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Occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in introduced and native species from two regions of France

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Abstract. To investigate the occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) in France, we examined 575 amphibians collected between 1875 and 2008 for the presence of the infection in the epidermis. We confirmed *Bd* in seven of the 15 stations investigated in the Aquitaine and Poitou-Charentes regions. Histological examination revealed the presence of the infectious agent associated with mild epidermal changes in 25 of 493 (5.1%) amphibians sampled in 2007-2008. The overall occurrence was 21.9% (21/96) in the introduced American bullfrog (*Lithobates catesbeianus*) and 2.3% (4/172) in the native European water frog complex (*Pelophylax* sp.). These prevalence data should be viewed as conservative giving the limitation of histology. Morbidity and mortality attributable to chytridiomycosis were not observed in these two species during this period. The introduced African clawed frog (*Xenopus laevis*) and all native museum specimens tested negative for chytrid infection. Unlike *L. catesbeianus*, *Pelophylax* sp. is broadly distributed at local and regional scales and whether or not this complex could act as a reservoir host in the dissemination of *Bd* remains to be determined. The presence of the chytrid fungus in amphibian populations from different locations in southwestern France calls for disease surveillance and for precautionary measures to avoid the spread of this emerging disease.

Keywords: chytridiomycosis, emerging infectious disease, France, histopathology, *Lithobates catesbeianus*, *Pelophylax* sp., *Xenopus laevis*.

Introduction

Amphibians are globally declining due to habitat loss and commercial exploitation. Among other factors, the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has recently raised major interest as an infectious disease threatening amphibian populations in many parts of the world (Berger et al., 1998; Carey et al., 2003; Skerratt et al., 2007; Dejean, Miaud and Ouellet, 2010). It infects the superficial layers of the amphibian epidermis. This fungus is enigmatic because it occurs in both declining and healthy amphibian populations and its recent emergence has lead to competing hypotheses regarding the endemic versus emerging nature of the pathogen (Ouellet et al., 2005; James et al., 2009; Padgett-Flohr and Hopkins, 2009). Morbidity and mortality vary among amphibian taxa and the outcome of the disease is dependent on host resistance and environmental factors. Different *Bd* lineages have been recently recognized and pathogen genotype may also be an important epidemiological determinant (Farrer et al., 2011). There is minimal host reaction to infection and pathological changes are somewhat inconsistent (Voyles, Rosenblum and Berger, 2011). The pathogenesis of chytridiomycosis is unclear but it may disrupt cutaneous osmoregulatory function in amphibians, leading to electrolyte imbalance and death in some species (Voyles et al., 2009). As a result of improvements in molecular diagnostics, many studies have been based solely on the magnitude of infection and few of them have actually performed detailed pathological investigations to link the emergence of the disease in amphibian populations with mortality rates large enough to cause the declines (Duffus, 2009). Yet, the negative effects of *Bd* on individuals do not necessar-

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ily translate into negative effects at the population level (Tobler, Borgula and Schmidt, 2012).

Among the different hypotheses trying to explain the dissemination of *Bd* into amphibian populations, the international trade sometimes leading to the introduction of invasive species and their pathogens has been proposed in recent years (Weldon et al., 2004; Fisher and Garner, 2007; Schloegel et al., 2009, 2010). Two prime suspects include the globally introduced American bullfrog (*Lithobates catesbeianus*) and the African clawed frog (*Xenopus laevis*). These species have been shown to be consistently carrying the fungus without any clinical signs of the disease both in their natural (Weldon et al., 2004; Ouellet et al., 2005) and feral ranges (Garner et al., 2006; Padgett-Flohr and Hopkins, 2009; Solís et al., 2010), and in the commercial trade (Schloegel et al., 2009; Bai, Garner and Li, 2010). Retrospective studies have shown that *Bd* has been infecting *L. catesbeianus* since the 1960s (Ouellet et al., 2005; Padgett-Flohr and Hopkins, 2009) and *X. laevis* since the 1930s (Weldon et al., 2004; Soto-Azat et al., 2010). In southwestern France, *L. catesbeianus* is assumed to have been introduced as ornamental garden animals in Arveyres (Gironde department) in 1968 and is now well established (Touratier, 1992; Berroneau, Détaint and Coïc, 2008). The origins of *X. laevis* are thought to be from a breeding centre release in western France (Deux-Sèvres department) in the early 1980s and the species is currently expanding in range (Fouquet, 2001; Fouquet and Measey, 2006).

