



# Assessing Risk and Guidance on Monitoring of *Batrachochytrium dendrobatidis* in Europe through Identification of Taxonomic Selectivity of Infection

VOJTECH BALÁŽ,<sup>\*</sup> \*\*\*\* JUDIT VÖRÖS,<sup>†</sup> \*\*\*\* PETR CIVIŠ,<sup>‡</sup> JIRI VOJAR,<sup>‡</sup> ATTILA HETTYEY,<sup>§\*\*</sup>  
 ENDRE SÓS,<sup>††</sup> RÓBERT DANKOVICS,<sup>‡‡</sup> ROBERT JEHLE,<sup>§§</sup> DITTE G. CHRISTIANSEN,<sup>\*\*\*</sup>  
 FRANCES CLARE,<sup>†††‡‡‡</sup> MATTHEW C. FISHER,<sup>‡‡‡</sup> TRENTON W. J. GARNER,<sup>†††</sup> §§§  
 AND JON BIELBY<sup>†††</sup>

<sup>\*</sup>Department of Biology and Wildlife Diseases, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1/3, 612 42, Brno Czech Republic

<sup>†</sup>Hungarian Natural History Museum, 1088 Budapest, Baross u. 13., Hungary

<sup>‡</sup>Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýčká 129, Prague CZ 165 21, Czech Republic

<sup>§</sup>Department of Integrative Biology and Evolution, Konrad Lorenz Institute of Ethology, University of Veterinary Medicine Vienna 1160 Vienna, Savoyenstr. 1A, Austria

<sup>\*\*</sup>Lendület Evolutionary Ecology Research Group, Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Herman Ottó út 15, 1022 Budapest, Hungary

<sup>††</sup>Budapest Zoo and Botanical Garden, 1146 Budapest, Állatkerti krt. 6-12, Hungary

<sup>‡‡</sup>Savaria Museum, 9700 Szombathely, Kisfaludy Sándor u. 9., Hungary

<sup>§§</sup>School of Environment and Life Sciences, University of Salford, M5 4WT Salford, Greater Manchester, United Kingdom

<sup>\*\*\*</sup>Institute of Evolutionary Biology and Environmental Studies, Ecology, Universität Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland

<sup>†††</sup>Institute of Zoology, Zoological Society of London, Regents Park, London, NW1 4RY, United Kingdom

<sup>‡‡‡</sup>Department of Infectious Disease Epidemiology, Imperial College, St. Mary's Hospital, London, United Kingdom, email trent.garner@ioz.ac.uk

**Abstract:** *Amphibians are globally threatened, but not all species are affected equally by different threatening processes. This is true for the threat posed by the chytridiomycete fungus (*Batrachochytrium dendrobatidis*). We compiled a European data set for *B. dendrobatidis* to analyze the trends of infection in European amphibians. The risk of infection was not randomly distributed geographically or taxonomically across Europe. Within countries with different prevalence, infection was nonrandom in certain amphibian taxa. Brown frogs of the genus *Rana* were unlikely to be infected, whereas frogs in the families *Alytidae* and *Bombinatoridae* were significantly more likely to be infected than predicted by chance. Frogs in the 2 families susceptible to *B. dendrobatidis* should form the core of attempts to develop spatial surveillance studies of chytridiomycosis in Europe. Ideally, surveys for *B. dendrobatidis* should be augmented by sampling the widespread genus *Pelophylax* because this taxon exhibits geographically inconsistent overinfection with *B. dendrobatidis* and surveillance of it may facilitate recognition of factors causing spatial variability of infection intensity. Several European amphibian taxa were not represented in our data set; however, surveillance of unsampled species should also occur when warranted.*

**Keywords:** amphibians < animals, conservation planning, disease, epidemiology, Europe, inventory and monitoring, risk assessment

Evaluación de Riesgo y Orientación para el Monitoreo de *Batrachochytrium dendrobatidis* en Europa Mediante la Identificación de la Selectividad Taxonómica de la Infección

§§§email trent.garner@ioz.ac.uk

\*\*\*\*These authors contributed equally to the study.

