1   # Climate change promotes hybridisation between deeply divergent

2   # species

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16

17 **Abstract**

18 Rare hybridisations between deeply divergent animal species have been reported for decades in

19 a wide range of taxa, but have often remained unexplained, mainly considered chance events

20 and reported as anecdotal. Here, we combine field observations with long-term data concerning

21 natural hybridizations, climate, land-use, and field-validated species distribution models for two

22 deeply divergent and naturally sympatric toad species in Europe (*Bufo bufo* and *Bufotes viridis*

23 species groups). We show that climate warming and seasonal extreme temperatures are

24 conspiring to set the scene for these maladaptive hybridisations, by differentially affecting life-

25 history traits of both species. Our results identify and provide evidence of an ultimate cause for

26 such events, and reveal that the potential influence of climate change on interspecific

27 hybridisations goes far beyond closely related species. Furthermore, climate projections suggest

28 that the chances for these events will steadily increase in the near future.

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Introduction

Hybridisation is a widespread phenomenon in nature (Mallet, 2005). However, its frequency, diversity of outcomes, underlying mechanisms, its role in the evolutionary process, and how to deal with it in conservation biology have been controversial topics for more than a century (Arnold, 2006; Schwenk et al., 2008). Much of our knowledge about the link between hybridisation dynamics in animals and climate changes, comes from studies of hybrid zones (Hewitt, 2011), where the reshuffling of species' ranges in response to changing climates brought into contact closely related and previously allopatric species. Pre-mating reproductive barriers could be incomplete between these species, and their genomes could still be porous to introgression, with several far reaching implications (Mallet, 2005; Arnold, 2006; Schwenk et al., 2008; Hewitt, 2011). Not surprisingly, species of ancient divergence and with a long-lasting history of sympatry have contributed the least to this body of knowledge (Mallet, 2005; Schwenk et al., 2008). These species have had ample opportunity to evolve strong pre-mating reproductive barriers, either as a by-product of a longer allopatric divergence or because of character displacement in response to natural selection (Coyne & Orr, 2004; Pfennig & Pfennig, 2009). Consequently, hybridisation events are extremely improbable between these species (e.g. Proietti et al., 2014), and their observation incidental in the wild.

We witnessed to one such event in southern Italy (on May 10th 2014) between two toad species, the common toad *Bufo bufo* and the green toad *Bufotes balearicus* (Figure 1). They belong to the *Bufo bufo* and *Bufotes viridis* species groups, whose divergence has been estimated to the Oligocene (around 20-30 million years ago; Maxson, 1981; Garcia-Porta et al., 2012), and which have largely overlapping distributions in central, eastern, and southern Europe

54 (Sillero et al., 2014). Although syntopy is not uncommon, especially in lowland areas, they

55 show distinct spatio-temporal patterns of habitat use (reviewed in Lanza et al., 2006), making

56 hybridisation at least three-times unexpected. First, they show markedly different breeding

57 phenologies: *Bufo bufo* is an early and explosive breeder, while *Bufotes balearicus* is a late and

58 prolonged breeder (Lanza et al., 2006). In Italy, breeding activities of *Bufo bufo* begin earlier in

59 the year (early winter to early spring, with variation among sites at different altitude and

60 latitude) and usually last 1–2 weeks. *Bufotes balearicus* starts breeding later (middle to late

61 spring), and this activity may last 2–3 months. Furthermore, breeding activities in syntopic

62 areas have been systematically reported as asynchronous, regardless of intraspecific differences

63 between sites (Lanza et al., 2006). Second, *Bufo bufo* and *Bufotes balearicus* display differences

64 in their altitudinal distribution. *Bufo bufo* breeding sites commonly occur from 0 to 2000 m

65 above sea level (MASL), while *Bufotes balearicus* shows marked preferences for sites in

66 lowland areas, rarely being observed above 1000 MASL (Romano et al., 2003; Spilinga et al.,

67 2007). Third, in spite of the largely polytopic habits of these two species, differences exist in

68 habitat and breeding site preferences. *Bufotes balearicus* favours open areas and bushlands and

69 breeds in temporary shallow waters, and *Bufo bufo* most commonly inhabits forested habitats

70 while using slow running or deeper, wider standing waters as breeding sites.

71 Remarkably, based on previous assessments (Carpino & Capasso, 2008), all these

72 differences applied to toad populations at our study site as well. This site is a high-altitude pond

73 located at the margins of a forested area (latitude: 40.9429° N; longitude: 14.7096° E; altitude:

74 1330 MASL; Figure 2). *Bufo bufo* was reported to breed at this site on February, whereas

75 *Bufotes balearicus* was absent here and in neighbouring areas above 800 MASL at least until

76 the year 2007, the year of the last herpetological assessment (Carpino & Capasso, 2008).

77 Here, we combine our field observation with data from previous reports of similar

78 hybridisation events for the *Bufo bufo* and *Bufotes viridis* groups in Europe along the last

79 century, in order to study the causation of these 'improbable' events To this end, we examined

80 the contribution of multiple factors, including all those commonly invoked to explain novel

81 interspecific hybridisations among animal species in the wild.

82

Materials & Methods

84

Assessing natural hybridisation

86 We sampled and carried to the laboratory a strip approximately 1.5 m in length from the clutch

87 laid by the hybrid pair on May 10th 2014. We visually searched for additional clues of

88 hybridisation at the breeding pond for the subsequent ten days. Although no further

89 heterospecific pairs were observed, we collected and carried to the laboratory two additional

90 egg-strings newly laid by unobserved parents. Fieldwork was approved by the Italian Ministry

91 of Environment (permission number: 0042634 dated 7 August 2013).

92 In order to confirm the hybrid nature of the egg-string laid by the heterospecific pair

93 (against the hypotheses of unfertilised eggs and of undetected homospecific paternity) and to

94 address the parental species of the other egg-strings, we monitored egg and tadpole

95 development under laboratory conditions and analysed the pattern of variation of individual

96 larvae at diagnostic genetic markers. Tadpoles were reared under standardised light and food

97 conditions, in plastic boxes ($0.8 \times 0.5 \times 0.2$ m) filled with oxygenated tap water. Larval

98 mortality was checked twice daily, from hatching to metamorphosis.

