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FEATURE ARTICLE

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

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Eastern Ukraine may constitute a chytrid coldspot for amphibians such as this *Pelophylax ridibundus* from habitat along the coast of the Azov Sea

Image credit: Natalia Suriadna

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

1. INTRODUCTION

Amphibia is the most imperilled class of vertebrates on Earth (Monastersky 2014, Luedtke et al. 2023), due in part to several infectious agents that have recently emerged (Stuart et al. 2004, Wake & Vredenburg 2008). These pathogens have caused sharp declines

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leading to population or even species extinctions (Martel et al. 2014, Jancovich et al. 2015, Scheele et al. 2019). Other pathogen-related declines, particularly in temperate areas, have been less dramatic, but nonetheless substantial and long-term (Murray et al. 2009, Bosch et al. 2018, Palomar et al. 2023). The most destructive of these pathogens, *Batrachochytrium dendrobatidis* (Longcore et al. 1999) (hereafter *Bd*), is a microscopic fungus that causes chytridiomycosis, a lethal skin disease affecting amphibians across the globe (Berger et al. 1998, Fisher et al. 2009). Although hundreds of species are susceptible to infection, the interactions between *Bd* and their amphibian hosts have highly variable outcomes (Fisher & Garner 2020), ranging from benign to lethal. Innate factors such as individual immune response, microbiome, life history and behaviour of the host (Lips 2016, Pabijan et al. 2020), as well as the genotype and phenotype of the infecting *Bd* strain (O'Hanlon et al. 2018), account for much of the variation. Environmental factors such as altitude and seasonal variation in temperature and precipitation shape the global distribution of the pathogen (Rödder et al. 2009, Liu et al. 2013, Olson et al. 2013), while smaller-scale climatic gradients as well as land use and water system properties affect local pathogen–host dynamics, among other factors (Kriger & Hero 2007a, Gervasi et al. 2017, Palomar et al. 2021). Recognizing the heterogeneity in the potential and realized distribution of *Bd*, species distribution models (SDMs) have become increasingly refined and have shown that the probability of occurrence of *Bd* in a particular region is non-uniform, with some areas considered as 'hotspots' with conditions favouring *Bd* persistence and spread, while others function as 'coldspots' in which the occurrence of *Bd* is unlikely (Xie et al. 2016, Zimkus et al. 2020). However, the predictive power of these models requires evaluation with field observations of the infection status of amphibians inhabiting areas of both high and low probability of occurrence.

Unfortunately, large parts of the globe remain unstudied for *Bd*, including vulnerable areas having diverse amphibian communities in potentially suitable habitat for chytrid fungi. Surveying efforts in Eastern Europe (defined here as Belarus, Moldova, western Russia, and Ukraine) have been patchy, insufficient or entirely missing. Tytar et al. (2023) recently published a regional-scale species distribution model (SDM) for *Bd* with a focus on Ukraine. They found that continentality, amongst other variables, had a particularly strong and parabolic relationship with *Bd* habitat suitability. High habitat suitability was found in the western and north-western parts of the country which are dominated by the Carpathian Mountains and their foothills (Ivano-Frankivsk, Transcarpathia, Lviv, Chernivtsi and Ternopil regions) or are lowland and mostly forest-covered (Volyn, Rivne, Zhytomyr and Kyiv regions). Low habitat suitability was found for the central and eastern areas of Ukraine with a distinctly more continental climate transitioning into steppe, suggesting that these areas may function as an environmental refuge for amphibians, countering *Bd* infection and spread (Tytar et al. 2023). However, these predictions remain untested.

