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**BROMELIGENOUS ANURAN SPECIES IN THE NORTHERN ATLANTIC FOREST:
bromeliad selection and assessment of *Batrachochytrium dendrobatidis* infection**

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Dissertação apresentada ao Programa de Pós-Graduação em Diversidade Biológica e Conservação nos Trópicos, Instituto de Ciências Biológicas e da Saúde da Universidade Federal de Alagoas, como requisito para obtenção do título de Mestre em CIÊNCIAS BIOLÓGICAS, área de concentração em Diversidade e ecologia de organismos tropicais

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***“To my mother Thelma Fajardo
Aguilar, my brother Pablo Ruano and
family.”***

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ABSTRACT

The chytrid fungus, *Batrachochytrium dendrobatidis* (hereafter *Bd*), is a pathogen that infects amphibians and may cause amphibian mass die-offs and populations declines in some regions. This disease was described from dying anurans from Australia and Panama in 1999. For almost a decade, the chytrid was thought to be genetically homogenous with a virulent strain (*Bd*-GPL) distributed worldwide. However, recent discoveries of chytrid ancient lineages, endemism, constant prevalence, oldest records and apparently non-declining populations suggest that the Atlantic forest might be the local of its origin. High diversity and endemism of many taxa occur in the Atlantic forest, and both amphibian and bromeliad diversity are not an exception. Tank bromeliads seem to be an ideal microhabitat for the chytrid fungus: many anuran species are bromeliad-dependent to complete their life cycle and bromeliad water reservoirs may help chytrid fungus to endure environmental constraints. A qPCR assay for detecting *Bd* in anurans was used to quantify the presence and infection loads of *Bd*. Specifically, three bromeligenous anurans in the Estação Ecológica de Murici, Alagoas State, Brazil were tested for *Bd*. Furthermore, bromeliad characteristics (phytotelm water temperature and pH, canopy closure, phytotelm diameter, leaf number and size, bromeliad maximum column depth to store water, phytotelm relative water volume) were measured and associated with *Phyllodytes edelmoi* occurrence. We performed a Logistic Generalized Linear Model (GLM) approach to analyze bromeliad variables selected by *P. edelmoi* adults, presence of tadpoles and the chytrid presence. Current knowledge on *B. dendrobatidis* in north Atlantic forest was added. Specifically, three bromeligenous species (*Phyllodytes acuminatus*, *P. edelmoi*, and *P. gyrinaethes*) tested positive for *Bd*. High prevalence on two of those species (*P. edelmoi* 31.8 % n=22; *P. gyrinaethes* 20.8 % n=24) were detected. Nevertheless, relative low intensity of zoospores infection was found. *Phyllodytes edelmoi* individuals selected smaller diameter bromeliads with more leaves and relative more volume of water. The chytrid presence was more frequent on individuals that inhabit smaller bromeliads with maximum capacity to store water. Bromeliad traits chosen by this bromeligenous frog showed that *P. edelmoi* optimum microhabitat may be related to a higher probability of presence of the chytrid fungus due to the higher number of individuals that prefer this trait.

Keywords: Brazil, Chytrid, ESEC de Murici, Pathogen, *Phyllodytes*, Phytotelmata.

RESUMO

O fungo quitrídeo (*Batrachochytrium dendrobatidis*) é um patógeno que infecta anfíbios e pode provocar mortalidade em massa e declínio de populações de anfíbios em algumas regiões. Essa doença foi descoberta em anuros moribundos da Austrália e Panamá em 1999. Por quase uma década, pensou-se que o quitrídeo era representado por uma linhagem virulenta geneticamente homogênea (*Bd-GPL*) no mundo todo. No entanto, descobertas recentes de linhagens antigas do fungo, endemismo, prevalência constante, registros mais antigos e, aparentemente, o não declínio de populações de anfíbios da Mata Atlântica sugerem que este seja o local de origem deste quitrídeo. Alta diversidade e endemismo de diversos táxons é característico do *hotspot* Mata Atlântica, e a diversidade de anfíbios e bromélias não são exceções. Bromélias com tanque parecem ser um microhabitat ideal para o fungo: muitas espécies de anuros dependem de bromélias para completar seu ciclo de vida e, o reservatório de água dessas plantas pode ajudar o fungo quitrídeo a resistir restrições ambientais. Um ensaio qPCR para detecção de *B. dendrobatidis* em anuros foi usado para avaliar a presença e quantificar a intensidade de zoósporos. Especificamente, as amostragens foram feitas em três espécies de anuros bromelígenas na Estação Ecológica de Murici, estado de Alagoas, Brasil. Além disso, as características das bromélias (temperatura e pH da água do fitotelmo, cobertura do dossel, diâmetro do fitotelmo, número e tamanho das folhas, profundidade máxima do reservatório de água, volume de água relativo da bromélia) foram medidas e associadas com a presença ou ausência da espécie bromelígena *Phyllodytes edelmoi*. Modelos lineares logísticos generalizados (GLM) foram utilizados para analisar as variáveis das bromélias que são selecionadas pelos indivíduos adultos de *P. edelmoi*, presença de girinos e, a conseqüente, presença do fungo quitrídeo. Informações ao conhecimento atual de *Batrachochytrium dendrobatidis* no norte da Mata Atlântica é acrescentado. O quitrídeo foi encontrado nas três espécies bromelígenas registradas nesse estudo (*Phyllodytes acuminatus*, *P. edelmoi*, e *P. gyrinaethes*). Altas prevalências foram detectadas para duas espécies (*P. edelmoi* 31,8% n = 22; *P. gyrinaethes* 20,8% n = 24). No entanto, a intensidade da infecção zoospórica encontrada foi relativamente baixa. *Phyllodytes edelmoi* seleciona bromélias com diâmetros menores, com mais folhas e maior volume relativo de água. A presença do quitrídeo foi mais frequente em indivíduos que habitam bromélias menores com a capacidade máxima de armazenar água. Características das bromélias escolhidas por esta espécie bromelígena revelou que o seu microhabitat ótimo pode estar relacionado com uma maior probabilidade da presença do fungo devido ao maior número de indivíduos que preferem essa característica..

