

## ***Batrachochytrium dendrobatidis* in Hungary: an overview of recent and historical occurrence**

JUDIT VÖRÖS<sup>1,2</sup>, DÁVID HERCZEG<sup>3,4,\*</sup>, ATTILA FÜLÖP<sup>5</sup>, TÜNDE JÚLIA GÁL<sup>1</sup>, ÁDÁM DÁN<sup>6</sup>, KRISZTIÁN HARMOS<sup>7</sup>, JAIME BOSCH<sup>8,9</sup>

<sup>1</sup> Department of Zoology, Hungarian Natural History Museum, 1088 Budapest, Hungary

<sup>2</sup> Laboratory for Molecular Taxonomy, Hungarian Natural History Museum, 1083 Budapest, Hungary

<sup>3</sup> Lendület Evolutionary Ecology Research Group, Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, 1022 Budapest, Hungary. \*Corresponding author. E-mail herczegdavid88@gmail.com

<sup>4</sup> Institute for Veterinary Medical Research, Centre for Agricultural Research, Hungarian Academy of Sciences, 1143 Budapest, Hungary

<sup>5</sup> MTA–DE Behavioural Ecology Research Group, Department of Evolutionary Zoology and Human Biology, University of Debrecen, 4032 Debrecen, Hungary

<sup>6</sup> Molecular Biology Department, Veterinary Diagnostic Directorate, National Food Chain Safety Office, 1143 Budapest, Hungary

<sup>7</sup> Bükk National Park Directorate, 3304 Eger, Hungary

<sup>8</sup> Museo Nacional de Ciencias Naturales, CSIC, 28006 Madrid, Spain

<sup>9</sup> Centro de Investigación, Seguimiento y Evaluación, Parque Nacional de la Sierra de Guadarrama, 28740 Rascafría, Spain

<sup>1, 2, 3, 4</sup> These authors contributed equally to this work

Submitted on: 2018, 5<sup>th</sup> February; revised on: 2018, 5<sup>th</sup> May; accepted on: 2018, 28<sup>th</sup> August

Editor: Marcello Mezzasalma

**Abstract.** *Batrachochytrium dendrobatidis* (*Bd*) is a fungal pathogen which causes the emerging infectious disease chytridiomycosis. *Bd* presents low host specificity and threatens amphibians worldwide, thus systematic inventory is the key in order to detect and mitigate the effects of the disease. Extensive data collection was conducted in Hungary in 2009-2015 from fourteen different areas. Combined data – recent field sampling on sixteen taxa and the examination of archived *Bombina* spp. specimens – from 1360 individuals were analysed with qPCR. Two sentinel taxa, *Bombina variegata* and the members of the *Pelophylax esculentus* complex were marked to monitor the occurrence of *Bd* in two core areas (Bakony Mts and Hortobágy National Park, respectively) of sampling. Climatic variables were also examined in core areas to test their effect on prevalence and infection intensity. Among the sixteen sampled amphibian taxa seven tested positive for *Bd* and the overall prevalence in Hungary was 7.46%. Among the ethanol-fixed *Bombina* spp. individuals *Bd* was not detected. In the first core area (Bakony Mts) the overall prevalence in *B. variegata* was 10.32% and juvenile individuals showed significantly higher prevalence than adults. On the other hand there was a significant negative relationship between infection prevalence and monthly mean air temperature. Finally, in the other core area (Hortobágy National Park) the overall prevalence in *P. esculentus* complex was 13.00%, and no differences were found in prevalence or infection intensity between sexes, sampling years or age classes.

**Keywords.** Chytridiomycosis, emerging infectious diseases, *Pelophylax esculentus* complex, *Bombina variegata*, inventory, Central-Europe.

### INTRODUCTION

Over the past decades several epidemics – caused by emerging infectious diseases – resulted in the large-

scale decline of numerous animal species globally (Dobson and Foufopoulos, 2001). One such emerging disease is chytridiomycosis in amphibians caused by the fungal pathogen *Batrachochytrium dendrobatidis* [hereafter, *Bd*

(Longcore et al., 1999)]. *Bd* is a highly generalist, water-borne pathogen which is primarily transmitted through direct contact with aquatic zoospores or infected individuals (Fisher et al., 2009). *Bd* is responsible for population declines, mass mortalities and even extinction of species, and presents one of the greatest threats to amphibians worldwide (Berger et al., 1998; Skerratt et al., 2007; Fisher et al., 2009).

*Bd* is widespread on all continents where amphibians occur (Olson et al., 2013), but the heaviest disease outbreaks were observed in the American Neotropics, Australia, North-America and Western Europe (Fisher et al., 2009). In Europe, the first detection of *Bd* related mass mortalities dates back to 1997 when the first recorded population decline as a result of mass die-off after the emergence of chytridiomycosis was observed in Central Spain, in the Guadarrama Mountain National Park, and targeted the Common midwife toad, *Alytes obstetricans* (Bosch et al., 2001). Though, as a result of the increased attention in the subsequent years, studies performed in the same region revealed that other species are highly susceptible to the disease as well (e.g. *Salamandra salamandra*, *Bufo spinosus*; Bosch and Martínez-Solano, 2006; Bosch et al., 2007). Moreover, the evidenced strong population declines of *A. obstetricans*, *A. muletensis* and *A. dickhilleni* in the Iberian Peninsula (Bosch et al., 2001; Walker et al., 2010; Bosch et al., 2013; Doddington et al., 2013; Rosa et al., 2013), and the high susceptibility of these species made the midwife toads the “flagship” species of European chytridiomycosis threat.

Central Europe harbours several amphibian species that might be susceptible to chytridiomycosis, such as *S. salamandra*, *B. bufo*, *Bombina bombina* or *Bombina variegata* (Baláz et al., 2014a,b). In the recent years *Bd* infection was detected in various areas of the Czech Republic, as a result of a systematic inventory (Civiš et al., 2012). Furthermore, the presence of the fungus was recently reported in low prevalence from Luxembourg (Wood et al., 2009), Poland (Sura et al., 2010; Kolenda et al., 2017), Germany (Ohst et al., 2013), Austria (Sztatecsny and Glaser, 2011), Slovakia (Baláz et al., 2014b) and Italy (Federici et al., 2008; Tessa et al., 2013). New data indicate that the fungus is present also in the Balkans, e.g., in Serbia (Mali et al., 2017), Albania, Montenegro and Macedonia (Vojar et al., 2017). Though, interestingly, no negative effects or *Bd*-linked population declines have been detected from Central-Eastern-Europe so far (Vörös et al., 2014).

Some aspects of chytridiomycosis epizootics show environmental correlates (Olson et al., 2013). *Bd* presents a reasonably wide environmental tolerance under a variety of temperature and precipitation regimes (Ron, 2005),

but previous studies postulated that climate (Berger et al., 2004; Bosch et al., 2007; Murray et al., 2009; Blaustein et al., 2010; Rohr et al., 2010; Rödder et al., 2010) and elevation (Lips et al., 2008; Walker et al., 2010; Becker and Zamudio, 2011) can significantly influence *Bd* outbreaks. Furthermore, large intra- and interspecific variations exist, especially in the prevalence (Gründler et al., 2012; Böll et al., 2014; Spitzen-Van Der Sluijs et al., 2014), but also in the intensity of infection (Van Sluys and Hero, 2009; Baláz et al., 2014a; Spitzen-Van Der Sluijs et al., 2014). In addition, behavioural differences influence the susceptibility to *Bd* which is further affected by the intraspecific variability related to sex and life stages (Blaustein et al., 2005; Garcia et al., 2006; Williams and Groves, 2014).

Hungary is situated in the Carpathian Basin, a region with high amphibian diversity due to different climatic and zoogeographical influences (Vörös et al., 2014). Previous findings about the occurrence of *Bd* in Hungary are restricted to a few areas and species where the presence was initially detected (Gál et al., 2012; Baláz et al., 2014b; Vörös et al., 2014; Drexler et al., 2017). Therefore, no large-scale distribution data on *Bd* presence is available to date from the country.

Our study displays multiple goals. First, we present a general overview on the occurrence of *Bd* in Hungary summarising data collected between the years 2009-2015. The data set includes the general occurrence of *Bd* on sixteen amphibian taxa with a special focus on the yellow-bellied toad *Bombina variegata* and water frogs belonging to the *Pelophylax esculentus* complex. We selected these two target taxa because these species may present high levels of infection intensity in Europe and so they may also act as sentinel taxa (Baláz et al., 2014b); in addition, they can play a role in the spread and the persistence of the disease (Baláz et al., 2014a).

Second, by studying *B. variegata* populations in Hungary we assessed whether distinct phylogenetic lineages – Alpine (West of the Danube) and Carpathian, occurring in the North Hungarian Range East of the Danube (Vörös et al., 2006) – express differences in prevalence and infection intensity. Moreover, to explore the historical distribution of *Bd* in Hungary field surveys were complemented with available archived samples of *Bombina* spp. from museum collections which comprise a dataset covering a 70 years’ time frame (1936-2005) prior to our field sampling.