Reports of the presence of *Bd* are few in France. Its presence, in particular, has recently been detected in the common midwife toad (*Alytes obstetricans*) and the fire salamander (*Salamandra salamandra*) from the Parc National des Pyrénées, Pyrénées-Atlantiques department (Dejean, Miaud and Ouellet, 2010; Walker et al., 2010; Farrer et al., 2011). In this study, we searched for the presence of *Bd* in amphibian populations from two regions of France across different introduced and native species. We processed by histological examination in order to evaluate the severity of epidermal changes in cases of positive infection. In a first attempt to explore the time and location of *Bd* emergence, we conducted a retrospective survey in two museum collections. Our objective was to assess the presence of the chytrid fungus in native amphibians before the first introductions of *L. catesbeianus* and *X. laevis* in southwestern and western France, respectively.

Materials and methods

We caught 493 postmetamorphic amphibians in April through August 2007 and February through July 2008 from 15 different stations located in the French departments of Deux-Sèvres (Poitou-Charentes) and Dordogne (Aquitaine) (Appendix 1, fig. 1, table 1). Among these specimens, 44 were in the juvenile and 449 in the subadult-adult life stages. They belonged to 6 anuran and 3 salamander species and included two introduced species, *L. catesbeianus* and *X. laevis*. Retrospectively, we also examined 79 amphibians (8 juveniles, 71 adults) of the Muséum National d'Histoire Naturelle (Paris) opportunistically collected between 1875 and 1967 from different localities in France (table 2). An additional 3 anurans (1 juvenile, 2 adults) captured in 1890 were analysed from the Muséum des Sciences Naturelles d'Angers. We restricted our museum sampling to amphibians collected before 1968. We clipped the fourth toe from the right hindlimb at the articulation proximal to the fourth phalange in wild specimens while it was the third toe from the left hindlimb at the articulation proximal to the third phalange in museum specimens. Tissue samples were stored in absolute alcohol. All wild animals were considered healthy at the time of sampling (no gross anomalies observed upon physical examination) and were subsequently released. We ensured that no contamination occurred between animals by changing gloves and sterilizing our surgical instruments. We also cleaned and disinfected all field equipment using a 1% Virkon solution between each sampling location (Dejean, Miaud and Ouellet, 2007).

Toes were later fixed in 10% neutral buffered formalin before processing for histopathology. Paraffin-embedded toes were sectioned longitudinally at 4 μm, and stained with hematoxylin-(eosine-phloxine)-saffron for light microscopic examination. Each slide was examined blind with no knowledge of species or date of collection. A second observer confirmed all positive cases and rejected occasional suspicious sections due to histologic artifacts. We diagnosed infection by *Bd* when zoosporangia (empty or with zoospores) were observed in the *stratum corneum* and/or *stratum granulosum* of the epidermis (Ouellet et al., 2005).

We performed Pearson chi-square tests to compare the proportions of infected individuals in the species examined. Data from the different stations and years were pooled for each species in order to increase the sample size. Significance level was set at $\alpha = 0.05$.

