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Countrywide screening supports model-based predictions of the distribution of *Batrachochytrium dendrobatidis* in Ukraine

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ABSTRACT: The chytrid *Batrachochytrium dendrobatidis* (Bd) is a widespread fungus causing amphibian declines across the globe. Although data on Bd occurrence in Eastern Europe are scarce, a recent species distribution model (SDM) for Bd reported that western and north-western parts of Ukraine are highly suitable to the pathogen. We verified the SDM-predicted range of Bd in Ukraine by sampling amphibians across the country and screening for Bd using qPCR. A total of 446 amphibian samples (tissue and skin swabs) from 11 species were collected from 36 localities. We obtained qPCR-positive results for 33 samples including waterfrogs (*Pelophylax esculentus* complex) and fire and yellow-bellied toads (*Bombina* spp.) from 8 localities. We found that Bd-positive localities had significantly higher predicted Bd habitat suitability than sites that were pathogen-free. Amplification and

42 sequencing of the internal transcribed spacer (ITS) region of samples with the highest Bd
43 load revealed matches with ITS haplotypes of the globally distributed BdGPL strain, and a
44 single case of the BdASIA-2/ BdBRAZIL haplotype. We found that Bd was nonrandomly
45 distributed across Ukraine, with infections present in the western and north-central forested
46 peripheries of the country with a relatively cool, moist climate. On the other hand, our results
47 suggest that Bd is absent or present in low abundance in the more continental central,
48 southern and eastern regions of Ukraine, corroborating the model-predicted distribution of
49 chytrid fungus. These areas could potentially serve as climatic refugia for Bd-susceptible
50 amphibian hosts.

51 KEY WORDS: Chytrid fungus · Chytridiomycosis · Species distribution model · Anura,
52 Amphibia

53 1. Introduction

54 Amphibia is the most imperilled class of vertebrates on Earth (Monastersky 2014,
55 Luedtke et al. 2023), due in part to several infectious agents that have recently emerged
56 (Stuart et al. 2004, Wake & Vredenburg 2008). These pathogens have caused sharp declines
57 leading to population or even species extinctions (Martel et al. 2014, Jancovich et al. 2015,
58 Scheele et al. 2019). Other pathogen-related declines, particularly in temperate areas, have
59 been less dramatic, but nonetheless substantial and long-term (Murray et al. 2009, Bosch et
60 al. 2018, Palomar et al. 2023). The most destructive of these pathogens, *Batrachochytrium*
61 *dendrobatidis* (Longcore et al. 1999) (hereafter *Bd*), is a microscopic fungus that causes
62 chytridiomycosis, a lethal skin disease affecting amphibians across the globe (Berger et al.
63 1998, Fisher et al. 2009). Although hundreds of species are susceptible to infection, the
64 interactions between *Bd* and their amphibian hosts have highly variable outcomes (Fisher &
65 Garner 2020), ranging from benign to lethal. Innate factors such as individual immune
66 response, microbiome, life history and behaviour of the host (Lips 2016, Pabijan et al. 2020),
67 as well as the genotype and phenotype of the infecting *Bd* strain (O’Hanlon et al. 2018),
68 account for much of the variation. Environmental factors such as altitude and seasonal
69 variation in temperature and precipitation shape the global distribution of the pathogen
70 (Rödger et al. 2009, Liu et al. 2013, Olson et al. 2013), while smaller-scale climatic gradients
71 as well as land use and water system properties affect local pathogen–host dynamics, among
72 other factors (Kriger & Hero 2007a, Gervasi et al. 2017, Palomar et al. 2021). Recognizing
73 the heterogeneity in the potential and realized distribution of *Bd*, species distribution models
74 (SDMs) have become increasingly refined and have shown that the probability of occurrence
75 of *Bd* in a particular region is non-uniform, with some areas considered as ‘hotspots’ with
76 conditions favouring *Bd* persistence and spread, while others function as ‘coldspots’ in which
77 the occurrence of *Bd* is unlikely (Xie et al. 2016, Zimkus et al. 2020). However, the
78 predictive power of these models requires evaluation with field observations of the infection
79 status of amphibians inhabiting areas of both high and low probability of occurrence.

80 Unfortunately, large parts of the globe remain unstudied for *Bd*, including vulnerable
81 areas having diverse amphibian communities in potentially suitable habitat for chytrid fungi.
82 Surveying efforts in Eastern Europe (defined here as Belarus, Moldova, western Russia, and
83 Ukraine) have been patchy, insufficient or entirely missing. Tytar et al. (2023) recently
84 published a regional-scale species distribution model (SDM) for *Bd* with a focus on Ukraine.
85 They found that continentality, amongst other variables, had a particularly strong and
86 parabolic relationship with *Bd* habitat suitability. High habitat suitability was found in the
87 western and north-western parts of the country which are dominated by the Carpathian
88 Mountains and their foothills (Ivano-Frankivsk, Transcarpathia, Lviv, Chernivtsi and
89 Ternopil regions) or are lowland and mostly forest-covered (Volyn, Rivne, Zhytomyr and

90 Kyiv regions). Low habitat suitability was found for the central and eastern areas of Ukraine
91 with a distinctly more continental climate transitioning into steppe, suggesting that these
92 areas may function as an environmental refuge for amphibians, countering *Bd* infection and
93 spread (Tytar et al. 2023). However, these predictions remain untested.

