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First record of *Batrachochytrium dendrobatidis* on amphibians in Romania

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Abstract. Here we report for the first time the presence of *Batrachochytrium dendrobatidis* in Romania. Seventy-seven amphibians belonging to four species were sampled in four regions of Transylvania: Sighisoara, Corund, Miercurea-Ciuc and Apuseni Mountains. Infected individuals were found in Sighisoara and Apuseni. Long term studies in Sighisoara region suggests that no unusual mass mortality occurs in the amphibian populations sampled for this study. We suspect that amphibians coexist with the chytrid. Further studies are urgently needed to understand the origin, ways of spreading, distribution, and potential impacts of *Bd* on amphibian populations in this country.

Key words: amphibian, chytrid, conservation, Romania.

Chytridiomycosis is an emerging infectious disease which is a major driver of amphibian decline in many parts of the world (e.g. Beebee & Griffiths 1995). It is caused by a fungus (*Batrachochytrium dendrobatidis* hereafter *Bd*) and it was first described on frogs from Australia and Central America in 1998 (Berger et al. 1998). The chytrid attacks the keratinised tissues of amphibians which are the mouthpart of the larvae and the skin of the post-metamorphic individuals (Berger et al. 1998, Daszak et al. 1999). The chytrid causes severe electrolyte imbalance (Voyles et al. 2006) and affects respiration ultimately resulting in the death of the individuals (Berger et al. 1998). Mass mortality due to *Bd* was reported for the adult stages as well as metamorphs (e.g. Bosch et al. 2001), and lower survival for larval stages (e.g. Garner et al. 2009). Having limited ability to swim, the fungus is likely carried in long distances by human activities, the animals or water flow (Johnson & Speare 2005).

The first record of *Bd* in the wild in European amphibians is from 1997 from Peňalara Mountains (Bosch et al. 2001). The occurrence of *Bd* in Europe was further reported from Portugal, Italy, France, Germany, Switzerland, Denmark, UK, Austria, Czech Republic, Poland and Hungary (Garner et al. 2005, Federici et al. 2008, Scalera et al. 2008, Sura et al. 2010, Ohst et al. 2011, Sztatecsny & Glaser 2011, Civis et al. 2012, http://www.bd-maps.net/).

Currently the Easternmost part of Europe where *Bd* was confirmed is Hungary (Gál et al. 2012). Here we report for the first time the presence of *Bd* in two regions of Romania.

Amphibians were sampled in four regions of Transylvania (Romania) (Fig. 1) the Apuseni mountains (hereafter Apuseni, with 26 individuals belonging to *Bombina variegata* and *Lissotriton vulgaris ampelensis*), Sighisoara area (41 individuals belonging to two species), Corund area (hereafter Corund, with one specimen belonging to *B. variegata*) and Miercurea Ciuc area (hereafter Ciuc, with 11 individuals belonging to *Rana temporaria*, *R. arvalis* and *L. vulgaris ampelensis*). Two sites were sampled in Sighisoara: the Breite wood-pasture (at an altitude of *ca* 430 m) and a deciduous forest (at *ca* 480 m altitude). In Apuseni six locations were sampled; two of them (Nucet, Baita) at an altitude of between 400-550 m, two sites at *ca* 800 m (Mununá, Valea largá) and two (Vartop, Padis) at *ca* 1100 m altitude. The site from Corund was at 650 m altitude and the site Ciuc at 700 m altitude (Table 1).

Data were collected in April 2011 (for Sighisoara, Corund and Ciuc) and in July 2011 (Apuseni).