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

2. MATERIALS AND METHODS

2.1. Sample collection and DNA extraction

Live amphibians were sampled at 36 localities across Ukraine (Table 1, Fig. 1; Table S1 in the Supplement at www.int-res.com/articles/suppl/d159p015_supp.xlsx) in 2017, 2020 and 2021. We collected tissue samples (toe clips and tail clips, n = 337) or skin swabs (n = 129) and occasionally both types of samples for the same individuals. For skin swabbing, we used sterile swabs (Puritan Medical Products, Ref 25-806 2WD) and followed the methods of Hyatt et al. (2007). Amphibian tissue samples were stored in 95% alcohol, whereas skin swabs were either stored dry or in 95% alcohol. Pathogen DNA extraction depended on sample type. For skin swabs, we used the PrepMan Ultra and zirconium beads procedure originally developed by Boyle et al. (2004). We cut off ~4 mm of swab (about half of the swab) with a sterile blade. After air-drying, the swab fragment was immersed in 100 µl PrepMan™

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

Locality	Lat (° N)	Long (° E)	Region	Year	N/*Bd*+/*Bd*−	Taxon	N tissues	N swabs	*Bd* hab. suit.
Chernihiv	51.48	31.3	Chernihiv	2020	4/0/4	Prid	0	4	0.624
Bahna	48.234	25.199	Chernivtsi	2017	48/9/39	Bvar	48	0	0.730
Krupianske	48.171	26.044	Chernivtsi	2017	10/4/6	Bvar	10	0	0.430
Mala Buda	48.179	26.107	Chernivtsi	2017	1/0/1	Bbom	1	0	0.430
Sadhora Forest	48.334	26.000	Chernivtsi	2017	6/0/6	*Bombina* spp.	6	0	0.569
Shypyntsi 1	48.371	25.735	Chernivtsi	2017	5/1/4	Bbom	5	0	0.634
Shypyntsi 2	48.378	25.734	Chernivtsi	2020	22/0/22	Pesc, Ples, Prid	22	2	0.562
Tsetsyno	48.306	25.847	Chernivtsi	2017	6/2/4	Bvar	6	0	0.781
Valia Kuzmyna	48.154	25.986	Chernivtsi	2017	12/0/12	Bvar	12	0	0.603
Zavoloka	48.247	25.888	Chernivtsi	2017	12/3/9	Bbom	12	0	0.584
Channel, Chornobyl Zone	51.392	30.156	Kyiv	2020, 2021	35/0/35	*Pelophylax* spp.	10	15	0.743
Ilia river, Chornobyl Zone	51.258	29.841	Kyiv	2021	9/0/9	*Pelophylax* spp., Bbom	0	9	0.311
Prypiat river, Chornobyl Zone	51.275	30.245	Kyiv	2021	7/0/7	*Pelophylax* spp., Bbom	0	7	0.609
Uzh river, Chornobyl Zone	51.206	30.129	Kyiv	2020, 2021	24/1/23	*Pelophylax* spp., Bbom	10	14	0.489
Kyiv	50.378	30.646	Kyiv	2020	18/0/18	Prid	18	3	0.684
Dobrytskyi pond	49.556	36.309	Kharkiv	2020	9/0/9	Pesc, Prid	9	0	0.062
Dvorichna	49.849	37.73	Kharkiv	2017	13/0/13	Prid, Pesc	12	1	0.370
Gaidary	49.625	36.325	Kharkiv	2021	3/0/3	Pvesp	0	3	0.132
Iskiv pond	49.627	36.282	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	49.615	36.312	Kharkiv	2020	6/0/6	Rarv, Pesc	5	1	0.132
Merchik river	50.069	35.278	Kharkiv	2021	2/0/2	*Pelobates* spp., Bbom	0	2	0.353
Mozh river	49.743	36.163	Kharkiv	2021	2/0/2	Pesc, Prid	2	0	0.235
Olkhova Balka	50.167	36.360	Kharkiv	2020	21/0/21	Bbom	21	0	0.138
Siverskyi Donets river	49.624	36.331	Kharkiv	2020	6/0/6	Pesc, Prid	6	0	0.132
Buzova	46.485	32.021	Kherson	2020	3/0/3	Pesc, Prid	3	0	0
Hola Prystan	46.540	32.550	Kherson	2020	14/0/14	Pesc, Prid	14	0	0.498
Buh river	47.996	31.002	Mykolaiv	2020	20/0/20	Prid	10	10	0.179
Paliivka	46.643	30.461	Odesa	2020	9/0/9	Prid	9	0	0.687
Turunchuk, Dnister River	46.468	30.190	Odesa	2020	17/0/17	Pesc, Prid	10	7	0.779
Udai River	50.165	32.756	Poltava	2017	7/0/7	Pesc, Ples,	7	0	0.135
Pishcha	51.608	23.822	Volyn	2021	8/1/7	Hori, Bvir, Pfus, Bbom	0	8	0.829
Svitiaz Lake	51.471	23.839	Volyn	2021	24/12/12	Pesc	12	12	0.851
Davydivka	46.506	35.123	Zaporizhzhia	2020	16/0/16	Pesc, Prid	16	3	0.172
Okhrymivka	46.501	35.3	Zaporizhzhia	2020	17/0/17	Pesc, Prid	17	5	0.421
Korostyshiv	50.320	29.068	Zhytomyr	2020	21/0/21	Prid	21	7	0.725
Total					446/33/413		337	129	

