model supports the status of full species for the adder population of Piemonte

213   (see Figure 1).

214   Examination of the nuclear genes demonstrated very different sequences between the

215   different analysed species (Figure 2). The genetic p-distance between *V. berus* of the

216   Piemonte and *V. berus* is 0.125% for BACH1 and 0.302% for RAG1; moreover, no

217   identical alleles have been detected between the adder from Piemonte and the other

218   analysed species.

219

220   **Fig. 1 & 2**

221

222   Genetic variability within the adder population from Piemonte

223 The analysis of the complete cytb of 23 samples produced three different haplotypes (max

224 2 mutations, 0.26% differences). The analyses of the 16S, cytb, CR and ND4 was

225 conducted on six individuals regrouping the three different haplotypes and all regions and

226 produced a combined sequence of 3254 bp with a maximal divergence of 2 bp (0.0092%)

227 within all vipers from Piemonte. From the six analysed individuals, four distinct

228 haplotypes were found, and three individuals share the same combined haplotype.

229

230 Morphology

231 The PerMANOVA of a set of morphological features, namely subcaudals, crownscales,

232 apicals, perioculars and parietals, revealed significant differences between the two species

233 (F = 11.75, p = 0.0001 in females and F = 4.35, p = 0.0002 in males; see Figure 3 and

234 Supplementary material Table 1). Discriminant analysis correctly classified 94% and

235 88% of females and males respectively. Hence, there are clear morphological differences

236 between individuals of the two species (Figure 4).

237

238 **Fig. 3 & 4**

239

240 Taxonomy

241

242 **Vipera walser** Ghielmi, Menegon, Marsden, Laddaga & Ursenbacher sp. nov. (Figure

243 1–4)

244

245 **Holotype.** Adult female: MSNG34485, collected in S. Giovanni d'Andorno, on the road

246 to Oropa in the Biella prealps, at about 1,300 m a.s.l. by A. Rosazza in the summer of

247 1930 (Figure 5).

10

248

249 **Fig. 5**

250

251 **Paratypes**. One adult male: MSNG33638M collected at Monte Rosso del Croso, on 30

252 August 1933. One juvenile male: MSNG33637B and one subadult male: MSNG30818C

253 collected at Alpe Finestre by Felice Capra, respectively on 28 July 1930 and 15 August

254 1928. One adult female: MSNG30818A, one subadult female: MSNG30818B, and two

255 juvenile females: MSNG33637C and MSNG33637D collected by Felice Capra at Alpe

256 Finestre between August 1928 and August 1939. One juvenile female: MSNG30286

257 collected by F. Capra at Monte Rosso del Croso on 12 September 1934; one adult female

258 MSNG33637A collected by F. Capra at Alpe le Piane on 5 August 1937; one adult female

259 MSNG41663 collected by A. Margiocco at Piedicavallo in September 1967.

260

261 **Type locality.** San Giovanni d'Andorno, strada per Oropa at 1,300 m a.s.l. in the Alps

262 north of town of Biella, a subrange of the Pennine Alps, Northwestern Italy.

263

264 **Differential Diagnosis.** *Vipera walser* sp. nov. is generally similar to the species of the

265 subgenus *Pelias*, and can be confused with *V. berus*, which co-occurs on the Alps in

266 allopatry (Figure 6, Table 2). The species differs in a generalized higher count of cephalic

267 scales, in particular the ones listed below (*V. berus* in parentheses): Higher number of

268 crown scales: 7-30, mean 17.4 (versus 4-22, mean 13.0); loreals: 4-15, mean 9.36 (versus

269 2-12, mean 6.72) and, to a lesser extent, perioculars: 16-23, mean 19.8 (versus 13-23,

270 mean 18.4) see Table 2. *V. walser*, in contrast to *V. berus*, also shows a marked tendency

271 towards fragmentation of the cephalic large shields: the parietal scales are often

272 completely broken down into several smaller scales: 2-14, mean 6.3 (versus 2-10, mean

11

273 2.4; see also Figure 7). Less commonly, also the frontal scale is fragmented into smaller