99   Tadpoles of *Bufo bufo* and *Bufotes balearicus* can be distinguished by larval morphology

100   (Ambrogio & Mezzandri, 2014) while hybrid tadpoles are usually heavily malformed

101   (Montalenti, 1932; Montalenti, 1933). However, in order to achieve correct identification and to

102   verify the absence of backcrosses between hybrids and parental individuals, we analysed

103   genetic variation at the following allozyme loci: malate dehydrogenase (Mdh-1 and Mdh-2; EC

104   1.1.1.37), isocitrate dehydrogenase (Icdh-1 and Icdh-2; EC 1.1.1.42), and malate dehydrogenase

105   NADP+-dependent (Mdhp-1; EC 1.1.1.40). Fifty tadpoles from each egg-string were euthanised

106   using a 200 mg/L solution of MS222, 10 days after hatching, and stored at -80°C until

107   subsequent analyses. The diagnostic value of each allozyme locus was verified through

108   preliminary analyses of 20 individuals per species, sampled from two sites in neighbouring

109   areas, where no evidence of potential hybridisation had been observed (*Bufo bufo*: 41.1737° N,

110   14.5834° E; *Bufotes balearicus*: 40.8866° N, 14.9318° E). Standard horizontal starch gel

111   electrophoresis and zymogram visualisation were carried out, following previously published

112   standard protocols (Harris & Hopkinson, 1976).

113

114   **Quantifying anthropogenic habitat change**

115 In order to assess anthropogenic habitat change as a possible explanation for the hybridisation

116   event we registered, we performed a land use change analysis considering an area of 119 km^2,

117   inside the Partenio Regional Park (Regione Campania, Italy). The clearing where the

118   hybridisation event was registered is located in the middle of the study area, approximately 15.6

119   km from the northern boundary and 2 km from the south-eastern boundary (Figure 2). We

120   obtained a map of the area from Google Earth Pro 7.1.5.1557 (Google Inc., Mountain View,

121   California). By using the "historical imagery" tool, and keeping the extent and resolution of the

122   map constant, we selected two images: one from 10 October 2014, five months after the

123   hybridisation event, and one from 31 October 2006, a few months before the last assessment

124   that confirmed the absence of *Bufotes balearicus* from the site (Carpino & Capasso, 2008). The

125   two images were imported into ArcGIS 10.3.1 (ESRI ©), with a resolution of 4.5 m per pixel,

126   and were georeferenced using administrative boundaries as reference points (RMS error = 6.23

127   m for the 2014 image; RMS error = 4.48 m for the 2006 image). The images were interpreted

128   using direct recognition (Campbell, 1978), considering five discrete land use classes that hold a

129   clear ecological importance for both toad species: agriculture, forests, natural clearings, natural

130   vegetation (other than forests), and artificial areas. For both images, a vector layer (format

131   shapefile, ESRI ©) was produced at a 1:25,000 scale. To perform the land cover change

132   analysis, following Falcucci et al. (2007), each vector layer was transformed into a raster layer

133   using 4 different pixel resolutions: 25 m, 50 m, 75 m, and 100 m. The change detection analysis

134   was performed for each pixel resolution, resulting in an average percentage change for every

135   land cover class.

136

137   **Climate influence on altitudinal distribution pattern**

138   Based on historical records of occurrence (Carpino & Capasso, 2008), and considering the

139   species' altitudinal distributions in peninsular Italy (Lanza et al., 2007; Guarino et al., 2012), the

140   presence of a *Bufo bufo* population at the study site was expected, whereas the presence of

141   *Bufotes balearicus* was not expected, either at this site or within neighbouring, high altitude

142   areas. Therefore, we focused the following analyses on the latter.

143   To address the plausibility of climate forcing on recent altitudinal distribution changes for

144   *Bufotes balearicus*, we calibrated a correlative species distribution model in peninsular Italy

145   considering the presence of the species across the 21st century. Then we projected the

146   distribution model to the current climate (average over 2007-2013) and to the future (average

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147   2070-2100). The model was calibrated considering six bioclimatic variables theoretically

148   important for the presence of the species: temperature seasonality, mean temperature of the

149   warmest quarter, mean temperature of the coldest quarter, temperature annual range,

150   precipitation seasonality, and precipitation of the coldest quarter. We obtained all climate

151   variables at 1 km resolution from WORLDCLIM (Hijmans et al., 2005), which provides climate

152   layers representative of the 1950–2000 time frame. To obtain the corresponding climate

153   variables for the 2007–2013 time frame, we followed the procedure presented in Maiorano et al.

154   (2013) and considered monthly temperature and precipitation values (spatial resolution equal to

155   50×50 km) from the Climatic Research Unit of the University of East Anglia (database: CRU

156   TS3.22; Harris et al., 2014). To downscale the CRU database to the 1 km^2 of the

157   WORLDCLIM database, we first calculated climate anomalies by contrasting monthly

158   temperature and precipitation values for 2007–2013 against the 1950–2000 climate data, as

159   obtained from the same CRU TS3.22 database. Anomalies were calculated as absolute

160   temperature difference ($\Delta°C$) and relative precipitation differences (% change). By using

161   bilinear resampling, we downscaled the anomalies to $0.0083°$ of spatial resolution ($\approx$ 1km).

162   Then, in order to obtain monthly maps of temperature and precipitation for 2007–2013, we

163   applied the anomaly corrections to the WORLDCLIM climate layers. Finally, we calculated all

164   the derived climate maps mentioned above.

165   The bioclimatic layers for 2070–2100 were obtained directly at the resolution of 1 km^2

166   from the WORLDCLIM database considering 3 emission scenarios (A1B, A2, and B1), and

167   many different global circulation models (24 GCMs for the A1B emission scenario, 19 GCMs

168   for A2, and 18 GCMs for B1) developed under the 4th Assessment Report of the

169   Intergovernmental Panel on Climate Change (IPCC, 2007).

170   To calibrate the models, we used the ensemble forecasting approach (Araújo & New,

171   2007) implemented in BIOMOD, a bioclimatic niche modelling package for the R environment

172   (Thuiller et al., 2009). We used the following eight models: (i) generalised linear models, (ii)

173   generalised additive models, (iii) classification tree analysis, (iv) artificial neural networks, (v)

174   generalised boosted models, (vi) random forests, (vii) flexible discriminant analysis, and (viii)

175   multivariate adaptive regression spline. All models were calibrated over the entirety of

176   peninsular Italy south of the Po river (212,460 km^2), with 350 points of presence for *Bufotes*

177   *balearicus* collected before 2000, plus 10,000 background points (see Supplemental

178   Information S1). All models were evaluated using a repeated split-plot procedure (70% of the

179   data used for calibration, 30% left apart for evaluation; the entire procedure repeated 10 times

180   for each model; Thuiller et al., 2009), and by measuring the area under the receiver operating

181   characteristic (ROC) curve (AUC) (Swets, 1988). All models with AUC values greater than 0.7

182   (Swets 1988) were projected over the entire study area using the 1950–2000, the 2007–2013,

183   and the 2070-2100 climate layers. We measured the minimum probability of presence obtained

184   in correspondence of the available points of presence for 1950–2000, and we used this threshold

185   to define areas of species presence (all areas above this minimum threshold of probability) in all

186   periods considered. Moreover, considering 100 m wide elevation classes, we calculated the

187   elevation-specific average probability of presence for all three periods, and obtained a model of

188   the probability of presence for *Bufotes balearicus* along the elevation range in peninsular Italy

189   (Figure 3).