94 In this study, we screened amphibians inhabiting Ukraine for the presence of *Bd* by
95 sampling at the country-wide scale and focusing mostly on waterfrogs (*Pelophylax* spp.) and
96 fire- and yellow-bellied toads (*Bombina* spp.). Western Palearctic species of these 2 genera
97 are well-known hosts of *Bd* that sometimes succumb to chytridiomycosis (Kolenda et al.
98 2017, Harnos et al. 2021) but are typically aclinical carriers of *Bd* infection, acting as
99 potential vectors of the pathogen due to their abundance and widespread occurrence (Baláz et
100 al. 2014). Our aims were 2-fold: (1) to assess the presence and distribution of *Bd* in Ukraine,
101 and (2) to use the field data to ground-truth the SDM model for *Bd* in Ukraine (Tytar et al.
102 2023). Our study provides the first surveillance data for *Bd* in Ukraine and, by supporting or
103 refuting model-based predictions on the spatial distribution of *Bd*, may facilitate future
104 amphibian management decisions and conservation actions.

105 2. Materials and Methods

106 2.1. Sample collection and DNA extraction

107 Live amphibians were sampled at 36 localities across Ukraine (Table 1, Fig. 1; Table
108 S1 in the Supplement at www.int-res.com/articles/suppl/d159p015_supp.xlsx) in 2017, 2020
109 and 2021. We collected tissue samples (toe clips and tail clips, $n = 337$) or skin swabs ($n =$
110 129) and occasionally both types of samples for the same individuals. For skin swabbing, we
111 used sterile swabs (Puritan Medical Products, Ref 25-806 2WD) and followed the methods of
112 Hyatt et al. (2007). Amphibian tissue samples were stored in 95% alcohol, whereas skin
113 swabs were either stored dry or in 95% alcohol. Pathogen DNA extraction depended on
114 sample type. For skin swabs, we used the PrepMan Ultra and zirconium beads procedure
115 originally developed by Boyle et al. (2004). We cut off ~4 mm of swab (about half of the
116 swab) with a sterile blade. After air-drying, the swab fragment was immersed in 100 μ l
117 PrepMan™ reagent with 0.04–0.05 g of 0.5 mm zirconia/silica beads (Biospec cat. No.
118 11079105z) in a 2.0 ml microtube (Sarstedt, REF 72.694.416). After centrifugation for 30 s at
119 $16\,000 \times g$, we homogenized the samples using a FastPrep-24 5G Bead Beating System (MP
120 Biomedicals) set at maximum speed (10 m s^{-1}) for 60 s, followed by another centrifugation
121 for 30 s at $16\,000 \times g$. Afterwards, the tubes were incubated for 15 min at 100°C , and then
122 centrifuged for 3 min at $16\,000 \times g$ and diluted 5–10 \times with distilled water. For tissue
123 samples, we used the Qiagen DNeasy Blood and tissue kit following the manufacturer's
124 instructions, except that incubation with Proteinase K was done at 70°C . Samples were eluted
125 in 200 μ l AE buffer and diluted 5–10 \times in distilled water. All extracted DNA samples
126 were stored at -20°C until further analysis.

127 2.2. Quantitative PCR

128 For quantitative PCR detection of *Bd* DNA in amphibian samples, we used the single-
129 plex Taqman protocol of Boyle et al. (2004), amplifying partial fragments of the internal
130 transcribed spacer 1 (ITS1) and 5.8S rRNA genes. We used PCR primers ITS1-3 Chytr and
131 5.8S Chytr and the Chytr MGB2 probe (Boyle et al. 2004) for qPCR on an Applied
132 Biosystems QuantStudio 5 Real-Time PCR machine. For quantitation, we constructed
133 standard curves using 10-fold dilutions of a synthetic genome template (gBlocks Gene
134 Fragment) containing target sequences of *Bd* and other amphibian pathogens (Standish et al.
135 2018). We report pathogen load as the number of *Bd* genomic equivalents (GE) per μ l of

136 sample. Each sample was run in duplicate on each plate. For swab samples, if at least 1
137 reaction returned a positive result, we extracted the sample again (from the remaining half of
138 the swab) and conducted another independent qPCR reaction in duplicate. For tissues, we
139 used another aliquot of the DNA extraction to replicate positive samples from the first qPCR.
140 In summary, we applied within-plate duplication for all samples as well as between-plate
141 duplication for those samples that returned at least 1 positive qPCR result. We spiked roughly
142 40% of the samples with a Taqman Exogenous Internal Positive Control (Thermo Fisher
143 Scientific) according to the manufacturer's instructions, to distinguish true negatives from
144 PCR inhibition.

145 **2.3. Data analysis**

146 We followed Hyatt et al. (2007) and Skerratt et al. (2008) in confirming positive
147 qPCR results by testing individuals on a second qPCR plate and using a second extraction if
148 possible (for swabs in our study). We considered amphibian samples as *Bd* positive (*Bd*+) if at
149 least 3 out of 4 qPCR replicates were positive, including samples showing consistent low
150 signals of the calculated zoospore equivalents (<1) estimated from the qPCR reactions,
151 following the experimental results of Bletz et al. (2015). Prevalence was calculated for
152 localities in which *Bd* was detected, and per taxon for the most numerous taxa: *Pelophylax*
153 spp., *Bombina bombina* and *B. variegata*. Prevalence was determined by dividing the number
154 of positive cases by the total number of samples per locality/taxon together with Clopper-
155 Pearson confidence intervals (CIs) calculated in the 'PropCIs' package in R version 3.3.1
156 (Scherer 2018). Using the regional SDM of Tytar et al. (2023), we checked for an association
157 between model-predicted suitability and *Bd* presence at the level of the sampling site. First,
158 we grouped our sampling sites into 2 predefined geographical areas: (1) localities in
159 peripheral western and northern Ukraine (Chernihiv, Chernivtsi, Chornobyl Zone, Volyn; 16
160 localities) which have generally high habitat suitability (mean suitability 0.68 ± 0.091) for *Bd*
161 according to Fig. 7 in Tytar et al. (2023), vs. (2) all other localities in central, eastern and
162 southern Ukraine with generally low habitat suitability (20 localities, mean suitability $0.25 \pm$
163 0.146). We then used Fisher's exact test to assess the hypothesis of no association between
164 geographic area and *Bd* presence. We also used the Kruskal-Wallis rank sum test to check
165 whether *Bd* habitat suitability values extracted from Tytar et al. (2023) (data with a resolution
166 of 3×3 km) were higher at sites with *Bd* infections vs. sites at which *Bd* was not detected.