274 scales. Some individuals exhibit a dorsum of the head covered in small, irregular scales,

275 like in *V. aspis*. *V. walser* has between 1.5 and 2 rows of subocular scales on both sides

276 of the head in 85% of the analyzed specimens (*V. berus* has typically one row of

277 suboculars, with the exception of some populations in the southern Alps). The dorsal

278 zigzag is often broken down into separate bars as in *Vipera aspis* (Linnaeus, 1758) or

279 *Vipera berus bosniensis* (see Figure 6). Despite the lack of a strictly diagnostic

280 morphological character, *V. walser* can be readily distinguished from populations of *V.*

281 *berus* from central and northern Europe by a combination of several characters (e.g. the

282 number of subocular scales, fragmentation of parietals and number of apicals).

283 Identification based solely on observation of external morphology is less obvious if

284 individuals of *V. berus* from Southern Alps are considered. Despite this, the species can

285 be identified unambiguously through a discriminant analysis based on the set of analyzed

286 characters in 94% and 88% of the females and males respectively (see Figures 2 and 3).

287 The mean p-distance, based on a combined dataset of about 3,000 base pairs of

288 mitochondrial genes, between *V. berus* and *V. walser* is 5.36%. Based on our current

289 knowledge of its distribution, *Vipera walser* is restricted to the Alps north of town of

290 Biella, a subrange of the Pennine Alps, west of the river Ticino, northwestern Italy (Figure

291 8).

292 　　　　Summary of the differences in cephalic scale count between *Vipera walser* and *V.*

293 *berus* (see also Table 2): Crown scales (females: $t_{45,49} = 4.81$, $p < 0.0001$; males: $t_{28,71} =$

294 5.20, $p < 0.0001$); loreals (females: $t_{94,59} = -7.52$, $p < 0.0001$; males: $t_{62,67} = -4.43$, $p <$

295 0.0001); and, in females only, perioculars (female: $t_{64,16} = 5.33$, $p < 0.0001$; males: $t_{17,25}$

296 $= -0.16$, $p = 0.87$) and apicals (females: $t_{32,86} = 2.14$, $p = 0.04$; males: $t_{18,08} = -0.12$, $p =$

297   0.91; the number of scales between the eyes and the supralabials are higher (females: $t_{66,40}$

298   = 5.85, p < 0.0001; males: $t_{37,93}$ = 7.90, p < 0.0001).

299

300   **Paratype variations.**

301   Details and meristics for the analysed individuals, including the type series, are

302   summarized in Table 3.

303

304   **Table 2 & 3.**

305

306   **Description of the holotype.**

307   Adult female conserved in 70% EtOH in rather good condition, with the body slightly

308   swollen probably due to preservation. Snout-vent length (SVL) 515.2 mm, tail 55.0 mm,

309   ratio of tail proportion (TL/SVL) 0.107. Two apical scales in contact with the rostral.

310   Head oval shaped, wider in the temporal region, neck not very distinct, snout rounded.

311   Frontal single, and larger than any other scale on head, five parietals. Rostral slightly

312   higher than broader; nasal roundish, nostril circular and approximately in the centre of

313   the nasal; one internasal on left side of the head and two on right side; perioculars 11–10;

314   two rows of suboculars on both sides of the head; circumoculars separated from nasals

315   by six and five loreal scales respectively on right and left side; supralabials 9–9, the fourth

316   and the fifth below the eye; 147 ventrals; 31 divided subcaudals (excluding spine); anal

317   entire; 21 scale rows at midbody. Dorsum is brown in colour with a continuous and

318   regular darker brown zig-zag. Head is reddish-brown with scattered, faint darker

319   markings, and a more obvious inverted V shaped ornamentation just before the neck.

320   Labials are paler with black markings bordering the edges. A wide black band is present

321   on both sides of the head between the postoculars and the neck. Ventrals are black, with

322 white, scattered speckling along the lower margin of the scales and, more consistently,

323 on both scale extremes by the first row of dorsals.

324

325 **Etymology**

326 *Vipera walser* sp. nov. is named after, and dedicated to, the Walser people with whom it

327 shares an extraordinary beautiful and wild area of the southwestern Alps.

