Chytrid fungus acts as a generalist pathogen infecting species-rich amphibian families in Brazilian rainforests

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ABSTRACT: The fungus *Batrachochytrium dendrobatidis* (*Bd*) is among the main causes of declines in amphibian populations. This fungus is considered a generalist pathogen because it infects several species and spreads rapidly in the wild. To date, *Bd* has been detected in more than 100 anuran species in Brazil, mostly in the southern portion of the Atlantic forest. Here, we report survey data from some poorly explored regions; these data considerably extend current information on the distribution of *Bd* in the northern Atlantic forest region. In addition, we tested the hypothesis that *Bd* is a generalist pathogen in this biome. We also report the first positive record for *Bd* in an anuran caught in the wild in Amazonia. In total, we screened 90 individuals (from 27 species), of which 39 individuals (from 22 species) were Bd-positive. All samples collected in Bahia (2 individuals), Pernambuco (3 individuals), Pará (1 individual), and Minas Gerais (1 individual) showed positive results for *Bd*. We found a positive correlation between anuran richness per family and the number of infected species in the Atlantic forest, supporting previous observations that *Bd* lacks strong host specificity; of 38% of the anuran species in the Atlantic forest that were tested for *Bd* infection, 25% showed positive results. The results of our study exemplify the pandemic and widespread nature of *Bd* infection in amphibians.

KEY WORDS: Pathogen · *Batrachochytrium dendrobatidis* · *Bd* · Geographic distribution · Atlantic forest · Amazon forest · Anurans

INTRODUCTION

Many anuran populations worldwide have experienced severe declines (Williams & Hero 1998, Bustamante et al. 2005), and anurans are justly considered among the most threatened vertebrates (Baillie et al. 2004, 2010). Infectious diseases are among the main causes of these population declines (Baillie et al. 2004, Fisher et al. 2012). Particularly, the aquatic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has led to the decline of several amphibian populations and species worldwide (La Marca et al. 2005, Lips et al. 2006, Skerratt et al. 2007), and it has mostly affected species with a high dependence on water (Bielby et al. 2008, Catenazzi et al. 2011). *Bd* is a generalist pathogen (Morgan et al. 2007, Walker et al.

2010, Olson et al. 2013), as it has infected more than 500 amphibian species in a wide variety of aquatic and terrestrial habitats (Gründler et al. 2012). *Bd* also has high tolerance to environmental variation, and it is found in diverse locations throughout Africa, Asia, Europe, and the Americas (Fisher et al. 2009, Olson et al. 2013).

Species diversity may affect the prevalence of infectious diseases because of increased interactions among the pathogen and potential hosts (Keesing et al. 2006). Data from a global amphibian assessment suggest that amphibian declines associated with *Bd* infection are greater in regions with higher species richness (Olson et al. 2013). However, at local scales, higher diversity tends to attenuate infection indices (Becker et al. 2014). Moreover, the effects of *Bd* vary among species and populations worldwide (Berger et al. 1999, Briggs et al. 2010, Kilpatrick et al. 2010, Searle et al. 2011), probably because of a combination of environmental factors and host traits that impact the spread and susceptibility of species to *Bd* (Walker et al. 2010, Bancroft et al. 2011). Life history traits have also been associated with higher *Bd* infection rates (Bancroft et al. 2011, Guayasamin et al. 2014) among families with high diversity, broad distributions, and with both aquatic and terrestrial stages (Corey & Waite 2008, Olson et al. 2013).

The Neotropical region of South and Central America contains the highest diversity of amphibians (Baillie et al. 2010). This area includes the Brazilian Atlantic forest and Cerrado (an open grassland physiognomy), 2 of the most endangered, species-rich (Myers et al. 2000) biomes and centers of endemism in the world (Haddad et al. 2013, Valdujo et al. 2013). Three distinct genotype lineages (including the most ancestral) of the *Bd* pathogen have been found in the Atlantic forest, and this biome may represent the center of origin for *Bd* (Schloegel et al. 2012, Rosenblum et al. 2013). To date, 2 anuran species in Cerrado and 110 species in several habitats of the Atlantic forest have been recorded as *Bd*-positive (Table S1 in the Supplement at www.int-res.com/articles/suppl/d114p061_supp.pdf), with records dating back to more than 100 yr ago (Rodriguez et al. 2014). However, the vast majority of these reports are from the south and southeast Atlantic forest (Lisboa et al. 2013, Rodriguez et al. 2014). In the northern portion of the biome, the distribution and prevalence of *Bd* are poorly documented (Carnaval et al. 2006, Verdade et al. 2012, Lisboa et al. 2013).