reagent with 0.04–0.05 g of 0.5 mm zirconia/silica beads (Biospec cat. No. 11079105z) in a 2.0 ml microtube (Sarstedt, REF 72.694.416). After centrifugation for 30 s at 16 000 × *g*, we homogenized the samples using a FastPrep-24 5G Bead Beating System (MP Biomedicals) set at maximum speed (10 m s^{-1}) for 60 s, followed by another centrifugation for 30 s at 16 000 × *g*. Afterwards, the tubes were incubated for 15 min at 100°C, and then centrifuged for 3 min at 16 000 × *g* and diluted 5–10× with distilled water. For tissue samples, we used the Qiagen DNeasy Blood and tissue kit following the manufacturer's instructions, except that incubation with Proteinase K was done at 70°C. Samples were eluted in 200 µl AE buffer and diluted 5–10× in distilled water. All extracted DNA samples were stored at −20°C until further analysis.

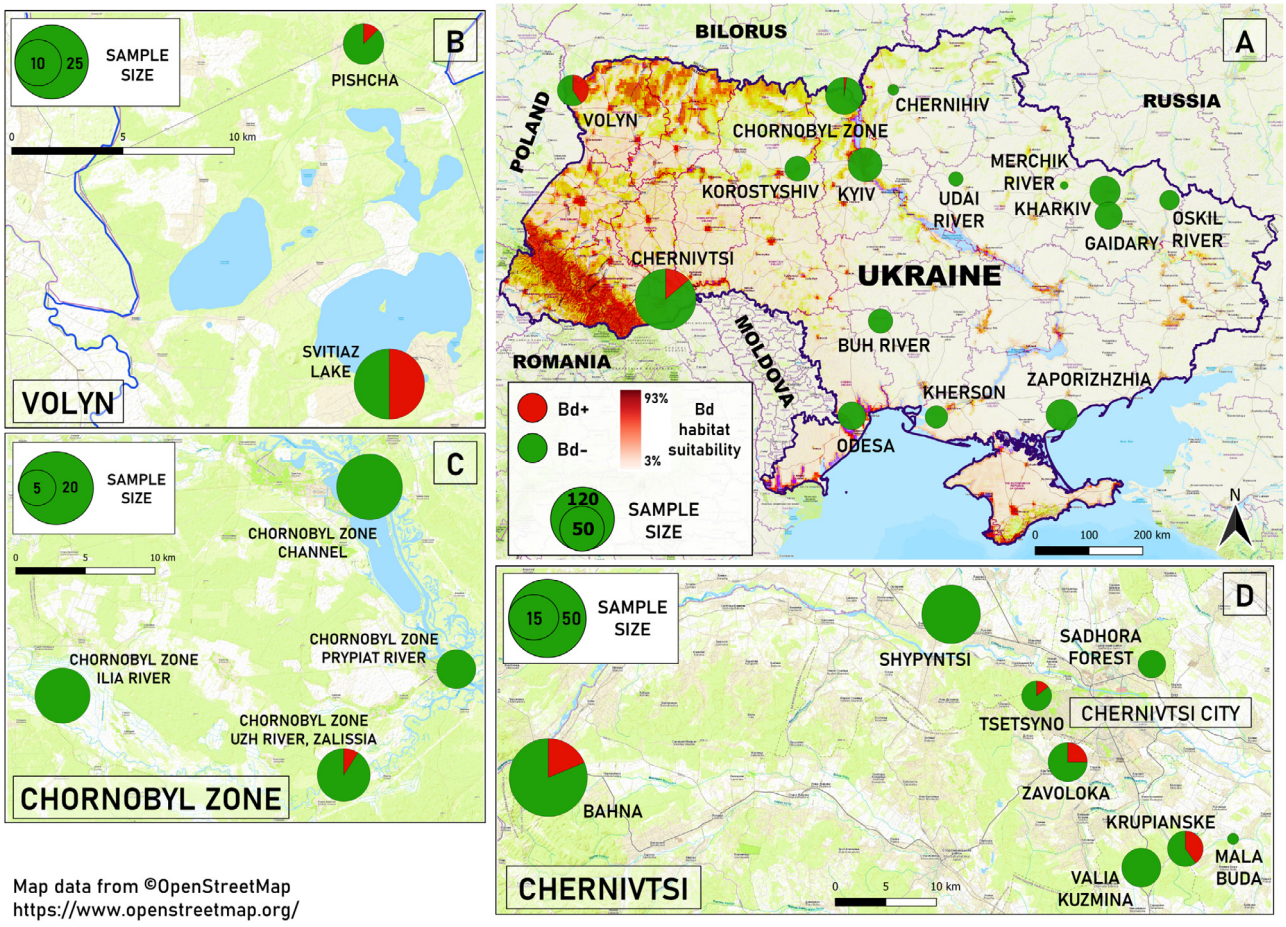

Fig. 1. (A) Sampled regions in Ukraine and selected localities in (B) Volyn, (C) Chornobyl Zone and (D) Chernivtsi regions. Pie charts show proportions of samples positive for *Batrachochytrium dendrobatidis*; chart size is proportional to sample sizes. For a detailed list of localities, see Table 1.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

2.2. Quantitative PCR

For quantitative PCR detection of *Bd* DNA in amphibian samples, we used the single-plex Taqman protocol of Boyle et al. (2004), amplifying partial fragments of the internal transcribed spacer 1 (ITS1) and 5.8S rRNA genes. We used PCR primers ITS1-3 Chytr and 5.8S Chytr and the Chytr MGB2 probe (Boyle et al. 2004) for qPCR on an Applied Biosystems QuantStudio 5 Real-Time PCR machine. For quantitation, we constructed standard curves using 10-fold dilutions of a synthetic genome template (gBlocks Gene Fragment) containing target sequences of *Bd* and other amphibian pathogens (Standish et al. 2018). We report pathogen load as the number of *Bd* genomic equivalents (GE) per µl of sample. Each sample was run in duplicate on each plate. For swab samples, if at least 1 reaction returned a positive result, we extracted the sample again (from the remaining half of the swab) and conducted another