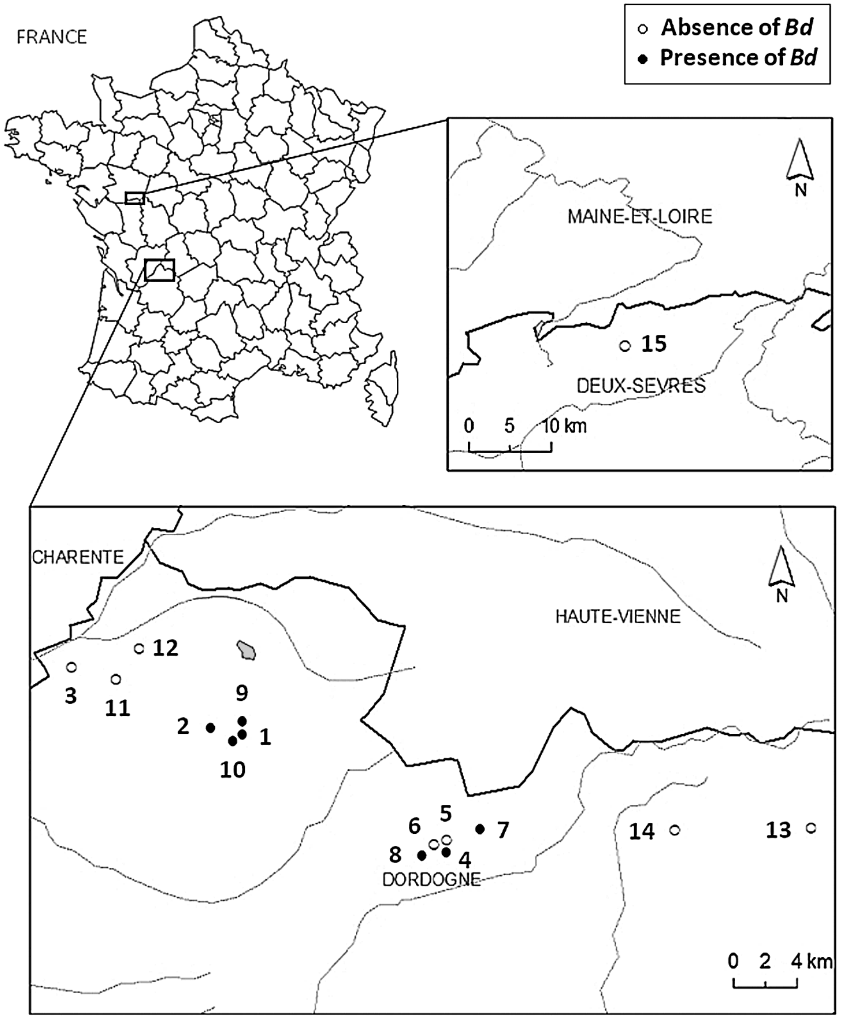

Figure 1. Map of France showing 15 stations investigated for the occurrence of *Batrachochytrium dendrobatidis* (*Bd*) in amphibian populations during 2007-2008.

Results

In total, 575 samples were tested for the presence of *Bd* (tables 1 and 2). We detected the infection in 25 of 493 (5.1%) amphibians from two species collected in the Dordogne department during the period 2007-2008, including the introduced *L. catesbeianus* and the native European water frog complex (*Pelophylax* sp.). We confirmed *Bd* in seven of the 15 stations investigated. Except for station 2, *L. catesbeianus* was present at the time of sampling in the other six positive stations. However, stations 1 through 14 were located in close proximity to a *L. catesbeianus*-invaded area. We did not detect a significant difference in the occurrence of *Bd* among the 7 native species ($\chi^2 = 3.204$, df $= 6$, $P = 0.783$). When adding the introduced species in the analysis, we detected a significant difference in the occurrence of *Bd* among species examined ($\chi^2 = 71.025$, df $= 8$, $P < 0.001$). Detailed infection prevalence was 21.9% (21/96) in *L. catesbeianus*, 2.3% (4/172) in *Pelophylax* sp., and 0% (0/225) in the seven other species. Among the 25 infected specimens, 8 were in the juvenile and 17 in the subadult-adult life stages. All museum speci-

Table 1. Occurrence of *Batrachochytrium dendrobatidis* in amphibian populations from two regions of France during 2007-2008. *Pelophylax* sp. represents a complex of water frogs that was not differentiated in the field.