Here we provide current information on *Bd* distribution in north and northeastern Brazil. We have included the first record of *Bd* in the state of Bahia, as

well as the first record in an anuran caught in the wild in Brazilian Amazonia. We also used data from previous chytrid studies in the Atlantic forest (south, southeast, and northern regions) to test the hypothesis that *Bd* is a generalist pathogen in this biome. If *Bd* is a generalist, we would expect that the number of species per family is positively correlated with the number of infected species in the family.

MATERIALS AND METHODS

We conducted fieldwork in 5 Brazilian localities: 4 in the Atlantic forest in the states of Pernambuco, Alagoas (June to December 2013 and February to May 2014), Bahia (July 2010), and Minas Gerais (August 2010) and 1 in the Amazon forest in the state of Pará (February 2013). At each location, we swabbed individuals from terrestrial (forest and open areas) and aquatic habitats (ponds and streams) during diurnal and nocturnal visual encounter surveys, as per the methods of Crump & Scott (2001).

To avoid cross-contamination, each collected anuran was immediately and individually maintained in a plastic bag. Individuals were swabbed for *Bd* detection by using sterile cotton swabs and wiping the forelimbs, hindlimbs, and pelvic region 5 times for each individual, as per the method described by Hyatt et al. (2007). DNA extraction was performed using 50 µl of PrepMan® ULTRA (Life Technologies, Thermo Fisher Scientific) for each swab, followed by Taqman® qPCR assays for detection and quantification of *Bd* loads, according to standard protocols (Boyle et al. 2004, Lambertini et al. 2013). *Bd* prevalence was estimated as the number of *Bd*-positive frogs divided by the total number of sampled frogs. Infection intensity was estimated as the number of zoospore equivalents (ZE) obtained using qPCR (see Lambertini et al. 2013). The strain used for the standard curve was CLFT 023 (from Monte Verde, Camanducaia, Minas Gerais), allowing for comparisons with other localities worldwide (Longo et al. 2013).

To test the hypothesis that *Bd* is a generalist pathogen (showing low species-specificity), we combined our data with records of previous Atlantic forest studies (Table S1 in the Supplement) to calculate the number of infected species. We then used a linear regression to test the correlation between the number of infected species and the total number of species registered in the biome (from Haddad et al. 2013). Differences between *Bd* prevalence and load among families were tested with analyses of variance (ANOVA). Analyses were performed using the R sta-

tistical computing environment (R Development Core Team 2013) after normalizing data through a log transformation.

RESULTS

Out of the 27 sampled species from 8 anuran families, 22 (81.5%) tested positive for *Bd* (Table 1). Out of the 90 sampled individuals from 5 localities (Fig. 1), 39 (43.3%) tested positive for *Bd* infection. All samples collected in Bahia (n = 2), Pernambuco (n = 3), Pará (n = 1), and Minas Gerais (n = 1) tested positive for *Bd* (Table 1).

Among families with the greatest number of samples, *Bd* prevalence was higher in Craugastoridae (56%; 2 species; 16 individuals) and Hylidae (42%; 15 species; 52 individuals) and lower in Leptodactylidae (38%; 3 species; 8 individuals) and Bufonidae (25%; 3 species; 8 individuals), although *Bd* prevalence among families did not differ statistically ($F = 0.80$, df = 3, p = 0.50). Infection load was the highest in Craugastoridae (log ZE = 1.39 ± 1.51, n = 9 individuals) and Hylidae (log ZE = 1.05 ± 0.87, n = 21 individuals), although loads did not differ between families ($F = 0.92$, df = 5, p = 0.47). The highest intensity of infection (log ZE = 1.79–5.20) was detected in *Crossodactylus*

Table 1. Anuran species sampled for *Batrachochytrium dendrobatidis* presence in the Amazon and Atlantic forests (see Fig. 1). Zoospore equivalents (mean and SD) were calculated only for positive individuals. For most species, all sampled individuals were adults; the exception was *Proceratophrys renalis*, for which an adult and a juvenile were sampled