Third, in order to further monitor *Bd* infection levels of amphibians in Hungary, we selected one population of two of the most susceptible taxa in Central-Eastern Europe, *B. variegata* and the *P. esculentus* complex (Baláz et al., 2014b), and extensively sampled these

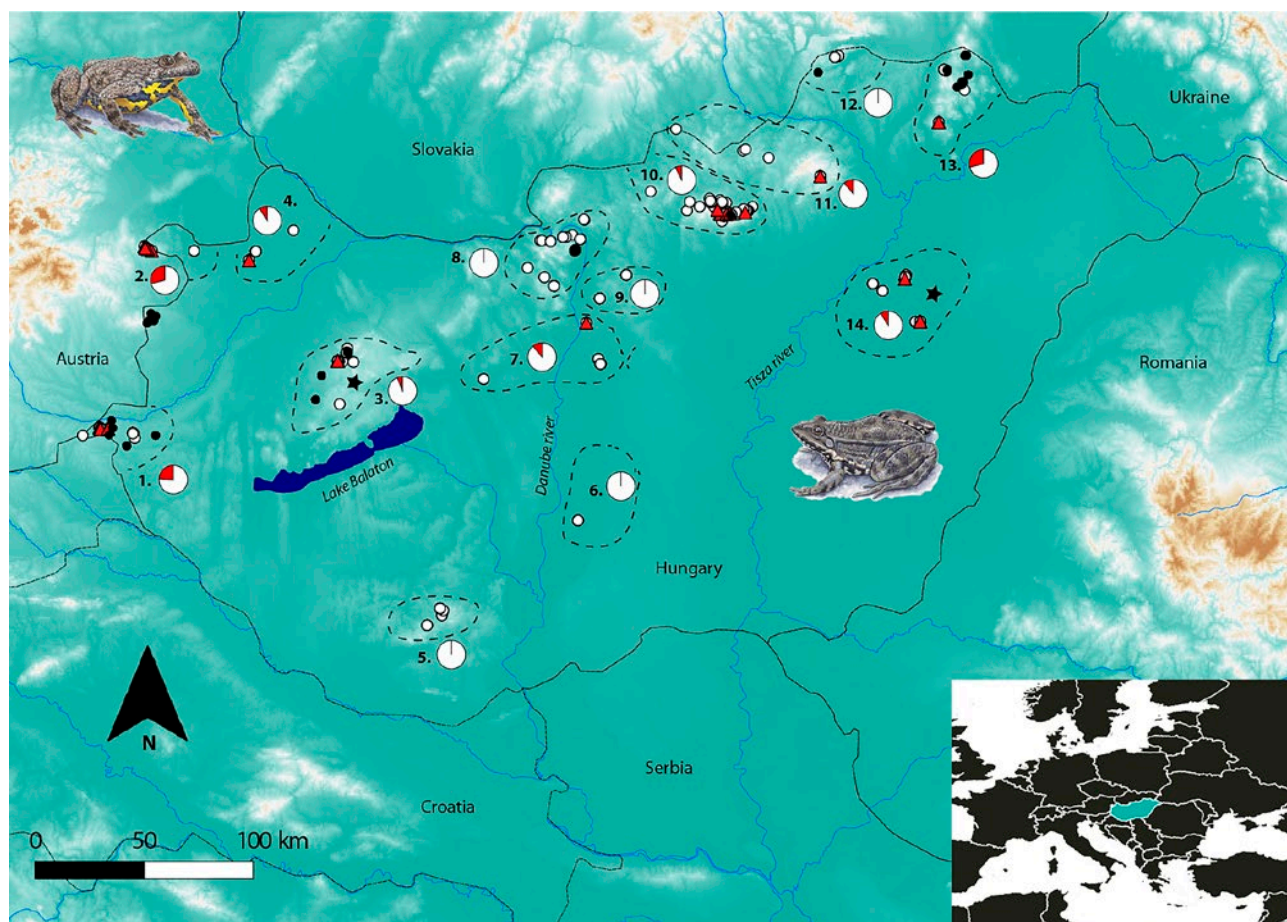
populations for three consecutive years in two core areas. Finally, we aimed to use climatic data (monthly mean precipitation and monthly mean air temperature) in these core areas to test if there is any correlation between the previously mentioned climatic variables and the occurrence of *Bd*.

## MATERIALS AND METHODS

### Data collection

Altogether 1233 specimens belonging to sixteen amphibian taxa were studied in the field between 2009-2015. Sampling was conducted in fourteen different regions in 45 distinct sampling points throughout Hungary, covering a great variety of wetland habitats (i.e. irrigation canals, streams, marshlands, ponds, fishponds, water reservoirs and temporary wetland habitats) and elevations ranging between 84 and 734 m a.s.l. (Fig. 1, Table 1). *Bombina variegata* was surveyed in five

regions from Transdanubia (Region 1, 2, 3, 5 and 8 in Table 1 and Fig. 1) representing the Alpine (Western) genetic lineage, and in three regions from the North Hungarian Mountains (Region 10, 12 and 13 in Table 1 and Fig. 1) representing the Carpathian (Eastern) genetic lineage, covering the distribution of the species in Hungary (Vörös et al., 2006). Identification of the two *Bombina* species and their hybrids was performed considering morphological characters plus genetic information provided by previous researches in Hungary (Vörös et al., 2006, 2007). Members of the *Pelophylax esculentus* complex were sampled in eight regions (Region 1, 3, 4, 7, 8, 9, 10 and 14 in Table 1 and Fig. 1). Age classes were characterized as tadpoles, juveniles and adults based on the external features of each species examined in the field. In those cases when we couldn't distinguish between age and sex of an individual we discarded the sample for further analysis. Additionally, 127 ethanol-fixed specimens of *Bombina* spp., deposited in the Hungarian Natural History Museum (Budapest, Hungary) and Savaria Museum (Szombathely, Hungary), collected between 1936 and 2005 from regions matching the current distribution of the species were swabbed (Supplementary table S1).



**Fig.1.** Map of Hungary showing sampling locations of *Bd* negative (black filled circles), *Bd* positive (red triangles) and archived (white circles) samples. Pie charts indicate *Bd* prevalence of the fourteen studied geographic regions. Numbers of regions correspond to Table 1. The two core areas are marked with asterisks (Region 3 and 14). Drawing of *Bombina variegata* and *Pelophylax ridibundus* are the courtesy of Márton Zsoldos.

**Table 1.** Summary of regions, sampling locations, coordinates and sampled species in our inventory. mtDNA lineages were indicated as Alpine (Alp) or Carpathian (Carp) in the case of *B. variegata*. Lat = Latitude; Long = Longitude; N = Number of individuals sampled; Prev = Prevalence; GE = Genomic equivalents.

Nr. of region	Alt	Lat	Long	Species	mtDNA lineage B. variegata	N	Positive/ Sampled	Prev (%)	Prev 95% CI (%)	GE mean	GE median	GE SD	GE range
1-Őrség	315.0	46.87	16.13	<i>Bombina variegata</i>	Alp	2	16 / 68	23.53	14.09-35.38	34.45	5.01	58.32	0.20-182.78
	264.0	46.87	16.45	<i>Bombina variegata</i>	Alp	7							
	253.0	46.89	16.43	<i>Hyla arborea</i>		1							
	253.0	46.89	16.43	<i>Lissotriton vulgaris</i>		1							
	253.0	46.89	16.43	<i>Rana arvalis</i>		1							
	253.0	46.89	16.43	<i>Rana dalmatina</i>		4							
	315.0	46.90	16.24	<i>Bombina variegata</i>	Alp	48							
	315.0	46.90	16.24	<i>Ichthyosaura alpestris</i>		1							
	267.0	46.91	16.23	<i>Pelophylax esculentus</i>		1							
	315.0	46.90	16.24	<i>Rana temporaria</i>		2							
2-Soproni Mts	493.0	47.65	16.48	<i>Bombina variegata</i>	Alp	14	4 / 14	28.57	8.38-58.10	2.05	2.40	1.13	0.48-2.90
	455.0	47.06	17.67	<i>Bombina bombina</i>		2	37 / 606	6.11	4.33-8.32	21.15	5.19	45.58	0.16-210.30
3-Bakony Mts	316.0	47.23	17.74	<i>Bombina variegata</i>	Alp	3							
	327.0	47.27	17.69	<i>Bombina variegata</i>	Alp	15							
	327.0	47.27	17.69	<i>Bufo bufo</i>		2							
	327.0	47.27	17.69	<i>Ichthyosaura alpestris</i>		12							
	327.0	47.27	17.69	<i>Lissotriton vulgaris</i>		19							
	327.0	47.27	17.69	<i>Rana dalmatina</i>		25							
	348.0	47.23	17.64	<i>Bombina bombina</i>		2							
	348.0	47.23	17.64	<i>Bombina variegata</i>	Alp	310							
	356.0	47.23	17.65	<i>Bufo bufo</i>		61							
	356.0	47.23	17.65	<i>Bufo viridis</i>		39							
4-Hanság	348.0	47.23	17.64	<i>Lissotriton vulgaris</i>		5							
	356.0	47.23	17.65	<i>Pelophylax ridibundus</i>		24							
	348.0	47.23	17.64	<i>Pelophylax sp.</i>		4							
	348.0	47.23	17.64	<i>Rana dalmatina</i>		83							
	113.0	47.66	16.74	<i>Bombina bombina</i>		4	3 / 33	9.09	1.92-24.33	0.56	0.16	0.70	0.15-1.37
	116.0	47.63	17.08	<i>Pelophylax ridibundus</i>	Alp	29							
	381.0	46.22	18.33	<i>Bombina variegata</i>	Alp	12	0 / 23	0.00	0.00-14.82				
	232.0	46.16	18.24	<i>Bombina variegata</i>	Alp	8							
	415.0	46.20	18.33	<i>Bombina variegata</i>	Alp	3							
	89.0	46.61	19.12	<i>Triturus dobrogicus</i>		13	0 / 13	0.00	0.00-24.71				
6-Kiskunság	100.0	47.18	18.53	<i>Bombina bombina</i>		4	2 / 18	11.11	1.38-34.71	36.77	36.77	50.11	1.34-72.20
	111.0	47.42	19.14	<i>Bufo viridis</i>		4							
7-Budapest	156.0	47.53	19.22	<i>Pelophylax ridibundus</i>		10							

