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First detection of chytrid fungus (*Batrachochytrium dendrobatidis*) in toads of the genus *Bombina* in Croatia

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Abstract. The global amphibian biodiversity crisis is significantly driven by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), contributing to declines in populations of hundreds of species and dozens of species extinctions over the last half-century. While the pathogen is widely distributed across at least 26 European nations, it remains undocumented in many regions of the Balkans. This study reports the first detection of Bd within populations of the genus *Bombina* in Croatia, based on field surveys conducted during the vernal and autumnal seasons of 2023 and 2024. Surveys targeted 19 distinct sites in Papuk Nature Park across an altitudinal gradient (87 m to 864 m), focusing on ephemeral aquatic microhabitats such as shallow temporary ponds and roadside ditches. Molecular screening of 82 individuals via quantitative PCR (qPCR) confirmed the pathogen in eight individuals across four ponds. Infections were identified in yellow-bellied toads (*Bombina variegata*) and their putative hybrids, while all sampled fire-bellied toads (*B. bombina*) tested negative. These results confirm the regional occurrence of Bd in Croatia. The presence of Bd in relatively isolated populations introduces a serious long-term threat to their viability, emphasizing the need for monitoring, targeted conservation, and in situ mitigation strategies to prevent sudden population collapses.

Keywords. amphibian pathogens; *Bombina bombina*; *Bombina variegata*; Croatia; wildlife disease monitoring

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The fungal pathogen *Batrachochytrium dendrobatidis* (Bd) is widely distributed across Europe, where it has been registered in nearly all Anuran species in at least 26 different nations (Tytar et al., 2023). Globally, this pathogen has altered amphibian biodiversity, contributing to the decline of at least 501 species and resulting in approximately 90 extinctions over the last half-century (Scheele et al., 2019). Bd causes lethal chytridiomycosis by invading the keratin-rich skin layers of its hosts, disrupting vital osmoregulation and electrolyte equilibrium, ultimately leading to cardiac arrest (Berger et al., 1998; Voyles et al., 2009). The rapid global spread of the fungus has been facilitated by anthropogenic activities, including the international trade of live animals (Fisher and Garner, 2007; O'Hanlon et al., 2018; Scheele et al., 2019). Biological vectors, like highly mobile and tolerant water frogs (*Pelophylax* spp.), act as regional reservoirs and facilitate the spread of Bd between disjointed aquatic habitats (Baláž et al., 2014 a, 2014 b; Tytar et al., 2023; Meletiadis et al. 2025).

Bombina variegata are recognized as a critical sentinel taxon for Bd surveillance in Central and Eastern Europe because they frequently carry the infection, often at high intensities (Baláž et al., 2014 a; Vörös et al., 2018; Tytar et al., 2023; Jakóbik et al., 2024). While populations in Poland appear to persist in a stable enzootic state, documented collapses in closely related (sub)species such as the Apennine yellow-bellied toad (*Bombina variegata pachypus* Bonaparte, 1838) in Italy, and a mortality event in Hungary underscore the potential for catastrophic outcomes (Stagni et al., 2004; Tobler et al., 2012; Canestrelli et al., 2013; Harmos et al. 2021; Razzetti et al., 2025). Survival of individuals is highly dependent on life stage, with newly metamorphosed individuals being exceptionally vulnerable to lethal infection (Vörös et al., 2018; Ujszegi et al., 2025).

We determine whether the fungal pathogen Bd is present within Croatian amphibian populations, utilizing the fire-bellied toad (*Bombina bombina* Linnaeus, 1758) and the yellow-bellied toad (*Bombina variegata* Linnaeus, 1758) as a model sentinel system.