| Taxon	Locality, state	Zoospore equivalents			Prevalence (infected/analyzed individuals)
		Mean	Min.–Max.	SD	
Bufonidae					
Frostius pernambucensis	Murici, Alagoas	62.17	–	–	1/2
Rhinella crucifer	Murici, Alagoas	4.79	–	–	1/3
Rhinella hoogmoedi	Murici, Alagoas	–	–	–	0/3
Craugastoridae					
Pristimantis vinhai	Itacaré, Bahia	2.37	–	–	1/1
Pristimantis ramagii	Murici, Alagoas	25.51	3.53–135.72	48.71	7/14
Dendrobatidae					
Adelphobates galactonotus	Santa Cruz Dos Martírios, Pará	1.56	–	–	1/1
Hemiphractidae					
Gastrotheca sp.	Murici, Alagoas	–	–	–	0/1
Hylidae					
Aplastodiscus sibilatus	Murici, Alagoas	–	–	–	0/1
Dendropsophus minutus	Murici, Alagoas	12.4	–	–	1/2
Hypsiboas albomarginatus	Murici, Alagoas	1.05	–	–	1/1
Hypsiboas crepitans	Poção, Pernambuco	34.62	2.2–67.05	45.85	2/2
Hypsiboas exastis	Murici, Alagoas	7.88	–	–	1/1
Hypsiboas semilineatuss	Murici, Alagoas	–	–	–	0/1
Phyllodytes cf. *acuminatus*	Murici, Alagoas	16.33	–	–	1/1
Phyllodytes cf. *edelmoi*	Murici, Alagoas	25.56	–	–	1/1
Phyllodytes edelmoi	Murici, Alagoas	60.22	2.95–227.27	111.37	4/12
Phyllodytes gyrinaethes	Murici, Alagoas	359.16	1.72–1276.49	555.53	5/24
Phyllodytes tuberculosus	Itacaré, Bahia	2.93	–	–	1/1
Phyllomedusa nordestina	Murici, Alagoas	72.63	3.30–141.95	98.04	2/2
Scinax cf. *eurydice*	Carangola, Minas Gerais	1.64	–	–	1/1
Scinax nebulosus	Murici, Alagoas	4.32	–	–	1/2
Scinax pachycrus	Poção, Pernambuco	1.88	–	–	1/1
Hylodidae					
Crossodactylus dantei	Murici, Alagoas	2793.2	264.98–5321.50	3575.55	2/2
Leptodactylidae					
Physalaemus cf. *cuvieri*	Murici, Alagoas	7.91	4.27–11.55	5.14	2/6
Leptodactylus natalensis	Murici, Alagoas	–	–	–	0/1
Leptodactylus cf. *mystaceus*	Murici, Alagoas	5.1	–	–	1/1
Odontophrynidae					
Proceratophrys renalis	Murici, Alagoas	6.01	–	–	1/2
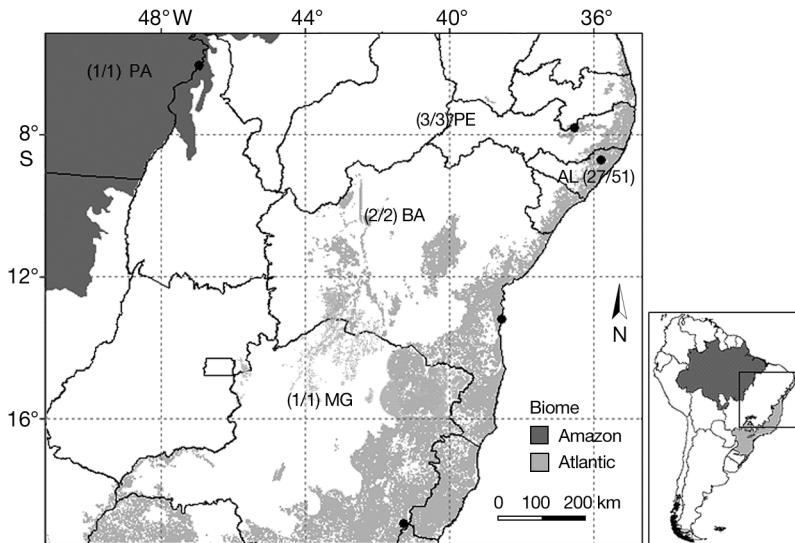
Fig. 1. Positive records of *Batrachochytrium dendrobatidis* infection reported in the present study for the Atlantic forest (light grey) of Brazil, at sampling sites (dots) in the states of Pernambuco (PE), Alagoas (AL), Bahia (BA), and Minas Gerais (MG), and for the Amazon forest (dark grey), in the state of Pará (PA). Shown in parentheses is the number of individuals that tested positive out of the total sampled. Source for remaining forest cover: SOS Mata Atlântica/INPE 2012 (www.sosmatatlantica.org.br)

DISCUSSION

Among the 6 main Brazilian biomes, 3 tested positive for *Bd* infection: Amazonia (present study), Cerrado (Ramalho et al. 2013), and the Atlantic forest (Toledo et al. 2006a, Lisboa et al. 2013, Rodriguez et al. 2014, present study). Our record is the first report of *Bd* infection in a frog caught in the wild; the previous record of *Bd* in Amazonia was in the tadpoles of an exotic bullfrog *Lithobates catesbeianus* obtained from a commercial frog farm (Schloegel et al. 2010). Although bullfrogs have been suggested as potential reservoirs and vectors of *Bd* (Schloegel et al. 2010), *Bd* was present in Brazil long before the introduction of bullfrogs (Rodriguez et al. 2014).