Station	Taxon	Year	Number infected/ number examined
1	*Bufo bufo*	2008	0/5
	Hyla arborea	2007	0/3
	Lissotriton helveticus	2007-2008	0/26
	Lithobates catesbeianus	2007	11/38
	Pelophylax sp.	2007-2008	2/31
	Rana dalmatina	2007-2008	0/5
	Triturus marmoratus	2007	0/12
2	*Lissotriton helveticus*	2007	0/1
	Pelophylax sp.	2007	1/75
	Triturus marmoratus	2007	0/18
3	*Lissotriton helveticus*	2007	0/19
4	*Lithobates catesbeianus*	2007	3/25
5	*Lithobates catesbeianus*	2007	0/1
6	*Lithobates catesbeianus*	2007	0/7
7	*Hyla arborea*	2008	0/10
	Lithobates catesbeianus	2007	2/14
8	*Lithobates catesbeianus*	2007	1/1
9	*Lithobates catesbeianus*	2007	4/10
10	*Pelophylax* sp.	2007	1/39
11	*Pelophylax* sp.	2007	0/9
12	*Pelophylax* sp.	2007	0/18
13	*Bufo bufo*	2008	0/14
	Rana dalmatina	2008	0/2
	Salamandra salamandra	2008	0/15
14	*Rana dalmatina*	2008	0/6
15	*Xenopus laevis*	2008	0/89
Total			25/493

mens collected between 1875 and 1967 tested negative (0/82). These data will be deposited at http://www.bd-maps.eu/.

Histological changes of the epidermis associated with *Bd* infection were mild and included focal hyperkeratosis and minimal *stratum corneum* sloughing. Most zoosporangia were empty spherical and ovoidal structures localized to areas of infection. Typical zoosporangia containing zoospores and/or empty zoosporangia were observed in the *stratum corneum* (fig. 2). These observations were in accord with the lack of clinical signs of disease observed in all amphibians examined under field conditions.

Discussion

Bd infection was observed with a prevalence of 21.9% in apparently healthy populations of the introduced *L. catesbeianus* in southwestern France. A lower prevalence of 2.3% was also diagnosed in the native *Pelophylax* sp. complex. This low prevalence in *Pelophylax* sp. was not statistically different from the apparent absence of *Bd* in the other native species. However, caution should be used when interpreting these results due to relatively small sample sizes where neither absence can be reliably inferred nor prevalence reliably estimated. In regard to the limitation of histological examination (Ouellet et al., 2005), our results probably underestimate the overall prevalence of the infection in the species examined. With species harbouring a low level of infection, molecular techniques (qPCR or real-time quantitative polymerase chain reaction) are usually considered as a more sensitive test for the detection of *Bd* zoospores, particularly at early stages of infection (Boyle et al., 2004). Accounting for the lower sensitivity of histology, we analysed the whole toe instead of a smaller patch of epidermal tissue. We also achieved our objective to evaluate the severity of epidermal changes associated to infection. Indeed, histology has the ability to detect epidermal changes associated with chytridiomycosis, not only the presence of zoospores (Smith, 2007). The absence of *Bd* in museum specimens collected before 1968 is of interest although further samples should be tested to better support this absence. Hence, we may have missed the pathogen considering the apparently low prevalence in the indigenous amphibians. Anurans of the *Pelophylax* sp. complex and *A. obstetricans* (high susceptibility to *Bd*) should be given priority, especially those collected in the early 1960s.