167 **2.4. *Bd* strain genotyping**

168 We used a second method, conventional PCR, to confirm the results of the qPCR
169 analysis. We selected 11 of the qPCR-positive samples with high infection loads representing
170 7 of 9 *Bd*+) localities (Table S1). We also randomly selected 10 *Bd*- samples as negative
171 controls. PCR amplification of a 300 bp fragment consisting of partial ITS1, 5.8S and partial
172 ITS2 genes (hereafter 'ITS') was performed with Bd1a and Bd2a primers (Annis et al. 2004).
173 We used DreamTaq Green PCR Master Mix (ThermoFisher) and set the amplification
174 conditions as follows: initial denaturation at 95°C for 10 min; followed by 42 cycles of
175 denaturation at 95°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 1 min; and
176 a final extension at 72°C for 10 min. As a positive control, we used *Bd* zoospores in a
177 concentration of 1000 GE, courtesy of An Martel (Ghent University). Afterwards, samples
178 were electrophoresed in a 1.5% agarose gel and purified using exonuclease/phosphatase. We
179 used the Big Dye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems) with
180 the PCR primers, followed by purification with the ExTerminator kit (A&A Biotechnology),
181 and commercial capillary sequencing at Genomed S.A. (Warsaw, Poland). Alignment of the

182 obtained sequences and representative ITS haplotypes from GenBank was carried out using
183 Clustal W (Thompson et al. 2003) in MEGA 11 (Tamura et al. 2021).

184 **2.5. Molecular identification of *Pelophylax* frogs**

185 Accurate identification of waterfrogs (*Pelophylax esculentus* complex) using
186 morphological traits is problematic in areas where hybrid frogs occur, requiring the use of
187 molecular methods to distinguish between taxa (e.g. Herczeg et al. 2017, Joško & Pabijan
188 2020, Mezhzherin et al. 2023). We used a molecular technique based on amplification of the
189 nuclear serum albumin intron 1 (SAI-1) described by Hauswaldt et al. (2012) and modified
190 by Ermakov et al. (2019). Amplification products obtained by using primers from Ermakov et
191 al. (2019) are size-specific for *P. lessonae*, *P. ridibundus* and *P. cf. bedriagae*, and therefore
192 allow for tentative discrimination between these taxa as well as their hybrids. We used this
193 method to distinguish between *Pelophylax* species with an emphasis on samples from *Bd*+
194 localities. SAI-1 genotyping was conducted only for waterfrog tissue samples.

195 **3. Results**

196 **3.1. *Bd* prevalence and distribution**

197 We obtained skin swabs and/or tissue samples of 11 amphibian species from 36
198 localities (Table 1; Table S1). Most of the tested individuals were waterfrogs of the
199 *Pelophylax esculentus* complex (303 individuals, 67.9%) or fire- or yellow-bellied toads
200 (*Bombina* spp., 126 individuals, 28.2%). A total of 15 tested individuals (all from Chernivtsi
201 and Volyn regions in western Ukraine) tested positive in 1 or 2 out of 4 qPCR reactions and
202 were treated as negative. These individuals had high cycle threshold (Ct) values (mean 41.5 ±
203 1.6) and low load (<1.0 GE). Out of 446 sampled amphibians, a total of 33 (7.4%) tested
204 positive in 3 or 4 out of 4 qPCRs and were treated as infected by *Bd* (Table 1). These
205 individuals occurred in 8 out of 36 localities (22.2%). Prevalence at the taxonomic level for
206 taxa with the largest sample sizes revealed the highest values for *B. variegata* at 19.7%
207 (11.5–30.4%), *B. bombina* at 9.1% (2.5–21%) and *Pelophylax* sp. at 4.3% (2.3–7.2%).
208 However, 12/13 infected *Pelophylax* individuals came from a single locality (Svitiaz Lake,
209 Volyn region) that contained mostly the hybrid *P. esculentus* as assessed by SAI-1 variation.
210 Four samples from 4 different localities in western Ukraine (Svitiaz Lake, Pishcha, Tsetsyno
211 and Zavoloka) revealed appreciable *Bd* loads of >100 GE μl⁻¹ (Table 2). Loads for most other
212 samples were generally low (<100 GE μl⁻¹) and were at the limits of detection (≤1.0 GE μl⁻¹)
213 for 6 samples. We calculated *Bd* prevalence at *Bd*+ sites with at least 10 samples. In the
214 Chernivtsi region, 3 sites met these criteria: Krupianske with 40% (12.1–73.7%) infected
215 individuals, Bahna with 18% (8.9–32%) and Zavoloka with 25% (5.4–57.2%); all of these
216 infected animals were fire- or yellow-bellied toads (*Bombina* spp.). The prevalence of *Bd*
217 among waterfrogs (*Pelophylax* spp.) at Svitiaz Lake in the Volyn region was remarkably high
218 at 50% (29.1–70.8%). Farther east, only 1 site along the Uzh River (Chornobyl zone)
219 revealed low *Bd* prevalence at 4.7% (0.1–23.8%) based on infection of a single individual,
220 although this frog also had low *Bd* load. PCR inhibition, evaluated by spiking with an internal
221 positive control, was not observed in any of the tested samples. The distribution of *Bd*+
222 amphibians in Ukraine was limited to sites sampled in the northwest (Volyn region) and
223 southwest (Chernivtsi region) and the single *Bd*+ frog from the north-central Uzh River
224 (Chornobyl zone, Kyiv region). We did not detect *Bd* in 291 amphibian samples originating
225 from the central, southern and eastern parts of the country. We found a significant association
226 ($p < 0.001$, Fisher's exact test) between geographic areas with high SDM-predicted habitat
227 suitability for *Bd* (Tytar et al. 2023) and *Bd* presence assessed by field sampling. We also

228 found that localities with *Bd* infections had significantly higher *Bd* habitat suitability indexes
229 (0.666 ± 0.157 vs. 0.382 ± 0.241) compared to localities without detectable *Bd* infections
230 (Kruskal-Wallis $\chi^2 = 8.04$, $df = 1$, $p < 0.01$) (Fig. 2).