Nr. of region	Alt	Lat	Long	Species	mDNA lineage B. <i>variegata</i>	N	Positive/ Sampled	Prev (%)	Prev 95% CI (%)	GE mean	GE median	GE SD	GE range
8-Pilis-Visegrádi Mts	168.0	47.78	19.04	<i>Bombina bombina</i>		1	0 / 78	0.00	0.00 – 4.62				
	418.0	47.78	19.00	<i>Rana dalmatina</i>		5							
	261.0	47.57	18.94	<i>Bufo bufo</i>		1							
	261.0	47.57	18.94	<i>Salamandra salamandra</i>		35							
	216.0	47.64	18.78	<i>Bombina bombina</i>		2							
	329.0	47.76	18.85	<i>Rana temporaria</i>		2							
	183.0	47.76	18.91	<i>Salamandra salamandra</i>		7							
	234.0	47.61	18.88	<i>Hyla arborea</i>		1							
	234.0	47.61	18.88	<i>Pelophylax</i> sp.		3							
	208.0	47.85	19.12	<i>Rana temporaria</i>		1							
	209.0	47.85	19.11	<i>Salamandra salamandra</i>		1							
	107.0	47.77	19.09	<i>Hyla arborea</i>		2							
	107.0	47.77	19.09	<i>Pelophylax</i> sp.		4							
9-Gödöllő Hills	358.0	47.72	19.06	<i>Bombina bombina</i> x <i>variegata</i>		1							
	358.0	47.72	19.06	<i>Bombina variegata</i>	Alp	2							
	301.0	47.78	18.99	<i>Pelophylax ridibundus</i>		8							
	301.0	47.78	18.99	<i>Rana temporaria</i>		2							
	224.0	47.63	19.38	<i>Lissotriton vulgaris</i>		20	0 / 56	0.00	0.00-6.38				
	156.0	47.53	19.22	<i>Pelophylax ridibundus</i>		1							
	111.0	47.76	17.34	<i>Rana arvalis</i>		1							
	96.0	47.26	19.23	<i>Rana arvalis</i>		17							
	96.0	47.26	19.23	<i>Rana dalmatina</i>		3							
	96.0	47.26	19.23	<i>Triturus dobrogicus</i>		14							
	492.0	47.90	19.98	<i>Bombina variegata</i>	Carp	2	7 / 103	6.80	2.78-13.50	6.93	2.13	9.19	0.61-23.55
	648.0	47.93	19.89	<i>Bombina variegata</i>	Carp	2							
	648.0	47.93	19.89	<i>Salamandra salamandra</i>		6							
10-Mátra Mts	598.0	47.90	19.97	<i>Bombina bombina</i>		2							
	587.0	47.85	19.96	<i>Bombina variegata</i>	Carp	3							
	316.0	47.97	19.52	<i>Salamandra salamandra</i>		1							
	720.0	47.90	19.93	<i>Bombina variegata</i>	Carp	4							
	403.0	47.92	19.97	<i>Bombina bombina</i>		2							
	304.0	47.93	19.98	<i>Bombina bombina</i> x <i>variegata</i>		1							
	636.0	47.87	19.97	<i>Bombina variegata</i>	Carp	32							
	727.0	47.88	20.01	<i>Bufo bufo</i>		1							
	727.0	47.88	20.01	<i>Ichthyosaura alpestris</i>		11							

Nr. of region	Alt	Lat	Long	Species	mDNA lineage B. <i>variegata</i>	N	Positive/ Sampled	Prev (%)	Prev 95% CI (%)	GE mean	GE median	GE SD	GE range
	411.0	47.93	19.96	<i>Pelophylax esculentus</i>		1							
	727.0	47.88	20.01	<i>Rana temporaria</i>		1							
	727.0	47.88	20.01	<i>Salamandra salamandra</i>		3							
	364.0	47.90	19.74	<i>Bombina bombina</i>		3							
	362.0	47.93	19.76	<i>Bombina variegata</i>	Carp	1							
	522.0	47.89	20.10	<i>Bombina bombina</i>		6							
	274.0	47.91	20.14	<i>Bombina bombina</i> x <i>variegata</i>		1							
	633.0	47.89	20.11	<i>Bombina variegata</i>	Carp	12							
	636.0	47.93	19.93	<i>Bombina bombina</i> x <i>variegata</i>		5							
	411.0	47.93	19.96	<i>Bombina variegata</i>	Carp	2							
	411.0	47.93	19.96	<i>Pelophylax esculentus</i>		1							
11-Bükk Mts	249.0	48.12	20.24	<i>Bufo bufo</i>		1	1 / 9	11.11	0.28-48.25	8.10	8.10	NA	NA
	320.0	48.15	20.10	<i>Rana temporaria</i>		1							
	443.0	48.04	20.56	<i>Ichthyosaura alpestris</i>		6							
	330.0	48.15	20.08	<i>Rana temporaria</i>		1							
12-Aggtelek Karst	286.0	48.54	20.66	<i>Bombina variegata</i>	Carp	6	0 / 12	0.00	0.00-26.46				
	238.0	48.53	20.64	<i>Salamandra salamandra</i>		6							
13-Zemplén Mts	468.0	48.27	21.29	<i>Bombina variegata</i>	Carp	10	6 / 22	27.27	10.73-50.22	244.00	101.15	328.43	13.03-882.54
	281.0	48.48	21.33	<i>Bombina variegata</i>	Carp	6							
	341.0	48.48	21.32	<i>Rana temporaria</i>		1							
	341.0	48.48	21.32	<i>Salamandra salamandra</i>		4							
	449.0	48.40	21.45	<i>Bombina variegata</i>	Carp	1							
14-Hortobágy	86.0	47.57	20.94	<i>Pelophylax esculentus</i>		18	16 / 178	8.99	5.23-14.19	10.48	1.48	17.98	0.64-57.91
	84.0	47.60	20.88	<i>Pelophylax lessonae</i>		1							
	86.0	47.57	20.94	<i>Pelophylax ridibundus</i>		2							
	85.0	47.62	21.08	<i>Pelophylax esculentus</i>		25							
	86.0	47.61	21.07	<i>Pelophylax ridibundus</i>		56							
	86.0	47.63	21.08	<i>Pelophylax</i> sp.		12							
	85.0	47.44	21.14	<i>Pelophylax esculentus</i>		20							
	85.0	47.44	21.14	<i>Pelophylax ridibundus</i>		42							
	84.0	47.45	21.17	<i>Pelophylax</i> sp.		2							
<b>Total</b>						<b>1233</b>							

### Systematic sampling of sentinel taxa in two core areas

Core areas were selected based on the prevalence found previously or in the first year of sampling (Gál et al., 2012; Baláz et al., 2014b). In Bakony Mts, *B. variegata* was systematically sampled in 2010–2012. Data of 2010 were published previously (Gál et al., 2012), thus our analyses includes a comparison of data from 2010 and new data from 2011 and 2012. Surveys were completed between March and September in 2010, April and September in 2011, May and July in 2012. The assigned locality, Iharkút (see asterisk on Fig. 1), is an old open bauxite mine, where human activities are common due to being a famous paleontological research site (Ósi et al., 2012). In Iharkút we were able to locate only two water bodies: a small lake and a nearby stream. Because of the close proximity (ca. 50 meters) and the presumed connection of the two habitats, all the toads belonged to the same population.

Members of the *P. esculentus* complex were screened for *Bd* in the Hortobágy National Park (HNP; see asterisk on Fig. 1). HNP is the largest continuous alkaline steppe in Europe covering 80,000 hectares. This natural reserve is abundant in wetland habitats like alkaline marshes, fishponds, wet grasslands and wet meadows (Ecsedi, 2004). *Pelophylax* species were sampled in three sites at HNP – Náduvar-Kösely canal near the city Náduvar, a fish pond system located eastwards to Hortobágy village and a marshland system at Egyek-Pusztakócs village – between April and October during three consecutive years (2012–2014).

### Taxonomic identification of *Pelophylax esculentus* complex

Water frog taxon identification was determined using the technique described by Hauswaldt et al. (2012), and is based on allele-size polymorphism in intron-1 of the serum albumin gene (SAI-1; Plötner et al., 2009) named RanaCR1, was identified in the serum albumin intron-1 (SAI-1, with a slight modification in PCR protocol (Herczeg et al., 2017)). To verify SAI-1 fragments we sequenced representative alleles on a Hitachi 3130 Genetic Analyzer (Applied Biosystems, UK). Consensus sequences were compiled using BioEdit version 7.0.9.0 (Hall, 1999) and aligned manually. If genetic samples were not available we referred to the individuals as *Pelophylax* sp.