independent qPCR reaction in duplicate. For tissues, we used another aliquot of the DNA extraction to replicate positive samples from the first qPCR. In summary, we applied within-plate duplication for all samples as well as between-plate duplication for those samples that returned at least 1 positive qPCR result. We spiked roughly 40% of the samples with a Taqman Exogenous Internal Positive Control (Thermo Fisher Scientific) according to the manufacturer's instructions, to distinguish true negatives from PCR inhibition.

2.3. Data analysis

We followed Hyatt et al. (2007) and Skerratt et al. (2008) in confirming positive qPCR results by testing individuals on a second qPCR plate and using a second extraction if possible (for swabs in our

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

2.4. *Bd* strain genotyping

We used a second method, conventional PCR, to confirm the results of the qPCR analysis. We selected 11 of the qPCR-positive samples with high infection loads representing 7 of 9 *Bd*+ localities (Table S1). We also randomly selected 10 *Bd*− samples as negative controls. PCR amplification of a 300 bp fragment consisting of partial ITS1, 5.8S and partial ITS2 genes (hereafter 'ITS') was performed with Bd1a and Bd2a primers (Annis et al. 2004). We used DreamTaq Green PCR Master Mix (ThermoFisher) and set the amplification conditions as follows: initial denaturation at 95°C for 10 min; followed by 42 cycles of denaturation at 95°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. As a positive control, we used *Bd* zoospores in a concentration of 1000 GE, courtesy of An Martel (Ghent University). Afterwards, samples were electrophoresed in a 1.5% agarose gel and purified using exonuclease/phosphatase. We used the Big Dye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems) with the PCR primers, followed by purification with the ExTerminator kit (A&A Biotechnology), and commercial capillary sequencing at Genomed S.A. (Warsaw, Poland). Alignment of the obtained sequences and representative ITS haplotypes from GenBank was carried out using Clustal W (Thompson et al. 2003) in MEGA 11 (Tamura et al. 2021).