231 3.2. Identification of *Bd* strains

232 PCR amplification of amphibian samples with the highest *Bd* loads resulted in ITS
233 sequences of *Bd* from 11 individuals (Table S1; GenBank accessions: PP301480–90). PCR
234 amplification failed in 10 randomly selected *Bd*- samples. Due to the low and noisy signal at
235 the 5' and 3' ends of the amplicons, we only obtained between 156 and 212 bp of high-
236 quality sequence data. Alignment of the obtained sequences revealed 2 ITS haplotypes (UA-
237 01, UA-02) differing by 11 nucleotide substitutions in the ITS1 region. UA-01 was PCR-
238 amplified from 10 individuals (Table S1): *B. bombina* from Shypyntsi 1 and Zavoloka
239 (Chernivtsi region), *B. variegata* from Bahna and Tsetsyo (Chernivtsi region), *Pelophylax* sp.
240 from Cherevatch (Chornobyl region) and Svitiyaz lake (Volyn region) and *Hyla orientalis*
241 from Pishcha (Volyn region). Haplotype UA-02 was found in a single *B. variegata* from
242 Bahna village (Chernivtsi region). NCBI Blast searches using the UA-01 haplotype as a
243 query showed 100% identity to haplotype CW34 (Schloegel et al. 2012) nested within Global
244 Panzootic Lineage 2 (Bd-GPL-2) and also to other *Bd* isolates from India and Japan (Table
245 3). NCBI Blast searches using UA-02 as a query showed 100% identity to haplotype KR36
246 (JX983075) of a Korean *Bd* clade readily distinguishable from Bd-GPL (Bataille et al. 2013),
247 as well as a close relationship (differing by a single 2 bp indel in our alignment) to isolate
248 UMI142 attributed to BdASIA-2/BdBRAZIL (Schloegel et al. 2012, O'Hanlon et al. 2018).

249 4. Discussion

250 Our main goal was to provide a preliminary assessment of *Bd* prevalence in Ukraine,
251 a country for which data were lacking until now. We detected *Bd* in 4 taxa (*Bombina*
252 *bombina*, *B. variegata*, *Pelophylax esculentus* complex and *Hyla orientalis*) at 8 of 36
253 localities, with an overall prevalence of 7.4%. However, the spatial distribution of *Bd* was
254 highly uneven, with infected amphibians being present in 2 western (Chernivtsi, Volyn)
255 regions, and a single detected infection in the north-central fringe (Chornobyl zone, Kyiv
256 region) of Ukraine. We failed to detect *Bd* at 20 localities (291 samples) broadly distributed
257 across the central, southern and eastern regions of the country.

258 4.1. Skewed spatial distribution of *Bd* infections in Ukraine

259 The spatial distribution of *Bd* infections in Ukraine has a clear geographical pattern,
260 with *Bd*+ animals occurring in the western and north-central peripheries of the country. We
261 could rule out false negatives as causing this skewed spatial pattern because we found no
262 indications of PCR inhibition as assessed by the application of an internal positive control.
263 Moreover, the Qiagen DNeasy kit that we used for most samples was previously found to be
264 the most efficient extraction method in terms of *Bd* detection (Bletz et al. 2015). One factor
265 that could have affected our results was the timing of sampling, i.e. *Bd* detection levels are
266 known to be lower in warmer months of the year, i.e. during late summer (Ouellet et al. 2005,
267 Kriger & Hero 2007b). In general, our sampling was opportunistically conducted from April
268 to October, with most samples collected in May and June (Table S1), although some
269 fieldwork in central and southern Ukraine took place in August (10/25 sites). Interpretation of
270 our results must therefore take into consideration the suboptimal timing of sampling at some
271 sites. Although late summer sampling could have contributed to the lack of *Bd* detection in
272 eastern and southern Ukraine, it cannot fully explain our findings, as most sampling was done
273 in cooler periods of the year.

274 The spatial pattern of *Bd* infection detected in Ukraine, limited to mostly the western
275 fringe of the country, is different to that of Central or Western Europe, in which *Bd* is
276 widespread with limited apparent geographical structuring (e.g. Ohst et al. 2013, Baláž et al.
277 2014). For instance, in neighbouring Poland, *Bd* is uniformly distributed throughout the
278 country (Palomar et al. 2021). In Hungary, *Bd* infects both *B. variegata* and *P. ridibundus*
279 (Vörös et al. 2018), although prevalence is relatively low. That *Bd* infects *P. esculentus* frogs
280 in the Volyn region of northwestern Ukraine is not surprising given the proximity of *Bd*+
281 sites in eastern Poland (about 90 km, Palomar et al. 2021). Likewise, the high prevalence of
282 *Bd* in the Chernivtsi region among *Bombina* toads is expected, considering frequent
283 infections detected in other parts of the ranges of these species (Baláž et al. 2014, Harnos et
284 al. 2021), and in particular, in *B. variegata* populations from the Carpathian Mountains
285 (Vörös et al. 2013, Palomar et al. 2021). Moreover, the occurrence of *Bd* in *P. esculentus* and
286 *B. variegata* in western Ukraine supports their roles as potential reservoir hosts of the chytrid
287 in the environment (Baláž et al. 2014, Vojar et al. 2017, Vörös et al. 2018, Palomar et al.
288 2021).