In Europe, *Bd* has previously been identified in introduced *L. catesbeianus* from France (Garner et al., 2006), Italy (Garner et al., 2006; Adams et al., 2008), and the United Kingdom (Garner et al., 2006). Garner et al. (2006) detected the presence of *Bd* in 61.5% (16/26) of

Table 2. Occurrence of *Batrachochytrium dendrobatidis* in museum amphibian specimens collected between 1875 and 1967 from different localities in France. *Pelophylax* sp. was originally identified as *Pelophylax* kl. *esculentus* and the exact localities were sometimes unknown in older specimens. *MNHN* = Muséum National d'Histoire Naturelle (Paris), *MSNA* = Muséum des Sciences Naturelles d'Angers.

Museum	Year	Taxon	Department (locality)	Number infected/ number examined
MNHN	1875-1962	*Salamandra salamandra*	Ariège, Pas-de-Calais	0/12
	1878-1900	*Ichthyosaura alpestris*	Vienne, Yonne	0/4
	1888-1890	*Triturus cristatus*	Indre (Argenton-sur-Creuse)	0/4
	1891-1962	*Rana dalmatina*	Calvados (Caen), Loiret	0/10
	1893-1963	*Lissotriton helveticus*	Somme	0/10
	1919	*Pelophylax* sp.	Indre-et-Loire	0/4
	1961	*Bufo calamita*	Vaucluse	0/4
	1962	*Bufo bufo*	(Berry)	0/3
	1962	*Hyla arborea*	Loiret	0/6
	1962-1967	*Pelodytes punctatus*	Bouches-du-Rhône (Gémenos, Marseille), Loiret, Lot-et-Garonne	0/8
	1963	*Triturus marmoratus*	Seine-et-Marne, Somme	0/14
MSNA	1890	*Hyla arborea*	Maine-et-Loire (Saumur)	0/2
	1890	*Pelophylax* sp.	Maine-et-Loire (Saumur)	0/1
Total				0/82

Figure 2. Histological section of the toe skin of an introduced American bullfrog (*Lithobates catesbeianus*) infected by *Batrachochytrium dendrobatidis* from Bridarias (station 1) in southwestern France. This asymptomatic specimen constituted the most infected individual in our study with localized areas of sloughed *stratum corneum* containing zoosporangia both empty and with zoospores. This figure is published in colour in the online version.

French *L. catesbeianus* specimens from three different *Bd*-positive locations in the Gironde and Loir-et-Cher departments. Our *L. catesbeianus* data expand the range of the amphibian chytrid fungus in five other locations in the Dordogne department. Thus, in all three French departments where populations of *L. catesbeianus* are currently observed (ACEMAV, 2003), individuals have been found to harbour *Bd* infection. In both its natural range and in introduced regions, *L. catesbeianus* is considered as a reservoir host of the chytrid fungus and relatively asymptomatic when naturally or experimentally-infected. For example, in native populations, a prevalence of 60.2% (80/133) was observed in *L. catesbeianus* from the Mont Saint-Hilaire Biosphere Reserve of Québec during the period 1960-2001 (Ouellet et al., 2005). The rate was 21.8% (39/179) for the same species in the northeastern United States during 2000-2002 (Longcore et al., 2007).

The presence of *Bd* infection in native European species has been reported in asymptomatic pool frogs (*Pelophylax lessonae*) from Italy (Simoncelli et al., 2005), edible frogs (*Pelophylax* kl. *esculentus*) from Italy (Adams et al., 2008; Federici et al., 2008), Denmark (Scalera, Adams and Galvan, 2008) and Luxembourg (Wood, Griffiths and Schley, 2009), and Perez's frogs (*Pelophylax perezi*) from Spain (Walker et al., 2010). It has been also observed in the hybridogenetic complex *Pelophylax lessonae-esculentus* from Switzerland with a mean proportion of individuals carrying *Bd* per positive site of 27% (Tobler, Borgula and Schmidt, 2012). In Québec, *Bd* has been detected in

