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New records of *Batrachochytrium dendrobatidis* in the state of Bahia, Brazil: histological analysis in anuran amphibian collections

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ABSTRACT: Infection caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) produces chytridiomycosis, a disease considered one of the main causes of amphibian population declines in the world. In Brazil, *Bd* has been recorded in several regions, but mainly in the Atlantic Forest biome. This study aimed to investigate the occurrence of *Bd* in amphibian species in Bahia State to test the hypothesis that *Bd* is widespread in other Brazilian biomes. Using histological analysis, we evaluated the skin of 190 anurans of 85 species preserved in herpetological collections. Based on these analyses, the distribution of *Bd* was extended approximately 400 km to the west, 150 km to the north and 105 km to the east in the state of Bahia. Of the 190 specimens analyzed, *Bd* infection was diagnosed in 16 individuals, from 14 species, with the earliest record from a specimen collected in 1996 in the Caatinga biome. We identified *Bd* in 13 adult specimens, including 2 individuals showing suggestive signs of the disease (loss of skin pigmentation). In tadpoles, we recorded fungal structures in the oral region and on the epidermis adjacent to the rows of teeth. The results of this study corroborate the prediction that *Bd* is widespread in the Atlantic Forest biome, and suggest that it is widespread in the other biomes of the state (Cerrado and Caatinga, at least since 1996). Conservation efforts should involve long-term studies aimed at providing information on the dynamics of the infection, its relationship with its host and its effect on amphibian populations.

KEY WORDS: Amphibians · Chytridiomycosis · Emerging infectious disease · Population decline of anurans · Extinction

1. INTRODUCTION

A decline in the amphibian populations has been recorded in several countries, generally associated with climate change, chemical contamination, habitat loss, infectious diseases, overexploitation, introduced species and ultraviolet radiation (Verdade et al. 2010, Costa et al. 2012, Fisher et al. 2012, O'Hanlon et al. 2018). Among these main causes, the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has emerged as an important infection agent, especially for amphibians in tropical regions (Daszak et al. 2003, Lips et al.

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2006, Pounds et al. 2006, Fisher et al. 2009, Voyles et al. 2009, Kolby & Daszak 2016, Carvalho et al. 2017).

Chytrids are soil fungi that are also found in water bodies, where they infect algae, protozoans, invertebrates and other fungi (Raven et al. 2007). The first recordings of vertebrates infected with chytrids occurred in Australia and Central America in the late 1990s, when *Bd* was described in association with deaths of anurans diagnosed with chytridiomycosis (Berger et al. 1998, Longcore et al. 1999). This disease was soon identified across other continents and recognized as a cause in the decline of many global amphibian populations (Olson et al. 2013), as it triggers lethal epidemics (Daszak et al. 2004, Ouellet et al. 2005, Voyles et al. 2009, Farrer et al. 2011, O'Hanlon et al. 2018).

Chytridiomycosis is transmitted via zoospores (flagellated spores adapted to aquatic environments) that generally survive at temperatures below 25°C and are disseminated through direct contact with other animals or contaminated water (Johnson & Speare 2003). The fungus is found only in the superficial epidermal layer of the amphibians. Behavioral symptoms are most commonly observed, and in some cases do not cause lesions or other symptoms that facilitate fungal identification (Burke 2011).

Bd does not target a specific host, being a generalist pathogen detected in more than 100 amphibian species in Brazil (Valencia-Aguilar et al. 2015). However, not all species that are infected are susceptible to the disease (Searle et al. 2011, Swei et al. 2011, Rodriguez et al. 2014), and resistant amphibians are capable of transporting the fungus to other areas, acting as reservoir hosts in the transmission of *Bd* (Johnson & Speare 2005).