Bd has been, and is still, broadly studied in the Atlantic forest, where the number of infected species has increased to 131 (Table S1); the majority of these reports are from the southern region, where 2 divergent strains of *Bd* (*Bd*-GPL and *Bd*-
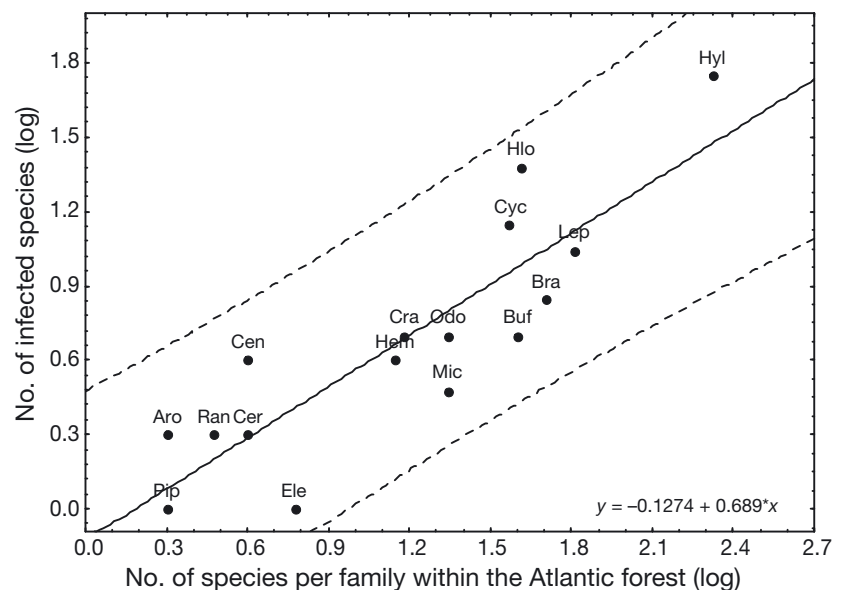
dantei and *Phyllodytes gyrinaethes*, followed by *Phyllomedusa nordestina*, *Frostius pernambucensis*, *Phyllodytes edelmoi*, *Hypsiboas crepitans*, and *Pristimantis ramagii.* The following species had the lowest loads: *Rhinella crucifer*, *Scinax nebulosus*, *Phyllodytes tuberculosus*, *Pristimantis vinhai*, *Scinax pachycrus*, *Scinax* cf. *eurydice*, *Adelphobates galactonotus*, and *Hypsiboas albomarginatus* (log ZE = 0–1.40). *Bd* was not detected in *Rhinella hoogmoedii*, *Gastrotheca* sp., *Aplastodiscus sibilatus*, *Hypsiboas semilineatus*, and *Leptodactylus natalensis* (only 1 individual was tested for each of these species). The sampled individual of *A. galactonotus* collected from Pará, Amazonia, was infected.

Anuran richness per family was positively correlated with the number of infected species in the Atlantic forest (r^2 = 0.753, p < 0.001, df = 14, Fig. 2). The richest families in the Atlantic forest (Hylidae, Hylodidae, and Leptodactylidae) had greater numbers of infected species (Table S2 in the Supplement).

Fig. 2. Linear regression of the number of species per family reported in the Atlantic forest of Brazil on the number of infected species (r^2 = 0.753, p < 0.001, df = 14). Data from studies listed in Table S1 in the Supplement. Dotted lines represent upper and lower 95% confidence intervals. Aro: Aromobatidae; Bra: Brachycephalidae; Buf: Bufonidae; Cen: Centrolenidae; Cer: Ceratoprhyidae; Cra: Craugastoridae; Cyc: Cycloramphidae; Ele: Eleutherodactylidae; Hem: Hemiphractidae; Hyl: Hylidae; Hlo: Hylodidae; Lep: Leptodactylidae; Mic: Microhylidae; Odo: Odontophrynidae; Pip:Pipidae, Ran: Ranidae