North American green frog specimens (*Lithobates clamitans*) collected as early as 1961 with an overall prevalence of 26.7% (135/506) during 1960-2001 (Ouellet et al., 2005). The prevalence was 25.5% (52/204) in *L. clamitans* from the northeastern United States in 2000-2002 (Longcore et al., 2007). In light of these findings, it will be interesting to determine in future studies whether anurans of the European water frog complex could act as a reservoir to more sensitive native species. At local and regional scales, *Pelophylax* sp. is broadly distributed (Pagano et al., 2001; ACEMAV, 2003) and, like *L. catesbeianus*, might play a role in the spread of *Bd*. The apparent absence of *Bd* in introduced *X. laevis* from station 15 also supports this hypothesis. The diversity of indigenous water frog assemblages may be already considered under threat due to the multiple introductions of the marsh frog (*Pelophylax ridibundus*) in some parts of France (Neveu, 1997; Schmeller et al., 2007).

Chytridiomycosis and ranaviral disease (an emerging viral disease in amphibians caused by ranaviruses, family *Iridoviridae*) (e.g., Teacher, Cunningham and Garner, 2010) are now listed as globally notifiable diseases by the World Organisation for Animal Health (OIE) with the aim to assure the sanitary safety of international trade in live amphibians and their products (Schloegel et al., 2010). Considering the many uncertainties surrounding the real significance of *Bd* infection into native and introduced amphibian populations, we recommend being proactive in France. Disease surveillance is thus warranted and any suspected cases of disease-induced mortality in the field should be followed by detailed pathological investigations. The presence of disease must be confirmed, not only the presence of an infection with fast molecular tests (Smith, 2007; Duffus, 2009). In Europe, rapid dissemination of *Bd* data can be made through the RACE (Risk Assessment of Chytridiomycosis to European Amphibian Biodiversity) project at http://www.bd-maps.eu/. Amphibian disease research is a rapidly evolving discipline and many diseases have yet to be discovered. Programs to eradicate or at least prevent the progression of introduced non-native amphibian species (e.g., Berroneau, Détaint and Coïc, 2008) should also be implemented in a concerted way. Finally, a more systematic application of precautionary measures (e.g., Dejean, Miaud and Ouellet, 2007), to avoid the dissemination of these emerging diseases through human activities in France and elsewhere in Europe, is also highly desirable.

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Appendix 1. Summary information of the 15 stations investigated for the occurrence of *Batrachochytrium dendrobatidis* in amphibian populations from the French departments of Deux-Sèvres (Poitou-Charentes) and Dordogne (Aquitaine) during 2007-2008.

Station	Locality	Commune	WGS84 coordinates (N/E)	Altitude (m)
1	Bridarias	Saint-Estèphe	45°36′42″/00°40′52″	237
2	Merigaud	Saint-Estèphe	45°36′46″/00°40′01″	276
3	Montouleix	Bussière-Badil	45°38′44″/00°35′18″	220
4	Pierres Blanches	Champs-Romain	45°32′44″/00°47′59″	294
5	Mazieras	Saint-Saud-Lacoussière	45°33′08″/00°47′59″	296
6	Le Verdoyer	Champs-Romain	45°32′50″/00°47′37″	303
7	La Gourgousse	Saint-Saud-Lacoussière	45°33′29″/00°49′08″	310
8	Les Bessines	Champs-Romain	45°32′49″/00°47′24″	296
9	Prieuraud	Piégut-Pluviers	45°36′58″/00°41′06″	235
10	Bridarias	Saint-Estèphe	45°36′33″/00°40′49″	240
11	Chez Jeamot	Bussière-Badil	45°38′20″/00°36′49″	224
12	Etapeau	Bussière-Badil	45°39′20″/00°37′36″	235
13	La Barde	La Coquille	45°33′32″/01°00′19″	310
14	Mamont	Mialet	45°33′26″/00°55′42″	301
15	La Vergnaie	Cersay	47°02′52″/00°23′02″	96