190   We further investigated the plausibility of a link between climate change and altitudinal

191   shifts by turning the model prediction into a working hypothesis. Based on the model results,

192   we selected a geographic area close to our study site, and carried out field searches for further,

193   unknown sites of occurrence of *Bufotes balearicus*, above 1200 MASL. To select the

194 geographic area, we adopted the following criteria: (i) location on a mountain massif, as close

195 as possible to our study site; (ii) presence of potential breeding sites (e.g., ponds) at altitudes

196 ≥1200 MASL; (iii) presence of *Bufotes balearicus* populations at lower elevations along the

197 same mountain; (iv) absence of obvious anthropogenic habitat discontinuities between low and

198 high altitude areas. Accordingly, we identified the Picentini Mountains (within the Picentini

199 Mountains Regional Park, roughly located 25 Km southeast of our study site) as an area of best

200 fit for our criteria. Field searches began on 2 May 2015, and lasted until the first evidence of

201 *Bufotes balearicus* in the area was found (21 May). The rationale underlying this experimental

202 integration was as follows: although failure to identify new high altitude sites of occurrence

203 would not be strong evidence against a role of climate change in promoting altitudinal shifts at

204 lake Campo Maggiore, or elsewhere, a positive result would provide support for the model

205 prediction, and therefore, support the hypothesis that our initial finding belongs to a suite of

206 events promoted by climate change.

207

208 **Climate influence on breeding phenology**

209 Our observation of the hybrid pair in May 2014 suggests delayed breeding activity of *Bufo bufo*

210 causing an overlap with the normal breeding period of *Bufotes balearicus* (Lanza et al., 2006;

211 Carpino & Capasso, 2008; Guarino et al., 2012). Therefore, subsequent analyses were focused

212 on *Bufo bufo*. Notably, while there is strong evidence for a link between the breeding phenology

213 of *Bufo bufo* and annual temperature cycles (Reading, 1998; Reading, 2003; Tryjanowski et al.,

214 2003), the same does not hold true for species of the *Bufotes viridis* group (including *Bufotes*

215 *balearicus*).

216   A search of academic and grey literature revealed five additional observations of hybrid

217 pairs among representatives of the two species groups in four different locations (Figure 3): two

218 sites located in the Czech Republic (Vlček, 1995; Vlček, 1997; Zavadil and Roth, 1997), one in

219 Sweden (Lang, 1926), and one in Austria (Duda, 2008).

220 The annual activity cycle of *Bufo bufo* populations can be affected by several

221 environmental features, including climate, and the five sites (including our observation) span a

222 wide range of latitudes. Thus, rather than considering average winter temperatures, we based

223 our analysis on the period when this species begins its breeding activity in each area, according

224 to regional atlases and databases (Gilsen & Kauri, 1959; Cabela, Grillitsch & Tiedemann, 2001;

225 Nečas et al., 1997; Guarino et al., 2012;). In addition, previous studies suggested that the

226 beginning of this activity is linked to the average temperatures of the preceding 1–2 months

227 (Reading, 1998). Therefore, in our testing for a link between hybridisation events and climate

228 anomalies, we set the period of interest to the two months preceding the usual start of the

229 breeding activity, for each geographic area. Accordingly, we analysed date ranges covering

230 December to January for the site in southcentral Italy, January to February for the sites in Czech

231 Republic and Austria, and February to March for the site in Sweden.

232 Long-term climate data for our study site were provided, by the Montevergine

233 Observatory (40.9360° N; 14.7288° E), as monthly averages since the year 1884. In order to

234 gain climate data for the four sites of past hybridisation, we searched the NOAA database

235 (available at http://gis.ncdc.noaa.gov) of monthly observational data using the following two

236 criteria: (i) climate station closest to the site of interest and (ii) time series of at least 40 years

237 before the year of the observed hybridisation event. The following stations best matched these

238 search criteria: Koebenhavn Landbohojskolen, Denmark (Id: DA000030380; Latitude: 55.683°

239 N; Longitude: 12.533° E); Praha Klementinum, Czech Rep. (Id: EZE00100082; Latitude:

240 50.090° N; Longitude: 14.419° E); Wien, Austria (Id: AU000005901; Latitude: 48.233° N;

241   Longitude: 16.35° E); OravskaLesna, (Id: LOE00116364; Latitude: 49.366° N; Longitude:

242   19.166° E).

243   For each climatic series retrieved, we analysed bimonthly average temperatures

244   considering the entire temporal series, and the 10 years preceding the hybridisation event, i.e. a

245   time-lapse approximating the average lifetime of a toad in the wild (Lanza et al., 2006).

246   To test the null hypothesis that an association between hybridisation events and

247   temperature anomalies was due to chance alone, we carried out binomial probability tests. We

248   set the probability threshold of a single event to 0.02, based on the highest value calculated for

249   the ratio between year rank (mildest = 1st rank) and climatic series length (i.e. the first out of 47

250   available years from the climatic station DA000030380). Since hybridisation events were both

251   spatially and temporally distant, data independence was assumed. However, to err on the side of

252   caution, we carried out the analyses considering the two observations in eastern Czech

253   Republic, as both independent and fully dependent (i.e. as a single observation); then we took

254   the highest value as the confidence level for accepting/rejecting the null hypothesis stated

255   above.

256   Finally, the paucity of hybridisation events recorded qualifies these events as rare, and

257   testifies to the strength of the pre-mating isolation mechanisms. On the other hand, given such

258   rareness, we cannot exclude the occurrence of potentially unobserved, unreported, or undetected

259   hybridisation events. Thus, we explored how potentially unknown events could affect the

260   significance of our test. To this aim, we carried out additional binomial probability tests by

261   progressively increasing the number of events while leaving the number of 'successes'

262   unchanged. The null hypotheses of no association was rejected at the nominal probability

263   threshold $\alpha = 0.05$.