We used sterile dry swabs (Biolab and Medical Wire and Equipment) to sample the back, belly, sides and legs of the specimens. The samples were then transferred to Hungary and were processed in the Laboratory for Molecular Taxonomy of the Hungarian Natural History Museum and the Veterinary Diagnostic Directorate of the National Food Chain Safety Office. To detect Bd we used the real-time PCR protocol by Boyle et al. (2004). We ran every sample in duplicate with a dedicated internal positive control (TaqMan Exogenous Internal Positive Control Reagents) for each sample that helps to identify inhibitors present in the DNA extractions. The templates were run

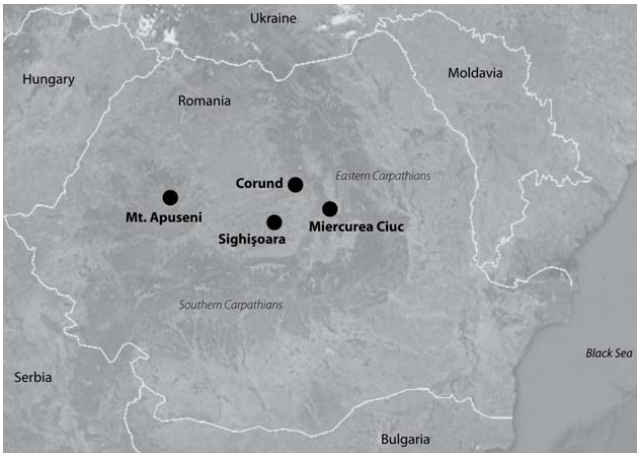

Figure1. The four sampling areas in Romania.

Table 1. Sampling from specimens collected in Romania. Lat=latitude, Long= longitude, Elv.=elevation, Ad= adult, Juv= juvenile, Tp= tadpole, GE= mean of genomic equivalent of zoospores. *R.=Rana, L=Lissotriton, B.=Bombina.* * indicates samples were three swabs were pooled in the analysis.

Locality	Lat °N	Long °E	Elv. (m)	Species	Life stage	Nr of individuals sampled (nr of positives)	Proportion of infected animals (with 95% CI values)	GE	Sampling Date
Miercurea Ciuc, Harghita	46.41000	25.80800	700	*R. temporaria*	Ad	4	(0-52)		04.2011
Miercurea Ciuc, Harghita	46.41000	25.80800	700	*R. arvalis*	Ad	6	(0-45)		04.2011
Miercurea Ciuc, Harghita	46.41000	25.80800	700	*L. vulgaris ampelensis*	Ad	1	(0-97)		04.2011
Firtos (Corund), Harghita	46.42344	25.13535	650	*B. variegata*	Ad	1	(0-97)		04.2011
Breite wood pasture reserve, Sighișoara	46.17708	24.97247	430	*B. variegata*	Ad	2	(0-84)		04.2011
Breite wood pasture reserve, Sighișoara	46.17708	24.97247	430	*L. vulgaris ampelensis*	Ad	4	(0-52)		04.2011
Breite wood pasture reserve, Sighișoara	46.17708	24.97247	430	*B. variegata*	Ad	20 (3*)	15%* (3.2-37.9)	0.6; 0.39; 1.22	04.2011
North Sighișoara	46.23104	24.79509	480	*L. vulgaris ampelensis*	Ad	15 (3*)	20%* (4.33-48)	0.23; 2.56; 50.34	04.2011
Nucet, Bihor, Mt. Apuseni	46.49035	22.55746	400	*B. variegata*	Juv (1), Ad (2)	3	(0-70)		07.2011
Baita, Mt. Bihor, Mt. Apuseni	46.48311	22.59726	530	*R. temporaria*	Ad	1 (1)	100% (2.49-100)	36.08	07.2011
Baita, Mt. Bihor, Mt. Apuseni	46.48311	22.59726	530	*B. variegata*	Ad (1), Juv (4)	5	(0-52)		07.2011
Vartop, Mt. Bihor, Mt. Apuseni	46.51376	22.67170	1150	*B. variegata*	Ad (2), Juv (1)	3 (1)	33% (0.84-90.58))	5.14	07.2011
Vartop, Mt. Bihor, Mt. Apuseni	46.51376	22.67170	1150	*B. variegata*	Tp	6	(0-45)		07.2011
Vartop, Mt. Bihor, Mt. Apuseni	46.51376	22.67170	1150	*R. temporaria*	Ad	1 (1)	100% (2.49-100)	0.92	07.2011
Padis, Mt. Bihor, Mt. Apuseni	46.58287	22.69917	1115	*B. variegata*	Ad	3 (2)	66% (9.42-99)	4.45; 0.17	07.2011
Mununá, Mt. Bihor, Mt. Apuseni	46.47116	22.81823	770	*B. variegata*	Ad	2	(0-84)		07.2011
Valea Larga, Mt. Metaliferi, Mt. Apuseni	46.35984	23.37932	780	*B. variegata*	Ad	2	(0-84)		07.2011