2.5. Molecular identification of *Pelophylax* frogs

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

3. RESULTS

3.1. *Bd* prevalence and distribution

We obtained skin swabs and/or tissue samples of 11 amphibian species from 36 localities (Table 1; Table S1). Most of the tested individuals were waterfrogs of the *Pelophylax esculentus* complex (303 individuals, 67.9%) or fire- or yellow-bellied toads (*Bombina* spp., 126 individuals, 28.2%). Out of 446 sampled amphibians, a total of 33 (7.4%) tested positive in 3 or 4 out of 4 qPCRs and were treated as infected by *Bd* (Table 1). These individuals occurred in 8 out of 36 localities (22.2%). A total of 15 tested individuals (all from Chernivtsi and Volyn regions in western Ukraine) tested positive in 1 or 2 out of 4 qPCR reactions and were treated as negative. These individuals had high cycle threshold (Ct) values (mean 41.5 ± 1.6) and low load (<1.0 GE). Prevalence at the tax-

onomic level for taxa with the largest sample sizes revealed the highest values for *B. variegata* at 19.7% (11.5–30.4%), *B. bombina* at 9.1% (2.5–21%) and *Pelophylax* sp. at 4.3% (2.3–7.2%). However, 12/13 infected *Pelophylax* individuals came from a single locality (Svitiaz Lake, Volyn region) that contained mostly the hybrid *P. esculentus* as assessed by SAI-1 variation. Four samples from 4 different localities in western Ukraine (Svitiaz Lake, Pishcha, Tsetsyno and Zavoloka) revealed appreciable *Bd* loads of >100 GE μl^{-1} (Table 2). Loads for most other samples were generally low (<100 GE μl^{-1}) and were at the limits of detection (⩽1.0 GE μl^{-1}) for 6 samples. We calculated *Bd* prevalence at *Bd*+ sites with at least 10 samples. In the Chernivtsi region, 3 sites met these criteria: Krupianske with 40% (12.1–73.7%) infected individuals, Bahna with 18% (8.9–32%) and Zavoloka with 25% (5.4–57.2%); all of these infected animals were fire- or yellow-bellied toads (*Bombina* spp.). The prevalence of *Bd* among waterfrogs (*Pelophylax* spp.) at Svitiaz Lake in the Volyn region was remarkably high at 50% (29.1–70.8%). Farther east, only 1 site along the Uzh River (Chornobyl zone) revealed low *Bd* prevalence at 4.7% (0.1–23.8%) based on infection of a single individual, although this frog also had low *Bd* load. PCR inhibition, evaluated by spiking with an internal positive control, was not observed in any of the tested samples. The distribution of *Bd*+ amphibians in Ukraine was limited to sites sampled in the northwest (Volyn region) and southwest (Chernivtsi region) and the single *Bd*+ frog from the north-central Uzh River (Chornobyl zone, Kyiv region). We did not detect *Bd* in 291 amphibian samples originating from the central, southern and eastern parts of the country. We found a significant association ($p < 0.001$, Fisher's exact test) between geographic areas with high SDM-predicted habitat suitability for *Bd* (Tytar et al. 2023) and *Bd* presence assessed by field sampling. We also found that localities with *Bd* infections had significantly higher *Bd* habitat suitability indexes (0.666 ± 0.157 vs. 0.382 ± 0.241) compared to localities without detectable *Bd* infections (Kruskal-Wallis $\chi^2 = 8.04$, df = 1, $p < 0.01$) (Fig. 2).