264

Results and Discussion

At the time of our observation (May 10th 2014), we counted 9 males, 3 females and 8 juveniles (22-26 mm long; presumably 1 year old) of *Bufotes balearicus*, plus 2 female *Bufo bufo,* and various newly spawned egg-strings.

All tadpoles from the putatively hybrid egg-string were identified as first-generation hybrids by their heterozygote status at all loci analysed. In line with previous findings (Montalenti, 1932; Montalenti, 1933), most of them were heavily malformed (see Figure 1), and none reached the metamorphosis. Instead, at all the loci analysed, tadpoles from the additional two clutches sampled at the breeding site were homozygotic for *Bufotes balearicus* diagnostic alleles, and were thus identified as belonging to this parental species. As expected, they did not show abnormalities, neither in the external morphology nor in the ontogenetic pathway.

Despite their wide sympatry, ease of observation, and more than a century-old knowledge of hybridisation in laboratory crosses, our literature searches for previous reports of interspecific breeding pairs in the wild, identified just 5 additional observations within a 94-year time span (Sweden, Lang, 1926; Czech Republic, Vlček, 1995, Vlček, 1997, Zavadil & Roth, 1997; Austria, Duda, 2008).

Three main hypotheses have been invoked to explain recently established interspecific hybridisations among animal species, and may have played a role in the present case by promoting syntopy and breeding season overlap (Crispo et al., 2011; Chunco, 2014): species translocations, anthropogenic habitat degradation (a derivation of the Anderson's 'hybridisation of the habitat' model; Anderson, 1948) and climate changes.

287   In the case of *Bufo bufo* and *Bufotes viridis*, a species translocation can be firmly

288   excluded in all the reported cases, based on the extensive knowledge of their natural geographic

289   distributions (Lanza et al., 2006; Sillero et al., 2014), as well as on the fossil data of both

290   species in Europe (Martin & Sanchiz, 2011).

291   Anthropogenic habitat degradation has been proposed as a main causative agent in some

292   case (Duda, 2008). By reducing the diversity and number of potential breeding sites in a given

293   area, physical alterations of habitat could promote syntopy of previously allotopic populations.

294   Although plausibly contributing, this hypothesis cannot explain the entire pattern, and it does

295   not apply to all cases. Our study site (but see also Zavadil & Roth, 1997) is located within a

296   protected area established in 1993, and an analysis of contemporary and historical aerial photos

297   of this site and neighbouring areas clearly show the absence of any physical alterations of

298   potential relevance for the two species (Figure 2). Moreover, habitat degradation could not

299   explain the overlap of the two breeding seasons. Climate changes, however, significantly

300   improve our ability to explain the occurrence of hybridisation events between these species.

301   By promoting a recent altitudinal migration of *Bufotes balearicus* from neighbouring,

302   lower altitude sites, the ongoing climate warming engendered the unexpected syntopy at our

303   study site. Support to this argument (the only alternative to recent translocation), comes from

304   our models of the distribution of *Bufotes balearicus* in peninsular Italy, based on a set of known

305   occurrences collected before year 2000, and projected to the average climate over the period

306   2007-2013. Indeed, our models indicated that the species' presence above 1200 MASL was

307   highly improbable under pre-2000 climate, but became plausible during 2007-2013 (Figure 3).

308   Furthermore, projecting the models under future climate projections for the time period 2070-

309   2100 under different emission scenarios the general pattern remains unchanged (Figure 3). The

310   reliability of the models was clearly confirmed by the field-validation procedure (see Methods).

311 Indeed, our field searches of *Bufotes balearicus* at high-altitude sites of predicted presence in

312 post-2000 projections were successful. We found a previously unreported site of occurrence

313 within the Picentini Mountains (Latitude: 40.8251°N; Longitude: 14.9864°E; roughly 25 Km

314 south-east of the study site), thus confirming that upward migrations of *Bufotes balearicus* are

315 ongoing, as predicted by our bioclimatic model (see also Zavadil & Roth, 1997).

316 Besides being a co-factor for syntopy, climate changes also contributed to the

317 hybridisation events by promoting an overlap of the breeding activities. Analysing long-term

318 climate series, we found that the years when hybridisation events were recorded in Europe

319 (including our observation) ranked 1st or 2nd hottest on record at most site, over time series

320 from 47 to 214 years-long. Moreover, bimonthly mean temperatures at these sites were 2.4 °C

321 to 5.5 °C above the 1961-1990 averages, and 1.9 °C to 4.3 °C above the preceding 10-years

322 averages (Figure 3). Binomial probability tests allowed us to reject the null hypothesis of

323 random association between hybridisation events and extremely mild winters, with very high

324 confidence (binomial probability: $P \leq 2.3 \times 10^{6}$). Also, additional binomial probability tests,

325 carried out in order to explore how unrecorded events could affect the significance of our test,

326 indicated that the null hypothesis of random association was rejected (at $\alpha = 0.05$) until the

327 number of events was ≥ 77, while leaving unchanged the number of 'successes'.

328 Although *Bufo bufo* is expected to bring forward its breeding activity after mild winters

329 (Reading, 1998; Reading, 2003; Tryjanowski et al., 2003), at least three lines of support may

330 help explaining this apparently counterintuitive pattern. In years when the breeding season

331 begins earlier (after a mild winter), breeding has been observed to last longer (Gittins et al.,

332 1980). Furthermore, a second and lower peak of breeding activity has been often observed later

333 in the season (Pages, 1984; Reading, 1998), especially after mild winters (Reading, 1998).

334 Finally, extensive ecophysiological investigations on bufonid toads, including *Bufo bufo*,

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335 indicate that increased temperatures during hibernation lead to significant alterations of several

336 processes affecting the breeding activity, including body size condition, annual ovarian cycle,

337 and seasonal synchronisation of breeding (Jørgensen, 1992).