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**Resumen:** *Los anfibios están amenazados globalmente, pero los diferentes procesos de amenaza no afectan a todas las especies de la misma manera. Esto es cierto para la amenaza que representa el hongo quitridiomiceto (*Batrachochytrium dendrobatidis*). Compilamos un conjunto de datos europeos de *B. dendrobatidis* para analizar las tendencias de la infección en anfibios de Europa. La distribución geográfica o taxonómica del riesgo de infección no se distribuyó aleatoriamente en Europa. En países con prevalencia diferente, la infección no fue aleatoria en ciertos taxa de anfibios. La infección fue poco probable en ranas del género *Rana*, mientras que en ranas de las familias *Alytidae* y *Bombinatoridae* la probabilidad de infección fue significativamente mayor. Ranas de las 2 familias susceptibles a *B. dendrobatidis* deberían formar el núcleo de intentos por desarrollar estudios de vigilancia espacial de la quitridiomycosis en Europa. Idealmente, se deben incrementar los monitoreos de *B. dendrobatidis* mediante muestreos del género *Pelodyx* por su distribución amplia y porque presenta una sobreinfección con *B. dendrobatidis* geográficamente inconsistente y su monitoreo puede facilitar el reconocimiento de factores que produce variación espacial de la intensidad de infección. Sin embargo, varios taxa de anfibios europeos no estaban representados en nuestro conjunto de datos y el monitoreo de especies no muestreadas también debe ocurrir cuando sea posible.*

## Introduction

Although a global net loss of amphibian biodiversity is not in question (Houlahan et al. 2000; Lips et al. 2008; Vredenburg et al. 2010), declines are not all-inclusive. Many populations and species seem relatively stable over the same period during which other species and populations have declined (Houlahan et al. 2000; Stuart et al. 2004; Sodhi et al. 2008). Populations of some species in areas affected by a threatening process even appear to have benefited from the decline of other species caused by this process (Bosch & Rincón 2008; Sodhi et al. 2008). The fact that there is considerable variation in response to a given driver of decline means the mechanisms behind amphibian declines do not affect all species equally.

*Batrachochytrium dendrobatidis* (Bd) is currently the only pathogen consistently identified as a key factor in loss of amphibian biodiversity and abundance at multiple geographic scales (e.g., Bosch et al. 2001; Lips et al. 2006; Vredenburg et al. 2010). *B. dendrobatidis* is a generalist parasite (Fisher et al. 2009), which shows strong variation in its ability to infect and cause disease in different hosts. Although the effect of multihost parasites on animal communities has been a topic of research for decades (Holt & Pickering 1985; van Riper 1986; Dobson & Hudson 1986), understanding of how host species diversity and community structure may influence disease dynamics is far from complete (Keesing et al. 2006; Begon 2008). For Bd, what does seem likely is that differences in species susceptibility to infection (Gahl et al. 2012) mean that phylogeny is expected to play an important role in determining pathogen effect and its distribution among hosts (e.g., Schmidt & Ostfeld 2001; Holt et al. 2003; Johnson & Thielges 2010).

Patterns of species declines in amphibian communities affected by lethal chytridiomycosis suggest that risk of infection may differ at the species, genus, and family level (e.g., Smith et al. 2009; Crawford et al. 2010; Bancroft et al. 2011), an observation supported by experimental studies of infection (Stockwell et al. 2010; Searle et al.

2011; Luquet et al. 2012). This variation in the distribution of infection across the Amphibia may have 2 explanations (Corey & Waite 2008; Smith et al. 2009). First, species with shared evolutionary history have similar ecologies, life-history traits, and environmental niches. Certain sets of these traits predispose species within a clade to decline, whereas a lack of these traits excuse others from the deleterious effects of a threatening process (e.g., Sodhi et al. 2008; Bielby et al. 2009; Bancroft et al. 2011). Second, a phylogenetic signal of decline may occur as a result of the geographic location of a clade because clades are not randomly distributed geographically and not all locations are exposed to equal levels of a given threat process (Bielby et al. 2006; Corey & Waite 2008). Heterogeneity in the distribution of Bd exists (Walker et al. 2010), but how species' susceptibility to infection varies within communities is unclear, yet it is extremely important information. If taxonomic variation in infection levels persists when geographic variation in threat intensity is accounted for, it would suggest that those differences are mainly a result of host biology. Identifying which species or clades are most likely to carry high levels of infection will therefore facilitate design and implementation of more efficient and standardized monitoring protocols by highlighting those focal taxa that may act as sentinel clades.

Heterogeneous host responses to Bd exposure are exhibited by European amphibians. Some species are susceptible to both infection and mortality attributable to chytridiomycosis, whereas others seem less likely to suffer from either (e.g., Bosch & Martínez-Solano 2006; Bielby et al. 2009; Sztatecsny & Glaser 2011). In Europe national data are being collected and collated for western countries and amphibian species native to these locations as part of the EU-funded project R.A.C.E. ([www.bd-maps.eu](http://www.bd-maps.eu)). Bd appears to be expanding its European range and causing local amphibian mortalities and declines (e.g., Walker et al. 2008; Garner et al. 2009; Walker et al. 2010). It is therefore important to extend sampling for Bd to include European countries and

European species for which current evidence of presence and prevalence of the fungus is lacking. Doing this and identifying sentinel clades are important steps toward developing a European risk assessment for Bd.