### Sampling protocol

We collected *Bd* samples following Hyatt et al. (2007) by either swabbing the skin of the individuals or clipping one of the toes. According to Hyatt et al. (2007) skin swabbing and toe clipping show similar performances in detectability of *Bd*. Skin swabbing was performed using two types of sterile swabs (SWA90006; Biolab, Budapest, Hungary, 5 mm diameter; and MW100-100; Medical Wire and Equipment, Wiltshire, England, 3 mm diameter). We collected each sample in a standardized way with three strokes on each side of the abdominal midline, the inner thighs, hands and feet. Toe clipping was performed using sterilized scissors and toe clips were stored in 70% EtOH in a freezer at -80 °C. Skin swabs were stored dry in individu-

ally labelled vials and transferred to a freezer for longer storage throughout the field season. For both sampling procedures we used a new pair of disposable gloves per individual, and after each sampling event we sterilized all the used sampling equipment in order to avoid cross-contamination. Mouthpart (oral disc) of larvae were swabbed following Hyatt et al. (2007). Ethanol-fixed specimens of *Bombina* spp. were screened by skin swabbing following methodology presented above.

### Genetic analysis of *Bd* samples

DNA was extracted using PrepMan Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the recommendations of Boyle et al. (2004). Because of size differences between swabs (i.e. 3 mm vs. 5 mm; see above), only the top 3 mm of the larger swabs was used in all cases. Extracted DNA was analysed using real-time quantitative polymerase chain reaction (qPCR) following the amplification methodology of Boyle et al. (2004) and Hyatt et al. (2007) targeting the partial ITS-1 – 5.8S rRNA regions. Samples were run in triplicate and an internal positive control was included (TaqMan exogenous internal positive control reagents; 4308323; Thermo Fisher Scientific, Waltham, Massachusetts, USA) to detect potential inhibitors present in the DNA extractions. We considered evidence of infection if genomic equivalents (GE) were  $\geq 0.1$  and we considered a sample positive if all three wells returned a positive reaction. When a sample returned an equivocal result, it was re-run. If it again returned an equivocal result, it was considered negative ( $N = 17$ , 1.3% of total samples). The templates were run on a Rotor-Gene 6000 real-time rotary analyser (Corbett Life Science, Sydney, Australia). GE were estimated from standard curves based on positive controls of 100, 10, 1, 0.1 developed from the *Bd* isolate IA 2011, from Acherito Lake, Spain. Finally, GE values of the three positive replicates were averaged.

In order to identify lineages of *Bd* found on amphibians in Hungary, 2  $\mu$ l of DNA extract from three individuals (one juvenile *P. ridibundus* plus one juvenile *B. variegata* from Bakony Mts, and one adult *B. variegata* from Órség) were selected as template for amplification of a partial fragment of ITS-1 rRNA. Nested PCR approach described by Gaertner et al. (2009) was performed. The amplified fragments were sequenced on an Applied Biosystems/Hitachi 3130 Genetic Analyser (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Sequences were aligned manually using BioEdit version 7.0.9.0. (Hall, 1999) and were blasted against available sequences from GenBank for identification.

### Climatic data

Climatic data were provided by the Hungarian Meteorological Service (OMSZ). For the core areas of *B. variegata* and *P. esculentus* complex climatic data were obtained from the closest meteorological station of each sampling site: Pápa city (47.29, 17.37), 135.5 m a.s.l, 21.5 km distance from Iharkút (Bakony Mts), and Kunmadaras village (47.46, 20.89), 88.8 m a.s.l. 12.5

km distance from Egyek-Pusztakócs (HNP), which is the closest sampling point to the station. We used monthly mean precipitation and monthly mean air temperature data for the period 2010-2014 to test if any relationship between climate and prevalence or infection intensity exists.

#### Statistical analyses

Statistical analyses were performed in R (version 3.4.4; R Core Team, 2018). Prevalence was expressed as a discrete binomial variable (uninfected vs. infected). Infection intensity was expressed through GE value. First, we calculated infection prevalence (%) of different amphibian species together with their 95% Clopper-Pearson confidence intervals (95% CI) as follows. Prevalence values were obtained by dividing the cumulative number of positive samples with the total number of samples per species and multiplied with 100 to obtain percentile values, while 95% CI values were calculated using the R package 'PropCIs' (function 'exactci'; Scherer, 2018). In *Bd* infected species we calculated the mean, median, SD and range of GE values as well. Second, we tested whether prevalence and infection intensity differed between phylogenetic lineages of *B. variegata*, and in the two sentinel taxa (i.e. *B. variegata* and *P. esculentus* complex) we also tested for differences between study years, sexes and age classes. Prevalence values were compared with Chi-square tests, while infection intensities were compared using Mood's median test, as implemented in the R package 'RVAideMemoire' (function 'mood medtest'; Hervé, 2018).

Finally, in the two sentinel taxa we tested the relationship between climatic variables and prevalence and infection intensity. We note here that the data set of the *P. esculentus* complex was restrained only on *P. ridibundus*, as the *Bd* infection of *P. esculentus* was very low (i.e. two infected individuals in total) and the sample size of *P. lessonae* was also not representative ( $N = 1$ ). The relationship between the climatic factors and infection prevalence was tested using generalized linear mixed models (GLMMs) with binomial error distribution term and the relationship between the climatic factors and infection intensity was analysed using linear mixed models with Gaussian distribution (LMMs). Prevalence and infection intensity, respectively, were entered as dependent variables in the models, while the focal climatic variable (i.e., air temperature or precipitation) was set as continuous predictor. In all models sampling year was entered as a random effect to control for the interannual variations in infection prevalence or intensity. Additionally, in the case of *P. ridibundus*, collection site ID within the HNP was entered also as a random factor to account for the variations in prevalence and intensity between collection sites. To assure the adequate distribution of model residuals, for the LMMs GE values were  $\log(x+1)$ -transformed. Prior entering into the models,  $\log(x+1)$ -transformed GE values and the continuous predictors (i.e. climatic variables) were scaled to mean = 0 and SD = 1 to improve model convergence (see also Schielzeth 2010). Model fits were checked visually by plot diagnosis. In all cases for the statistical comparison of infection intensities only infected individuals were used. Mixed models were constructed using the 'lme4' package for R (Bates et al., 2015), and P-values for the

linear mixed models were obtained using the function 'Anova' (type III) from the R package 'car' (Fox and Weisberg, 2011). We used a significance level of  $P \leq 0.05$  throughout.

## RESULTS

### *Bd* occurrence in Hungary

In Hungary, nine regions were infected with *Bd* and the overall prevalence was 7.46% (95% CI: 6.05-9.07), indicating a low presence of the fungus in the country (Table 1). Among the sixteen sampled amphibian taxa seven were found infected with *Bd*, including one unidentified *Pelophylax* individual (Table 2). Details on prevalence and summary statistics of GE values are presented in Table 2; while the geographic distribution of the sampling sites with the site-specific prevalence is shown in Fig. 1.

### *Bd* occurrence in *Bombina variegata*

In *B. variegata* the overall prevalence was 12.69% (95% CI: 9.91-15.92). Details on prevalence and summary statistics of GE values for the different regions are presented in Table 3. We found no significant difference between the two lineages of *B. variegata* in infection prevalence ( $N_{\text{Alpine}} = 422$ ,  $N_{\text{Carpathian}} = 82$ ;  $\chi^2 = 0.155$ ,  $df = 1$ ,  $P = 0.693$ ) and intensity ( $N_{\text{Alpine}} = 52$ ,  $N_{\text{Carpathian}} = 12$ ,  $P = 0.750$ ). *Bd* was not detected among the ethanol-fixed *B. variegata* specimens.

In Bakony Mts between 2010 and 2012 we sampled 310 individuals of *B. variegata*, among which 32 individuals were found to be infected with *Bd*. Here the overall prevalence was 10.32 % (95% CI: 7.16-14.25), and the mean, median, SD and range of GE values were 15.92, 5.09, 38.60 and 0.159-210.3, respectively. There was no significant difference in infection prevalence ( $N_{2010} = 80$ ,  $N_{2011} = 144$ ,  $N_{2012} = 86$ ;  $\chi^2 = 4.980$ ,  $df = 2$ ,  $P = 0.082$ ) nor in intensity between the three study years ( $N_{2010} = 13$ ,  $N_{2011} = 14$ ,  $N_{2012} = 5$ ,  $P = 0.201$ ), and we found no significant difference in prevalence ( $N_{\text{males}} = 113$ ,  $N_{\text{females}} = 90$ ;  $\chi^2 = 0.241$ ,  $df = 1$ ,  $P = 0.623$ ) and infection intensity between sexes ( $N_{\text{males}} = 8$ ,  $N_{\text{females}} = 2$ ,  $P = 0.545$ ). However, there was a significant difference in prevalence between the two age classes ( $N_{\text{juveniles}} = 105$ ,  $N_{\text{adults}} = 204$ ;  $\chi^2 = 11.563$ ,  $df = 1$ ,  $P < 0.001$ ), with juveniles being more infected than adults (proportion of individuals infected: 19.04% versus 5.88%). Differences in infection intensity between the two age classes were not significant ( $N_{\text{juveniles}} = 20$ ,  $N_{\text{adults}} = 12$ ,  $P = 0.273$ ). There was significant negative relationship between infection prevalence and monthly mean air temperature ( $\chi^2 = 4.482$   $df = 1$ ,  $P =$



**Table 2.** *Batrachochytrium dendrobatidis* (*Bd*) infection in amphibian species sampled in Hungary between the years 2009 and 2015. Prev = prevalence; GE = genomic equivalents of zoospores.