338 Our analyses do not indicate climate change as the single explanatory factor. The

339 environmental contexts in which interspecific interactions occur, the diverse forms of habitat

340 disturbance, or behavioural changes during altitudinal migrations (Canestrelli et al., 2016a;

341 Canestrelli et al., 2016b), might be locally influential. Nonetheless, these analyses clearly show

342 that climate changes played a fundamental part in promoting hybridisation events. In light of the

343 direction of these changes (IPCC, 2014), and of the results of our modelling exercise, we

344 hypothesise that these events will become progressively more common in the near future. Most

345 importantly, our results reveal a wider potential influence of climate changes on interspecific

346 reproductive interactions, particularly in the many instances where climate-driven asynchrony

347 and/or allotopy are integral components of the reproductive isolating barriers.

348 Hybridisation events among non-closely related species are generally believed to yield

349 events that are transient, and potentially affecting local population demography at most, because

350 strongly maladaptive (Rhymer & Simberloff, 1996; Malone & Fontnot, 2008). Nevertheless,

351 there may be exceptions, whereby the effects of maladaptive processes propagate from

352 population to community level (Farkas, 2015). Moreover, as revealed by years of investigation

353 on the hybridisation process in several animal taxa, including amphibians, new evolutionary

354 pathways have been sometime opened by such rare and maladaptive events (Arnold, 2006).

355

Acknowledgments

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360

361 **Figures**

362

363 **Figure 1. Interspecific hybridisation between the common toad (*Bufo bufo*) and the green toad**

364 **(*Bufotes balearicus*) in the wild.** The hybrid pair (a) was found spawning (b) on 10 May 2014, at

365 lake Campo Maggiore, a high-elevation pond within the Partenio Regional Park, southern Italy

366 (latitude: 40.9429° N; longitude: 14.7096° E; altitude: 1330 MASL). The majority of tadpoles from

367 the hybrid egg-string reared under standard laboratory conditions were heavily malformed (inset),

368 and none survived until metamorphosis; this pattern was not observed for control tadpoles from

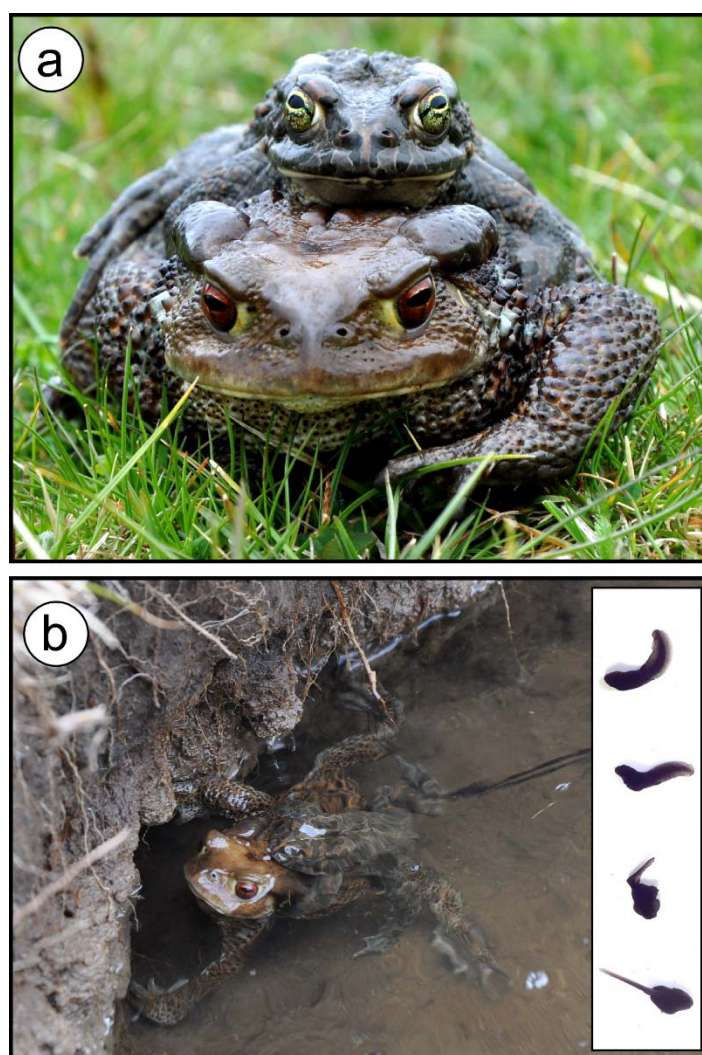369 con-specific matings. (Photos: M. Zampiglia)

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Figure 2. Land-use change detection analysis. (a) Location of the study area in Italy. (b) map of the land use in 2006, as obtained from direct interpretation of an aerial photo collected on 31 October 2006; in the same map, the exact location where the hybridisation event has been registered is indicated, as well as all areas where land use was different when compared to a second aerial photo collected on 9 October 2014. Both aerial photos were obtained from Google Earth Pro 7.1.5.1557 (Google Inc., Mountain View, California). (c) Average percent change (range of percent change in parenthesis) in land use classes from 2006 to 2014; total km^2 area for each land cover class in 2006 is provided in the last column. Aerial photos of the breeding site and its neighbourhoods collected on 31 October 2006 (d), and 9 October 2014 (e).