328

329 **Fig. 6**

330

331 **Fig. 7**

332

333 **Discussion**

334 Delineating species boundaries correctly is crucial for the discovery of life's diversity

335 because it determines whether or not different individual organisms are members of the

336 same entity (Dayrat, 2005). Most evolutionary biologists now agree that species are

337 separately evolving lineages of populations or meta-populations, with disagreements

338 remaining only about where along the divergence continuum separate lineages should be

339 recognized as distinct species (Padial et al., 2010). The Mitochondrial Tree–

340 Morphological Character Congruence (MTMC) approach has been formalized by

341 Miralles & Vences (2013) and represents the most common practice in zootaxonomic

342 studies, combining evidence from DNA sequences and morphological data. Integrative

343 taxonomy has been also proposed as a framework to bring together conceptual and

344 methodological developments aimed to describe, classify, and name new taxa (Padial et

345 al., 2010). The integration by congruence approach of integrative taxonomy follows the

346 principle that different lines of evidence should be combined to delimit species, such as

14

347 genetic (mtDNA and nuclear), morphological, distributional and ecological data. The

348 genetic differentiation between *V. walser* and *V. berus*, both on mitochondrial and nuclear

349 DNA, is beyond known values between well-established species within the same

350 subgenus. The status of full species is further confirmed by the bPTP analysis, and as a

351 morphological line of evidence by the discriminant analysis. Furthermore, there is no

352 evidence of introgression from, for example, *V. berus* as confirmed by the numerous

353 individuals analysed for mtDNA, and the strong difference between these two species on

354 the two nuclear genes sequenced. The species, within the alpine context, inhabits an

355 ecologically peculiar area, characterized by some the highest rainfall of the whole alpine

356 region (Mercalli et al., 1998).

357   The discovery of the *V. walser* lineage was particularly unexpected, especially in

358 this biologically well-known and densely sampled region of Western Europe. The species

359 shows closer genetic affinities with, on one hand, *V. darevskii* and *V. kaznakovi*, species

360 occurring in the Caucasus and, on the other, with the *V. ursinii* complex (see Table 1),

361 than with the *V. berus* complex. Limited phylogenetic support suggests a simultaneous

362 split between *V. ursinii* complex, *V. kaznakovi* (Georgia) complex and *V. walser* (possible

363 trichotomy). Moreover, the ML phylogenetic reconstruction regrouped *V. walser* with the

364 *V. kaznakovi* (Georgia) complex, whereas the genetic distance displayed more affinities

365 with the *V. ursinii* complex.

366   Until now, it was believed that Western Europe was colonised from the *Pelias*

367 subgenus only by *V. berus* (including *V. seoanei* Lataste, 1879, restricted to the Iberian

368 peninsula), and the *V. ursinii* group, which occupy distinct habitats (cold forest for *V.*

369 *berus* and steppe areas for *V. ursinii*; Saint Girons, 1980). The presence of a new distinct

370 lineage, more related to the Caucasian vipers, strongly suggests an additional, more

371 recent, colonisation of Western Europe (from the *V. kazankovi*-complex or during the

15   1

372　split between the *V. kaznakovi* complex and *V. ursinii* complex) than the one involving

373　the *V. berus* group, and possibly one that was concurrent with that of *V. ursinii* (Early

374　Pliocene; Ferchaud et al., 2012).

375　　　　Given that the European viper species tend to exclude each other geographically,

376　resulting in limited portions of overlapped ranges (Saint Girons, 1980), we can assume

377　that *V. walser* found refugial areas different from those of *V. berus* during the numerous

378　glaciations of the Pleistocene. Currently, both *V. berus* and *V. walser* seem to occupy very

379　similar habitats, suggesting a possible competition (or ecological differentiation as that

380　between *V. aspis* and *V. berus*; Guillon et al., 2014). It is, however, possible that, like *V.*

381　*kaznakovi*, *V. walser* can tolerate warmer temperatures than can *V. berus* so long as

382　sufficient humidity is present. However, this possibility needs to be investigated as it

383　could have important implications for future conservation programmes.