In Brazil, the complete geographic distribution of *Bd* is unknown (Carvalho et al. 2017). The largest incidence of *Bd* was recorded in the south and southeast regions of Brazil, especially in the Atlantic Forest biome (Carnaval et al. 2006, Toledo et al. 2006, Vieira et al. 2012, Lisboa et al. 2013, Valencia-Aguilar et al. 2015). In this biome, 15 species are considered Threatened, and populations of another 24 species have been reduced due to *Bd* infection (Carvalho et al. 2017, IUCN 2018, O'Hanlon et al. 2018). There are also records of *Bd* in the Amazon (Becker et al. 2016, Carvalho et al. 2017), Cerrado (Ramalho et al. 2013, Carvalho et al. 2017), Caatinga and Pampa (Carvalho et al. 2017) regions, but in lesser numbers.

The record in the Cerrado biome reinforces the hypothesis that *Bd* is not only restricted to humid environments. In northeastern Brazil, records are limited to the states of Pernambuco (Carnaval et al. 2006), Alagoas (Lisboa et al. 2013), Bahia (Valencia-Aguilar et al. 2015) and Piauí (Carvalho et al. 2017), generally occurring in anuran populations living in high-altitude forests (Berger et al. 1998, Ron 2005, Lips et al. 2006, Gründler et al. 2012).

The state of Bahia has a territory of 567 295 km^2, and contains remnants of Atlantic Forest and Cerrado biomes, as well as a large area of the Caatinga biome (Ab'Sáber 2003, IBGE 2013). Despite 3 records of *Bd* in Bahia (Valencia-Aguilar et al. 2015, Carvalho et al. 2017), information concerning its distribution in this state is still unknown.

To diagnose the occurrence of *Bd*, a number of methods may be used, including the routine histological examination of the skin of specimens preserved in formalin or ethanol (Berger et al. 1999, Hyatt et al. 2007). Using this technique, *Bd* has already been found in amphibian species in Australia, the USA, Panama and Ecuador (Nichols et al. 1998, Berger et al. 1999, Carey et al. 1999, Burrowes et al. 2004). In Australia, this method has been used to map chytrid distribution and to determine its focal geographical occurrence (Berger et al. 1999). The present study aimed to investigate the occurrence of *Bd* in anuran species from 3 biomes (Atlantic Forest, Cerrado and Caatinga) with different phytophysiognomies and climatic conditions, in the state of Bahia, Brazil, based on the assumption that *Bd* can occur in areas with different climatic conditions.

2. MATERIALS AND METHODS

We analyzed 190 specimens from 85 anuran species collected from municipalities in the Atlantic Forest, Caatinga and Cerrado biomes (MMA 2007). We used 2 criteria to choose the specimens: (1) representativeness in the biome and (2) representativeness in the collection. Among the specimens selected for analysis, we included some with signs of chytridiomycosis suggestive of *Bd* infection (e.g. loss of pigmentation or white marks on the skin).

All selected specimens had originally been fixed in 10% formalin, preserved in 70% ethanol and stored in the Amphibian and Reptile Division of the Zoological Museum of the State University of Feira de Santana and the Amphibian Collection of the Natural History Museum of the Federal University of Bahia, both in the state of Bahia, Brazil. We obtained information concerning biomes, average temperatures and altitudes of the Brazilian municipalities in which the *Bd* fungus was recorded from the Brazilian Institute of Geography and Statistics (IBGE 2013).

We collected skin samples of approximately 1 cm^2 from the pelvic region of the selected individuals (using pincers and surgical scissors). These samples were processed using standard histological protocols (Berger et al. 1999), adapted by the pathological Veterinary Sector of the Veterinary Hospital of the University of the Recôncavo of Bahia, where the histological analyses were conducted. To dehydrate these tissue samples, we used a sequence of ethanol solutions of 70, 80, 90, 95 and 100%, for a period of 20 min in each solution. In the next step, the samples were washed in 2 xylol solutions for 50 min each, followed by washing for another 2 h in 2 paraffin solutions, 1 h for each. All procedures took place in an oven at 60°C.