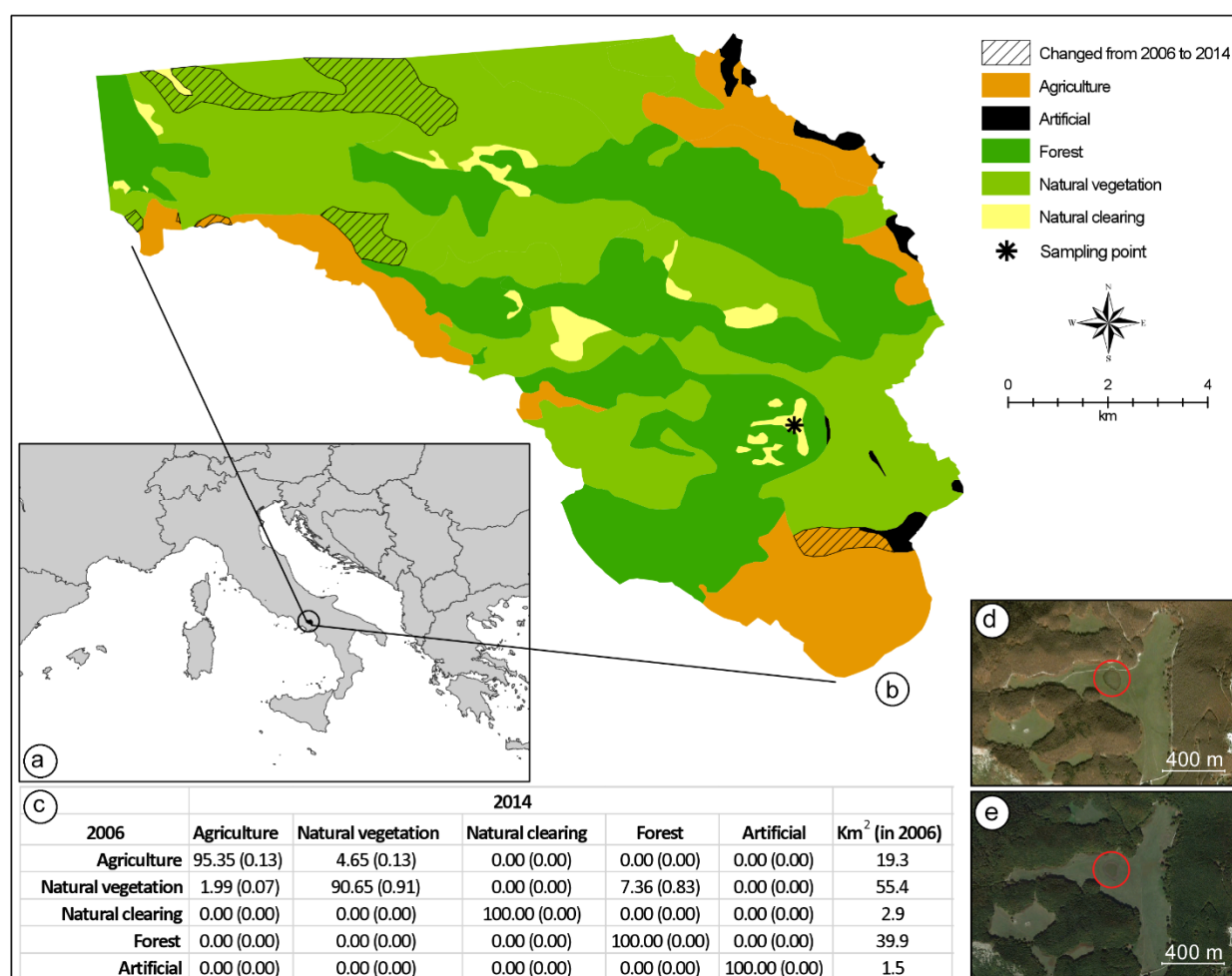| 2006	2014 Agriculture	Natural vegetation	Natural clearing	Forest	Artificial	Km^2 (in 2006)
Agriculture	95.35 (0.13)	4.65 (0.13)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	19.3
Natural vegetation	1.99 (0.07)	90.65 (0.91)	0.00 (0.00)	7.36 (0.83)	0.00 (0.00)	55.4
Natural clearing	0.00 (0.00)	0.00 (0.00)	100.00 (0.00)	0.00 (0.00)	0.00 (0.00)	2.9
Forest	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	100.00 (0.00)	0.00 (0.00)	39.9
Artificial	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	100.00 (0.00)	1.5

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Figure 3. Climate correlates of the interspecific hybridisation events observed in the wild between species of the common toad (*Bufo bufo*) and the green toad (*Bufotes viridis*) species groups. Bar plots showing frequency distribution (percent) of bimonthly mean temperature deviations (ΔTm) from the 1961–1990 average, compared to the two months preceding the breeding activity at each geographic region: (a) December to January (Italy, this study), (b) February to March (Sweden, Lang, 1926), and (c-e) January to February (Czech Republic, Vlček, 1995, Vlček, 1997, Zavadil & Roth, 1997, respectively). Values for the years when hybrid mates were observed are marked using red arrows. Optimal bar width was computed for each climatic series following the Freedman–Diaconis rule. (f) Average probability of presence vs elevation at sea level (m) as modelled for the pre-2000 climate (red line), the 2007–2013 climate (solid black line), and the 2070-2010 climate (blue line); the black dotted line indicates the minimum plausible level of probability of presence, above which the species can be considered present, while below is considered absent. (g) Mean temperature data for each site, and year of observation of interspecific mates. CsL: climatic series length, in years, before the observed event; b-MT: bimonthly mean temperature; R: rank over the entire climatic series (1= mildest); ΔTm: deviation from the 1961–1990 average temperature (°C); $\Delta T10y$: deviation from the preceding 10-year average temperature (°C).
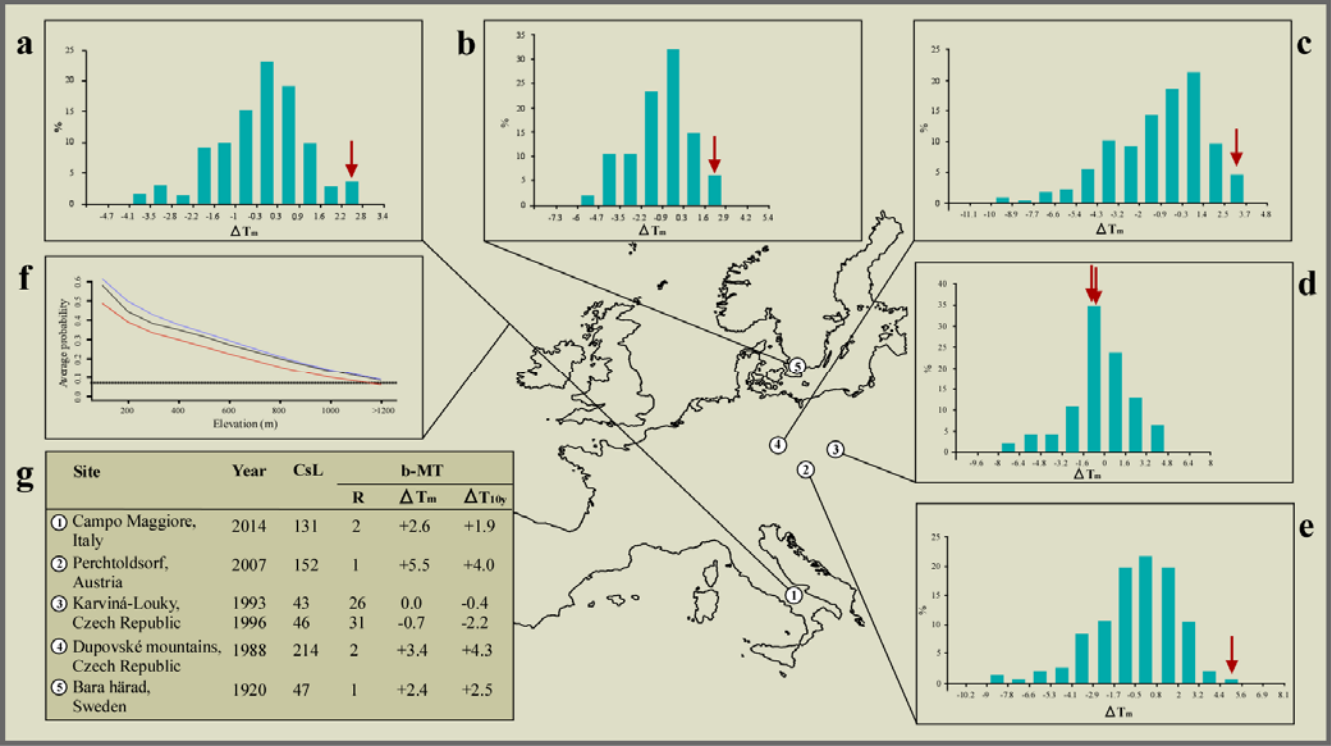
References