Species	Positive/ Sampled	Prev (%)	Prev 95% CI (%)	GE mean	GE median	GE SD	GE range
<b>Order Anura</b>							
<b>Family Bombinatoridae</b>							
<i>Bombina bombina</i>	1 / 29	3.45	0.09-17.76	16.41	16.41	NA	NA
<i>Bombina variegata</i>	64 / 504	12.70	9.92-15.92	40.08	4.96	120.76	0.16-882.54
<i>Bombina bombina</i> x <i>variegata</i>	0 / 8	0.00	0.00-36.94				
<b>Family Bufonidae</b>							
<i>Bufo bufo</i>	0 / 66	0.00	0.00-5.44				
<i>Bufo viridis</i>	2 / 43	4.65	0.57-15.81	36.77	36.77	50.11	1.34-72.20
<b>Family Hylidae</b>							
<i>Hyla arborea</i>	0 / 4	0.00	0.00-60.24				
<b>Family Ranidae</b>							
<i>Pelophylax esculentus</i>	2 / 66	3.03	0.37-10.52	1.07	1.07	0.41	0.78-1.36
<i>Pelophylax lessonae</i>	0 / 1	0.00	0.00-97.5				
<i>Pelophylax ridibundus</i>	21 / 164	12.80	8.10-18.91	20.21	1.59	41.72	0.15-164.30
<i>Pelophylax</i> sp.	1 / 33	3.03	0.08-15.76	15.75	15.75	NA	NA
<i>Rana dalmatina</i>	0 / 120	0.00	0.00-3.03				
<i>Rana arvalis</i>	0 / 19	0.00	0.00-17.65				
<i>Rana temporaria</i>	0 / 11	0.00	0.00-28.49				
<b>Order Caudata</b>							
<b>Family Salamandridae</b>							
<i>Salamandra salamandra</i>	0 / 63	0.00	0.00-5.69				
<i>Triturus dobrogicus</i>	0 / 27	0.00	0.00-12.77				
<i>Lissotriton vulgaris</i>	0 / 45	0.00	0.00-7.87				
<i>Ichthyosaura alpestris</i>	1 / 30	3.33	0.08-17.22				
Total	92 / 1233	7.46	6.05-9.07				

**Table 3.** *Batrachochytrium dendrobatidis* (*Bd*) detection in regions representing the surveyed local populations of *B. variegata* in Hungary. Prev = prevalence; GE = genomic equivalents of zoospores.

Genetic lineage	Region	Positive/ Sampled	Prev (%)	Prev 95% CI (%)	GE mean	GE median	GE SD	GE range
Alpine	Órség	16 / 57	28.07	16.97-41.54	34.45	5.01	58.32	0.20-182.78
	Soproni Mts	4 / 14	28.57	8.39-58.10	2.05	2.40	1.13	0.48-2.90
	Bakony Mts	32 / 328	9.76	6.77-13.49	15.93	5.09	38.61	0.16-210.30
	Mecsek Mts	0 / 23	0.00	0.00-14.82				
	Pilis-Visegrádi Mts	0 / 2	0.00	0.00-84.19				
Carpathian	Mátra Mts	6 / 58	10.34	3.89-21.17	5.36	1.86	8.97	0.61-23.55
	Aggtelek Karst	0 / 6	0.00	0.00-45.93				
	Zemplén Mts	6 / 16	37.50	15.20-64.57	244.00	101.15	328.43	13.03-882.54
Total		64 / 504	12.59	9.83-15.80				

0.034), and a marginally significant positive relationship between prevalence and monthly mean precipitation ( $\chi^2 = 3.611$ ,  $df = 1$ ,  $P = 0.057$ ). There was no significant rela-

tionship between infection intensity and monthly mean air temperature ( $\chi^2 = 0.180$ ,  $df = 1$ ,  $P = 0.671$ ). However, there was a significant positive relationship between

infection intensity and monthly mean precipitation ( $\chi^2 = 4.227$ ,  $df = 1$ ,  $P = 0.039$ ); though, this significant relationship disappeared after removing one outlier GE value from the data set ( $\chi^2 = 1.510$ ,  $df = 1$ ,  $P = 0.219$ ).

All the three sequences (i.e. sequences obtained from juvenile *P. ridibundus* and *B. variegata* from Bakony Mts, and one adult *B. variegata* from Órség) were identified as ITS-1 rRNA of *Bd*, belonging to the globally dispersed *Bd*-GPL lineage (GenBank accession numbers: MH745069-71). One sequence showed 100% identity with *Bd* from Cape Cod (GenBank accession number: FQ176489.1, FQ176492.1), South Africa (JQ582903-4, 15, 37), and Italy (FJ010547). The second sequence was 100% identical with a sequence of *Bd* from Ecuador (FJ232009.1), and the third sequence represented a unique haplotype. Genetic distance (p-distance) among sequences ranged between 0.005-0.035.

#### *Bd* occurrence in *Pelophylax ridibundus*

In Hortobágy between 2012 and 2014 we sampled 100 individuals of *P. ridibundus*, among which 13 were found to be infected with *Bd*. Here the overall prevalence was 13.00% (7.10-21.20), and the mean, median, SD and range of GE values were 11.52, 1.59, 19.63 and 0.635–57.905, respectively. We found a significant difference in infection prevalence between years ( $N_{2012} = 35$ ,  $N_{2013} = 48$ ,  $N_{2014} = 17$ ;  $\chi^2 = 27.750$ ,  $df = 2$ ,  $P < 0.001$ ); all the infected individuals being captured in 2012 (prevalence: 37.14%), while no infected individuals being found in 2013-2014. We found no significant difference in prevalence ( $N_{males} = 42$ ,  $N_{females} = 30$ ;  $\chi^2 = 0.002$ ,  $df = 1$ ,  $P = 0.958$ ) and infection intensity between sexes ( $N_{males} = 7$ ,  $N_{females} = 6$ ,  $P = 1.000$ ). Age classes did not differ in infection prevalence ( $N_{juveniles} = 9$ ,  $N_{adults} = 72$ ;  $\chi^2 = 0.827$ ,  $df = 1$ ,  $P = 0.363$ ). Infection intensities of the different age classes cannot be compared because no infected juveniles were captured. We found no significant relationship between infection prevalence and monthly mean air temperature ( $\chi^2 = 2.375$ ,  $df = 1$ ,  $P = 0.123$ ), and between prevalence and monthly mean precipitation ( $\chi^2 = 0.010$ ,  $df = 1$ ,  $P = 0.920$ ). Since infection prevalence was relatively low in the *P. esculentus* complex and infected individuals were captured in the same month and year, the relationship between climatic variables and infection intensity could not be tested in this taxa.

## DISCUSSION

Low *Batrachochytrium dendrobatidis* prevalence was experienced throughout the country (Table 1, Table 2),

with similar or slightly lower values than in neighbouring countries e.g. Czech Republic (Baláz et al., 2014a; 19% average at country level), Austria (Sztatecsny and Glaser, 2011; 5.9-45% at country level) or Poland (Kolenda et al., 2017; 18% average at country level). Overall, seven taxa carried the infection: *Bombina bombina*, *Bombina variegata*, *Bufo viridis*, *Pelophylax ridibundus*, *Pelophylax esculentus*, *Pelophylax* sp. and *Ichthyosaura alpestris*. In accordance with previous studies in Central Europe (Ohst et al., 2013; Baláz et al., 2014a,b; Kolenda et al., 2017), *B. variegata* and the members of the *P. esculentus* complex showed the highest prevalence and *Bd* infection intensity in Hungary. On the other hand, there was no difference in prevalence and infection intensity was detected between the two ancient phylogenetic lineages of *B. variegata*. *Bd* was present in nine of the fourteen studied regions. The highest prevalence was experienced in the Alpine foothills at Órség (Region 1), Soproni Mts (Region 2), and in the Zemplén Mts (Region 13). These three regions represent the margins of the Alps and Carpathians (respectively) hosting populations with continuous distribution towards the higher regions. On the other hand, the remnant mountain regions, where prevalence was much lower (Regions 3, 10 and 11), are geographically isolated from other higher elevations. In contrast, amphibians from five regions (Regions 5, 6, 8, 9 and 12) seemed to not carry *Bd*. This either indicates that *Bd* has not reached these parts of the country yet, or more comprehensive sampling would be needed to locate its presence.