Palavras-chave: Brasil, quitrídeo, ESEC de Murici, patógeno, *Phyllodytes*, Fitotelmata

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1 INTRODUCTION

In the last 30 years, amphibian populations have been declining worldwide (LIPS et al., 2008; STUART et al., 2008; WAKE; VREDENBURG, 2008). Declines have been associated with several factors such as deforestation, environmental pollution, climate change, introduction of exotic species, diseases and a complex synergy among these forces in a geographic context (BLAUSTEIN et al., 2011; HOF et al., 2011). However, the most enigmatic declines in pristine forests are linked to the presence of the fungal pathogen *Batrachochytrium dendrobatidis* (hereafter *Bd*), which often leads to chytridiomycosis (STUART et al., 2008). *Bd* is a chytrid fungus (Class Chytridiomycetes: Order Chytridiales) that infects keratinized epidermal cells of adult amphibians and keratinized mouthparts (jaw sheaths and teeth) of tadpoles (BERGER et al., 1998). Specifically, adult amphibians with high zoospore infection may die by cardiac arrest due to attenuated Na⁺ absorption through the skin (CAMPBELL et al., 2012; VOYLES et al., 2009). In contrast, tadpoles' mortality is scarce (KILPATRICK; BRIGGS; DASZAK, 2010), but *Bd* can affect their capability of food intake, foraging efficiency, behavior, growth and development (HAN; BRADLEY; BLAUSTEIN, 2008; VENESKY; WASSERSUG; PARRIS, 2010). This disease was described in 1999 in amphibians from Australia, Panama and North America (LONGCORE; PESSIER; NICHOLS, 1999), and has been detected in all continents except Antarctica (FISHER; GARNER; WALKER, 2009). Moreover, the chytrid fungus has been detected in ca. 500 amphibian species around the world (OLSON et al., 2013). Despite of been recently discovered, *Bd* earliest record is 1894 in anurans from Rio Grande do Sul State, Brazil (RODRIGUEZ et al., 2014).

In Brazil, many anurans have shown *Bd* infection (ca. 130 species) and these records are mainly from the southern region of the Atlantic forest (CARNAVAL et al., 2006; GRÜNDLER et al., 2012; LISBOA et al., 2013; RODRIGUEZ et al., 2014; TOLEDO, 2006; VALENCIA-AGUILAR et al. *in press*; VIEIRA et al., 2013). Furthermore, high diversity and long term endemism of *Bd* genotypes have been found in the southern Atlantic forest (RODRIGUEZ et al., 2014; ROSENBLUM et al., 2013; SCHLOEGEL et al., 2012). However, chytridiomycosis outbreaks have not been directly attributed so far to amphibian populations declines in the Atlantic forest (HADDAD et al.

2013). Conversely, enigmatic declines occurred in the late 70's in Brazil with similar characteristics as the mass mortality event reported in Costa Rica (LIPS, 1998). This finding raises concern in how temporal dynamics and impacts of *Bd* on Brazilian amphibian populations with a long history of co-occurrence fit in the puzzle (LIPS, 2014). Until now, the northern region of the Atlantic forest has not been sampled throughout regarding *Bd* presence and the *Bd* genotypic strain is unknown. This distributional gap is ca. 1,700 km ranging from Alagoas State (LISBOA *et al.*, 2013) to Minas Gerais State (RODRIGUEZ *et al.*, 2014).

Batrachochytrium dendrobatidis follows a dynamic disease progression with epizootic and enzootic stages. For instance, during an epizootic stage, *Bd* affects naïve amphibian populations and may behave as a killing wave that can reduce 50% of host species richness and produce mass population declines (e.g. BREM; LIPS, 2008). On the other hand, *Bd* enzootic stage may have a constant prevalence and reduce amphibian mortality (e.g. BATAILLE *et al.*, 2013; RODRIGUEZ *et al.*, 2014). Recent studies reinforce the need to include open areas, which are less favorable sites for *Bd*, and enzootic sites (FLECHAS; SARMIENTO; AMÉZQUITA, 2012) as the Atlantic forest of Brazil. In addition, the inclusion of these sites can help understand how environment–host dynamics at different scales have been shaped in a long term interaction (LIPS, 2014; LONGO *et al.*, 2013). Moreover, the recognition of factors that influence *Bd* and amphibians at optimal and suboptimal fine scale microhabitats are needed to understand the dynamic of *Bd* within a habitat. For instance, some amphibians' natural history, or behavioral traits in specific microhabitats as bromeliad tanks can help to model such dynamics.

Bromeliads have been considered as perfect reservoirs for *Bd* across Neotropical regions (MCCRACKEN *et al.*, 2009; RODRIGUEZ *et al.*, 2014; STUCKERT *et al.*, 2009). For example, lowland rainforests in Ecuador have shown a high prevalence of *Bd* in bromeliads (MCCRACKEN *et al.*, 2009) and Atlantic forest anurans that use phytotelmata have been found with relatively high *Bd* prevalence (RODRIGUEZ *et al.*, 2014). Although bromeliads are diverse and abundant in the Atlantic forest and many anurans complete their lifecycle within this microhabitat (HADDAD; PRADO, 2005; PEIXOTO,

1995), bromeligenous species in the Atlantic forest have not been specifically surveyed for *Bd*.

Here we screened bromeligenous anurans in the northern Atlantic forest for *Bd*. The genus *Phyllodytes*, a Neotropical endemic, was targeted as model sample. Specifically, *P. acuminatus* Bokermann, 1966, *P. edelmoi* Peixoto, Caramaschi & Freire, 2003 and *P. gyrinaethes* Peixoto, Caramaschi & Freire, 2003 that are endemic to the Northern Atlantic forest (PEIXOTO; CARAMASCHI; FREIRE, 2003) were chosen. These species are considered as Data Deficient in IUCN red List (IUCN, 2014) and information on ecology and *Bd* risk assessment are needed to guide conservation efforts. In addition, the assessment of these bromeligenous anurans for *Bd* may shed light on the dynamic of this disease. Therefore, we proposed: 1) to assess the presence and quantify intensity of *Bd* infection in *Phyllodytes* species in Estação Ecológica de Murici, Alagoas state, Brazil 2) to determine whether there are bromeliad characteristics with *P. edelmoi* occurrence which favor *Bd* presence 3) to bring baseline data on the bromeliad selection of this poorly known anuran species.

2 LITERATURE REVIEW

2.1 Amphibian declines

In the past 30 years, the alarming population decline of amphibians worldwide has been reported and discussed (BLAUSTEIN *et al.*, 2011; HOF *et al.*, 2011). Wake & Vredenburg (2008) argue that biological diversity is entering in a new period of mass extinction that could be listed as the sixth mass extinction on earth's history. This crisis is uneven among vertebrates, amphibians are the most impacted group whereas 42% of ca. 7,000 species worldwide have been declining (FROST, 2014; STUART *et al.*, 2008). Indeed, amphibian declines are complex to understand because they differ among geographic locations, species, populations, life stages and are dependent on many factors which may interact together with natural stress (BLAUSTEIN *et al.*, 2011).