Ambrogio A, Mezzadri S. 2014. *Girini d'Italia –Tadpoles of Italy*. Piacenza: Gavia Edizioni.

Anderson E. 1948. Hybridization of the habitat. *Evolution* **2:**1-9. DOI: 10.2307/2405610.

Araújo MB, New M. 2007. Ensemble forecasting of species distributions. *Trends in Ecology and Evolution* **22**:42-47. DOI: 10.1016/j.tree.2006.09.010

Arnold ML. 2006. *Evolution Through Genetic Exchange.* Oxford: Oxford Univerity Press.

Cabela A, Grillitsch H, Tiedemann F. 2001. *Atlas zur Verbreitung und Ökologie der Amphibien und Reptilien in Österreic*. Wien: Naturhistorischen Museums.

Campbell JB. 1978. A geographical analysis of image interpretation methods. *Professional Geographer* **30**:264-269. DOI: 10.1111/j.0033-0124.1978.00264.x

Canestrelli D, Bisconti R, Carere C. 2016a. Bolder takes all? The behavioral dimension of biogeography. *Trends in ecology & evolution* **31**:35-43. DOI: 10.1016/j.tree.2015.11.004.

Canestrelli D, Porretta D, Lowe WH, Bisconti R, Carere C, Nascetti G. 2016b. The tangled evolutionary legacies of range expansion and hybridization. *Trends in Ecology & Evolution* **31**:677-688. DOI: 10.1016/j.tree.2016.06.010.

Carpino F, Capasso S. 2008. *I vertebrati terrestri del Parco regionale del Partenio.* Napoli: Edizioni Scientifiche Italiane.

Chunco AJ. 2014. Hybridization in a warmer world. *Ecology and Evolution* **4**:2019-2031. DOI: 10.1002/ece3.1052.

Coyne JA, Orr. H.A. 2004. *Speciation*. Sunderland: Sinauer Associates.

Crispo E, Moore JS, Lee-Yaw JA, Gray SM, Haller BC. 2011. Broken barriers: human-induced changes to gene flow and introgression in animals. *BioEssays* **33**:508-518. DOI: 10.1002/bies.201000154.

Duda M. 2008. First record of a natural male hybrid of *Bufo (Pseudepidalea) viridis* Laurenti, 1768 and *Bufo (Bufo) bufo* Linnaeus, 1758 from Austria. *Herpetozoa* **20**:184-186.

427 Falcucci A, Maiorano L, Boitani L. 2007. Changes in land-use/land-cover patterns in Italy and their

428   implications for biodiversity conservation. *Landscape Ecology* **22:**617-631. DOI:10.007/s10980-006-

429   9056-4.

430 Farkas TE, Hendry AP, Nosil P, Beckerman AP. 2015. How maladaptation can structure biodiversity:

431   eco-evolutionary island biogeography. *Trends in Ecology and Evolution* **30**:154-160.

432   DOI:10.1016/j.tree.2015.01.002.

433 Garcia-Porta AJ, Litvinchuk SN, Crochet PA, Romano A, Geniez PH, Lo-Valvo M, Lymberakis P,

434   Carranza S. 2012. Molecular phylogenetics and historical biogeography of the west-palearctic

435   common toads (*Bufo bufo* species complex). *Molecular Phylogenetics and Evolution* **63**:113-130.

436   DOI: 10.1016/j.ympev.2011.12.019

437 Gilsen T, Kauri H. 1959. Zoogeography of the Swedish amphibians and reptiles. *Actavertebratica* **1**:191-

438   397.

439 Gittins SP, Parker AG, Slater FM. 1980. Population characteristics of the common toad (*Bufo bufo*)

440   visiting a breeding site in mid-Wales. *Journal of Animal Ecology* **49**:161-173. DOI: 10.2307/4281.

441 Guarino FM, Aprea G, Caputo V, Maio N, Odierna G, Picariello O. 2012. *Atlante degli Anfibi e dei*

442   *Rettili della Campania*. Napoli: Massa Editore.

443 Harris H, Hopkinson DA. 1976. *Handbook Of Enzyme Electrophoresis In Human Genetics*. New York:

444   Elsevier/North-Holland.

445 Harris I, Jones PD, Osborn TJ, Lister TH. 2014. Updated high resolution grids of monthly climatic

446   observation: the CRU TS3.1 dataset. *International Journal of Climatology* **34**:623-642.

447   DOI: 10.1002/joc.3711

448 Hewitt GM. 2011. Quaternary phylogeography: the roots of hybrid zones. *Genetica* **139**:617-638.

449   DOI:10.1007/s10709-011-9547-3.

450 Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A. 2005. Very high resolution interpolated climate

451   surfaces for global land areas. *International Journal of Climatology* **25**:1965–1978.

452   DOI**:** 10.1002/joc.1276.

453   Intergovernmental Panel on Climate Change. *Climate change 2007: the physical science basis.*

454   *Contribution of Working Group I to the Fourth Assessment Report of the IPCC*. Cambridge

455   University Press, Cambridge, 2007.

456   IPCC, 2014: Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to

457   the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team,

458   R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.

459   Jørgensen CB. 1992. Growth and reproduction. In: Feder M. Burggren W. eds. *Environmental*

460   *Physiology of the Amphibians*. Chicago: University of Chicago Press, 439-466.