We generated primary data on infection of amphibians sampled in 6 central and eastern European countries and integrated them with previously published and unpublished data for Central and western European countries. Using these data, and other infection data from a range of sources, we looked for taxonomic patterns of infection prevalence with the goal of identifying clades that may be unusually susceptible to infection when level of exposure to Bd is accounted for. To do so we first aimed to identify those countries with an identified presence of Bd, with a view to conducting taxonomic-level analyses within and among those countries to test the hypothesis that there would be significant taxonomic variation in probability of infection regardless of the relative level of infection within that country. Given the presence of a nonrandom pattern of infection, we aimed to identify those clades that were significantly over- and underinfected relative to background prevalence at a European level and to account for the broad-scale variation in exposure to Bd within countries. For clades we identified as over- or underinfected, we tested the hypothesis that there are consistent patterns in taxonomic susceptibility to infection by determining whether the direction of the trends in those clades was consistent across countries. In doing so and by extension, we aimed to determine which taxa would be most suitable to act as sentinel clades for detecting presence and effect of Bd across Europe.

## Methods

We collected toe clips and superficial skin swabs (Dryswab, MW100; MWE, Wiltshire) for the molecular detection of Bd infection from 6 central and eastern European countries between 2002 and 2009 (Table 1). We identified Bd infection with quantitative PCR (qPCR) amplification following the protocol of Boyle et al. (2004). All samples were run against a set of standards of known concentration (0.1, 1, 10, and 100 genomic equivalents [GEs]). Samples were run in duplicate and positive amplification in both wells and a mean GE score  $\geq 0.05$  was considered evidence of infection.

For analyses we combined our primary data with data sets derived from Garner et al. (2005) (data archived at [www.bd-maps.eu](http://www.bd-maps.eu)) and extraction of primary data published in the literature. To generate the latter, we performed a literature search in Web of Science, Biological Abstracts, and Zoological Record with the keywords *Batrachochytrium* and *chytridiomycosis*. We manually searched the results for publications containing prevalence data on Bd infection in wild amphibians in Europe. The overall data set included only data that had been gen-

erated using Bd-specific DNA detection methods (e.g., standard PCR by Annis et al. [2004] and Simoncelli et al. [2005] or real-time qPCR by Boyle et al. [2004]) and for which prevalence of infection could be extracted. We incorporated literature published before 31 April 2011 (see Supporting Information for a full list of publications). We extracted sampling location, country, sampling effort, and prevalence for each amphibian species from each publication. The final data set (Supporting Information) included 44 of 75 native European amphibian species listed by Speybroeck et al. (2010) and one introduced species, the North American Bullfrog (*Lithobates catesbeianus*).

Although ecological boundaries are more likely to affect patterns of Bd prevalence than national borders (e.g., Walker et al. 2010), we used political boundaries because in this case we believe they are more appropriate for informing conservation. *B. dendrobatidis* is 1 of 2 amphibian pathogens that require notification of the World Organisation for Animal Health (OIE) when infection is detected ([http://www.oie.int/eng/maladies/en\\_classification2010.htm?e1d7](http://www.oie.int/eng/maladies/en_classification2010.htm?e1d7)). According to the Aquatic Animal Health Code of the OIE, responsibility lies with the signatory countries, which include all EU member and associated states. Countries are responsible for disease monitoring, so policy to mitigate Bd or control ongoing spread is predominantly set on the basis of political boundaries.

To assess whether Bd infection was randomly distributed among countries, we used a  $\chi^2$  test on count data of positive and negative samples from countries with sufficient sample sizes according to our criteria ( $>10$  samples/country and Bd detected at least once [Supporting Information]). We then assessed nonrandom distribution of infection in some host clades (see below for a definition) with a  $\chi^2$  test implemented on counts of Bd infections for selected clades and by combining data across countries where clades were sampled and Bd was detected (Supporting Information).

Use of surveillance data makes it impossible to unambiguously determine whether clades consistently testing negative for infection are resistant to Bd (Luquet et al. 2012) or have never been exposed to Bd. To account for this to some degree, we limited the analyses of nonrandomness among clades to countries where infection had been detected. We also restricted this analysis to clades that were well sampled and monophyletic, following the phylogeny of Frost et al. (2006) and Frost (2011). We considered sampling effort sufficient if the clade of interest had been sampled in a minimum of 4 countries, if sample size for each country was  $>50$  individuals, and if overall sampling effort exceeded 500 individuals (with one exception, see below [Supporting Information]). Focal clades for the Anura included *Pelophylax* (European green frogs), *Rana* (European brown frogs), and Bufonidae (toads). We also selected the

Table 1. Previously unpublished primary data on *Batrachochytrium dendrobatidis* surveillance for Central and Eastern Europe.