Samples were embedded in liquid paraffin that was polymerized at room temperature as soon as the tissue was introduced. We made 5 µm wide sections in all processed tissues and then stained the samples with hematoxylin and eosin (H&E). This coloring technique is used to document the general tissue morphological aspects and to identify signs of possible infection. To confirm diagnosis, samples that showed signs of infection underwent a second staining using the Grocott-Gomori methenamine silver nitrate procedure, widely used for the detection of fungal cell walls, as it permits a better visualization for staining glycogen and mucin, polysaccharides that are components of the basement membrane of the fungus (Junqueira & Junqueira 1983). We did not conduct the second coloring method in those cases in which a positive diagnosis was discarded after the first procedure. Such histological procedures are routinely used to diagnose *Bd* (e.g. Nichols et al. 1998, Berger et al. 1999, Burrowes et al. 2004).

3. RESULTS

We detected spores of *Batrachochytrium dendrobatidis* in anuran skin from all sampled biomes. We identified the fungus in samples from 13 municipalities distributed throughout the Atlantic Forest (8 species), Caatinga (5 species) and Cerrado (1 species) biomes, suggesting that *Bd* is widely distributed throughout the state of Bahia, Brazil (Fig. 1).

We found the infection in 16 individuals (13 adults and 3 tadpoles), belonging to 14 species and 6 families (Table 1; also see Table S1 in the Supplement at www.int-res.com/articles/suppl/d136p147_supp.pdf). Positive cases corresponded to approximately 8% of the total sample (190 specimens). The oldest sample containing *Bd* obtained from the examined collections dates back to 1996 and belongs to a specimen of *Physalaemus albifrons* from the municipality of Barra; the most recent infected sample dates from the year 2014, found in a tadpole of *Bokermannohyla* sp. collected in a stream in the municipality of Piatã. Both samples were obtained in the Caatinga biome (Table 1).

Among infected individuals in the Caatinga biome, we detected *Bd* in specimens from the families Hylidae, Leptodactylidae and Bufonidae (Table 1). In this study, we recorded the fungus for the first time in the Cerrado biome of Bahia (Barreiras municipality), in *Leptodactylus* cf. *chaquensis* (Leptodactylidae). Furthermore, the study extended records of *Bd* in the Atlantic Forest of Bahia to include the municipalities of Camamu, Ibirapitanga, Igrapiúna, Ituberá and Salvador. As such, these findings extended the occurence of *Bd* in Bahia approximately 400 km to the west, 150 km to the north (both from the pre-existing record in the Lençóis municipality, in the center of the state), and 105 km to the east (from the pre-existing record in the municipality of Itacaré, on the south coast from Bahia Atlantic Forest) (Fig. 1).

Of the 13 adult anuran specimens diagnosed with *Bd*, 1 specimen of *B. capra* and 1 specimen of *L.* cf. *chaquensis* presented signs of chytridiomycosis, such as loss of skin pigmentation (Fig. 2). Zoosporangia (spore cases) were recorded in 3 different phases of development (Fig. 3): (1) initial stage, in which a central spherical basophilic mass is observed; (2) second stage, in which zoospores, generally 4–10 rounded structures, are observed; and (3) third stage, in which the zoospores are rounded, colorless and/or with internal septation. The colors of the walls of these structures were clearly altered by the staining method used (Grocott-Gomori methenamine silver nitrate procedure) (Fig. 3E,F).

In tadpoles, we recorded the fungal structures indicative of *Bd* in the oral region. The zoospores were observed in greater density in the epidermis close to the tadpole tooth-line, where the histological examination revealed more abnormalities, such as deformities caused by hyperkeratosis and loss of pigmentation.