289 **4.2. Fieldwork verifies model-based predictions of *Bd* distribution in Ukraine**

290 We found a significant association between sites with *Bd*+ amphibians from our field
291 survey and areas of high habitat suitability for *Bd* (Figs. 1 & 2) from the SDM of Tytar et al.
292 (2023). Our chytrid screening results and the model-based predictions (Tytar et al. 2023)
293 suggest that *Bd* is absent or present in low abundance in the central, eastern and southern
294 regions of Ukraine. The landscape of these areas is, in general, open, non-forested,
295 predominantly agricultural and transitioning towards steppe in the eastern- and southern-most
296 peripheries. These areas are associated with lower precipitation during warmer months and
297 increased continentality (lower minimum and higher maximum temperatures). The optimal
298 thermal range for *Bd* growth is relatively narrow (17–25°C), while temperatures close to
299 30°C, freezing and desiccation are lethal for the fungus (Johnson & Speare 2003, Piotrowski
300 et al. 2004). High summer temperatures, low precipitation and cold winters may prevent
301 individual infections from reaching thresholds inducing disease and mortality (Carey et al.
302 2006). Higher environmental temperatures during the summer season may also increase the
303 survival rates of infected amphibians (Woodhams et al. 2003, Retallick & Miera 2007, Andre
304 et al. 2008). Because environmental temperature is an important determinant of *Bd*
305 distribution and host–pathogen dynamics, we expect epidemic outbreaks of chytridiomycosis
306 to be unlikely in the more continental climate of central, eastern and southern Ukraine.
307 Within these generally less favourable areas for *Bd*, there are quite large forests, swamps and
308 riverine habitats with relatively good conditions for amphibians, e.g. at sites situated in
309 national parks in the vicinities of the Udai and Merchik Rivers, in the surroundings of
310 Gaidary in the Kharkiv region, in floodplain forests of the lower Dnieper River of Pravi
311 Solontsi in the Kherson region and the Danube Delta of Vylkove in the Odessa region
312 (Suriadna & Mykytynets 2023). These areas may contain large amphibian populations, and
313 our findings suggest they may be free from *Bd*. We propose that amphibian habitats in
314 central, eastern and southern Ukraine may act as potential climatic refuges for amphibians
315 (Puschendorf et al. 2009), allowing species susceptible to *Bd* at least seasonal respite from
316 infection and morbidity. Of particular interest are regions in central Ukraine at the edges of
317 environmental space deemed unsuitable for *Bd*. Due to their proximity to predicted areas of
318 *Bd* occurrence, these potential refugia may have an important role in amphibian conservation
319 by supplementing, through migration, populations undergoing chytrid-mediated decline.

320 Although SDMs are widely used and have become important tools for species
321 conservation, relatively few studies have tested the predictive ability of the models by

322 conducting field studies in areas of high and low suitability (Searcy & Shaffer 2014, Rhoden
323 et al. 2017, Fois et al. 2018). In our case, the SDM for *Bd* was based on data collected prior to
324 our field research. Indeed, none of the localities used by Tytar et al. (2023) for the *Bd* SDM
325 were from Ukraine. Despite the lack of training data from the area of interest, the model
326 performed notably well, as all of our localities with *Bd*+ amphibians were collected in, or
327 adjacent to, areas of high predicted habitat suitability. Conversely, we did not detect any
328 infections from localities predicted to have low habitat suitability for *Bd*. Our study thus
329 emphasizes the value of SDMs in determining the distribution of an important amphibian
330 pathogen, and more generally, the utility of local SDMs in refining range predictions in
331 geographically restricted areas (Searcy & Shaffer 2014, Johnson et al. 2023).

332 **4.3. Tentative identification of *Bd* strains**

333 DNA sequence analysis of the obtained ITS haplotypes suggests that amphibians from
334 western Ukraine are infected by at least 2 *Bd* lineages: the globally distributed *Bd*GPL strain
335 and the *Bd*ASIA-2/*Bd*BRAZIL strain reported from South Korea, Brazil and a single isolate
336 from Michigan, USA, originating from a market-traded *Lithobates catesbeianus* (Schloegel et
337 al. 2012). The implications of these results suggest that the epizootic-causing *Bd*GPL of
338 potentially high virulence infects Ukrainian amphibians at least in the western and northern
339 fringes of the territory. Although all amphibians handled by us were asymptomatic, *Bd*GPL
340 infections have the potential to trigger chytridiomycosis and inflict morbidity and mortality
341 (Farrer et al. 2011, O’Hanlon et al. 2018). Our tentative assignment of a single isolate to the
342 *Bd*ASIA-2/*Bd*BRAZIL strain is a surprising although not unprecedented finding, as several
343 *Bd* strains thought to be endemic to specific regions have been shown to have intercontinental
344 distributions (including *Bd*ASIA-2/*Bd*BRAZIL) following more extensive sampling
345 (O’Hanlon et al. 2018, Byrne et al. 2019). However, phylogeographic inference based solely
346 on ITS is problematic, as ITS gene trees can be highly discordant with genealogies based on
347 other parts of the *Bd* genome (O’Hanlon et al. 2018), and *Bd* strains often contain dozens of
348 ITS haplotypes (Schloegel et al. 2012). Thus, our assignment of the *Bd* isolates to particular
349 strains based on ITS haplotypes should be considered as preliminary and in need of
350 verification. Clearly, more sampling and multilocus or genomic studies are needed to unveil
351 the diversity of *Bd* strains in Eastern Europe.