and analyzed on a *Rotor-Gene 6000* real-time rotary analyzer (Corbett Life Science). Genomic equivalents (GE) for all positive samples were estimated from standard curves based on known positive controls, considering 0.1 as the minimum value indicative of infection. The analytical sensitivity of the Taqman assay is 0.1 zoospore equivalents (Boyle et al. 2004). The samples from Sighisoara were analysed pooling three swabs while those from Apuseni, Corund and Ciuc individually. The maximum number of swabs that can be pooled without lowering the sensitivity of the Taqman assay is 5 (Hyatt et al. 2007).

In Sighisoara wood-pasture *Bd* was detected in three groups of *B. variegata* suggesting that at least 14% of the sampled individuals were infected. None of the *L. vulgaris ampelensis* individuals tested positive for *Bd* at this site. In the forest site of Sighisoara *Bd* was found in three groups, suggesting that at least 20% of the individuals were carrying the fungus in this site. In Apuseni 20% of the analysed *B. variegata* samples and the *R. temporaria* individual were found to be infected. Infected individuals of *B. variegata* were found both in the temporary ponds and the spring. None of the amphibians tested positive for *Bd* from Corund and Ciuc. In general GE values were low both for individuals sampled in early spring as well as in summer (Table 1). It is in accordance with previous findings from Central Europe which indicated that European *Bombina* species carry the fungus with low infection load (Civiš et al. 2012, Gál et al. 2012, Sztatecsny & Glaser 2010).

Several hypotheses have been proposed about the origin of *Bd* in wild populations, whether it is a novel or an introduced pathogen (Rachowitz et al. 2005). Recently Farrer et al. (2011) reported that *Bd* shows much higher genetic and phenotypic diversity linked to virulence than it was previously thought. They identified three deeply diverged lineages of *Bd*. One lineage is endemic to Switzerland (*Bd*CH), one lineage found in two continents (Africa and Europe) might have been endemic to South Africa but was introduced to Mallorca (*Bd*CAPE), and a globally distributed hypervirulent, recombinant lineage (*Bd*GPL). A fourth lineage is distinguished that seems to be endemic to Japan (Goka et al. 2009). Among the four lineages only the interlineage recombinant *Bd*GPL is associated with epizootics on five continents, populations infected with non-*Bd*GPL lineages do not show *Bd*-associated decline. These results suggest that *Bd* vectored by human is capable to recombine with local *Bd* lineage and become hypervirulent. However the study of Farrer et al. (2011) was based only on 20 *Bd* genomes and no samples were analysed from Central and Eastern Europe, thus origin of *Bd* in Romania still needs to be determined.

The regions surveyed in Romania are largely rural and the culture of importing exotics for pets, research, education, medicinal purposes is still not developed. Thus human-mediated introduction by exotic species of non-endemic *Bd* into the areas are not very likely. *Bd* kills the infected individuals within days and usually causes mass mortality between amphibians which is visible (Berger et al. 1998, Daszak et al. 1999). At the studied populations no unusual mass mortality was observed in amphibians. Contrary, long term studies e.g. in Sighisoara region suggests that amphibian populations are stable and the yearly variations of temporary pond occupancy are influenced by climatic conditions (Hartel et al. 2007, Hartel et al. 2011).

Our results show that *Bd* is present in amphibian populations from two regions in Romania and at least in Southern Transylvania amphibian populations seem not to be affected by the pathogen (see above). Since many regions of Romania harbour rich amphibian communities and connected populations, this may represent good ground for the spread of *Bd*. Further studies are urgently needed to understand the genetic origin, ways of spreading, distribution, and potential impacts of *Bd* on amphibian populations in this country.

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