Amphibian declines are not a single-cause phenomenon, they rather exist as a synergetic interaction among factors provoking different scenarios (HOF *et al.*, 2011). However, the most significant factor is habitat destruction (BLAUSTEIN *et al.*, 2011). Specifically, deforestation exposes amphibians to strong changes in microclimatic regimes, homogenizes habitat complexity and splits populations apart (HUSSAIN; PANDIT, 2012). For instance, negative effects of habitat split have been associated with loss of anuran richness with aquatic larvae on the Atlantic forest (BECKER *et al.* 2007). Another cause is related to exposure to a variety of contaminants (pesticides, herbicides, fungicides, fertilizers, etc.). Certainly, contaminants can cause changes in locomotion activity, reproduction, feeding and growth (BLAUSTEIN *et al.*, 2011; HAYES *et al.*, 2010b). For example, atrazine exposure can induce complete feminization and chemical castration in *Xenopus laevis* males (HAYES *et al.*, 2010a). Also climate change has been pointed out for affecting survival, growth, reproduction and dispersal capabilities of anurans as well as altering vegetation, soil, and hydrology (BLAUSTEIN *et al.*, 2010), causing unsuitable habitats for amphibian population viability. Altogether, these forces can contribute to amphibian declines but also may influence amphibian susceptibility to disease by altering host-community structure, transmission pathways, pathogen persistence and virulence (PATZ *et al.*, 2000). For instance, diseases such as

ranavirus and chytridiomycosis are linked to enigmatic amphibian declines (HOF *et al.*, 2011; LESBARRÈRES *et al.*, 2012) and are part of these complex phenomena as well.

Batrachochytrium dendrobatidis is the fungal pathogen that may cause chytridiomycosis. This emergent disease was directly linked to mass die-offs of amphibian populations in cloud forests of Australia and Panama (BERGER *et al.*, 1998; LIPS *et al.*, 2008). Furthermore, it was also associated with the decline of frog populations in South America, North America, Central America, New Zealand, Europe and Africa (BERGER *et al.*, 1998; FISHER; GARNER; WALKER, 2009; LIPS *et al.*, 2008). Certainly, declines were most unexplainable in the tropics because they were limited to forest areas with no apparent habitat degradation (BERGER *et al.*, 1998; LIPS, 1998; LIPS *et al.*, 2008). In several of these areas, *Bd* was found in adults and declines occur abruptly in a few months (BERGER *et al.*, 1998). For instance, Gillespie *et al.* (2014) documented real-time arrival of *Bd* and the decline of an amphibian population in Australia showing lethal outbreaks of the disease. On the contrary, apparently enigmatic declines are not always explained by *Bd*. For example, a population of poison frog *Atelopus spumarius* in Ecuador showed decline that apparently could be explain by *Bd* (TARVIN; PEÑA; RON, 2014), due to the high susceptibility of this genus (STUART *et al.*, 2008). However, this decline was explained by an event of selective logging (TARVIN; PEÑA; RON, 2014).

Amphibian population extinctions and declines by emergent diseases are a major concern for scientist and conservationist worldwide due to its complex causes (RÖDDER *et al.*, 2009; SCHEELE *et al.*, 2014). Assessments for threats on amphibian diversity suggest that most affected amphibians are aquatic species (STUART *et al.*, 2008) and those species inhabiting mountainous areas restricted to medium and high altitudes. Furthermore, pristine tropical forests have higher disease risk than habitats characterized by habitat loss (BECKER; ZAMUDIO, 2011). This suggests a synergistic interaction between the infectious agent and environmental conditions, which varies with elevation and usually affects more species associated with riparian systems than to terrestrial habitats (FISHER; GARNER; WALKER, 2009; KILPATRICK; BRIGGS; DASZAK, 2010). Among families, decline patterns arise in a phylogenetic perspective. For example, Bufonidae has shown by far the highest percentage of species in serious

decline, specifically in the genus *Atelopus* (ca. 78% of 145 species). Also there are many species affected in Hylidae and Leptodactylidae (STUART et al., 2008).

2.1.1 *Batrachochytrium dendrobatidis*: Description and Pathology

Also known as the chytrid fungus, *Batrachochytrium dendrobatidis* (Chytridiomycota; Chytridiales) is the first pathogen of the order Chytridiales to be reported in vertebrates (LONGCORE; PESSIER; NICHOLS, 1999). This fungus was identified in the early 90's and described by Longcore et al. (1999). Specifically, *Bd* grows in keratinized epidermal cells of adult amphibians (BERGER et al., 1998) and infects mouthparts with keratinized surfaces as jaw sheaths and teeth of tadpoles. Highly infected adults may die by cardiac arrest due to interruption of electrolytes transport across the epidermis (VOYLES et al., 2009). Moreover, typical pathological signs include epidermal hyperplasia, hyperkeratosis and shedding of the epidermis (VOYLES et al., 2009). On tadpoles, *Bd* can affect food intake, foraging efficiency, behavior, growth and development (e.g. HAN; BRADLEY; BLAUSTEIN, 2008; VENESKY; WASSERSUG; PARRIS, 2010) and generally is not lethal at this stage (BLAUSTEIN et al., 2005).

As a typical chytrid, *Bd* has two life stages: a sessile stage, reproductive thallus with a single zoosporangium, and a motile stage, with an aquatic uniflagellated zoospores (BERGER et al., 2005). *In situ*, experiments showed that zoospores can swim for up to 24 h covering a distance of 2 cm (LONGCORE; PESSIER; NICHOLS, 1999; PIOTROWSKI; ANNIS; LONGCORE, 2004). Zoosporangia produce zoospores via asexual reproduction. Although sexual reproduction is rare, the evidence for *Bd* first sexual reproduction was recently found in the southern Atlantic Forest. This was a hybrid genotype strain CLFT02402 produced from recombination between *Bd*-GPL and *Bd*-Brazil genotypes (SCHLOEGEL et al., 2012).

The chytrid fungus grows in culture under a range of temperatures (4 – 25 °C), and pH (4 – 8). Specifically, *in vitro* optimal growth occurs between 17 – 25°C and a pH of 6 – 7 (PIOTROWSKI; ANNIS; LONGCORE, 2004), but tradeoffs exist between the zoosporangium maturation rate which increases with temperature, and the number of produced zoospores per zoosporangium which decreases with temperature

(WOODHAMS et al., 2008). Nevertheless, several strains show reduced growth and/or mortality above 28°C (FISHER; GARNER; WALKER, 2009; PIOTROWSKI; ANNIS; LONGCORE, 2004), whereas higher temperature variability has shown to increase *Bd* impact (HAMILTON et al., 2012) and strains appear to respond differently to temperature patterns (e.g. STEVENSON et al., 2013). In addition, *Bd* growth can also be regulated by biotic interactions. For example, microorganisms such as daphnia, ciliates and rotifers are effective for regulating *Bd* infection because they consume *Bd* zoospores in aquatic environments (e.g. BUCK; TRUONG; BLAUSTEIN, 2011; SCHMELLER et al., 2013).