352 **4.4. Implications for amphibian conservation**

353 Our results show that potentially virulent strains of *Bd* are present in Ukraine, and
354 therefore conservationists and natural resource managers should consider chytridiomycosis as
355 a possible threat to Ukrainian amphibian populations, at least in the western parts of the
356 country. Of particular interest are areas situated in the ‘forest zone’ of Ukraine, encompassing
357 the Carpathian Mountains and the northwestern regions that are relatively moist and are
358 mostly exempt from high summer temperatures. The highest regional species diversity of
359 amphibians in Ukraine is in the Carpathian Mountains and their foothills (comprising 19
360 species; Pysanets 2012), and this area is therefore vulnerable to chytrid-induced infection,
361 morbidity and mortality. Our documentation of *Bd* in the Chernivtsi region substantiates
362 concerns (Tytar et al. 2023) that amphibian declines on account of chytridiomycosis may
363 threaten this area. Future studies should prioritize regions in western Ukraine, especially in
364 the Carpathian Mountains, for chytrid screening. More importantly, biosecurity measures
365 aimed at curbing the human-mediated spread of *Bd* should be put in place especially in
366 western Ukraine. Our countrywide assessment should now be followed up by more detailed
367 studies of *Bd* prevalence at the local scale. Replicated transects spanning the transitions
368 between areas of high and low habitat suitability for *Bd*, e.g. from the Carpathian Mountains

369 into west-central Ukraine (Ternopil/Khmelnytskyi region) may be particularly informative on
370 the value of climatic refugia for amphibians.

371 Our work also accentuates the high value of amphibian habitat enclaves in the more
372 continental parts of Ukraine, especially the remaining natural complexes in the territory on
373 either side of the middle course of the southern Dnieper River ranging from the mouth of the
374 Desna River to Khortytsia Island, as well as the area of the drained Kakhovka reservoir as a
375 result of the destruction of the Kakhovka dam in June 2023 by Russian forces (see Glanz et
376 al. 2023), which now seems to have high nature conservation potential. As climatic refugia
377 for amphibians, these habitats should be afforded protection or managed to avoid habitat
378 degradation. Since land use in these parts is predominantly agricultural, a potential concern is
379 the effect of heightened pesticide contamination of aquatic habitats. Agricultural pesticide
380 exposure may exacerbate *Bd* transmission and progression of chytridiomycosis (McCoy &
381 Peralta 2018), and, paradoxically, exposure to some fungicides at the tadpole stage may
382 increase susceptibility to *Bd* after metamorphosis (Rohr et al. 2017). Another major concern
383 is environmental destruction brought about by the Russian invasion of Ukraine that began in
384 February 2022. In affected areas such as the Siverskyi Donets and lower Dnieper River
385 valleys, direct habitat destruction and contamination of soils and water bodies with complex
386 mixtures of explosives, oil, burning products, heavy metals from shells and flooding after
387 dam destruction have exacerbated other risks. The shortage of resources for biomonitoring in
388 Ukraine due in part to the war effort prevents rapid assessment and mitigation measures
389 which may have enormous long-term effects on amphibians and nature in general.

390 By implementing comprehensive protection measures, including establishing
391 protected areas, promoting sustainable agriculture (reducing reliance on harmful chemicals)
392 and addressing the threat of chytridiomycosis, we can safeguard these crucial ecosystems and
393 the amphibian species they harbour. Integrating these efforts into broader biodiversity and
394 climate-resilient conservation strategies will ensure a more comprehensive and long-term
395 approach to protecting Ukraine's natural heritage in the face of ongoing challenges.

396

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412

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635 Table 1. Sampling localities and their geographical positions (latitude, longitude), region and year sampled. Number of samples (N), number of
 636 *Bd* positive (*Bd*+) and *Bd* negative (*Bd*-) samples, amphibian taxa sampled (taxon), number of tissue samples (N tissues) and number of swabs
 637 (N swabs); both swab and tissue samples were analysed for some individuals. Habitat suitability indexes for *Batrachochytrium dendrobatidis*
 638 extracted from Tytar et al. (2023) are given for each locality in the last column. Bbom: *Bombina bombina*; Bvar: *Bombina variegata*; Bvir:
 639 *Bufo viridis*; Hori: *Hyla orientalis*; Lvulg: *Lissotriton vulgaris*; Pfus: *Pelobates fuscus*; Pvesp: *P. vespertinus*; Pesc: *Pelophylax esculentus*;
 640 Ples: *P. lessonae*; Prid: *P. ridibundus*; Rarv: *Rana arvalis*

Locality	Lat (° N)	Long (° E)	Region	Year	N/ <i>Bd</i> +/ <i>Bd</i> -	Taxon	N tissues	N swabs	<i>Bd</i> hab. suit.
Chernihiv	51.48	31.3	Chernihiv	2020	4/0/4	Prid	0	4	0.624
Bahna	48.23	25.19	Chernivtsi	2017	48/9/39	Bvar	48	0	0.730
Krupianske	48.17	26.04	Chernivtsi	2017	10/4/6	Bvar	10	0	0.430
Mala Buda	48.17	26.10	Chernivtsi	2017	1/0/1	Bbom	1	0	0.430
Sadhora Forest	48.33	26.00	Chernivtsi	2017	6/0/6	<i>Bombina sp.</i>	6	0	0.569
Shypyntsi 1	48.37	25.73	Chernivtsi	2017	5/1/4	Bbom	5	0	0.634
Shypyntsi 2	48.37	25.73	Chernivtsi	2020	22/0/22	Pesc, Ples, Prid	22	2	0.562
Tsetsyno	48.30	25.84	Chernivtsi	2017	6/2/4	Bvar	6	0	0.781
Valia Kuzmyna	48.15	25.98	Chernivtsi	2017	12/0/12	Bvar	12	0	0.603
Zavoloka	48.24	25.88	Chernivtsi	2017	12/3/9	Bbom	12	0	0.584
Channel, Chornobyl Zone	51.39	30.15	Kyiv	2020, 2021	35/0/35	<i>Pelophylax sp.</i>	10	15	0.743
Iliia river, Chornobyl Zone	51.25	29.84	Kyiv	2021	9/0/9	<i>Pelophylax sp.</i> , Bbom	0	9	0.311
Prypiat river, Chornobyl Zone	51.27	30.24	Kyiv	2021	7/0/7	<i>Pelophylax sp.</i> , Bbom	0	7	0.609