The Carpathian Basin combines the characteristics of the neighbouring regions. Despite the relatively small extent of Hungary, the climatic elements have distinct temporal and spatial characters (Mezősi, 2017). Although the majority of the country has an elevation of less than 300 m a.s.l., Hungary has several moderately high ranges of mountains and the highest peak located in the Mátra Mts at 1014 m a.s.l. (Table 1, Region 10). Overall, our results rather supporting the relationship between the measured climatic variables and prevalence or infection intensity. We found significant relationship regarding *B. variegata* individuals in the Bakony Mts core area, where prevalence was negatively affected by monthly mean temperature. Furthermore, the monthly mean precipitation positively affected the *Bd* infection intensity. Nonetheless, the robustness of the latter result is questionable, since the relationship disappeared when we excluded an outlier value from the analysis. This substantial effect of one outlier value could have on the outcomes of this analysis suggests the need for an extensive sampling in order to test whether this result is a statistical artefact or a real biological phenomenon.

To determine the time and location of the emergence or introduction of *Bd* in different regions worldwide, it is important to study archived specimens deposited to museum collections. To examine the historical presence of the fungus in Hungary we screened archived specimens of *Bombina* spp. collected in the regions 1, 2, 3, 8, 10, 12, 13 and the Kőszeg Mts (archived data only) between 1936 and 2005. In total 127 specimens were analysed and all of the samples were *Bd* negative. Both for field and for museum samples we used the same detection methodology, following Hyatt et al. (2007). The detection probability with qPCR is more sensitive and accurate compared to conventional PCR or histology (Annis et al., 2004; Boyle et al., 2004; Kriger et al., 2006). There is no difference in regard of *Bd* detectability between sample collection techniques (i.e., skin swabbing, brushing or scraping). Nonetheless, preservation methodology and storage history may have influence on the results (Soto-Azat et al., 2009). The Amphibian Collection of the Hungarian Natural History Museum is stored in ethanol, but no record is available about the mode of initial preparation. As formaldehyde is known to inhibit PCR reaction, there is therefore a slight chance that qPCR reactions failed to detect *Bd* in our archived samples; however, this may be an unlikely possibility.

Although with testing archived specimens we did not find evidence on when *Bd* might have been introduced into the country, our genetic analyses showed that the fungus found on amphibians in Hungary is a member of the *Bd*-GPL lineage. This was confirmed by a recent study tracking the origin of *Bd* using a full genome approach, which detected *Bd*-GPL lineage in Hungary (from Iharkút, Bakony Mts; O'Hanlon et al., 2018) and is in line with previous findings reporting that this lineage has a widespread distribution in Europe (Farrer et al., 2007).

During the surveys in the core area of Bakony Mts (Region 3, Table 1) juvenile *B. variegata* individuals showed a significantly higher prevalence compared to adults. The same pattern was observed for two *B. variegata* populations in a seven-year period study in the Netherlands, which the authors explained by the less developed immune responses, or immunosuppression, following the stress of metamorphosis (Spitzen-van der Sluijs et al., 2017). Quite surprisingly, during our study, two juveniles changed infection state once (recovered from *Bd* positive). It is a relatively common phenomenon in the field, when infected adult frogs lose and regain the infection which may be caused by overwintering tadpoles or larvae acting as reservoirs (Briggs et al., 2010; Spitzen-van der Sluijs et al., 2017). In contrast, it is less frequent with juvenile individuals as it was experienced in our study. Similar pattern was observed for *Epidalea calamita*

in Spain, where juveniles changed infection state towards the end of metamorphosis, possibly mediated by the increasing water temperature in permanent ponds (Bosch et al., unpublished).

In Iharkút (Bakony Mts), during our study period the environmental conditions changed unexpectedly. The lake which hosted most of the amphibian species – including *B. variegata* – dried out after the first season of sample collection. In the second year only four individuals of *B. variegata* were captured around this locality, however the rest of the specimens (N = 181) found shelter in a nearby stream unsuitable for breeding. During the third year the lake kept dry and only seven out of 87 individuals were found in or around the lake. Even though there was no difference in prevalence between the three years, they showed a downward trend towards significance. Already low prevalence (23%) dropped down to 11% in the second and to 5% in the third year. This trend could be associated with the differences in habitat type, as it was observed for *Salamandra salamandra* in the Guadarrama National Park, Spain (Medina et al., 2015). Here, *Bd* infection was greater in salamander larvae from permanent ponds, while it was absent or weak in temporary water bodies and permanent streams. Also, infection intensity in larval cohorts was reduced when water was flowing rather than standing. Same authors suggested that increased water flow rate reduce the likelihood of successful pathogen transmission.

Chytridiomycosis is limited to the keratinized tissues of the host individual, therefore tadpoles and post-metamorphic amphibians are mostly affected by the disease (Rachowicz and Vredenburg, 2004). Our dataset covered all life stages of amphibians and the presence of the infection was not detected in tadpoles of *B. bufo* and *R. dalmatina* (N = 39). On the other hand, post-metamorphic and juvenile individuals were found infected in the regions 1, 3, 10 and 13 of *B. variegata* and the members of the *P. esculentus* complex, even though all sampled individuals apparently didn't display any clinical sign of chytridiomycosis.

In Central Europe the *P. esculentus* complex is formed by two sexual species, the *P. ridibundus* and the *P. lessonae* and their interspecific mating produces the hybridogenetic *P. esculentus*. Overall, our results in the core area of Hortobágy National Park showed higher *Bd* prevalence in *P. ridibundus* compared to the hybrid *P. esculentus* (Table 2) which is related to the fact that the hybrids have more effective peptide defence system against *Bd* and have a richer peptide repertoire than both parental species (Daum et al., 2012). Further, contrary to what was observed in *B. variegata* in the Bakony Mts core area, we did not find differences in *Bd* infection between

life stages and sexes in *P. ridibundus* individuals.

Our results fit into the general pattern showing significant variability in the effects of chytridiomycosis across Europe. The marked difference in species susceptibility between amphibian species/communities of Western and Central-Eastern Europe might be determined by multiple linked factors, e.g. virulence of different *Bd* strains (Farrer et al., 2007), genotype (Savage and Zamudio, 2011), behaviour (Williams and Groves, 2014), microbial skin community compound of host species (Bletz et al., 2013), or structure of amphibian communities (Becker et al., 2014). In the Iberian Peninsula – that received the most attention due to mass amphibian mortalities caused by chytridiomycosis – infection was clustered within high-altitude areas, where environmental conditions are the most optimal for growth of *Bd* (Piotrowski et al., 2004). In contrast, Hungary harbours only low-elevation mountains, where environmental conditions might be less favourable for *Bd*-linked epidemics. Differences in elevation might explain the relatively lower impact and infection values of amphibians in Hungary, than it was reported for surrounding countries in Central and Eastern Europe (e.g., Austria, Sztatecsny and Glaser, 2011; Czech Republic, Baláz et al., 2014a or Poland, Kolenda et al., 2017).

Since *Bd*-related disease outbreak have been proven to be climate-driven (Bosch et al., 2007), amphibians of Central-Eastern Europe might be heavily impacted in the future due to global climate change. Changes in the climate might alter *Bd* diffusion and make it's spreading less predictable, thus areas not yet affected by epidemics require particular attention and constant monitoring.

#### ACKNOWLEDGEMENTS

We thank B. Halpern, K. Szabó, I. Kiss, E. Jáger, K. Suri, R. Dankovics, B. Velekei, A. Ósi, G. Deák, Cs. Tóth, A. Bérczes, G. Magos, L. Urbán, B. Bándli, M. García-París, P. Mikulíček, C. Gabor, C. Serrano-Laguna, Zs. Végvári, D. R. Brooks, E. Vörös, L. Vörös, E. Kovács and F. Hock for providing samples or helping in the field. We are grateful to T. Garner (Zoological Society of London) for important initial and then continuous help with *Bd* research in Hungary. T. Papp and M. Benkő (Institute for Veterinary Medical Research, Budapest) kindly provided facility and reagents for qPCR. We gratefully acknowledge the excellent technical assistance of Á. Juhász and E. Ottinger (National Food Chain Safety Office), as well as M. Tuschek and V. Krízsik (HNHM). Savaria Museum Szombathely provided toad specimens for sampling. During the project

JV was supported by the Hungarian Scientific Research Fund (OTKA K77841) and by the Bolyai János Research Scholarship of the Hungarian Academy of Sciences (BO/00579/14/8). DH was supported by the European Union and co-financed by the European Social Fund through the Social Renewal Operational Programme under the projects TÁMOP-4.2.2/B-10/1-2010-0024 and SROP-4.2.2.B-15/1/KONV-2015-0001. AF was supported by the Hungarian National Research, Development and Innovation Office (OTKA grant no. K112527). Research permit was issued by the National Inspectorate of Environment, Nature Conservation and Water Management (14/3535/2/2010) and the Tisza Region Inspectorate of Environment, Nature Conservation and Water Management (4633/OH/2012).

#### SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <<http://www.unipv.it/webshi/appendix>> manuscript number 22611.