4. DISCUSSION

This study expands the distribution of *Bd* in the state of Bahia, Brazil, with records for all biomes represented in the state, including the Cerrado, in which the fungus had not previously been registered for the state. The results of this study corroborate the prediction that *Bd* is widespread in the Atlantic Forest (Car-

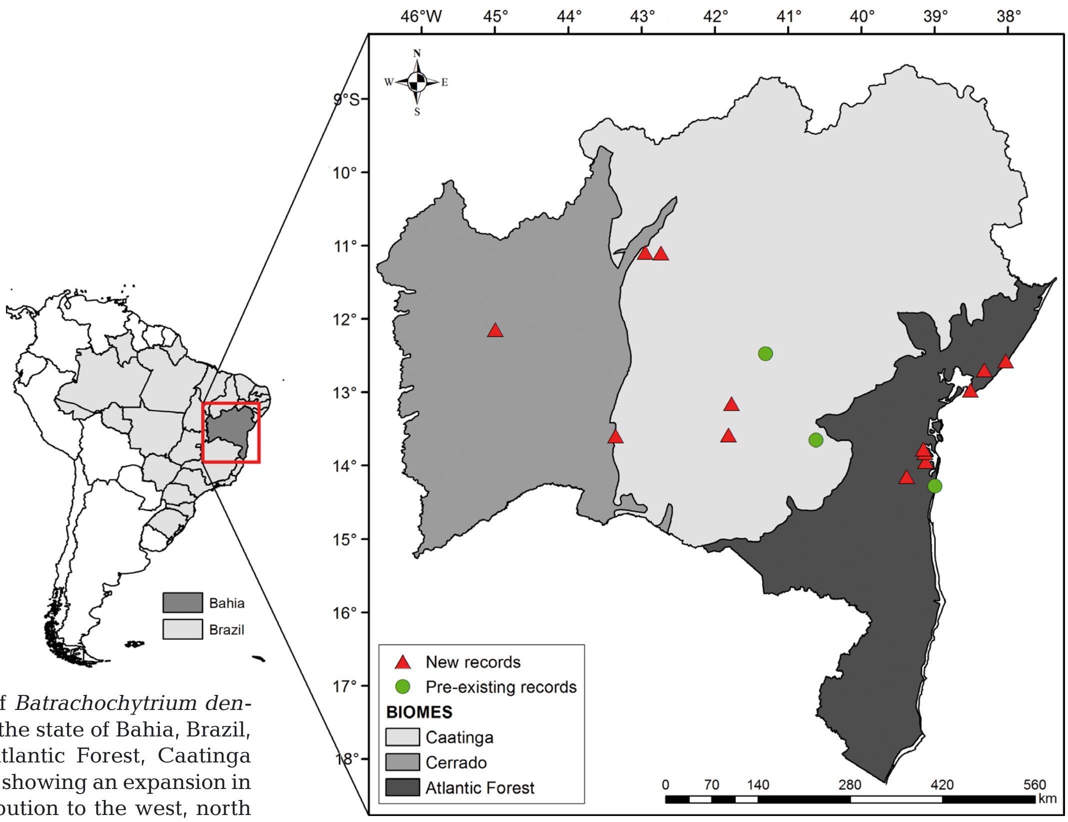

Fig. 1. Distribution of *Batrachochytrium dendrobatidis* fungus in the state of Bahia, Brazil, distributed in the Atlantic Forest, Caatinga and Cerrado biomes, showing an expansion in its geographic distribution to the west, north and east of pre-existing records

naval et al. 2006, Toledo et al. 2006, Lisboa et al. 2013, Valencia-Aguilar et al. 2015) and confirm the inferences predicted by models of *Bd* geographic distribution proposed by Ron (2005), which includes the 3 biomes in the state of Bahia.