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This study was conducted within the Papuk Nature Park, Croatia (45°30'18"N, 17°41'56"E), and its immediate periphery in May 2023 and 2024, and only one population (2) was sampled in September 2023. Papuk Nature Park is a UNESCO Global Geopark, and a national protected area designated by the Croatian Parliament and managed by a state-founded Public Institution. The research area encompasses approximately 1,221 km², with an altitudinal gradient ranging from 87 m to 864 m (mean elevation = 204 m). Surveys targeted ephemeral aquatic microhabitats, specifically focusing on shallow temporary ponds, puddles on unpaved roads, and roadside ditches. Due to the expansive nature of the site (~50 km) and the challenging topography, an opportunistic search strategy was implemented via vehicle-based reconnaissance to locate suitable water bodies. Presence of *Bombina* toads was determined through visual encounter surveys conducted during both diurnal and nocturnal periods, with night sampling ending a few hours after nightfall. Adult toads were captured either manually or using dip nets. Consistent with established biosafety protocols (Hyatt et al., 2007; Bacigalupe et al., 2017; Vörös et al., 2018), all specimens were handled using a new pair of gloves to minimize the risk of pathogen transmission. Except some co-occurrence of skin shedding potentially associated with Bd infection, no clearly visible signs of chytridiomycosis were seen, and no lethargy or mortality was observed. Following the completion of sampling, the toads were returned to their precise coordinates of capture to ensure minimal population disruption.

Species identification was based on phenotype using photographs of the ventral and dorsal sides of the individuals. While phenotypic criteria may not always fully reflect underlying genetic ancestry, ventral morphology (Fig. 1) constitutes a robust and widely accepted method for species discrimination in this group (Szymura, 1993; Vörös et al., 2007).

Skin swabs were obtained following the protocols from Boyle et al., (2004) and Hyatt et al., (2007), with the modification that before swabbing the animal was rinsed with

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distilled/demineralised water to dispose of surface debris (*e.g.* mud). The swabbing was conducted with single-use sterile FLOQSwabs (Copan Diagnostics, Italy) moistened with milliQ water, 60 seconds on the dorsal side and 60 seconds on the ventral side of the animal. The swabs were stored in tubes containing 1 mL of DNA Shield (Zymo, cat. Number R1100-250) and kept in -20ºC until DNA extraction.

We disrupted the swabs with Lysing Matrix E (1,2 MBiomedicals, cat nr. 116984010), vortexing, and sample homogenisation with the TissueLyser II (Qiagen) twice for six minutes at 30 Hz following the EHI standardised protocol (Lauritsen et al., 2025). We extracted DNA from 200 µl of the supernatant using solid-phase reversible immobilisation (SPRI) following the in-house protocol for DNA extraction (Lauritsen et al., 2025).

Bd presence was assessed using a real-time TaqMan PCR assay performed on a MyGo Pro qPCR system (IT-IS Life Science Ltd), following the protocol described by Blooi et al. (2013). DNA extracts were diluted 1:10 and analysed alongside negative controls and positive standards consisting of synthetic GeneArt DNA fragments (Thermo Fisher Scientific) containing the target sequence. Serial ten-fold dilutions were prepared to generate standards ranging from 1 to 10,000,000 (10^0–10^7). Copy numbers were estimated from DNA concentration and fragment length using the Thermo Fisher Scientific DNA Copy Number Calculator. A sample was considered positive when the estimated infection load was equal to, or greater than, that of the lowest positive standard and when amplification curves displayed a clear sigmoidal shape.

A total of 79 samples were collected from *Bombina* toads (Table 1, Supplementary Table 1). In three locations *B. bombina* were present, in 16 locations *B. variegata* were present and in six locations putative hybrid individuals were present. Only in pond 6 both parental species and putative hybrid individuals were present. Molecular screening confirmed the presence of Bd in eight individuals from four ponds (6, 13, 17 & 19; Fig. 2). Infection was found in *B.*

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variegata specimens as well as a putative hybrid individual giving an overall prevalence of 10.1% (8/79 individuals; 95 CI: 5 - 18.1%) and mean infection intensity of 112.5 GE (range: 48.6 - 305.1 GE). All the Bd loads detected are considered to be low and are consistent with the reported Bd loads for the genus in Europe (Baláž et al., 2014 a; Jakóbik et al., 2024). All *B. bombina* individuals sampled during the survey (at ponds 6, 8 and 9) tested negative. The fungus remained undetected in the remaining 15 sampling localities; however, the sample size is too small to infer anything about the status of infection.