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

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

3.2. Identification of *Bd* strains

PCR amplification of amphibian samples with the highest *Bd* loads resulted in ITS sequences of *Bd* from 11 individuals (Table S1; GenBank accessions: PP301480–90). PCR amplification failed in 10 randomly selected *Bd*– samples. Due to the low and noisy signal at the 5′ and 3′ ends of the amplicons, we only obtained between 156 and 212 bp of high-quality sequence data. Alignment of the obtained sequences revealed 2 ITS haplotypes (UA-01, UA-02) differing by 11 nucleotide substitutions in the ITS1 region. UA-01 was PCR-amplified from 10 individuals (Table S1): *B. bombina* from Shypyntsi 1 and Zavoloka (Chernivtsi region), *B. variegata* from Bahna and Tsetsyo (Chernivtsi region), *P. esculentus* from Svitiaz lake (Volyn region) and *Hyla orientalis*

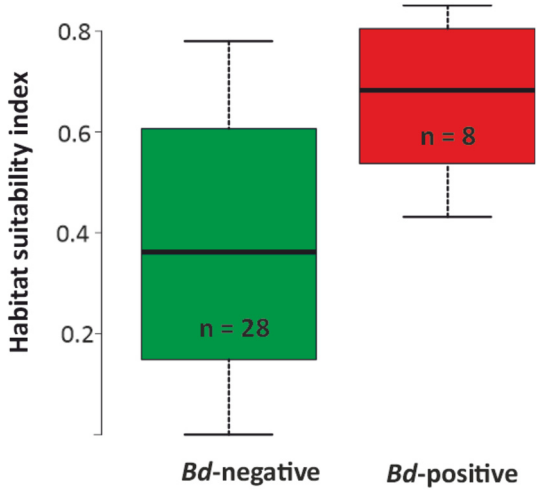

Fig. 2. Localities in which amphibians were infected by *Batrachochytrium dendrobatidis* (*Bd* positive) had higher model-predicted habitat suitability indexes than localities in which infection was not detected (*Bd* negative). Boxes show the interquartile range representing 50% of the data, with the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

from Pishcha (Volyn region). Haplotype UA-02 was found in a single *B. variegata* from Bahna village (Chernivtsi region). NCBI Blast searches using the UA-01 haplotype as a query showed 100% identity to haplotype CW34 (Schloegel et al. 2012) nested within Global Panzootic Lineage 2 (*Bd*-GPL-2) and also to other *Bd* isolates from India and Japan (Table 3). NCBI Blast searches using UA-02 as a query showed 100% identity to haplotype KR36 (JX983075) of a Korean *Bd* clade readily distinguishable from *Bd*-GPL (Bataille et al. 2013), as well as a close relationship (differing by a single 2 bp indel in our alignment) to isolate UMI142 attributed to *Bd*ASIA-2/*Bd*BRAZIL (Schloegel et al. 2012, O'Hanlon et al. 2018).

4. DISCUSSION

Our main goal was to provide a preliminary assessment of *Bd* prevalence in Ukraine, a country for which data were lacking until now. We detected *Bd* in 4 taxa (*Bombina bombina*, *B. variegata*, *Pelophylax esculen-*