Country	Species	No.	Pos. <sup>a</sup>	Age <sup>b</sup>	Locality	Lat	Long	Habitat <sup>c</sup>	Year	Elevation (m)	Genomic equivalent (SD)	Percent prevalence (CI)
Croatia	<i>R. latastei</i>	8	—	ad	Istarske Toplice	45.46	13.88	NA	2003	NA	—	—
Czech Republic	<i>Lisotriton vulgaris</i>	14	—	NA	358 01 Kraslice	50.35	12.50	pond	2002	approximately 600	—	—
Estonia	<i>Pelophylax lessonae</i>	20	—	NA	Tolkuse Raba	58.14	24.52	moor	2004	NA	—	—
Estonia	<i>Pelophylax lessonae</i>	19	—	NA	Laeva	58.43	26.32	newly restored pond	2004	NA	—	—
Slovenia	<i>R. latastei</i>	29	—	ad	Gozd Pavonec	45.95	13.67	NA	2003	NA	—	—
Hungary	<i>Salamandra salamandra</i>	2	—	ad	Újhuta, Zemplén Mountains	48.38	21.45	captured on land	2007	315	—	—
Hungary	<i>Bufo bufo</i>	5	—	ad	Szentendre, Pilis-mountains	47.71	19.04	temporary pond	2004	266	—	—
Hungary	<i>Bufo bufo</i>	2	—	ad	Vörös János-séd, Bakony	47.27	17.69	stream	2007	450	—	—
Hungary	<i>Rana dalmatina</i>	7	—	ad	Vörös János-séd, Bakony	47.27	17.69	stream	2007	450	—	—
Hungary	<i>Pelophylax esculentus</i>	2	—	ad	Túrkeve, Great Hungarian Plain	47.10	20.75	stream	2004	81	—	—
Hungary	<i>Pelophylax ridibundus</i>	4	—	ad	Dinnyés, Lake Velence	47.18	18.54	stream	2004	102	—	—
Hungary	<i>Rana temporaria</i>	5	1	ad	Visegrad, Apatkúti-völgy	47.77	18.98	stream	2004	176	38.815 (0.02)	20 (1.03–75.74)
Hungary	<i>Bombina variegata</i>	5	—	ad	Gigányrudas, Órség	46.90	16.24	temporary pond	2008	313	—	—
Hungary	<i>Bombina variegata</i>	13	—	ad	Felsőszónók, Órség	46.86	16.15	temporary pond	2007–2008	300	—	—
Hungary	<i>Bombina variegata</i>	5	1	ad	Felsőszónók, Hampó-völgy, Órség	46.87	16.14	temporary pond	2007	295	31.5 (9.99)	20 (1.03–65.74)
Hungary	<i>Bombina variegata</i>	6	—	ad	Grajka, Órség	46.91	16.23	temporary pond	2008	255	—	—
Hungary	<i>Bombina variegata</i>	36	18	juv	Iharkút, Bakony Mountains	47.23	17.64	pond	2008	400	1.58 (0.64)–406 7.41 (1260)	50 (33.4–66.6)
Hungary	<i>Bombina variegata</i>	8	2	ad	Cák, Kőszegi Mountains	47.37	16.50	temporary pond	2008	438	0.73 (0.47)–4.469 (1.1)	25 (4.64–63.53)
Hungary	<i>Bombina variegata</i>	5	—	ad	Rostalló, Zemplén Mountains	48.46	21.45	temporary pond	2007	250	—	—
Hungary	<i>Bombina variegata</i>	13	—	ad	Szakonyfalu, Órség	46.91	16.22	temporary pond	2007–2008	310	—	—