Even though *Bd* is considered as an amphibian specialist (VOYLES; ROSENBLUM; BERGER, 2011), the capability of survival outside the host is an important aspect for the transmission dynamics of this chytrid. For instance, *in vitro* studies show that *Bd* has the capacity to grow on other substrates, such as boiled snake skin, 1% keratin agar, peptonized milk, triptone and feather meal (PIOTROWSKI; ANNIS; LONGCORE, 2004; SYMONDS et al., 2008). Furthermore, other studies suggest that *Bd* can persist for up to three months in sterile sand or lake water (JOHNSON; SPEARE, 2003). Moreover, wild caught crayfish (*Procambarus alleni* and *P. clarkia*) can retained *Bd* in their gastrointestinal tracts for at least three months, possibly causing mortality in crayfish and transmitting the infection to amphibian tadpoles (MCMAHON et al., 2013). Also *Bd* may be carried on algae, terrestrial reptiles, waterfowl bird, and nematodes (e.g. GARMYN et al., 2012; JOHNSON; SPEARE, 2005; KILBURN; IBÁÑEZ; GREEN, 2011; SHAPARD; MOSS; SAN FRANCISCO, 2012).

Bd temporal and spatial dynamics that regulate its presence and quantity deserve further investigation. In one hand, *Bd* may be in an enzootic stage, characterized by a constant prevalence and reduced amphibian mortality. On the other hand, the chytrid may be in an epizootic stage affecting naïve amphibian populations provoking mass die-offs (BREM; LIPS, 2008; LIPS et al., 2008). Part of those dynamics, that shapes how the disease affects amphibians in a given site, are probably due to a complex interaction between climatic conditions, *Bd* strain, amphibian susceptibility, historical presence, associated microfauna and non-amphibian species (SCHMELLER et al., 2013; VOYLES; ROSENBLUM; BERGER, 2011; VOYLES et al., 2012; WALKER et al., 2010).

The mechanism for the introduction and spread of *Bd* is still in debate, but the most accepted hypotheses is that *Bd* was spread out worldwide from its origin by live trade of amphibians for human consumption (CATENAZZI; VREDENBURG; LEHR, 2010; SCHLOEGEL et al., 2012; WELDON et al., 2004). For example, the role of the trade of North American bullfrog (*Lithobates catesbeianus*) as a carrier of *Bd* and spreading virulent *Bd* genotypes in a global panel can support this idea (SCHLOEGEL et al., 2012).

2.1.2 *Batrachochytrium dendrobatidis*: Distribution and Evolutionary History

The chytrid fungus has been detected in at least 500 of 1,240 (42%) analyzed amphibian species around the world, except Antarctica (Figure 1). Many distributional sampling gaps around the world exist regarding amphibians infected by *Bd*. For example, the chytrid fungus has been detected in 52 of 82 countries in which sampling was reported (OLSON et al., 2013). In other words, the need of more detailed studies for *Bd* distribution, risk assessment and conservation efforts are still a priority (RÖDDER et al., 2009; SCHEELE et al., 2014).

Recent emergence of this disease, that can cause catastrophic population loss, has led to a constant debate of its possible origin and dispersion (LIPS et al., 2008; VENESKY et al., 2014). Thus, two hypotheses attempt to explain chytridiomycosis in wild populations. In the first place, the novel pathogen hypothesis suggests that *Bd* is a new emerging pathogen that is expanding its geographic distribution and encountering hosts. Second, the endemic pathogen hypothesis proposes that is a widespread pathogen whose emergence was triggered by a change in host, pathogenicity, or environment (RACHOWICZ et al., 2005; VENESKY et al., 2014). Nevertheless, these hypotheses are in constant debate and new evidence suggests that geographic historical distribution of *Bd* may support both in different context. For example, genome data suggest that *Bd* is likely endemic in some areas (e.g. Brazilian Atlantic forest) and a novel pathogen in others (RODRIGUEZ et al., 2014; ROSENBLUM et al., 2013).

Figure 1 - Global distribution of *Batrachochytrium dendrobatidis*.