The majority of the infected anuran species belong to the Hylidae family, which consists of species that use mostly vertical strata as habitat (principally bushes and herbaceous plants) near bodies of water (streams, dams and pools) (Bertoluci & Rodrigues 2002). We highlight that results based on a histological diagnosis may be susceptible to underestimation, due to the age of the samples, their preservation in alcohol and the nature of the fungus, which infects the epidermal surface (Berger et al. 1999, Puschendorf & Bolaños 2006). Nonetheless, the results presented in this study are similar to those found in Costa Rica (Lips et al. 2003), South Africa (Lane et al. 2003) and in the Brazilian Atlantic Forest (Carnaval et al. 2006), all of which used histological methods for diagnosing the presence of *Bd.*

The first *Bd* record in South America dates from 1894 in Brazil (Rodriguez et al. 2014), where studies have been developed on the historical declines associated with the occurrence of different *Bd* strains (Jenkinson et al. 2016, Carvalho et al. 2017). In North America, the first record of *Bd* occurred in Canada in 1960 (Ouellet et al. 2005), and in South Africa in 1930 in a species of the genus *Xenopus* (Rachowicz et al. 2005).

Our results evidenced that *Bd* has been present in the Caatinga biome since at least the 1990s, as registered in a specimen of *Physalaemus albifrons* (Leptodactylidae) in the municipality of Barra, in the Ecoregion of the São Francisco River Dunes. Based on these new records for the state of Bahia, we corroborate the hypothesis that the fungus has been disseminated for decades, not only in the Atlantic Forest, but also in other biomes, such as the Caatinga and the Cerrado. It is possible that the fungus has occurred in the state since long before, since our oldest samples are limited to the 1990s (13 samples, 6 earlier than the earliest in 1996); however, previous studies have recorded the fungus in more recent specimens collected in the last decade (Lisboa et al. 2013, Valencia-Aguilar et al. 2015, Carvalho et al. 2017).

The widespread occurrence of *Bd* in the Atlantic Forest presents a challenge for the conservation of threatened anurofauna (Myers et al. 2000, Galindo-Leal & Câmara 2005, Silvano & Segalla 2005, ICMBIO 2014), as wet and cool environments are optimal

Table 1. Anuran species infected by *Batrachochytrium dendrobatidis*, together with corresponding region, vegetation type, collection period, life stage and collection number. Vegetation types follow Veloso et al. (1991). Ecoregions for the Caatinga biome follow Velloso et al. (2002). UFBA: Natural History Museum of the Federal University of Bahia; MZFS: Zoological Museum of the State University of Feira de Santana

Family/species	Municipality (locality)	Vegetation type	Annual average air temp (°C)	Altitude (m)	Biome (ecoregion)	Year collected	Life stage	Collection number
Craugastoridae								
Pristimantis paulodutrai (3) (Bokermann, 1975)	Mata de São João (Reserva Sapiranga) (1) / Salvador (Ondina) (2)	Dense ombrophilous forest	29/28	31/8	Atlantic Forest	2005/2004	Adult	UFBA 5635, 2158, 2179
Bufonidae								
Rhinella rubescens (Lutz, 1925)	Rio de Contas (Serra da Mesa)	Seasonal deciduous forest	25	1050	Caatinga (Chapada Diamantina)	2002	Adult	MZFS 1151
Hemiphractidae								
Gastrotheca megacephala Izecksohn, Carvalho-e-Silva & Peixoto, 2009	Camamu	Dense ombrophilous forest	28	23	Atlantic Forest	2005	Adult	MZFS 2094
Hylidae								
Aplastodiscus ibirapitanga (Cruz, Pimenta & Silvano, 2003)	Ibirapitanga	Dense ombrophilous forest	23	113	Atlantic Forest	2010	Tadpole	MZFS 1029
Aplastodiscus sibilatus (Cruz, Pimenta & Silvano, 2003)	Igrapiúna	Dense ombrophilous forest	28	22	Atlantic Forest	2009	Tadpole	MZFS 772
Bokermannohyla sp.	Piatã	Steppe	24	1280	Caatinga (Chapada Diamantina)	2014	Tadpole	MZFS 1290
Bokermannohyla capra Napoli & Pimenta, 2009	Igrapiúna (Reserva Ecológica Michelin)	Dense ombrophilous forest	27	22	Atlantic Forest	2008	Adult	MZFS 3180
Corythomantis greeningi Boulenger, 1896	Serra do Ramalho	Seasonal deciduous forest	33	438	Caatinga	2007	Adult	MZFS 3022
Scinax eurydice (Bokermann, 1968)	Mata de São João (Reserva Sapiranga)	Dense ombrophilous forest	29	31	Atlantic Forest	2004	Adult	UFBA 4073
Leptodactylidae								
Leptodactylus cf. *chaquensis*	Barreiras	Seasonal deciduous forest	32	454	Cerrado	2009	Adult	UFBA 12908
Physalaemus albifrons (Spix, 1824)	Barra (Ibiraba)	Steppe	33	406	Caatinga (São Francisco Dunes)	1996	Adult	UFBA 1562
Physalaemus cuvieri Fitzinger, 1826	Camaçari	Dense ombrophilous forest	28	36	Atlantic Forest	2014	Adult	MZFS 4605
Pleurodema diplolister (Peters, 1870)	Gentio do Ouro	Steppe	25	687	Caatinga	2000	Adult	MZFS 1556
Phyllomedusidae								
Phyllomedusa bahiana Lutz, 1925	Mata de São João	Dense ombrophilous forest	29	31	Atlantic Forest	2009	Adult	UFBA 9777