Continued

Table 1. continued

Country	Species	No.	Pos. <sup>a</sup>	Age <sup>b</sup>	Locality	Lat	Long	Habitat <sup>c</sup>	Year	Elevation (m)	Genomic equivalent (SD)	Percent prevalence (CI)
Hungary	<i>Bombina variegata</i>	8	1	ad	Szakonyfalu, Cigányrudas, Órség	46.90	16.24	temporary pond	2007	313	4.01 (2.87)	12.5 (0.64–50)
Hungary	<i>Bombina variegata</i>	2	–	ad	Újhuta, Zemplén Mountains	48.38	21.45	temporary pond	2007	315	–	–
Hungary	<i>Bombina variegata</i>	8	–	juv	Vörös János-séd, Bakony Mountains	47.27	17.69	temporary pond	2007–2008	450	–	–
Hungary	<i>Bombina variegata</i>	26	–	ad	Vörös János-séd, Bakony Mountains	47.27	17.69	temporary pond	2007–2009	450	–	–
Slovakia	<i>Bombina variegata</i>	8	1	ad	Banská Bystrica, Podlavické	48.75	19.10	spring	2008	550	28.7(5.6)	12.5 (0.64–50)
Slovakia	<i>Salamandra salamandra</i>	4	–	juv	Banská Bystrica, Podlavické	48.75	19.10	spring	2008	550	–	–
Czech Republic	<i>Bufo bufo</i>	2	–	ad	Prague, Prokopské údolí	50.04	14.35	pond	2008	272	–	–
Czech Republic	<i>Bufo bufo</i>	45	1	juv	Prague, Albertov	50.07	14.43	city park	2008	226	0.75 (0.094)	2.2 (0.12–11.83)
Czech Republic	<i>Bufo bufo</i>	20	–	ad	Prague, Albertov	50.07	14.43	city park	2008	226	–	–
Czech Republic	<i>Bufo bufo</i>	7	–	juv	Prague, Peřín	50.08	14.40	city park	2008	300	–	–
Czech Republic	<i>Rana temporaria</i>	5	–	ad	Pregue, Říčanský potok	50.05	14.59	several ponds connected by a stream	2008	270	–	–
Czech Republic	<i>Pelophylax esculentus</i>	26	2	ad	Pregue, Říčanský potok	50.05	14.59	several ponds connected by a stream	2008	270	1.72 (0.48)–37.56 (10.1)	7.7 (1.39–24.6)
Czech Republic	<i>Pelophylax esculentus</i>	33	–	ad	Prague, Prokopské údolí	50.04	14.35	pond	2008	272	–	–
Czech Republic	<i>Lissotriton vulgaris</i>	5	–	ad	Prague, Albertov	50.07	14.43	city park	2008	226	–	–
Czech Republic	<i>Bufo bufo</i>	31	–	ad	Most	50.56	13.58	temporary pool on spoil bank	2009	240	–	–
Czech Republic	<i>Bufo bufo</i>	30	–	ad	Tuchoraz	50.05	14.84	pond	2009	310	–	–
Czech Republic	<i>Bufo bufo</i>	2	–	ad	Tuchoraz	50.05	14.84	stream bank	2009	310	–	–

Continued

Table 1. continued

Country	Species	No.	Pos. <sup>a</sup>	Age <sup>b</sup>	Locality	Lat	Long	Habitat <sup>c</sup>	Year	Elevation (m)	Genomic equivalent (SD)	Percent prevalence (CI)
Czech Republic	<i>Bombina bombina</i>	1	–	ad	Most, Ruzodolska spoil bank	50.56	13.58	temporary pool on spoil bank	2009	240	–	–
Czech Republic	<i>Bombina bombina</i>	15	2	juv	Most, Ruzodolska spoil bank	50.58	13.62	temporary pool on spoil bank	2009	240	0.07 (0.006)–0.34 (0.06)	20 (5.69–46.57)
Czech Republic	<i>Lissotriton vulgaris</i>	24	–	juv	Most, Ruzodolska spoil bank	50.58	13.62	temporary pool on spoil bank	2009	240	–	–
Czech Republic	<i>Lissotriton vulgaris</i>	7	–	ad	Most, Ruzodolska spoil bank	50.58	13.62	temporary pool on spoil bank	2009	240	–	–
Czech Republic	<i>Triturus cristatus</i>	30	–	ad	Most, Ruzodolska spoil bank	50.58	13.62	temporary pool on spoil bank	2009	240	–	–

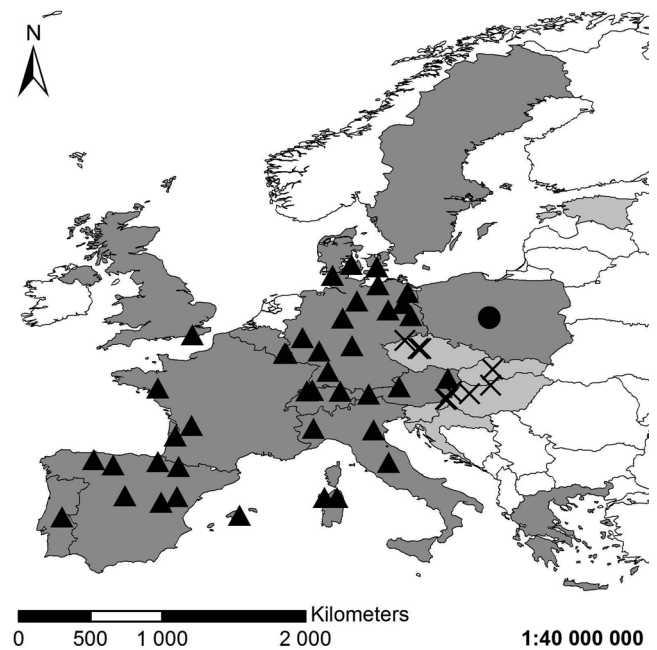
<sup>a</sup>Number of individuals testing positive.<sup>b</sup>Abbreviations: ad, adult; NA, not available; juv, juvenile.<sup>c</sup>Spoil bank is waste from mining excavation.

monophyletic clade formed by Alytidae (*Alytes* [midwives] and *Discoglossus* [disc-tongued frogs]) and Bombinatoridae (European members of *Bombina* [fire-bellied toads]) (Frost et al. 2006), which we refer to as Alytidae *sensu lato*. The only caudate clade meeting our criteria was the Pleurodelinae. Although sampling effort for Salamandrinae was not sufficient according to our criteria, we included this subfamily because in the wild one member can be infected and die from the infection (Bosch & Martínez-Solano 2006).