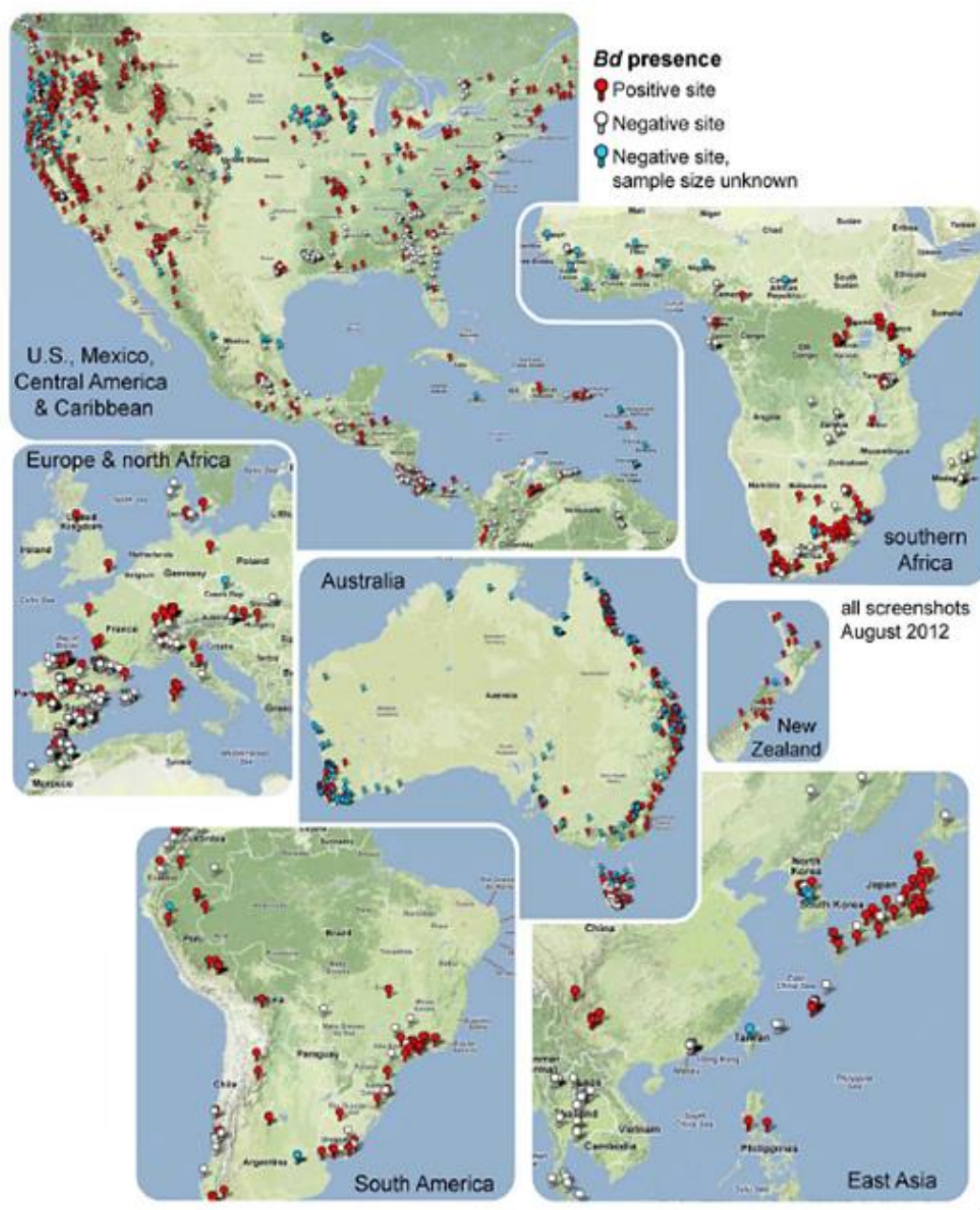


Figure from Olson et al. (2013).

Chytridiomycosis origin was thought to be in Africa for some specific reasons. First, earliest *Bd* record (until then) had been found in *Xenopus laevis* individuals since 1938. Second, wild individuals of *X. laevis* did not show clinical signs, nor experienced any sudden die-offs. Finally, an assay for human pregnancy, developed in the 1930's, that involved capture and international trade of huge amounts of *X. laevis* individuals

could disperse the fungus (WELDON et al., 2004). However, recent phylogenetic studies based on complete genome of several strains of *Bd* worldwide revealed a complex and different evolutionary history of this fungus (BATAILLE et al., 2013; ROSENBLUM et al., 2013). For instance, there is a virulent *Bd*-global panzootic lineage (*Bd*-GPL) geographically widespread (FARRER et al., 2011), and three other endemic lineages refer as the Swiss lineage (*Bd*-CH), the Cape lineage (*Bd*-Cape) and *Bd*-Brazil (FARRER et al., 2011; ROSENBLUM et al., 2013; SCHLOEGEL et al., 2012). Furthermore, the discovery that *Bd*-Brazil strain diverged earliest in the phylogenetic history of the pathogen (ca. 104,700 years ago), and the record of the earliest occurrence of *Bd* (1884) in Atlantic forest suggests that this hotspot is the possible geographic origin of this fungus (RODRIGUEZ et al., 2014).

2.2 Atlantic forest

The Atlantic forest domain is distributed along the Brazilian Atlantic coast, and extended through inland areas of Argentina and Paraguay, between latitude 3° S to 30° S and longitude 35° W to 60° W. This domain has boundaries in the south with the pampas, and drier inland formations such as the Bolivian Chaco, the Pantanal in the southwest, the South American savanna Cerrado in the west and the Caatinga in the northwest. The Atlantic forest is characterized by a complex topography that varies from sea level to 2,700 meter above the sea level (m.a.s.l.) (RIBEIRO et al., 2011). In general, coastal areas receive ca. 4,000 mm/year of rain and inland forests receive ca.1,000 mm/year of rain (CÂMARA, 2003). The Atlantic forest is extremely heterogeneous with an evergreen to semi-deciduous forests, but also deciduous forests, mangroves, swamps, *restingas*, inselbergs, high-altitude grasslands and mixed Araucaria pine forests (SCARANO, 2009).

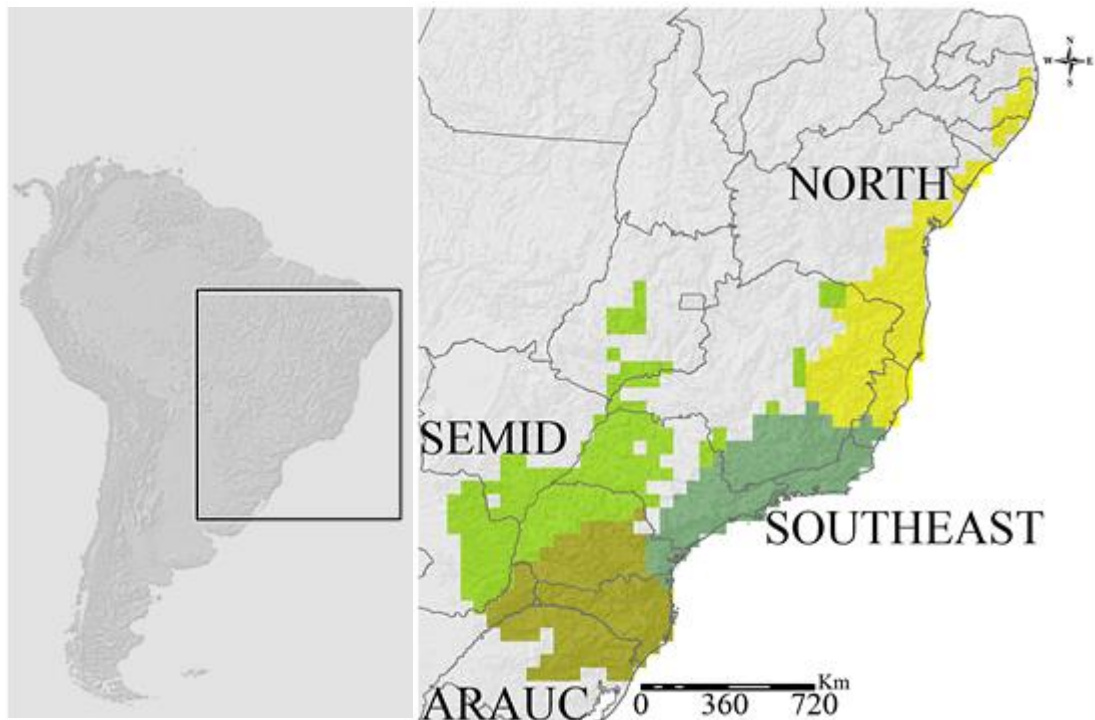
2.2.1 Diversity and endemism

The Atlantic forest of Brazil is one of the most diverse and endemic hotspot for biodiversity conservation in the world (MYERS et al., 2000). This hotspot possesses nearly 20,000 species of plants, 500 of bromeliads, 263 of mammals, 936 of birds, 306 reptiles and 540 amphibians (MITTERMEIER et al. 2005; HADDAD et al., 2013;