Uzh river, Chornobyl Zone	51.20 6	30.12 9	Kyiv	2020, 2021	24/1/23	<i>Pelophylax</i> sp., Bbom	10	14	0.489
Kyiv	50.37 8	30.64 6	Kyiv	2020	18/0/18	Prid	18	3	0.684
Dobrytskyi pond	49.55 6	36.30 9	Kharkiv	2020	9/0/9	Pesc, Prid	9	0	0.062
Dvorichna	49.84 9	37.73	Kharkiv	2017	13/0/13	Prid, Pesc	12	1	0.370
Gaidary	49.62 5	36.32 5	Kharkiv	2021	3/0/3	Pvesp	0	3	0.132
Iskiv pond	49.62 7	36.28 2	Kharkiv	2021	1/0/1	Pvesp	0	1	0.160
Kharkiv	49.98	36.23	Kharkiv	2021	8/0/8	Lvulg, Pesc, Prid,	3	5	0.257
Koriakiv pond	49.61 5	36.31 2	Kharkiv	2020	6/0/6	Rarv, Pesc	5	1	0.132
Merchik river	50.06 9	35.27 8	Kharkiv	2021	2/0/2	<i>Pelobates</i> sp., Bbom	0	2	0.353
Mozh river	49.74 3	36.16 3	Kharkiv	2021	2/0/2	Pesc, Prid	2	0	0.235
Olkhova Balka	50.16 7	36.36 0	Kharkiv	2020	21/0/21	Bbom	21	0	0.138
Siverskyi Donets river	49.62 4	36.33 1	Kharkiv	2020	6/0/6	Pesc, Prid	6	0	0.132
Buzova	46.48 5	32.02 1	Kherson	2020	3/0/3	Pesc, Prid	3	0	0
Hola Prystan	46.54 0	32.55 0	Kherson	2020	14/0/14	Pesc, Prid	14	0	0.498
Buh river	47.99 6	31.00 2	Mykolaiv	2020	20/0/20	Prid	10	10	0.179
Paliivka	46.64 3	30.46 1	Odesa	2020	9/0/9	Prid	9	0	0.687
Turunchuk, Dnister River	46.46 8	30.19 0	Odesa	2020	17/0/17	Pesc, Prid	10	7	0.779
Udai River	50.16 5	32.75 6	Poltava	2017	7/0/7	Pesc, Ples,	7	0	0.135

Pishcha	51.60 8	23.82 2	Volyn	2021	8/1/7	Hori, Bvir, Pfus, Bbom	0	8	0.829
Svitiaz Lake	51.47 1	23.83 9	Volyn	2021	24/12/12	Pesc	12	12	0.851
Davydivka	46.50 6	35.12 3	Zaporizhzhia	2020	16/0/16	Pesc, Prid	16	3	0.172
Okhrymivka	46.50 1	35.3	Zaporizhzhia	2020	17/0/17	Pesc, Prid	17	5	0.421
Korostyshiv	50.32 0	29.06 8	Zhytomyr	2020	21/0/21	Prid	21	7	0.725
Total					446/33/413		337	129	

641 Table 2. *Batrachochytrium dendrobatidis* (*Bd*) load as measured by cycle threshold (Ct) and
 642 quantities of *Bd* genome equivalents (GE) in amphibian samples (swabs and tissues) from
 643 Ukraine. Values are given as means \pm SD. Species abbreviations as in Table 1. ID refers to a
 644 unique identifier for each individual