384

385　Near-future threats and conservation

386　*V. walser* appears to occur only in a very limited area in the Alps north of Biella (Figure

387　8). It is very likely that all native populations of adder south of the Alps and west of the

388　river Ticino belong to the species herein described. Based on the Italian Atlas of

389　Amphibians and Reptiles (Sindaco et al., 2006), the current distribution area ('extent of

390　occurrence') is almost certainly < 1,000 km^2. Consequently, *V. walser* should be

391　classified as 'Endangered' according to IUCN Red List criteria B1a/B2a. If we consider

392　that the population is strongly fragmented, or that the actual area of occupancy is probably

393　< 500 km^2 and fragmented (IUCN Red List Categories and Criteria: Version 3.1. Second

394　edition), then *V. walser* appears to be among the most threatened vipers in the world. The

395　new taxon's sister species *V. darevskii*, with area of occupancy < 10 km^2, is now listed as

396　'Critically Endangered' (Tuniyev et al., 2009), whereas *V. kaznakovi* (related to *V.*

16

397   *darevskii* and thus to *V. walser*) is considered 'Endangered', meaning that the entire clade

398   is highly threatened with extinction.

399

400   **Fig. 8**

401

402   Within its restricted range, *V. walser* appears to be quite common in suitable

403   habitat. However, to date, no systematic survey has been undertaken, either to estimate

404   its population density or identify its habitat requirements. Such surveys are clearly a

405   priority for the future research. Estimates of current abundance, using mark-recapture or

406   distance sampling (e.g. Mazerolle et al., 2007) would be useful to determine total

407   population size and trends, and to more precisely assign the species to a Red List category.

408   Occupancy modelling (Larson, 2014) might also be suitable to determine areas of

409   occupancy at appropriate scales.

410   Perhaps more important would be detailed studies of the species' precise habitat

411   requirements, to determine how past and current land use changes have affected the

412   species, and how they might be altered to benefit the species in the near future. Based on

413   our preliminary observations, this species inhabits open areas, often with rocky outcrops

414   (Figure 9), and may not tolerate woodland unless it is very sparse. European mountains

415   experienced a long period of agricultural/agropastoral expansion from the Late Middle

416   Ages to the 19th Century, with large areas of the Alps converted to upland grasslands and

417   heathlands (e.g. Vives et al., 2014). These open landscapes were presumably beneficial

418   for *V. walser*. However, the decline in agropastoral activities in the last 100 years and

419   associated afforestation (Carlson et al., 2014; Garbarino et al. 2014) is probably the

420   greatest threat to the species, and it is an urgent priority to assess such changes within the

421   range of *V. walser*. More immediate and major threats in the short term are culling and

17

422 collection. Indeed, the description of several new vipers species (e.g. *V. kaznakovi* and

423 *Montivipera wagneri* (Nilson & Andrén, 1984)), as well as the attraction of being peculiar

424 and rare (e.g. *Macrovipera schweizeri* (Werner, 1935)) have led to the illegal collection

425 of numerous individuals for the international pet trade (Nilson et al., 1999,

426 http://www.iucnredlist.org), causing local extinctions. Because this species occurs only

427 in Italy, we strongly suggest that a specific legal protection for the species should be

428 implemented very quickly.

429

430 **Fig. 9**

431

432 Longer term prospects and climatic change

433 Of course, it can be argued that *V. walser*, as a restricted-range relic species, is likely

434 heading down an evolutionary dead end path (Allendorf and Luikart, 2007), in the sense

435 of Darwin's "wreck of ancient life" (Darwin, 1859), or Jeannel's "fossiles vivants"

436 (Jeannel, 1943). Its eventual natural extinction may take many millennia, but its ability to

437 survive the next 100 years may hang on two important aspects of its biology. First, there

438 is a real lack of genetic variability within the population as compared to that in other

439 vipers (e.g. Ferchaud et al., 2011; Ursenbacher et al., 2006). The population is already

440 fragmented into two main subpopulations, and, presumably, the complex topography of

441 ridges and valleys may work to further isolate populations, as in *V. berus* (Ursenbacher

442 et al., 2009). A high priority for future study is an examination of habitat suitability at the

443 landscape scale coupled with research on dispersal mechanisms and ability in the species.