SIQUEIRA; TABARELLI, 2006). Furthermore, endemism rates range from 30% in birds, 44% in plants (MITTERMEIER et al. 2005) and 80% in amphibians (HADDAD et al., 2013). Recent studies attempt to explain the evolutionary process that shaped the high diversity in the Atlantic forest. For example, Carnaval *et al.* (2014) with a phylogeographic endemism approach suggests that different processes explain its complexity: 1) contemporary climatic heterogeneity for the south Atlantic forest; and 2) past climate dynamics, in areas of relative stability, in the north Atlantic forest. Similarly, Vasconcelos *et al.* (2014) split the Atlantic forest domain by biogeographic regions based on amphibians distribution, climatic conditions and vegetation structure (Figure 2): a) SEMID: less species diversity, where the weather is hot and seasonally dry; b) ARAUC: southern species from the Araucaria forest, where the weather is cooler and rainy all through the year; c) SOUTHEAST: high diverse and small-ranged species from the southeastern Brazilian, where the climate is cooler and has complex topography; and d) NORTH: northern small-ranged species that presumably evolved within the Pleistocene forest refugia, where the climate currently is hot. This regionalization has not taken into account habitat destruction (VASCONCELOS et al., 2014). However, both approaches support the northern Atlantic forest as a different region.

Since the arrival of European colonist in the sixteenth century deforestation has been a major threat in the Atlantic forest domain. For instance, reduction of a total 1,360,000 km² of the original forest to only 11.7% (163,377 km²) in Brazil, 24.9% (11,618 km²) in Paraguay and 38.7% (9,950 km²) in Argentina shows an alarming decrease. Moreover, small fragments of the forest represent 12.59% of the total original Brazilian Atlantic forest (RIBEIRO et al., 2011). In total, 22,603.5 km² of forest are protected by 249 natural reserves. However, the total amount of protected forest includes only 9.3% of the remaining forest cover (RIBEIRO et al., 2009). In fact, one of the most devastated region is the north Atlantic forest, specifically north of *São Francisco* river (excluding *Brejos nordestinos*), which represent only 12.1% (3,798.18 km²) of its original forest. Certainly, conservation efforts, as connecting fragments, stimulating restoring actions and declaring protected areas in this region, should be major priorities (RIBEIRO et al., 2009).

Figure 2 - Biogeographic regions based on the anuran distribution in the Atlantic forest fauna



Modified from Vasconcelos et al. (2014).

Notes: ARAUC = Araucaria forest, NORTH = semideciduous/deciduous and ombrophilous forests, SEMID = semideciduous forest and savanna-like vegetation and SOUTHEAST = coastal ombrophilous forest and adjacent semideciduous forest.

2.2.2 Estação Ecológica de Murici (ESEC de Murici)

One of the most important complex of forest fragments for conservation in the northern Atlantic forest region, specifically in the state of Alagoas, is the *Estação Ecológica de Murici* (ESEC de Murici). These forest fragments are located in the Murici, Flexeiras e Messias municipalities, Alagoas State, Brazil (Figure 3). Specifically, the largest fragment in this ESEC is the Mata da Bananeira with an extension of 26.28 km². The climate is defined as subtropical with dry summer (Köppen's climate classification), being hot and humid, with a rainy season between April and August, and the drier period between November and January (ALVARES et al., 2013). This fragment is located at NORTH biogeographic region of the Atlantic forest (VASCONCELOS *et al.*,

2014). Alternatively, based on endemism of birds, butterflies and primates this region is defined as Pernambuco sub-region (SILVA; CASTELETI, 2003).

Figure 3 - Inset map is South America with Atlantic forest in green. Square highlights the north portion of Atlantic forest. Satellite image where yellow represents the boundary of the Estação Ecológica de Murici (ESEC Murici) and A represents *Mata da Bananeira* forest fragment.