#### REFERENCES

- Annis, S.L., Dastoor, F.P., Ziel, H., Daszak, P., Longcore, J.E. (2004): A DNA-based assay identifies *Batrachochytrium dendrobatidis* in amphibians. *J. Wildl. Dis.* **40**: 420-428.
- Baláz, V., Vojar, J., Civiš, P., Andera, M., Rozínek, R. (2014a): Chytridiomycosis risk among Central European amphibians based on surveillance data. *Dis. Aquat. Organ.* **112**: 1-8.
- Baláz, V., Vörös, J., Civiš, P., Vojar, J., Hettyey, A., Sós, A., Dankovics, R., Jehle, R., Christiansen, D.G., Clare, F., Fisher, M.C., Garner, T.W.J., Bielby, J. (2014b): Assessing risk and guidance on monitoring of *Batrachochytrium dendrobatidis* in Europe through identification of taxonomic selectivity of infection. *Conserv. Biol.* **28**: 213-223.
- Bates, D., Maechler, M., Bolker, B., Walker, S. (2015): Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* **67**: 1-48.
- Becker, C.G., Rodriguez, D., Toledo, L.F., Longo, A.V., Lambertini, C., Correa, D.T., Leite, D.S., Haddad, C.F.B., Zamudio, K.R. (2014): Partitioning the net effect of host diversity on an emerging amphibian pathogen. *Proc. R. Soc. B* **281**: 20141796.
- Becker, C.G., Zamudio, K.R. (2011): Tropical amphibian populations experience higher disease risk in natural habitats. *Proc. Natl. Acad. Sci. USA* **108**: 9893-9898.

- Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., Slocombe, R., Ragan, M.A., Hyatt, A.H., McDonald, K.R., Hines, H.B., Lips, K.R., Marantelli, G., Parkes, H. (1998): Chytridiomycosis causes amphibian mortality associated with population declines in the rain forest in Australia and Central America. *Proc. Natl. Acad. Sci. USA* **95**: 9031-9036.
- Berger, L., Speare, R., Hines, H.B., Marantelli, G., Hyatt, A.D., McDonald, K.R., Skerratt, L.F., Olsen, V., Clarke, J.M., Gillespie, G., Mahony, M., Sheppard, N., Williams, C., Tyler, M.J. (2004): Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Aust. Vet. J.* **82**: 31-36.
- Blaustein, A.R., Walls, S.C., Bancroft, B.A., Lawler, J.J., Searle, C.L., Gervasi, S.S. (2010): Direct and indirect effects of climate change on amphibian populations. *Diversity* **2**: 281-313.
- Blaustein, A.R., Romansic, J.M., Scheesele, E.A., Han, B.A., Pessier, A.P., Longcore, J.E. (2005): Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus *Batrachochytrium dendrobatidis*. *Conserv. Biol.* **19**: 1460-1468.
- Bletz, M.C., Loudon, A.H., Becker, M.H., Bell, S.C., Woodhams, D.C., Minbiole, K.P.C., Harris, R.N. (2013): Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.* **16**: 807-820.
- Böll, S., Tobler, U., Geiger, C.C., Günter, H., Schmidt, B.R. (2014): Unterschiedliche *Bd*-Prävalenzen und -Befallsstärken verschiedener Amphibienarten und Entwicklungsstadien an einem Chytridpilz belasteten Standort in der bayerischen Rhön. *Z. Feldherpetol.* **21**: 183-194.
- Bosch, J., Martínez-Solano, I. (2006): Chytrid fungus infection related to unusual mortalities of *Salamandra salamandra* and *Bufo bufo* in the Peñalara Natural Park (Central Spain). *Oryx* **40**: 84-89.
- Bosch, J., Martínez-Solano, I., García-París, M. (2001): Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biol. Conserv.* **97**: 331-337.
- Bosch, J., Carrascal, L.M., Durán, L., Walker, S., Fisher, M.C. (2007): Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain: is there a link? *Proc. R. Soc. B.* **274**: 253-260.
- Bosch, J., García-Alonso, D., Fernández-Beaskoetxea, S., Fisher, M.C., Garner, T.W. (2013): Evidence for the introduction of lethal chytridiomycosis affecting wild Betic midwife toads (*Alytes dickhilleni*). *EcoHealth* **10**: 82-89.
- Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A.T., Hyatt, A.D. (2004): Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Dis. Aquat. Org.* **60**: 141-148.
- Briggs, C.J., Knapp, R.A., Vredenburg, V.T. (2010): Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proc. Natl. Acad. Sci. USA* **107**: 9695-9700.
- Civiš, P., Vojar, J., Literák, I., Balaž, V. (2012): Current state of *Bd*'s occurrence in the Czech Republic. *Herpetol. Rev.* **43**: 75-78.
- Daum, J.M., Davis, L.R., Bigler, L., Woodhams, D.C. (2012): Hybrid advantage in skin peptide immune defenses of water frogs (*Pelophylax esculentus*) at risk from emerging pathogens. *Infect. Genet. Evol.* **12**: 1854-1864.
- Dobson, A.P., Foufopoulos, J. (2001): Emerging infectious pathogens of wildlife. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **356**: 1001-1012.
- Doddington, B.J., Bosch, J., Oliver, J.A., Grassly, N.C., García, G., Benedikt, R.S., Garner, T.W.J., Fisher, M.C. (2013): Context-dependent amphibian host population response to an invading pathogen. *Ecology* **98**: 1795-1804.
- Drexler, T., Ujszegi, J., Németh, Z.M., Vörös, J., Hettyey, A. (2017): A susceptibility and sensitivity to chytridiomycosis of two anuran species native to Hungary. *Természetvédelmi Közlemények* **23**: 14-23. (in Hungarian with English abstract)
- Ecsedi, Z. (2004): A Hortobágy Madárvilága. Hortobágy Természetvédelmi Egyesület, Winter Fair, Balmazújváros-Szeged. (in Hungarian)
- Farrer, R.A., Weinert, L.A., Bielby, J., Garner, T.W.J., Balloux, F., Clare, F., Bosch, J., Cunningham, A.A., Weldon, C., Preez, L.H., Anderson, L., Pond, S.L.K., Shahar-Golan, R., Henk, D.A., Fisher, M.C. (2007): Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *P. Natl. Acad. Sci. USA* **108**: 18732-18736.
- Federici, S., Clemenzi, S., Favelli, M., Tessa G., Andreone, F., Casiraghi, M., Crottini, A. (2008): Identification of the pathogen *Batrachochytrium dendrobatidis* in amphibian populations of a plain area in the Northwest of Italy. *Herpetology Notes* **1**: 33-37.
- Fisher, M.C., Garner, T., Walker, J. (2009): Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time and host. *Ann. Rev. Microbiol.* **63**: 291-310.