ID	Species	Locality	Sample type	Ct	GE
MPFC5667	Hori	Pishcha	Swab	32.5 \pm 0.5	120.5 \pm 19.2
MPFC5675	Pesc	Svitiaz Lake	Swab	35.9 \pm 0.8	14.1 \pm 2.6
MPFC5676	Pesc	Svitiaz Lake	Swab	37.9 \pm 2.0	4.4 \pm 4.3
MPFC5677	Pesc	Svitiaz Lake	Swab	38.6 \pm 1.8	2.3 \pm 1.3
MPFC5678	Pesc	Svitiaz Lake	Swab	38.2 \pm 0.8	3.1 \pm 0.9
MPFC5682	Pesc	Svitiaz Lake	Swab	37.6 \pm 0.6	4.7 \pm 0.7
MPFC5686	Pesc	Svitiaz Lake	Swab	39.0 \pm 1.2	2.4 \pm 2.6
MPFC5707	Pesc	Svitiaz Lake	Tissue	33.1 \pm 0.3	65.9 \pm 25.8
MPFC5709	Pesc	Svitiaz Lake	Tissue	37.5 \pm 0.5	3.4 \pm 1.4
MPFC5711	Pesc	Svitiaz Lake	Tissue	31.4 \pm 0.9	259.9 \pm 185.0
MPFC5715	Pesc	Svitiaz Lake	Tissue	36.5 \pm 0.8	5.3 \pm 1.6
MPFC5716	Pesc	Svitiaz Lake	Tissue	32.8 \pm 1.2	66.4 \pm 22.3
MPFC5717	Pesc	Svitiaz Lake	Tissue	39.7 \pm 1.3	0.7 \pm 0.6
MPFC5704	Pesc	Uzh river	Tissue	43.0 \pm 1.4	0.2 \pm 0.1
B.UA17.07	Bvar	Tsetsyno	Tissue	37.9 \pm 4.5	39.3 \pm 34.5
B.UA17.08	Bvar	Tsetsyno	Tissue	33.8 \pm 0.8	127.8 \pm 148.9
B.UA17.13	Bbom	Zavoloka	Tissue	37.5 \pm 2.7	27.7 \pm 26.0
B.UA17.17	Bbom	Zavoloka	Tissue	39.5 \pm 3.1	11.1 \pm 13.8
B.UA17.19	Bbom	Zavoloka	Tissue	36.4 \pm 4.4	189.2 \pm 218.1
B.UA17.44	Bbom	Shypyntsi 1	Tissue	37.1 \pm 2.3	22.4 \pm 25.9
B.UA17.47	Bvar	Krupianske	Tissue	39.0 \pm 1.3	6.6 \pm 3.4
B.UA17.51	Bvar	Krupianske	Tissue	39.3 \pm 1.5	5.9 \pm 4.5
B.UA17.52	Bvar	Krupianske	Tissue	38.9 \pm 1.0	6.6 \pm 3.0
B.UA17.54	Bvar	Krupianske	Tissue	37.2 \pm 1.4	8.4 \pm 8.1
B.UA17.73	Bvar	Bahna	Tissue	42.3 \pm 0.4	0.7 \pm 0.5
B.UA17.76	Bvar	Bahna	Tissue	41.7 \pm 0.6	1.0 \pm 0.4
B.UA17.79	Bvar	Bahna	Tissue	38.0 \pm 1.8	14.9 \pm 11.3
B.UA17.80	Bvar	Bahna	Tissue	41.2 \pm 3.3	4.8 \pm 7.5
B.UA17.84	Bvar	Bahna	Tissue	40.7 \pm 1.9	1.5 \pm 2.0
B.UA17.93	Bvar	Bahna	Tissue	37.7 \pm 0.7	8.9 \pm 7.7
B.UA17.96	Bvar	Bahna	Tissue	41.7 \pm 1.7	0.5 \pm 0.6
B.UA17.99	Bvar	Bahna	Tissue	41.6 \pm 3.0	0.7 \pm 1.0
B.UA17.102	Bvar	Bahna	Tissue	38.3 \pm 2.4	6.1 \pm 7.2

645 Table 3. Best NCBI Blast hits to Ukrainian internal transcribed spacer (ITS) haplotypes (UA-01, UA-02) based on percent identity and E-value
 646 (all queries had 100% coverage). Haplotype identifiers (haplotype ID), GenBank accession numbers (acc. num.), host species (host) and
 647 sampling environment (environment, if known), locality from which the host was sampled (locality) and references are listed. NCBI Blast was
 648 accessed on 24 January 2024

	Haplotype ID	Acc. num.	% Identity/e-value	Host	Environment	Locality	Reference
UA-01	IN12	MG252085	100.00 / 1×10^{-101}	Unspecified anuran	Wild	India: Western Ghats	Mutnale et al. (2018)
	CW34	JQ582906	100.00 / 1×10^{-101}	<i>Xenopus laevis</i>	--	South Africa	Schloegel et al. (2012)
	Bd-04	AB435214	100.00 / 1×10^{-101}	<i>Rana rugosa</i>	--	Japan: Ibaraki, Sakuragawa	Goka et al. (2009)
	Isolate 350	JX993751	99.52 / 5×10^{-100}	<i>Lithobates catesbeianus</i>	Pet trade	Singapore	Gilbert et al. unpublished
	Bd-56	AB724257	99.52 / 5×10^{-100}	<i>Lithobates catesbeianus</i>	--	Japan: Ibaraki	Goka et al. unpublished
	CN5	JN870742	99.52 / 5×10^{-100}	<i>Fejervarya limnocharis</i>	Wild	China: Zhejiang	Bai et al. (2012)
	Not design.	HM153084	99.52 / 2×10^{-99}	<i>Anaxyrus houstonensis</i>	--	USA: Texas	Gaertner et al. unpublished
UA-02	KR36	JX983075	100.00 / 2×10^{-104}	<i>Bombina orientalis</i>	Wild	South Korea	Bataille et al. (2013)
	KR35	JX983074	99.53 / 8×10^{-103}	<i>Bombina orientalis</i>	Wild	South Korea	Bataille et al. (2013)
	Bd-33, 34	AB723969-70	99.07 / 1×10^{-101}	<i>Cynops ensicauda</i>	--	Japan: Okinawa	Goka et al. unpublished
	UMI 142	JQ582936	99.07 / 1×10^{-101}	<i>Lithobates catesbeianus</i>	Market	USA: Michigan	Schloegel et al. (2012)
	Bd-12	AB435222	99.07 / 1×10^{-101}	<i>Afrixalus fornasinii</i>	Pet trade	Japan: Shizuoka, Numazu	Goka et al. (2009)

649 Fig. 1. (A) Sampled regions in Ukraine and selected localities in (B) Volyn, (C) Chernobyl
650 Zone and (D) Chernivtsi regions. Pie charts show proportions of samples positive for
651 *Batrachochytrium dendrobatidis*; chart size is proportional to sample sizes. For a detailed list
652 of localities, see [Table 1](#). Habitat suitability values from [Tytar et al. \(2023\)](#) are overlaid on
653 the map in panel A

654 Fig. 2. Localities in which amphibians were infected by *Batrachochytrium dendrobatidis* (*Bd*
655 positive) had higher model-predicted habitat suitability indexes than localities in which
656 infection was not detected (*Bd* negative). Boxes show the interquartile range representing
657 50% of the data, with the median value depicted as a thick horizontal line; whiskers
658 encompass the remaining quartiles, and minimum and maximum values are shown by thin
659 horizontal lines. Habitat suitability values were extracted from [Tytar et al. \(2023\)](#)