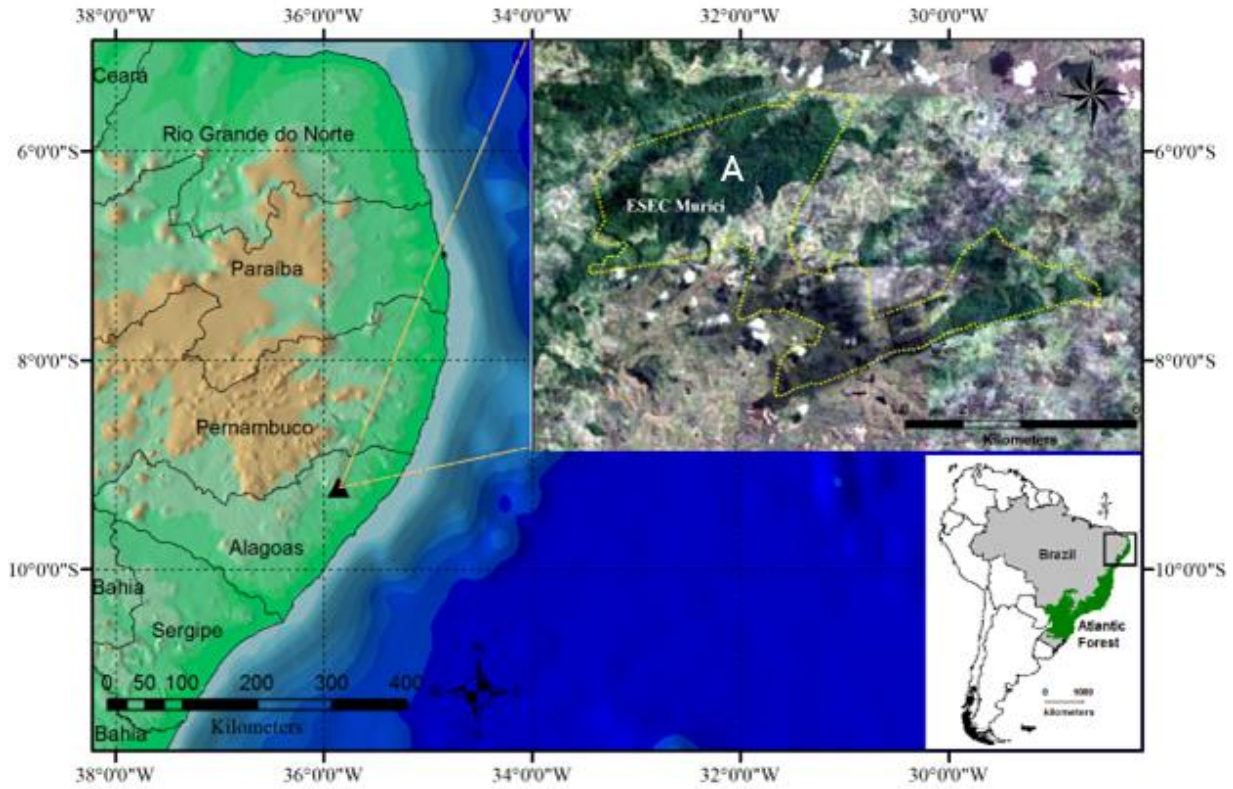


Figure: Author (2014).

Note: Square highlights the north portion of Atlantic forest. Satellite image with yellow contour represents Estação Ecológica de Murici (ESEC Murici) whereas A represents *Mata da Bananeira* forest fragment.

2.2.3 Atlantic forest and *Batrachochytrium dendrobatidis*

Recently, the Atlantic forest has shaken the research of *Bd* worldwide for many reasons. First, *Bd* is highly widespread and apparently has not been linked to amphibian declines (GRÜNDLER *et al.*, 2012; VERDADE *et al.*, 2012). Second, the discovery of endemic strains with 76 unique haplotypes (FARRER *et al.*, 2011; RODRIGUEZ *et al.*, 2014; ROSENBLUM *et al.*, 2013; SCHLOEGEL *et al.*, 2012). Finally, *Bd* has been present in this area for the past 100 years with a steady moderate prevalence

(RODRIGUEZ *et al.*, 2014). However, these evidences were obtained from the south Atlantic forest. For the north Atlantic forest there is a gap of knowledge regarding to types of strain, prevalence, presence and general epidemiology of *Bd*.

Figure 4 - Distribution of studied sites of *Batrachochytrium dendrobatidis* in Brazilian Atlantic rainforest.

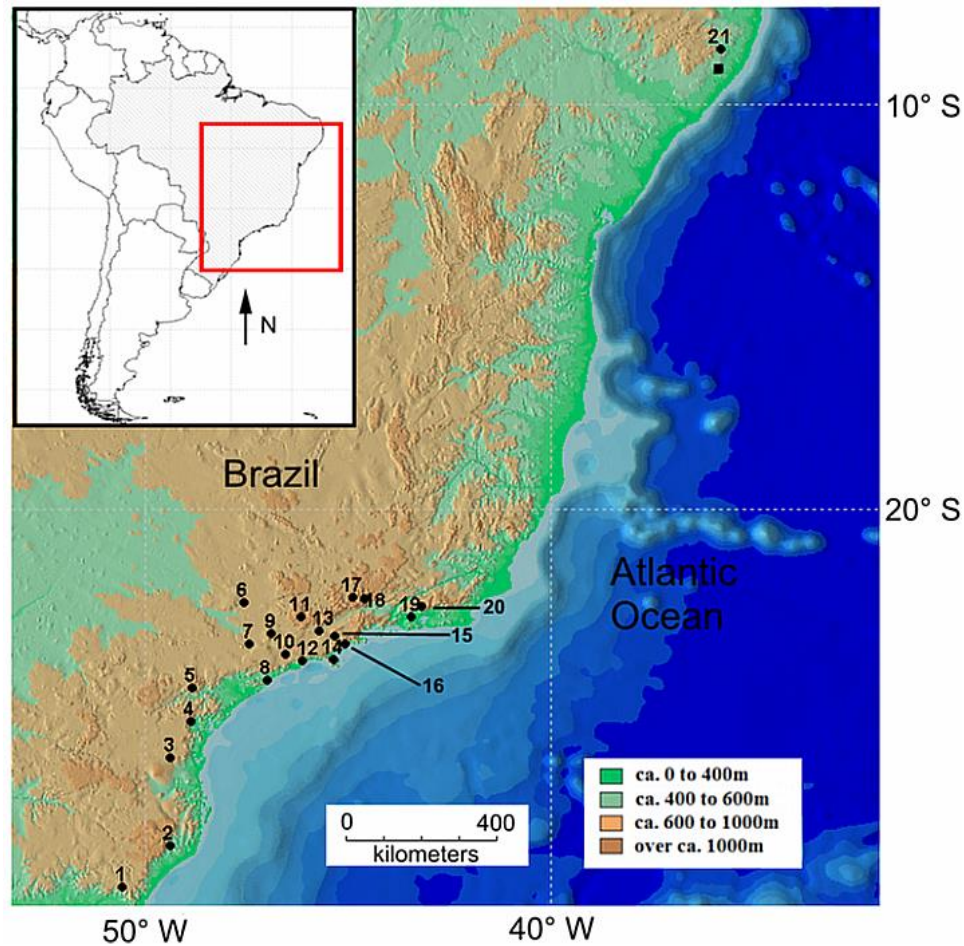


Figure from Lisboa *et al.* (2013).

Until now ca.130 anuran species have been positive to *Bd*, mainly inhabiting the southern region of the Atlantic Forest (BECKER; ZAMUDIO, 2011; CARNAVAL, *et al.*, 2006; GRÜNDLER *et al.*, 2012; LISBOA *et al.*, 2013; RODRIGUEZ *et al.*, 2014; TOLEDO, 2006; VALENCIA-AGUILAR *et al.*, *in press*; VIEIRA *et al.*, 2012; VIEIRA *et al.*, 2013). For the northern Atlantic Forest, *Bd* has been found in tadpoles of *Hypsiboas freicanecae*, in Jaqueira municipality, state of Pernambuco (CARNAVAL *et al.*, 2006)

and recently, in tadpoles of *Aplastodiscus sibilatus* and *H. freicanecae* from Murici municipality, state of Alagoas (LISBOA et al., 2013). Basic information of *Bd* presence is still needed in the northern Atlantic forest. For instance, there is a wide geographic gap (ca. 1,700 km) between south and northern portions of Atlantic Forest regarding *Bd* surveys (Figure 3).