444   Second, and related to the above, its ability to withstand or adapt to climatic

445 change expected to take place within its range will be crucial. The current habitat of *V.*

446 *walser* is restricted to an area of around 800 km^2 within a few valleys, which experience

18

447 some of the highest rainfall in the Alps (Mercalli et al., 1998). Point estimates of annual

448 rainfall from presence locations within its area of occupancy range from 1,018 to 1,604

449 mm (mean = 1,348 mm ± 111 SD) and mean minimum temperature between May and

450 October from 3.1 to 10.0 ^{0} C (mean = 6.1^0 ± 1.2 C SD). Climate models (CMIP5 IPPC

451 Fifth Assessment; www.worldclim.org) indicate that in the next 20 years, these valleys

452 will become far wetter (mean = 1,536 mm ± 129 SD) and warmer (mean = 8.5 ± 1.2^0 C

453 SD; Figure 10). Consequently, species distribution modelling, and how this distribution

454 might change under realistic climate change scenarios, especially including the influence

455 of habitat and habitat change and dispersal ability (e.g. Pearson & Dawson, 2003), is

456 clearly a priority.

457

458 **Fig. 10**

459

460 Conclusion

461 The present study described and named a new viper species, *V. walser*, which shows

462 strong genetic and morphological differentiation from all other known European viper

463 species. The new taxon occurs in a restricted area of the southwestern Italian Alps and

464 shows close affinities with the Caucasian species *V. dinniki*, *V. darevskii* and *V.*

465 *kaznakovi*, opening unexpected and interesting biogeographic scenarios. The very small

466 extent of occurrence of the new species implies a particularly high threat level, and thus

467 conservation managements should be developed. The protection of its habitat, the

468 limitation of the forest regrowth, but also the evaluation of its likely future distribution

469 given climatic changes (for the long term) or struggle against culling (short term) are key

470 elements to investigate. Involvement of local authorities, foundations and other

471 stakeholders will be crucial in realising effective protection of this species.

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739　Table 1. Genetic distance (p-distance) between *Vipera walser* nov. sp. and the different

740　taxa of European vipers calculated on 3254 bp of the mtDNA (see Material and methods

741　for more details about the genes analysed)

species	p-distance
V. u. ursinii	3.97%
V. eriwanensis	3.97%
V. darevskii	4.24%
V. dinniki	4.76%
V. kaznakovi (Russia)	5.34%
V. berus	5.36%
V. u. graeca	5.60%
V. kaznakovi (Georgia)	5.62%
V. seoanei	5.82%
V. anatolica	6.76%
V. aspis	8.73%
V. ammodytes	10.06%
M. lebetina	13.46%

742
743

744 Table 2. Mean sizes, general and head scalation of *Vipera walser* sp. nov. and other

745 related species, with standard deviations and minimal/maximal values, when provided.

746 Origin of the data: 1) this study; 2) Ursenbacher et al., 2005; 3) Scali & Gentilli, 1998; 4)

747 Joger & Stümpel, 2005; 5) Nilson et al., 1995; 6) Orlov & Tuniyev, 1990; 7) Geniez &

748 Teynié, 2005; 8) Göçmen et al., 2014; 9) Avcý et al. 2010. The number of males and

749 females are indicated except for the last two species, where data have been gathered from

750 studies and the information were not available.