The identification of Bd within Croatian *Bombina variegata* and putative hybrid individuals, specifically in the Papuk Nature Park, marks a significant expansion of the pathogen's documented range in the Balkans (Vojar et al., 2017; Vörös et al., 2018). Previous surveys done in Croatia (Vörös and Jelić, 2011) reported no infections (n = 13, of which n = 11 were *B. variegata*), however, the limited sample size in this region may explain the lack of Bd detection at the time. Regional surveillance has shown Bd prevalence ranging from 14.3% in Albania, Montenegro and Republic of North Macedonia (Vojar et al., 2017) to over 40% in Bd hotspots in Northwestern Italy (Meletiadis et al., 2025). Because the Croatian detection sites are relatively isolated from other known *B. variegata* habitats and given that the pet trade can be considered an unlikely factor in this case, transmission has likely occurred through alternative biological or environmental pathways. As we cannot account for temporal or other sources of variability, further targeted research is needed to draw any relevant conclusions about the mode of arrival and the epidemiological status of Bd in Croatia (Olson et al., 2013; Vörös et al., 2018; Jakóbik et al., 2024). While there is a legitimate chance that Bd may not have a major negative impact on Croatian populations, as many *Bombina* populations in Central Europe persist in a stable enzootic state, coexisting with the fungus without documented declines (Tobler et al., 2012, Baláž et al., 2014 b; Vörös et al., 2018; Jakóbik et

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al., 2024), dedicated long term studies are needed to assess the actual impact on local populations. Further research conservation efforts should be taken on the national level to use these records as an early warning and work on the establishment of a national Bd monitoring system.

The presence of Bd in these populations potentially introduces a serious long-term threat to their viability, particularly as habitat isolation increases the risk of local extinction due to limited recolonization potential (Bacigalupe et al., 2017; Scheele et al., 2019). The species remain highly susceptible during the metamorphic stage where Bd-induced mass mortality in several consecutive years can lead to rapid population collapses (Vörös et al., 2018; Ujszegi et al., 2024, 2025). These risks are further compounded by environmental synergies; extreme drought and temperature fluctuations can concentrate individuals in shrinking pools, increasing transmission rates and physiological stress (Adams et al., 2017; Vörös et al., 2018; Buttimer et al., 2025; Thumsová et al., 2025). The effect of Bd on Croatian populations warrants caution, since the presence of Bd in isolated habitats increases the risk of local extinction due to limited potential for natural recolonization (Bacigalupe et al., 2017; Scheele et al., 2019).

Habitat preservation may focus on maintaining environmental refugia that provide seasonal protection from chytridiomycosis, and bolsters host resistance (Forrest and Schlaepfer, 2011; Jakóbik et al., 2024). Protecting mature forests and high-quality wetlands associated with lower pathogen prevalence ensures habitat connectivity to facilitate natural recolonization (Bacigalupe et al., 2017; Kärvemo et al., 2018; Heard et al., 2018). Conservation efforts must also prioritize ecosystem integrity by supporting diverse aquatic microfauna that act as biological controls by preying on infectious zoospores (Schmeller et al., 2014).

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at Zenodo: 10.5281/zenodo.21216385.