Table 3. Best NCBI Blast hits to Ukrainian internal transcribed spacer (ITS) haplotypes (UA-01, UA-02) based on percent identity and expectation value (E-value) (all queries had 100% coverage). Haplotype identifiers (haplotype ID), GenBank accession numbers (acc. num.), host species (host) and sampling environment (environment, if known), locality from which the host was sampled (locality) and references are listed. NCBI Blast was 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}	*Xenopus laevis*	—	South Africa	Schloegel et al. (2012)
	Bd-04	AB435214	100.00 / 1×10^{-101}	*Rana rugosa*	—	Japan: Ibaraki, Sakuragawa	Goka et al. (2009)
	Isolate 350	JX993751	99.52 / 5×10^{-100}	*Lithobates catesbeianus*	Pet trade	Singapore	Gilbert et al. unpubl.
	Bd-56	AB724257	99.52 / 5×10^{-100}	*L. catesbeianus*	—	Japan: Ibaraki	K. Goka et al. unpubl.
	CN5	JN870742	99.52 / 5×10^{-100}	*Fejervarya limnocharis*	Wild	China: Zhejiang	Bai et al. (2012)
	Not design.	HM153084	99.52 / 2×10^{-99}	*Anaxyrus houstonensis*	—	USA: Texas	J.P. Gaertner et al. unpubl.
UA-02	KR36	JX983075	100.00 / 2×10^{-104}	*Bombina orientalis*	Wild	South Korea	Bataille et al. (2013)
	KR35	JX983074	99.53 / 8×10^{-103}	*B. orientalis*	Wild	South Korea	Bataille et al. (2013)
	Bd-33, 34	AB723969-70	99.07 / 1×10^{-101}	*Cynops ensicauda*	—	Japan: Okinawa	K. Goka et al. unpubl.
	UMI 142	JQ582936	99.07 / 1×10^{-101}	*L. catesbeianus*	Market	USA: Michigan	Schloegel et al. (2012)
	Bd-12	AB435222	99.07 / 1×10^{-101}	*Afrixalus fornasinii*	Pet trade	Japan: Shizuoka, Numazu	Goka et al. (2009)

tus and *Hyla orientalis*) at 8 of 36 localities, with an overall prevalence of 7.4%. However, the spatial distribution of *Bd* was highly uneven, with infected amphibians being present in 2 western (Chernivtsi, Volyn) regions, and a single detected infection in the north-central fringe (Chornobyl zone, Kyiv region) of Ukraine. We failed to detect *Bd* at 20 localities (291 samples) broadly distributed across the central, southern and eastern regions of the country.

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

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

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

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

We found a significant association between sites with *Bd*+ amphibians from our field survey and areas of high habitat suitability for *Bd* (Figs. 1 & 2) from the SDM of Tytar et al. (2023). Our chytrid screening results and the model-based predictions (Tytar et al. 2023) suggest that *Bd* is absent or present at low levels in the central, eastern and southern regions of Ukraine. The landscape of these areas is, in general, open, non-forested, predominantly agricultural and transitioning towards steppe in the eastern- and southern-most peripheries. These areas are associated with lower precipitation during warmer months and increased continentality (lower minimum and higher maximum temperatures). The optimal thermal range for *Bd* growth is relatively narrow (17–25°C), while temperatures close to 30°C, freezing and desiccation are lethal for the fungus (Johnson & Speare 2003, Piotrowski et al. 2004). High summer temperatures, low precipitation and cold winters may prevent individual infections from reaching thresholds inducing disease and mortality (Carey et al. 2006). Higher environmental temperatures during the summer season may also increase the survival rates of infected amphibians (Woodhams et al. 2003, Retallick & Miera 2007, Andre et al. 2008). Because environmental temperature is an important determinant of *Bd* distribution and host–pathogen dynamics, we expect epidemic outbreaks of chytridiomycosis to be unlikely in the more continental climate of central, eastern and southern Ukraine. Within these generally less favourable areas for *Bd*, there are quite large forests, swamps and riverine habitats with relatively good conditions for amphibians, e.g. at sites situated in national parks in the vicinities of the Udai and Merchik Rivers, in the surroundings of Gaidary in the Kharkiv region, in floodplain forests of the lower Dnieper River of Pravi Solontsi in the Kherson region

and the Danube Delta of Vylkove in the Odesa region (Suriadna & Mykytynets 2023). These areas contain large amphibian populations, and our findings suggest they may be free from *Bd*. We propose that amphibian habitats in central, eastern and southern Ukraine may act as potential climatic refuges for amphibians (Puschendorf et al. 2009), allowing species susceptible to *Bd* at least seasonal respite from infection and morbidity. Of particular interest are regions in central Ukraine at the edges of environmental space deemed unsuitable for *Bd*. Due to their proximity to predicted areas of *Bd* occurrence, these potential refugia may have an important role in amphibian conservation by supplementing, through migration, populations undergoing chytrid-mediated decline.

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

4.3. Tentative identification of *Bd* strains

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

4.4. Implications for amphibian conservation

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

ticularly informative on the value of climatic refugia for amphibians.

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

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

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