3

	Vipera walser sp. Nov [1]		Vipera berus (Italian clade) [1]		Vipera berus (Italian clade) [2]		Vipera berus (Po Plain) [3]		Vipera berus (Northern clade) [2]		Vipera berus bosniensis [4]		Vipera kaznakovi [4] [5] [6]		Vipera darevskii recalculated from [6] [7] [8] and [9]	
	female (31)	male (17)	female (70)	male (65)	female (35)	male (28)	female (13)	male (11)	female (54)	male (47)	female	male	female	male	female	male
ventral scales	148.50 ±3.45 141-156	143.33 ±3.28 138-149	145.13 ±4.96 131-165	142.68 ±4.49 131-158	145.77 ±3.23 137-153	142.43 ±3.73 134-149	145.9 ±1.3 140-154	142.1 ±1.1 136-148	147.15 ±3.55 139-155	144.11 ±3.54 135-152	144.90 ±2.81 139-159	141.75 ±3.19 136-149	136.2 ±2.60 130-139	135.0 ±1.81 133-139	136.5 ±4.4 132-144	134.6 ±3.5 129-136
subcaudal scales	27.96 ±2.41 23-32	35.06 ±2.41 30-38	28.39 ±3.28 22-41	35.05 ±3.27 27-43	27.63 ±2.13 23-34	34.36 ±2.39 29-39	33.8 ±1.1 29-42	41.5 ±1.1 35-47	30.50 ±3.59 21-39	36.82 ±2.71 30-42	36.87 ±2.71 32-42	28.60 ±2.18 24-32	28.4 ±1.69 26-32	33.6 ±2.80 23-41	28.9 ±2.76 25-33	33.4 ±3.68 29-38
loreals (both side)	9.45 ±2.16	9.19 ±1.72	7.29 ±2.29	6.12 ±2.18	6.71 ±2.47	5.89 ±2.60			5.54 ±2.40	4.47 ±1.99			11.06 ±3.13			
perioculars (both side)	20.00 ±1.51	19.50 ±1.83	18.20 ±1.69	18.55 ±1.94							18-22		20.0 ±1.98		18.04 ±1.55	
apicals	2.29 ±0.74	1.94 ±0.43	2.00 ±0.24	1.95 ±0.21									1.50 ±0.54		1.57 ±0.5	
crownscales	18.07 ±4.41	16.00 ±2.30	13.70 ±3.51	12.26 ±3.27									14.94 ±3.79		7.5 ±2.4	
subocular ranks	1.55 ±0.30	1.50 ±0.16	1.14 ±0.31	1.09 ±0.23												
total length (mm)	455.56 ±167.1	386.00 ±50.83	491.83 ±71.79	451.88 ±71.25			420.0 ±37.1	437 ±21.3					479.4 ±45.8	466.4 ±40.4	382.1 ±46.7	376.8 ±32.0
tail length (mm)	43.42 ±17.69	50.83 ±14.16	52.27 ±8.75	60.02 ±10.19			56.6 ±5.7	76.3 ±5.0					52.0 ±6.9	64.0 ±5.7	45.1 ±6.3	55.1 ±5.3
% tail	9.90% ±0.70%	12.8% ±1.09%	10.7% ±1.25%	13.3% ±1.54%			11.9%	14.9%					10.8%	13.7%	11.8% 1.10%	14.7% 1.71%

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ID	ID locality	age	Total length (in mm)	Tail length (in mm)	Crown scales	Rostral	Loreals (mean)	Perioculars (sum left+right)	Suboculars (mean)	apicals	Frontal	Parietals (sum left+right)	Subcaudals	Ventrals	MSR
M1	v.stronam1	ad			14	1	4	20	1,5	2	1	3	36		
M2	v.stronam2	ad			18	1	4,5	21	1,5	2	2	3	36		
M3	v.elvom1	ad			10	1	4,5	16	1,5	2	1	9	34		
M4	v.olocchiam1	ad			14	1	5,5	22	1,25	2	1	2	38		
M5	v.mastallonem1	ad			17	2	4	20	1,25	2	1	6	31		
M6	v.dolcam1	juv			20	1	5,5	21	2	1	1	3	38		
M7	a.meggianam1	ad	410	61	17	2	5,5	21	1,5	2	1	4	38	142	22
M8	oropam1	ad					5			3		7			
M9	v.elvom2 (MRSN)(MZUT R 2069)	ad			16	1			1,5	2	1	7	36		
M10	m.rossodelcrosom1(MSNG33638M)	ad	481	61	15	1	2,5	18	1,5	1	1	2	33	149	21
M11	cimarascàm1(MSNG32286)	ad	480	60				19	1,5	2	1	2	35	143	21
M12	a.finestrem1(MSNG30818C)	subad.	306	40	15	1	3,5	16	1,5	2	1	2	37	143	21
M13	a.finestrem2(MSNG33637B)	juv	224	27	16	1	4,5	19	1,5	2	1	2	36	147	21
M14	sesseram1(MISN N° cat.2)	ad			17	1	5,5	19	1,5	2	1	6	36	138	21
M15	v.stronam3	ad	505		16	1	4,5	18	1,5	2	1	2	33	141	19
M16	v.stronam4	juv	210		17	1	5	22	1,5	2	1	4	34	142	21
M17	v.riobachm1	ad	472	56	16	1	5,5	20	1,5	2	1	4	30 (28-32)	145	21
F1	v.chiobbiaf1	juv			21	1	6	21	1,5	2	1	5	31		