Brazil) have been present at least since 1897 (Rodriguez et al. 2014) in terrestrial and aquatic habitats without lethal effects on anuran populations (Toledo et al. 2006b, Gründler et al. 2012, Rodriguez et al. 2014). In the northern region, information on *Bd* has been limited to reports of anuran infection from the states of Alagoas, Bahia, and Pernambuco (Carnaval et al. 2006, Lisboa et al. 2013, present study). Our findings of *Bd* in Bahia fill a gap of 1600 km between Minas Gerais and Alagoas, indicating that *Bd* is widespread across a broad host range throughout the Atlantic forest. *Bd* prevalence and load (Table 1) among anurans in the northern region may be regarded as moderate. However, there is a clear need for more surveys in this region, since small sample sizes and differences in sampling effort may lead to a bias in estimating prevalence and pathogen load (Ohst et al. 2013). Climatic and forest characteristics can affect *Bd* infection (Rödder et al. 2008, Fisher et al. 2009, Gründler et al. 2012), and the differing climate dynamics in northern and southern parts of the Atlantic forest may therefore affect *Bd* dynamics in these regions (Câmara 2003, Carnaval et al. 2014).

Our *Bd* data for the northern region of the Atlantic forest are consistent with an enzootic pattern where there is no detectable effect of *Bd* on anuran survival (Briggs et al. 2010). The same pattern was found in the south Atlantic forest (Rodriguez et al. 2014) and other areas (Canada: Forzán et al. 2010; Cameroon: Doherty-Bone et al. 2013; Germany: Ohst et al. 2013). These findings contradict epizootic events reported in Australia (Berger et al. 1998), Costa Rica (Lips et al. 2003), Mexico (Lips et al. 2004), Panama (Lips 1999), Spain (Bosch et al. 2001), the USA (Vredenburg et al. 2010), and Venezuela (La Marca 2005), where high prevalence (Lips et al. 2006) and high *Bd* infection loads (Vredenburg et al. 2010) have been associated with local mass extinctions and fatal declines.

The Atlantic forest is among the most threatened forests in the world, where more than 80% of the remaining fragments are smaller than 0.5 km^2 (Ribeiro et al. 2009). Nevertheless, this biome has high anuran species richness (522 species) in Brazil, presumably because of its diverse geographical and climatic characteristics (Ribeiro et al. 2009, Toledo & Batista 2012, Haddad et al. 2013, Toledo et al. 2014). This high diversity of species and habitats may favor *Bd* infections by providing diverse hosts and microhabitats (Becker & Zamudio 2011). As in Costa Rica and Australia (Becker & Zamudio 2011), the number of species per family was positively correlated with the number of infected species in the Atlantic forest (Fig. 2). Even though several anuran species in the Atlantic forest still need to be screened for *Bd* infection, our data support the hypothesis that this fungus is a generalist pathogen (Fisher et al. 2009) that spreads rapidly (Morgan et al. 2007) and infects many different host species types (Crawford et al. 2010, Walker et al. 2010). In contrast, *Bd* infections in Europe and the USA are associated with amphibian families that display similar life-history traits (Corey & Waite 2008, Bancroft et al. 2011, Baláž et al. 2014). For example, species of the family Ranidae are more susceptible to *Bd* infection because of their similarities in body shape and habitat use for breeding (Bancroft et al. 2011).

We found that Hylidae and Leptodactylidae have the greatest number of *Bd*-positive species in the Atlantic forest (Table S2 in the Supplement). These families exhibit the greatest diversity of species and reproductive modes in this biome and include species that spawn aquatic and terrestrial eggs and use different types of lotic, lentic, and terrestrial habitats during the breeding season (Haddad & Prado 2005). In southern and southeastern Brazil, species that breed in lentic and terrestrial habitats are characterized by a higher *Bd* prevalence when compared with species that breed in lotic habitats (Gründler et al. 2012, Rodriguez et al. 2014). Likewise, North American ranids (another species-rich family) that are dependent on water bodies (i.e. lentic habitats) for laying eggs and tadpole development seem to be more prone to *Bd* infection than strictly terrestrial or direct-development species (see also Becker et al. 2014).

The Atlantic forest extends from 3° to 31° S and 35° to 60° W in Brazil (Ribeiro et al. 2009); to date, only 38% of the anuran species present in 10 states have been screened for *Bd* infection, of which 25% tested positive. Several taxa (mainly in the north and northeastern regions) have not been sampled, and long-term monitoring of anurans in the Brazilian biomes is clearly needed to fill geographical, taxonomic, and ecological gaps. These data reveal infection trends in the wild, providing sine qua non information that can be directly integrated into amphibian conservation plans.

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