Using data from countries where distribution of infection prevalence was taxonomically nonrandom, we ran permutation tests (Bielby et al. 2006) to show which focal clades were over- and underinfected as follows. We used binomial tests to calculate the minimum sample size necessary to detect a significant difference in prevalence. For each focal clade, we generated a null distribution of expected infections for that clade with permutation tests. To do this, we used the total number of samples for the focal clade across Europe (including samples only from countries where infection had been detected) and, as the background level of infection, the overall prevalence of infection for those infected countries where the focal taxon had been sampled. Because sampling effort and the countries where each clade was sampled varied, background prevalence and total counts varied among clades. If the observed number of infections for the focal clade lay in the 2.5% tail at either end of the null distribution or outside this range, we categorized it as under- or overinfected accordingly.

To ascertain whether patterns of over- or underinfection were consistent among countries, we implemented the same cross-clade analysis but restricted the analysis to data from 10 selected countries. Thus, we could identify whether taxonomic selectivity in risk of infection persisted when threat intensity was relatively homogeneous. Criteria for country selection were presence of infected amphibians in our data set, multiple species sampled in the country, and an overall country sample size >100. We first conducted a  $\chi^2$  test (or Fisher's exact test if any of the expected values for  $\chi^2$  test were <5) for each of the 10 countries to determine whether infection was evenly distributed among the focal clades (Supporting Information). If we detected significant variation of infection among clades within a country, we again used permutation tests, as described previously, to determine which clades were over- and underinfected given the background prevalence of infection in that country.

After determining country-level patterns of over- and underinfection for each clade in each country, we used Fisher's method of combining *p* values on the data in Supporting Information to determine whether clades were consistently over- or underinfected across countries and therefore whether our results were robust to variation in sampling effort and threat intensity. To discount the possibility that observed taxonomic patterns of infection



**Figure 1.** European countries sampled for *Batrachochytrium dendrobatidis* (*Bd*) and locations where infected individuals were detected (dark gray, previously surveyed countries; light gray, countries sampled for the first time during this study; triangles, approximate locations where *Bd* was detected in western Europe previous to this study; crosses, locations where *Bd* was detected in this study; circle, location in Poland lacking georeferenced data).

were solely a result of interclade variation in exposure to *Bd* (e.g., that underinfected clades occurred only in locations in which *Bd* had not been introduced), we performed a  $\chi^2$  test to determine whether frequency of positive and negative samples varied across focal taxa. We did this with the data from localities with confirmed presence of the pathogen. We then used the data in permutation tests, with background prevalence counted from all positive samples including nonfocal taxa, to ascertain which clades were over- and underinfected once intensity of exposure to *Bd* was accounted for (Supporting Information).

## Results

We generated primary data for 547 individuals representing 12 amphibian species and 42 sites (Fig. 1). Thirty-two amphibians tested positive for *Bd* (Table 1). Infected amphibians were sampled in Slovakia, the Czech Republic, and Hungary (9/42 sites) and included the common toad (*Bufo bufo*, Bufonidae), European fire-bellied toad (*Bombina bombina*), yellow-bellied toad (*Bombina variegata*) (Bombinatoridae), common frog (*Rana*

*temporaria*), and green frogs (*Pelophylax* kl. *esculentus*). Infection was detected only in adult frogs except for recent metamorphs of *B. variegata* sampled at a site in the Bakony Mountains, Hungary, and juveniles of *B. bombina* sampled on spoil banks near Most in the Czech Republic (Table 1). The most commonly infected species sampled in this component of the study was *B. variegata*, which tested positive for Bd at 4 localities in Hungary and 1 locality in Slovakia. Prevalence at these 5 locations was highest in the Bakony Mountains (18/36 individuals). GE values ranged from 0.71 to 4067.41 (mean = 255.55). The heaviest infections (GE = 4067.41 [SD 1260]) occurred in a *B. variegata* juvenile sampled in the Bakony Mountains.