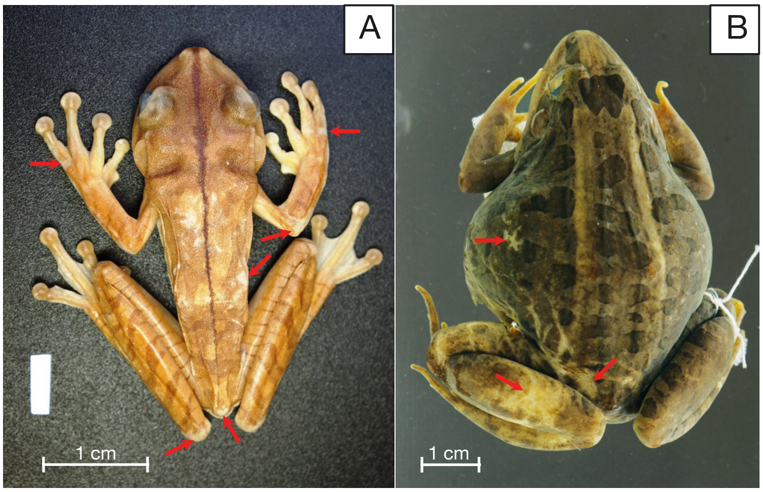

Fig. 2. Species infected by *Batrachochytrium dendrobatidis* with suggestive macroscopic signs of chytridiomycosis (white marks indicated by arrows) on dorsal skin and limbs. (A) *Bokermannohyla capra* (MZFS 3180), collected in 2008 from Igrapiuna municipality, state of Bahia, Brazil; photo by Ednei Mercês. (B) *Leptodactylus* cf. *chaquensis* (UFBA 12908), collected in 2009 from Barreiras municipality, state of Bahia, Brazil; photos by Carina G. M. Santos

for the survival of *Bd* zoospores, for periods of several months (Johnson & Speare 2003). On the other hand, hot and dry environments are less suitable for the survival of spores, as the dissemination of *Bd* zoospores is dependent on water, wet or damp material or direct skin contact with infected amphibians (Johnson & Speare 2005, Carnaval et al. 2006, Pessier & Mendelson 2010, Phillott et al. 2010). Despite this, we recorded *Bd* in the Caatinga and Cerrado biomes, whose annual average temperature and rainfall are 25°C and 1.175 mm.

In the Caatinga biome, we recorded *Bd* in 2 municipalities (Piatã and Rio de Contas) that belong to the region of the Chapada Diamantina mountain complex, with an average altitude of more than 1000 m (Velloso et al. 2002). This region includes humid enclaves within the Caatinga biome (Veloso et al. 1991). It is worth noting that it is a vast region, >1520 km^2, where many endemic anuran species can be found, including the genus *Bokermannohyla* (Leite et al. 2008, Magalhães et al. 2016), in which this study recorded the *Bd* fungus in a tadpole.