- Fox, J., Weisberg, S. (2011): An R companion to applied regression, second edition. Sage, Thousand Oaks, CA. <http://socserv.socsci.mcmaster.ca/jfox/Books/Companion>.
- Gaertner, J.P., Forstner, M.R.J., O'Donnell, L., Hahn, D. (2009): Detection of *Batrachochytrium dendrobatidis* in endemic salamander species from Central Texas. *EcoHealth* **6**: 20-26.
- Gál, J.T., Szabó, K., Vörös, J. (2012): Kitridiomikózis vizsgálata egy magas-bakonyi vizes élőhely kételtű közösségén. *Állattani Közlemények* **97**: 47-59.
- Garcia, T.S.J., Romansic, M., Blaustein, A.R. (2006): Survival of three species of anuran metamorphs exposed to UV-B radiation and the pathogenic fungus *Batrachochytrium dendrobatidis*. *Dis. Aquat. Organ.* **72**: 163-169.
- Gründler, C.M., Toledo, L.F., Parra-Olea, G., Haddad, C.F.B., Giasson, L.O.M., Sawaya, R.J., Prado, C.P.A., Araujo, O.G.S., Zara, F.J., Centeno, F.C., Zamudio, K.R. (2012): Interaction between breeding habitat and elevation affects prevalence but not infection intensity of *Batrachochytrium dendrobatidis* in Brazilian anuran assemblages. *Dis. Aquat. Organ.* **97**: 173-184.
- Hall, T.A. (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* **41**: 95-98.
- Hauswaldt, J.S., Höer, M., Ogielska, M., Christiansen, D.G., Dziewulska-Szwajkowska, D., Czernicka, E.A., Vences, M. (2012): A simplified molecular method for distinguishing among species and ploidy levels in European water frogs (*Pelophylax*). *Mol. Ecol. Resour.* **12**: 797-805.
- Herczeg, D., Vörös, J., Christiansen, D.G., Benovics, M., Mikulíček, P. (2017): Taxonomic composition and ploidy level among European water frogs (Anura: Ranidae: *Pelophylax*) in eastern Hungary. *J. Zool. Syst. Evol. Res.* **55**: 129-137.
- Hervé, M. (2018): RVAideMemoire: Testing and Plotting Procedures for Biostatistics. R package version 0.9-69-3. <https://CRAN.R-project.org/package=RVAideMemoire>
- Hyatt, A.D., Boyle, D.G., Olsen, V., Boyle, D.B., Berger, L., Obendorf, D., Dalton, A., Kriger, K., Hero, M., Hines, H., Phillot, R., Campbell, R., Marantelli, G., Gleason, F., Coiling, A. (2007): Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Dis. Aquat. Org.* **73**: 175-192.
- Kolenda, K., Najbar, A., Ogielska, M., Baláž, V. (2017): *Batrachochytrium dendrobatidis* is present in Poland and is associated with reduced fitness in wild population of *Pelophylax lessonae*. *Dis. Aquat. Org.* **124**: 241-245.
- Kruger, K.M., Hines, H.B., Hyatt, A.D., Boyle, D.G., Hero, J.M. (2006): Techniques for detecting chytridiomycosis in wild frogs: comparing histology with real-time Taqman PCR. *Dis. Aquat. Org.* **71**: 141-148.
- Lips, K.R., Diffendorfer, J., Mendelson, J.R., Sears, M.W. (2008): Riding the wave: reconciling the roles of disease and climate change in amphibian declines. *PLoS Biol.* **6**: e72.
- Longcore, J.E., Pessier, A.P., Nichols, D.K. (1999): *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia* **91**: 219-227.
- Mali, I., Villamizar-Gomez, A., Krizmanić, I., Ajtić, R., Forstner, M.R.J. (2017): Evidence of *Batrachochytrium dendrobatidis* infection in amphibians from Serbian lowlands. *J. Wildl. Dis.* **53**: 686-689.
- Medina, D., Garner, T.W., Carrascal, L.M., Bosch, J. (2015): Delayed metamorphosis of amphibian larvae facilitates *Batrachochytrium dendrobatidis* transmission and persistence. *Dis. Aquat. Org.* **117**: 85-92.
- Mezősi, G. (2017): Climate of Hungary. In: *The Physical Geography of Hungary*, pp. 101-119. Mezősi, G., Ed, Springer, Cham.
- Murray, K.A., Skerratt, L.F., Speare, R., McCallum, H. (2009): Impact and dynamics of disease in species threatened by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*. *Conserv. Biol.* **23**: 1242-1252.
- O'Hanlon, S.J., Rieux, A., Farrer R.A. et al. (2018): Recent Asian origin of chytrid fungi causing global amphibian declines. *Science* **360**: 621-627.
- Ohst, T., Gräser, Y., Plötner, J. (2013): *Batrachochytrium dendrobatidis* in Germany: distribution, prevalences, and prediction of high risk areas. *Dis. Aquat. Org.* **107**: 49-59.
- Olson, D.H., Aanensen, D.M., Ronnenber, K.L., Powell, C.I., Walker, S.F., Bielby, J., Garner, T.W.J., Weaver, G., Fisher, M.C. (2013): Mapping the Global Emergence of *Batrachochytrium dendrobatidis*, the Amphibian Chytrid Fungus. *PLoS ONE* **8**: e56802.
- Ósi, A.L., Makádi, M., Rabi, Z., Szentesi, G., Botfalvai, P., Gulyás, P. (2012): The Late Cretaceous continental vertebrate fauna from Iharkút, western Hungary: a review. In: *Bernissart Dinosaurs and Early Cretaceous Terrestrial Ecosystems*, pp. 533-568. Godefroit, P., Ed, Indiana University Press.
- Piotrowski, J.S., Annis, S.L., Longcore, J.E. (2004): Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia* **96**: 9-15.
- Plötner, J., Köhler, F., Uzzell, T., Beerli, P., Schreiber, R., Guex, G.-G., Hotz, H. (2009): Evolution of serum

- albumin intron-1 is shaped by a 5' truncated non-long terminal repeat retrotransposon in western Palearctic water frogs (Neobatrachia). *Mol. Phylogenetic Evol.* **53**: 784-791.
- R Core Team (2018): R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org>.
- Rachowicz, L.J., Vredenburg, V.T. (2004): Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. *Dis. Aquat. Organ.* **61**: 75-83.
- Rödger, D., Kielgast, J., Lötters, S. (2010): Future potential distribution of the emerging amphibian chytrid fungus under anthropogenic climate change. *Dis. Aquat. Organ.* **92**: 201-207.
- Rohr, J.R., Raffel, T.R. (2010): Linking global climate and temperature variability to widespread amphibian declines putatively caused by disease. *P. Natl. Acad. Sci. USA* **107**: 8269-8274.
- Ron, S.R. (2005): Predicting the distribution of the amphibian pathogen *Batrachochytrium dendrobatidis* in the New World. *Biotropica* **37**: 209-221.
- Rosa, G.M., Anza, I., Moreira, P.L., Conde, J., Martins, F., Fisher, M.C., Bosch, J. (2012): Evidence of chytrid-mediated population declines in common midwife toads (*Alytes obstetricans*) in Serra da Estrela Portugal. *Anim. Conserv.* **16**: 306-315.
- Savage, A.E., Zamudio, K.R. (2011): MHC genotypes associate with resistance to a frog-killing fungus. *P. Natl. Acad. Sci. USA* **108**: 16705-16710.
- Scherer, R. (2018): PropCIs: Various confidence interval methods for proportions. R package version 0.3-0. <https://CRAN.R-project.org/package=PropCIs>
- Schielzeth, H. (2010): Simple means to improve the interpretability of regression coefficients. *Methods Ecol. Evol.* **1**: 103-113.
- Skerratt, L.F., Berger, L., Speare, R., Cashins, S., McDonald, K., Hines, A., Kenyon, N. (2007): Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth* **4**: 125-134.
- Soto-Azat, C., Clarke, B.T., Fisher, M.C., Walker, S.F., Cunningham, A.A. (2009): Non-invasive sampling methods for the detection of *Batrachochytrium dendrobatidis* in archived amphibians. *Dis. Aquat. Organ.* **84**: 163-166.
- Spitzen-Van Der Sluijs, A., Canessa, S., Martel, A., Pasmans, F. (2017): Fragile coexistence of a global chytrid pathogen with amphibian populations is mediated by environment and demography. *Proc. R. Soc. B.* **284**: 20171444.
- Spitzen-Van Der Sluijs, A., Martel, A., Hallmann, C.A., Bosman, W., Garner, T.W.J., Van Rooij, P., Jooris, R., Haesebrouck, F., Pasmans, F. (2014): Environmental determinants of recent endemism of *Batrachochytrium dendrobatidis* infections in amphibian assemblages in the absence of disease outbreaks: environmental determinants promote *Bd* endemism. *Conserv. Biol.* **28**: 1302-1311.
- Sura, P., Janulis, E., Profus, P. (2010): Chytridiomikoza – śmiertelne zagrożenie dla płazów (Chytridiomycosis – a mortal danger for amphibians). *Chrońmy Przyrodę Oczyszczaj* **66**: 406-421.
- Sztatecsny, M., Glaser, F. (2011): From the eastern lowlands to the western mountains: first records of the chytrid fungus *Batrachochytrium dendrobatidis* in wild amphibian populations from Austria. *Herpetol. J.* **21**: 87-90.
- Tessa, G., Angelini, C., Bielby, J., Bovero, S., Giacoma, C., Sotgiu, G., Gardner, T.W.J. (2013): The pandemic pathogen of amphibians, *Batrachochytrium dendrobatidis* (Phylum Chytridiomycota), in Italy. *Ital. J. Zool.* **80**: 1-11.
- Van Sluys, M., Hero, J.M. (2009): How does chytrid infection vary among habitats? The case of *Litoria wilcoxii* (Anura, Hylidae) in SE Queensland, Australia. *EcoHealth* **6**: 576-583.
- Vojar, J., Havlíková, B., Solský, M., Jablonski, D., Iković, V., Baláž, V. (2017): Distribution, prevalence and amphibian hosts of *Batrachochytrium dendrobatidis* in the Balkans. *Salamandra* **53**: 44-49.
- Vörös, J., Alcobendas, M., Martínez-Solano, I., García-París, M. (2006): Evolution of *Bombina bombina* and *Bombina variegata* (Anura: Discoglossidae) in the Carpathian Basin: a history of repeated mt-DNA introgression across species. *Mol. Phylogenetic Evol.* **38**: 705-715.
- Vörös, J., Szalay, F., Barabás, L. (2007): A new computer-aided videomorphological method for the quantitative analysis of ventral pattern in *Bombina* spp. (Anura: Discoglossidae). *Herpetol. J.* **17**: 97-103.
- Vörös, J., Bosch, J., Dán, A., Hartel, T. (2012): First Record of *Batrachochytrium dendrobatidis* in Romania. *North-West. J. Zool.* **9**: 446-449.
- Vörös, J., Kiss, I., Puky, M. (2014): Amphibian declines and conservation in Hungary. In: *Amphibian Biology: Status of decline of amphibians: Eastern hemisphere*. Amphibian, pp. 99-130. Heatwole, H., Wilkinson, J.W., Eds, Pelagic Publishing.
- Walker, S.F., Bosch, J., Gomez, V., Garner, T.W.J., Cunningham, A.A., Schmeller, D.S., Ninyerola, M., Henk, D.A., Ginestet, C., Arthur, C.-P., Fisher, M.C. (2010): Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. *Ecol. Lett.* **13**: 372-382.

- Williams, L.A., Groves, J.D. (2014): Prevalence of the amphibian pathogen *Batrachochytrium dendrobatidis* in eastern hellbenders (*Cryptobranchus A-Alleganien-sis*) in western North Carolina, USA. *Herpetol. Conserv. Bio.* **9**: 454-467.
- Wood, L.R., Griffiths, R.A., Schley, L. (2009): Amphibian chytridiomycosis in Luxemburg. *Bull. Soc. Nat. luxemb.* **110**: 109-114.