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FIGURES & TABLES

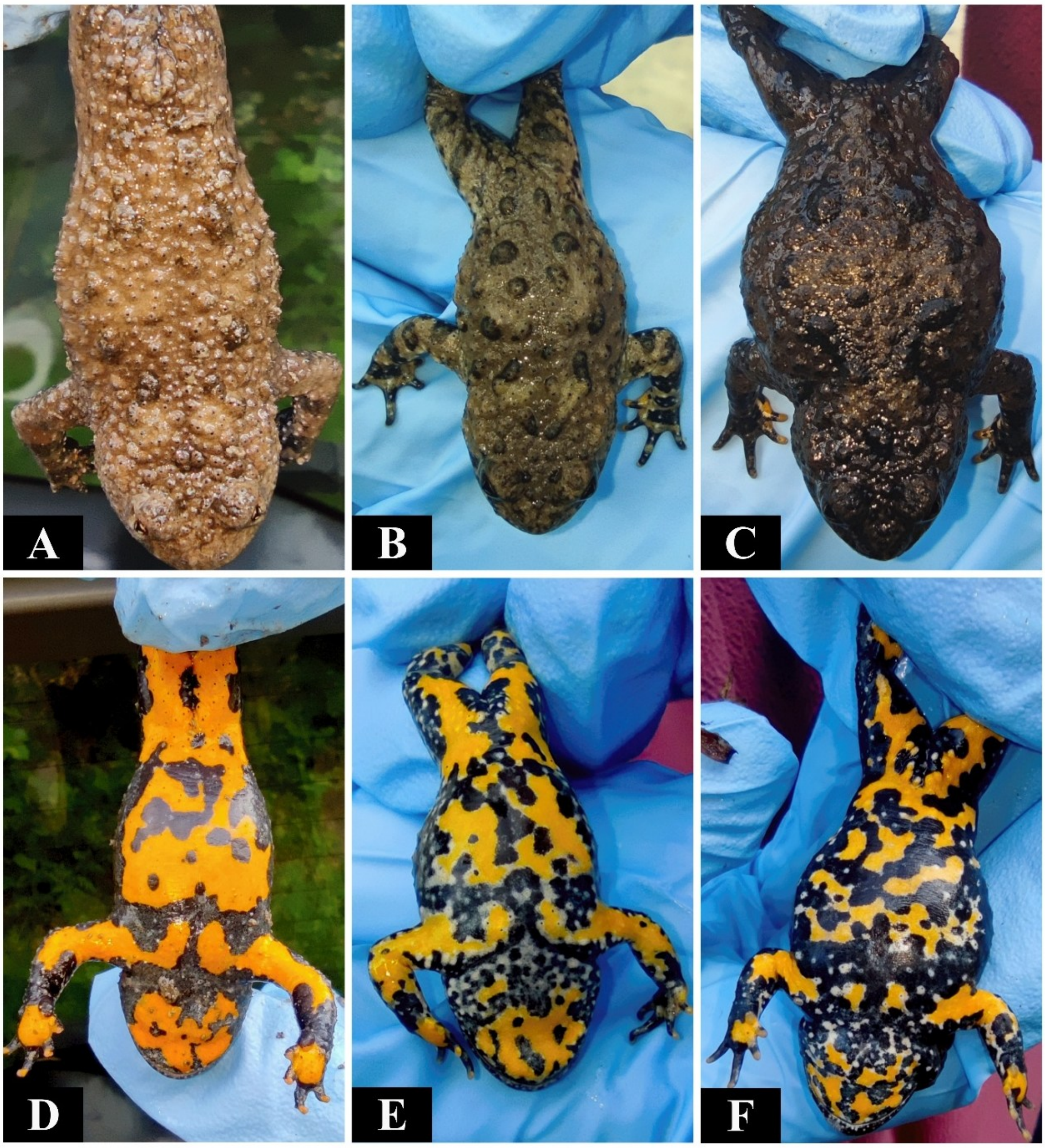

Fig. 1. Dorsal (top) and ventral (bottom) photographs of sampled individuals from different localities in Croatia. Photograph A/D. is *B. variegata*, B/E. is a putative hybrid and C/F. is *B. bombina*.

Table 1. Pond number, number of sampled individuals, infected individuals, infection load per pond (considering only positive individuals) and prevalence with 95% CI.