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F2	v.stronaf1	ad			17	1	4,5	21	1,5	2	1	9	26		
F3	v.stronaf2	ad			20	1	5,5	21	2	3	1	3	27		
F4	v.masttallonef1	juv			19	1	5,5	19	1,625	2	1	4	30		
F5	v.stronaf3	subad.			26	1	5	23	1,5	2	1	10	27		
F6	v.stronaf4	ad			25	1	7,5	22	2	4	1	13	27		
F7	v.dolcaf1	ad			16	1	6	19	1,5	1	1	12	32		
F8	v.mastallonef2	juv			16	1	4	20	1,5	3	1	8	27		
F9	v.mastallonef3	ad			18	1	4	20	1,5	2	1	6	29		
F10	v.elvof1	ad	610	65	18	1	5	18	1,5	3	2	14	32	148	21
F11	v.vognaf1	ad	527	55	13	1	4,5	18	1	1	1	2	25	149	21
F12	v.vognaf2	ad	548	59	13	1	4,5	21	1,5	2	1	4	28	150	21
F13	a.lepianef1(MSNG33637A)	ad	588	59	14	1	2	19	1,5	2	1	2	29	147	21
F14	s.giovannidandornof1(MSNG34485)	ad	570	55	16	1	5,5	21	2	2	1	5	31	147	21
F15	a.finestref1(MSNG30818B)	subad.	263	27,5	17	1	5	20	2	2	1	4	29	148	21
F16	m.rossodelcrosof1(MSNG30286)	juv	232	24,5	17	1	4,5	22	1,5	2	1	6	27	147	21
F17	a.finestref2(MSNG33637C)	juv	213	21	20	1	4,5	19	1,5	2	1	2	28	154	19
F18	a.finestref3(MSNG33637D)	juv	191	17,5	16	1	2,5	20	1	1	1	6	23	149	21
F19	v.sesiaf1(MSNG2171A)	ad	593	55	7			17	1	1	1	3	30	156	21
F20	r.valdobbiaf1(MSNG2171B)	juv	219	22				20	1,5	2	1		29	151	23
F21	a.finestref4(MSNG30818A)	ad			19	1	5	18	1,5	3	1	6			
F22	oropaf1	ad			17	1	5,5	19	2	3	1	3			
F23	oropaf2	juv			19	1	5	23	2	3	1	6			
F24	sesseraf1	ad			15	1	3	20	1	2	1	3			
F25	v.stronaf5	ad	535	50	17	1	4	18	1,5	3	1	9	30	148	21
F26	v.stronaf6	ad	520		20	1	5,5	21	2	3	1	3	27	145	
F27	v.stronaf7	ad	460		22	1	4	20	1,5	3	1	7	24	141	
F28	v.stronaf8	ad			16	1	5,5	20	1,5	3	1	7	27	151	
F29	v.stronaf9	ad	580		21	1	4,5	22	1,5	3	2	4	26	145	21
F30	sesseraf2	ad	640	54	14	1	4,5	19	1,5	2	1	2	24	146	
F31	piedicavallof1(MSNG41663)	ad			16	1	4,5	19	1,5	2	1	10	30	151	

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