The complete data set was composed of infection data for 7403 individuals (45 species sampled in 19 countries) (Supporting Information). Bd infections were detected in 788 individuals (approximately 11%), and they exhibited a nonrandom pattern geographically and taxonomically. At the country level, count data of infection, with all species pooled and analyses restricted to countries where Bd was detected, showed a significant variation from that expected by chance ( $n = 7032$ ;  $p < 0.001$ ;  $df 13$ ;  $\chi^2 = 565.74$ ) (Supporting Information), as did count data of infection among focal clades pooled across all countries ( $n = 6517$ ;  $p < 0.001$ ;  $df 5$ ;  $\chi^2 = 394.151$ ). Across all countries combined, *Pelophylax* and Alytidae s.l. were overinfected relative to their respective null distributions. All other focal clades were underinfected (Supporting Information). Taxonomic patterns of infection in Austria, Czech Republic, Germany, Italy, Spain, and Switzerland (see Supporting Information for country-level test results) differed significantly from the null model, but no single clade exhibited a consistent pattern in all countries for which sufficient data were available. Nevertheless, combining  $p$  values of individual binomial tests (Bielby et al. 2006) indicated *Rana* was consistently underinfected, whereas the Alytidae s.l. was the only clade consistently overinfected (Table 2). These trends were confirmed by results of the analysis of data derived exclusively from positive localities (Supporting Information) and suggest the observed pattern was not the result of certain clades existing in areas that were not amenable to Bd invasion or persistence.

## Discussion

The consequences of Bd infection for European amphibians may be more subtle and heterogeneous than for central American and Australian amphibians (e.g., Bielby et al. 2008; Tobler & Schmidt 2010; Garner et al. 2011). Given the substantial variation in response to Bd exposure in Europe, there is an urgent need to understand what dictates risk of infection and disease in European amphibians. The initial step in assessing risk of a pathogen is describing the distribution (Reingold 1998). Here, we

extended surveillance for Bd infection into previously unsampled central and eastern European countries. Sampling effort for Croatia, Slovenia, and Estonia was admittedly poor, so our lack of Bd positive samples in these countries should not be considered indicative of disease-free status. For Croatia and Slovenia, the sampled species, *Rana latastei*, is probably a poor target for detecting Bd (see below). Data for other countries (Slovakia, Czech Republic, Hungary) showed Bd is not uncommon in Central Europe (Table 1). Only 1 site was sampled in Slovakia, but of the 19 sites sampled in Hungary, 26% contained infected hosts, and 18% of Czech sites had infected amphibians. These percentages are relatively consistent with those reported for Iberia (Walker et al. 2010), Germany (Ohst et al. 2011), and Austria (Sztatecsny & Glaser 2011), but they appear relatively low in comparison with prevalence observed during decline events in Australia and the Neotropics (Lips et al. 2008; Murray et al. 2010).

The geographic patterns of infection provide little guidance for designing European monitoring schemes. Widespread but spatially heterogeneous Bd distributions instead indicate that sampling effort should not be spatially or topographically restricted (Walker et al. 2010; Sztatecsny & Glaser 2011; Ohst et al. 2011). In comparison, our analyses of heterogeneity of risk among hosts did provide insight. With our results, surveillance can be developed for most European countries and ecosystems because our focal taxa cover broad geographic ranges and can be found in most areas that support breeding populations of amphibians. Depending on the regional amphibian community composition and the goals of a spatial study of Bd in Europe, strategies can be designed that focus on species, species complexes, genera, families, and even higher taxonomic rankings.

Brown frogs (*Rana*) are poor candidates for spatial surveillance in Europe due to their consistent level of underinfection. Ranid samples taken at high-elevation sites where other species are known to be lethally infected with Bd (Walker et al. 2010) commonly test negative for Bd with molecular diagnostics, but not always (F. Clare, unpublished data). This is in sharp contrast to what is seen in some brown ranids in North America (Vredenburg et al. 2010; Searle et al. 2011). Prevalence of Bd in Salamandridae, irrespective of the subfamily, and toads (*Bufo*) in North America does not differ significantly from background prevalence in Europe. This is surprising given that Bd-related mortalities occur in common toads (*Bufo bufo*) and the one species (*Salamandra salamandra*) of Salamandrinae included in our data set (Bosch & Martinez-Solano 2006; Garner et al. 2009). Furthermore, data for *S. salamandra* were predominantly available for the one country where mortality in this species has been described (Bosch & Martinez-Solano 2006).

Results for *Pelophylax* often showed overinfection or prevalence near the background rate. Despite this inconsistency in the trend among countries, sampling



**Table 2.** Results of Fisher's combined test for focal taxa on the basis of data from localities with confirmed presence of Bd.

Clade	df	$\chi^{2a}$	p	Direction of variation from random
<i>Pelophylax</i>	10	14.916	0.1352	no variation
<i>Rana</i>	10	32.505	0.0003	decrease
Bufo	4	8.910	0.0634	no variation
Alytidae s.l.	12	31.910	0.0014	increase
Pleurodelinae	8	4.663	0.7337	no variation

<sup>a</sup>Obtained by combining p values resulting from Fisher's method.