Pond	**Sampled**	**Infected**	**Infection load (GE; mean [min, max])**	**Prevalence (%)**	**95% CI (%)**
01	2	0	0	0	0
02	1	0	0	0	0
03	12	0	0	0	0
04	10	0	0	0	0
05	2	0	0	0	0
06	8	2	181.4 (57.8, 305.1)	25.00	3.1 - 65.09
07	1	0	0	0	0
08	6	0	0	0	0
09	1	0	0	0	0
10	1	0	0	0	0
11	5	0	0	0	0
12	9	0	0	0	0
13	8	4	64.1 (48.6, 90.1)	50	15.70 - 84.30
14	5	0	0	0	0
15	1	0	0	0	0
16	1	0	0	0	0
17	2	1	213.8	33.33	0.84 - 90.57
18	2	0	0	0	0
19	1	1	52.9	100	2.5 - 100

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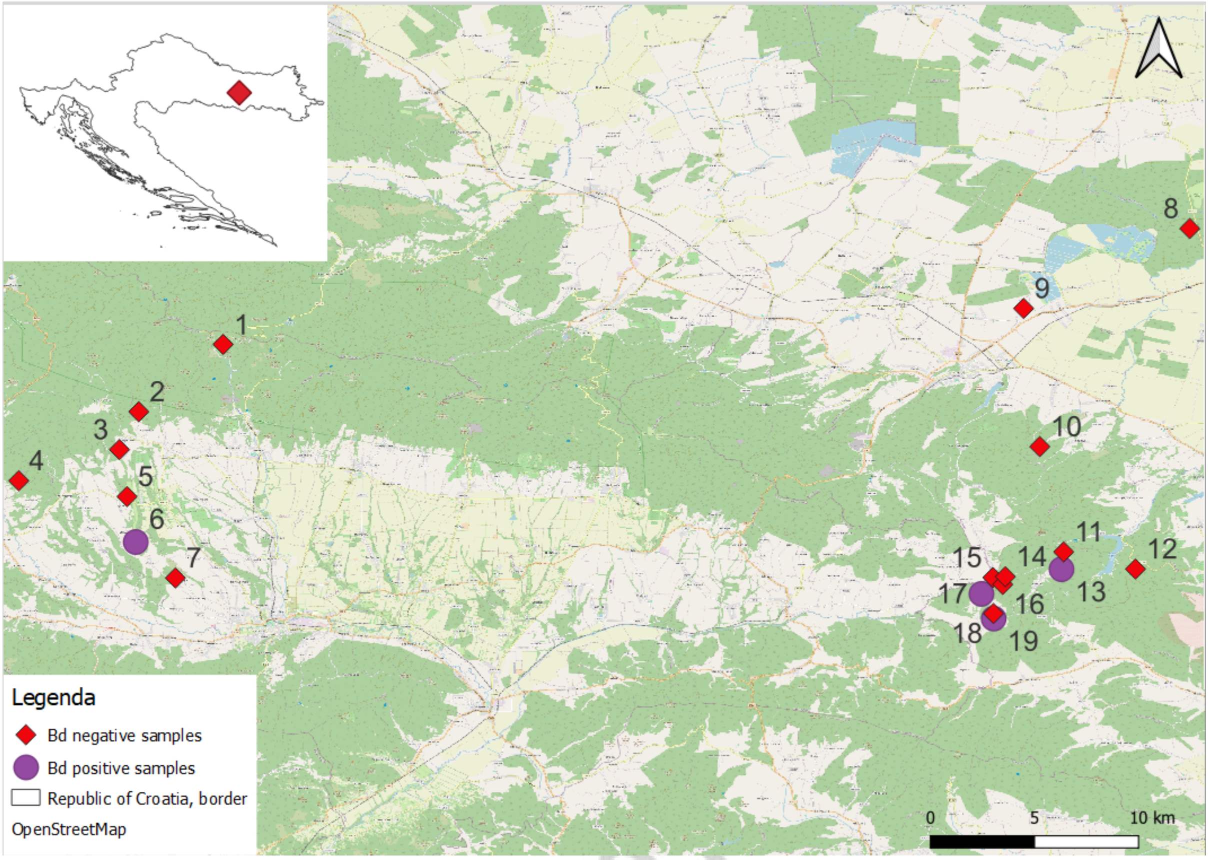

Fig. 2. Map showing population locations in Papuk Nature Park and its surrounding area. The box in the left corner shows the relative position of the research area in Croatia. Map background is from openstreetmap.org.