However, we also recorded *Bd* in an area of lower altitude (between 400 and 600 m a.s.l.) in the Caatinga biome, in the Serra do Ramalho municipality, in the Ecoregion of the São Francisco River Dunes (Ibiraba, municipality of Barra) (Velloso et al. 2002), and in the municipality of Barreiras, in the Cerrado biome, whose temperatures are high throughout the year (minimum 19°C and maximum 35°C). This record demonstrates the plasticity of the fungus when faced with climatic conditions at the limit of its survival threshold, considered to be between 16 and 26°C (Gründler et al. 2012).

Despite the infected anuran species found in this study having different reproductive strategies, the majority (63%) spend at least part of their development in bodies of water. In addition, with the exception of *Phyllomedusa bahiana*, the adults enter into the water to vocalize, lay eggs and escape from predators (Duellman & Trueb 1994). It is in this aquatic environment that the fungal spores may remain active for weeks. However, species that use habitats such leaf cover (*Pristimantis paulodutrai*) and scrub and tree canopy (*Phyllomedusa bahiana* and *Gastrotheca megacephala*, respectively), which are non-aquatic environments, were also infected by *Bd*. This result could be explained by the humidity that exists in these microhabitats within the Atlantic Forest, sustaining the presence of fungal zoospores (Johnson & Speare 2003).

Among the infected species, some are from xeric and sub-humid areas, such as *Corythomantis greeningi*, which occurs widely in the dry regions of the Caatinga and subhumid regions of northeastern Brazil and northern Minas Gerais; *Rhinella rubescens*, mainly found in the core region of the Cerrado biome; *Pleurodema diplolister*, a typical species from the Caatinga and Cerrado biomes, but also inhabiting restinga environments on the northern coast of Bahia; and *Physalaemus albifrons*, an inhabitant of open areas in the Cerrado and Caatinga biomes, but also observed in dune and restinga environments located on the northern coast of Bahia (IUCN 2018). All of these species are widely distributed within these environments.

These species inhabit areas that were previously considered unsuitable for *Bd* (Berger et al. 2004, Gründler et al. 2012, IUCN 2018). Understanding how environmental, genetic and ecological factors interact with chytridiomycosis is fundamental given that the pathogen responsible for this disease has been recorded in several anuran communities in Brazil (Carnaval et al. 2006, Toledo et al. 2006, Lisboa et al. 2013, Ramalho et al. 2013, Valencia-Aguilar et al. 2015, Becker et al. 2016). Furthermore, conservation efforts should involve long-term studies, with the objective of providing information about the dynamics of *Bd* infection, the interaction between *Bd* and its hosts and its effect on amphibian populations.