*Pelophylax* species may still be informative because of their widespread distribution and abundance, particularly where Alytidae and Bombinatoridae species are not present. When all data are considered, members of this genus appeared to be overinfected (Supporting Information) and thus may be a better candidate for detecting Bd than toads or the Salamandrinae. However, *Pelophylax* at positive sites were not significantly overinfected (Supporting Information). This result suggests that although the clade is suitable for including in national schemes for Bd surveillance, comparisons among European countries may reveal environmental factors that may affect prevalence of infection, as have been described for *Alytes* sp. (Bosch et al. 2007; Walker et al. 2010).

Efforts to detect the presence and prevalence of Bd in European anurans should focus on sampling Alytidae and Bombinatoridae. This was the only consistently overinfected clade in our study at all scales of analysis, and the natural ranges of its members cover most of Europe (IUCN 2012). Alytidae appear to be highly susceptible to lethal chytridiomycosis (e.g., Bosch et al. 2001; Walker et al. 2008; Bielby et al. 2009), and sampling members of this family should form the core of any attempt to detect lethal disease. The native European distribution of this family ends in western Switzerland and central Germany, precluding inclusion in surveys of much of central and eastern Europe (Gasc et al. 1997). However, the eastern border of the Alytidae distribution overlaps with western limit of the distribution of the Bombinatoridae. The combined distributions form a relatively continuous range in most of Europe of species included in the one consistently overinfected clade.

Lethal chytridiomycosis has been implicated in the sudden decline of the Apennine yellow-bellied toad, *B. variegata pachypus* (taxonomy after Speybroek et al. 2010) in Bologna (Stagni et al. 2004). Although our surveys did not detect evidence of mortality in the field for either *Bombina* species we sampled, infection was detected in both species and a relatively high prevalence was detected in postmetamorphic juveniles of *B. variegata* in Hungary. Captive mortality in *B. v. pachypus* was described in recently metamorphosed and juvenile individuals, so further surveillance of *B. variegata*, and perhaps captive observations of animals collected in the field (sensu Tobler & Schmidt 2010), are warranted in or-

der to determine whether lethal chytridiomycosis occurs beyond Western Europe.

Geographic variation in threat intensity and sampling effort could have introduced a source of bias into our results. Our specific aim was not to identify countries with high levels of infection; rather, we sought to determine whether, among countries with different levels of infection and sampling, we could identify focal clades that seem to carry unusual levels of infection and may be suitable candidates to act as sentinel taxa for monitoring Bd infection levels or changes in distribution of infection. By analyzing patterns from a range of countries and comparing the consistency of the direction of over- and underinfection for a given clade, we were therefore testing whether those patterns remained when variation in threat and sampling intensity were included in the analyses. For example, the country-level results (Supporting Information) indicated that Spain and the Czech Republic had very different levels of sampling and infection prevalence ( $n = 1149$ , prevalence = 0.25, and  $n = 297$ , prevalence = 0.02, respectively), yet identification of Alytidae s.l. as a consistently overinfected taxa suggests this pattern is robust to sampling biases. Our results showed that *Rana* and Alytidae *sensu lato* were, respectively, consistently under- and overinfected. This result suggests that our findings are a result of host-pathogen biology rather than variation in sampling or pathogen intensity, both of which seem to exist in Europe. By identifying possible sentinel clades, our findings have important implications for addressing some of the sampling biases present in current data sets and for directing efforts to fill data gaps in countries (e.g., Croatia, Slovakia, Denmark) for which we had too few data to include in clade-level analyses.

Geographical gaps in knowledge regarding the distribution of Bd and its various lineages are being identified by a consortium of researchers (Project R.A.C.E., <http://www.bd-maps.eu/>). Taxonomic gaps are also being addressed, but both cannot be fully covered in a single project. There is a need to develop national schemes that survey for Bd, and we are aware of efforts to develop such projects in 17 EU member and associate states. Our results provide guidelines for initiating new and expanding current Bd surveillance schemes in a targeted manner by focusing on broadly distributed and readily infected hosts. However, we also urge that, in the context of

the conservation of threatened species, understudied amphibian species not be ignored for the sake of minimizing sampling effort and other forms of expediency.

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## Supporting Information

Literature search bibliography (Appendix S1), final data set (Appendix S2), summary data (Appendix S3), focal-taxa infection data (Appendix S4), and results of taxon-level (Appendix S5), country-level (Appendix S6), and site-specific (Appendix S7) permutation and Fisher's exact tests are available as part of the online article. The authors are solely responsible for the content and functionality of these materials. Queries (other than absence of the material) should be directed to the corresponding author.

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