461   Lang K. 1926. Groddjurens och kraldjurens utbredning inom Bara harad (Malmohuslan). *Fauna och*

462   *Flora Uppsala* **21**:145-150.

463   Lanza B, Andreone F, Bologna MA, Corti C, Razzetti E. 2007. *Amphibia—Fauna d'Italia, Vol XLII*.

464   Bologna: Calderini Editore.

465   Lanza B, Nistri A, Vanni S. 2006. *Iconography of ItalianAmphibia*. Modena: Grandi e Grandi editori.

466   Maiorano L, Cheddadi R, Zimmermann NE, Pellissier L, Petitpierre B, Pottier J, Laborde H, Hurdu BI,

467   Pearman PB, Psomas A, Singarayer JS, Broennimann O, Vittoz P, Dubuis A, Edwards ME, Binney

468   HA, Guisan A. 2013. Building the niche through time: using 13,000 years of data to predict the

469   effects of climate change on three tree species in Europe. *Global Ecology and Biogeography* **22**:302-

470   317. DOI: 10.1111/j.1466-8238.2012.00767.x.

471   Mallet J. 2005. Hybridization as an invasion of the genome. *Trends in Ecology and Evolution* **20**:229–

472   237. DOI:10.1016/j.tree.2005.02.010.

473   Malone JH, Fontenot BE. 2008 Patterns of Reproductive Isolation in Toads. *PLoS ONE* **3**:e3900.

474   http://dx.doi.org/10.1371/journal.pone.0003900.

475   Martín C, Sanchiz B. 2011. *Lisanfos KMS. Version 1.2*. Madrid: Museo Nacional de Ciencias Naturales.

476   Maxson LR 1981. Albumin evolution and its phylogenetic implications in toads of the genus Bufo. II.

477   Relationships among Eurasian Bufo. *Copeia* **3**:579-583. DOI: 10.2307/1444561.

478   Montalenti G. 1932. Sull'embriogenesi degli ibridi di *Bufo vulgaris* e *Bufo viridis*. Rendiconti
479   dell'Accademia Nazionale dei Lincei, Classe di Scienze Fisiche, Matematiche e Naturali, Roma,
480   15:994-1000.

481   Montalenti G. 1933. L'ontogenesi degli ibridi fra *Bufo vulgaris* e *Bufo viridis. Physiological zoology*
482   **6**:329-395.

483   Nečas, P., Modrý, D., Zavadil. V. 1997. *Czech Recent and Fossil Amphibians and Reptiles: Atlas and*
484   *Field Guide*. Frankfurt Am Main: Edition Chimaira.

485   Pages JM. 1984. Ecologie des amphibiens du sud-ouest de la Montagne Noire. *Alytes* **3**:56-69.

486   Pfennig KS, Pfennig D. 2009. Character displacement: ecological and reproductive responses to a
487   common evolutionary problem. *The Quarterly Review of Biology* **84**:253-276.

488   Proietti MC, Reisser J, Marins LF, Marcovaldi MA, Soares LS, Monteiro DS, Wijeratne S, Pattiaratchi
489   C, Secchi ER. 2014. Hawksbill × loggerhead sea turtle hybrids at Bahia, Brazil: where do their
490   offspring go? *PeerJ* **2**:e255 DOI 10.7717/peerj.255.

491   Reading CJ. 1998. The effect of winter temperatures on the timing of breeding activity in the common
492   toad *Bufo bufo*. *Oecologia* **117**:469-475. DOI: 10.1007/s004420050682.

493   Reading CJ. 2003. The effects of variation in climatic temperature (1980–2001) on breeding activity and
494   tadpole stage duration in the common toad, *Bufo bufo*. *Science of The Total Environment* **310**:231-
495   236. DOI: 10.1016/S0048-9697(02)00643-5.

496   Rhymer JM, Simberloff D. 1996. Extinction by hybridization and introgression. *Annual Review of*
497   *Ecology, Evolution, and Systematics* **27**:83-109.

498   Romano A, De Cicco M, Utzeri C. 2003. Bufo viridis Laurenti, 1768 in Monti Simbruini Regional Park:
499   altitude record for Peninsular Italy. *Herpetozoa* 91-93.

500   Schwenk K, Brede N, Streit. B. 2008. Introduction. Extent, processes and evolutionary impact of
501   interspecific hybridization in animals. *Philosophical Transactions of the Royal Society B* **363**:2805-
502   2811. DOI:10.1098/rstb.2008.0055.

503   Sillero N, Campos J, Bonardi A, Corti C, Creemers R, Crochet PA, Isailovic JC, Denoël M, Ficetola GF,

504   Gonçalves J, Kuzmin S, Lymberakis P, de Pous P, Rodríguez A, Sindaco R, Speybroeck J, Toxopeus

505   B, Vieites DR, Vences M. 2014. Updated distribution and biogeography of amphibians and reptiles

506   of Europe. *Amphibia-Reptilia* **35**:1-31. DOI: 10.1163/15685381-00002935

507   Spilinga C, Esposito C, Forcina G, Romano A. 2007. Nuovi dati sulla distribuzione di alcuni anfibi nel

508   Lazio. *Aldrovandia* **3**: 125 – 126

509   Swets J.A. 1988. Measuring the accuracy of diagnostic systems. *Science* **240**:1285–1293. DOI:

510   http://dx.doi.org/10.1126/science.3287615.

511   Thuiller W, Lafourcade B, Engler R, Araujo MB. 2009. BIOMOD: a platform for ensemble forecasting

512   of species distributions. *Ecography* **32**:369–373. DOI: 10.1111/j.1600-0587.2008.05742.x.

513   Tryjanowski P, Rybacki M, Sparks T. 2003 Changes in the first spawning dates of common frogs and

514   common toads in western Poland in 1978—2002. *Annales Zoologici Fennici* **40**:459–464.

515   Vlček P. 1995. Mexidruhovy amplexus ropuch. *Živa* **43**:83.

516   Vlček P. 1997. Poznatky z křížení ropuchy obecné s ropuchou zelenou. *Živa* **45**:132-133.

517   Zavadil V, Roth P. 1997 Natural hybridisation between *Bufo viridis* and *Bufo bufo* in the Douposkéhory

518   hills (northwest Bohemia, Czech Republik) with general comments on hybridisation of European

519   green and common toads. In *Herpetologiabonnensis* (eds Böhme, W., W. Bischoff, T. Ziegler)

520   Proceedings of the 8th Ordinary General Meeting of the Societas Europaea Herpetologica, Bonn.