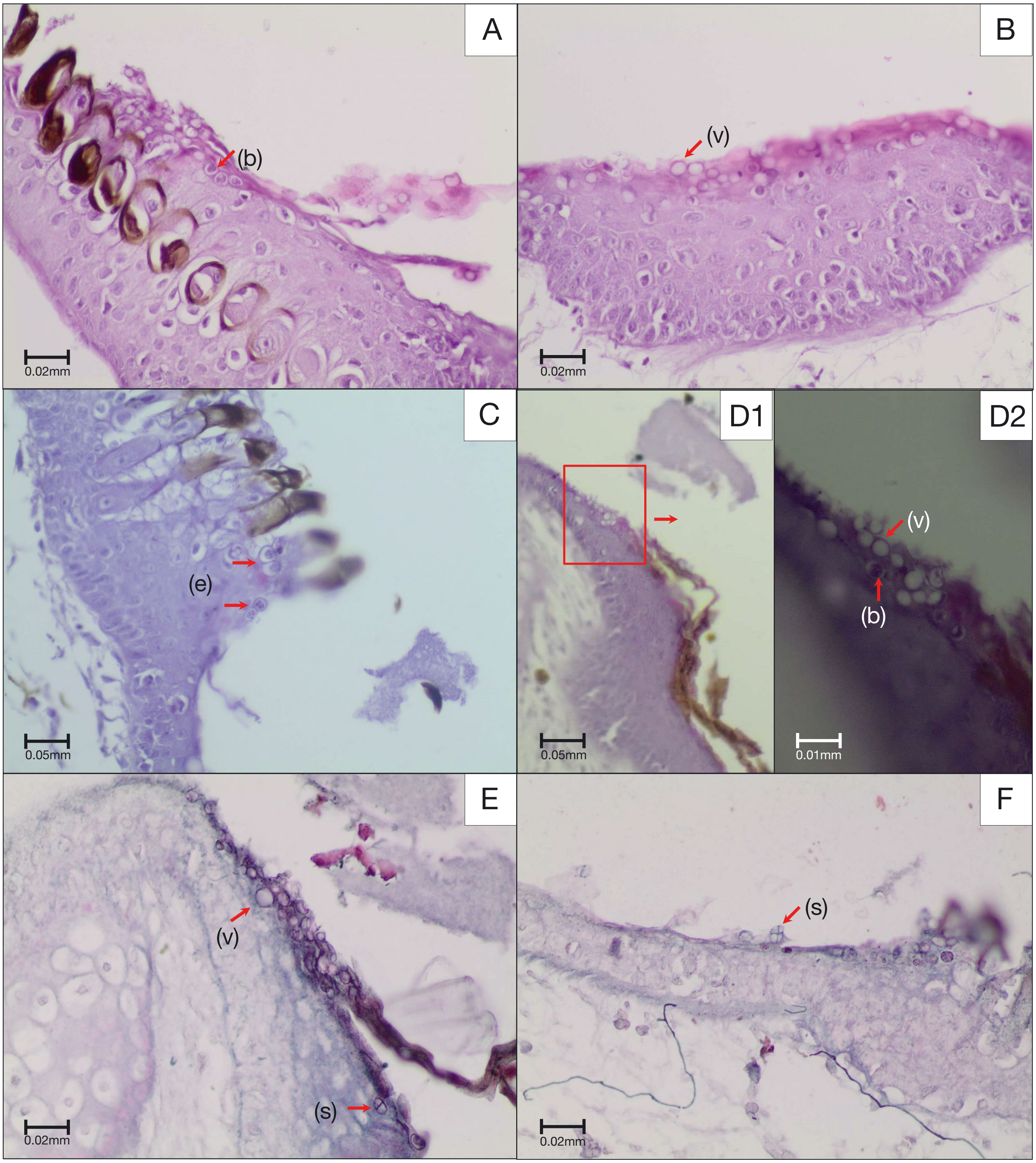

Fig. 3. Skin sections from the oral disc of 2 tadpoles collected in the south of Bahia state, Brazil, infected by *Batrachochytrium dendrobatidis*, showing the different stages of the fungus. (A,B) Transversal sections from the oral disc of an *Aplastodiscus ibirapitanga* (MZFS 1029) tadpole from the Ibirapitanga municipality, showing initial stage with basophilic mass (b) and empty sporangia (v), zoospores already ejected. (C–F) Transversal sections from the oral disc of an *A. sibilatus* (MZFS 772) tadpole from the Igrapiuna municipality, showing a keratinized superficial epidermal layer (corneal stratum) containing numerous *B. dendrobatidis* sporangia ovals (e), indicated by the arrow in panel C. (D1) Section overview; (D2) detail from D1, showing empty sporangia (v) and others containing basophilic structures in their interior (b). (A–D) stained by hematoxylin and eosin. (E,F) Details of septate sporangia (s) and empty sporangia (v), stained with Grocott methenamine silver nitrate (a specific dye for fungi)

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