2.3 Bromeliads and anurans

Bromeliads (Bromeliaceae) is a plant family mostly distributed in the Neotropics, with the exception of one species that is found in Africa (KITCHING, 2000). Specifically, the Atlantic forest is considered the evolutionary core of this family, with approximately 500 species (SIQUEIRA; TABARELLI, 2006). Their ecological importance is due to their phytotelm (plural phytotelmata = “plants that holds water”). This morphological structure maintains water and nutrients (KITCHING, 2000) being considered as “biodiversity amplifiers” with their ability to buffer environmental fluctuations generating and harboring high levels of species diversity (GONÇALVES-SOUZA et al., 2010). Indeed, phytotelmata is used by a wide range of taxonomically diverse groups, including bacteria, fungi, insects, amphibians, reptiles, and other plants (BUOSI et al., 2014; GONÇALVES-SOUZA et al., 2010; KITCHING, 2001; LEHTINEN, 2004; LEROY et al., 2013).

The importance of bromeliads for amphibians is associated with the presence of phytotelm that may provide stability and protection against desiccation, offering shelter, and food resource (KITCHING, 2000; LEHTINEN, 2004). In fact, some amphibians have different degrees of association with bromeliads: some anurans use bromeliads during its entire life-cycle (bromeligenes or bromeligenous) and others use them sporadically as shelter or reproduction (bromeliculas) (PEIXOTO, 1995). For example, Drummond (2009) analyzed the effects of forest fire on anuran populations in a rocky outcrop of the Brazilian Cerrado and recorded an increase of amphibian individuals in bromeliads. Certainly, environmental simplification caused by fire, turned this microhabitat an important shelter.

Several bromeliad traits are associated with the presence of anurans, including humidity, water availability and tank size (BANDONI; NAVAS, 2004; PEDERASSI *et al.*, 2012; STUCKERT *et al.*, 2009). Generally, bromeliad morphological traits are chosen by anurans to guarantee their survival. For example, Pederassi et al. (2012) found association between morphological traits of the bromeliads and the presence of *Scinax argyreornatus* (Anura, Hylidae), a bromelicula species, but also reports that relative humidity is an important condition. Similarly, Bandoni & Navas (2004) showed a non-random choice of bromeliads by a bromeligenous species, *Scinax perpusillus*, whose individuals preferred larger, and clustered bromeliads in the southern Atlantic forest of Brazil. Moreover, anuran species have other types of selection conditions (i.e. niche partitioning) triggered by reproductive ecological traits, such as behavior, parental care, food availability, avoidance of predators, avoidance of competitive traits with phylogenetically related species, among others (BROWN; MORALES; SUMMERS, 2008; POELMAN; WIJNGAARDEN; RAAIJMAKERS, 2013; RYAN; BARRY, 2011; ZHOU et al., 2008)..

2.3.1 The genus *Phyllodytes* Wagler 1830

Table 1- Composition of *Phyllodytes* species groups based on dorsal color pattern.

Group	Species	Distribution
<i>P. luteolus</i> group	<i>Phyllodytes acuminatus</i> Bokermann, 1966	AL, PE
	<i>Phyllodytes brevirostris</i> Peixoto and Cruz, 1988	PA
	<i>Phyllodytes edelmoi</i> Peixoto, Caramaschi and Freire, 2003	AL, PE
	<i>Phyllodytes kautskyi</i> Peixoto and Cruz, 1988	ES
	<i>Phyllodytes luteolus</i> (Wied-Neuwied, 1824)	PE, BA, ES
	<i>Phyllodytes melanomystax</i> Caramaschi, Silva and Britto-Pereira, 1992	BA
<i>P. tuberculosus</i> group	<i>Phyllodytes tuberculosus</i> Bokermann, 1966	SE, BA
	<i>Phyllodytes punctatus</i> Caramaschi and Peixoto, 2004	SE
<i>P. auratus</i> group	<i>Phyllodytes wuchereri</i> (Peters, 1873)	BA
<i>P. gyrinaethes</i> group	<i>Phyllodytes gyrinaethes</i> Peixoto, Caramaschi and Freire, 2003	AL, PE
Without group*	<i>Phyllodytes maculosus</i> Cruz, Feio and Cardoso, 2007	BA, MG

Modify from Caramaschi et al. (2004)

Note: Alagoas (AL), Bahia (BA), Minas Gerais (MG), Espírito Santo (ES), Paraíba (PA), Pernambuco (PE), Sergipe (SE).

The genus *Phyllodytes* is exclusively bromeligenous and includes 11 species of tree frogs (Hylidae) endemic to Eastern Brazil (FROST, 2014). Although interspecific

relationships remain unknown, these species have been allocated in four species groups (Table 1) based mostly on their color patterns (CARAMASCHI; PEIXOTO; RODRIGUES, 2004). The *P. luteolus* group has plain dorsal color pattern, a more or less defined dorsolateral dark line in the canthus rostralis and/or behind the corner of the eyes. The *P. auratus* group possesses two dorsolateral white or yellowish stripes bordered by a dark line. The *P. tuberculosus* group is characterized by pale brown dorsum with scattered brown dots. Finally, *P. gyrinaethes* group that has two irregularly patterned dorsolateral light areas in contrast with a marbled dorsum (CARAMASCHI; PEIXOTO; RODRIGUES, 2004). More recently *P. maculosus* was described (CRUZ; NEVES; CARDOSO, 2006) and could not be appropriately allocated in existing groups (table 1). Molecular study showed that *Phyllodytes* is a paraphyletic genus and raised *Phyllodytes auratus* (From Trinidad & Tobago) to a new genus *Phytotriades* Jower, Downie & Cohen, 2008. Because few *Phyllodytes* species (only two actual species and *Phytotriades auratus*) were included in this analysis, the interspecific relationships

Three species are known only from their type-localities (*P. brevirostris*, *P. kautskyi*, *P. punctatus*), and two are known from a few localities (*P. gyrinaethes* and *P. maculosus*). Furthermore, the two species restricted to the most northern part of the Atlantic forest are poorly known. For instance, almost all natural history of *Phyllodytes edelmoi* and *P. gyrinaethes* are based on their description manuscript and some recent descriptions of advertising calls (DE LIMA et al., 2008; ROBERTO; AVILA, 2013). Data on *Bd* infection, systematics, ecology and natural history are still lacking for these species.

2.3.1.1 *Phyllodytes gyrinaethes* Peixoto, Caramaschi & Freire 2003

This species is known from two localities. Specifically, its type locality at Fazenda Bananeiras, ESEC de Murici, Murici municipality, Alagoas State, Brazil, and Usina Colonia, Pernambuco State, Brazil. Population status is unknown and is consider as data deficient species in the IUCN Red list (IUCN, 2014).

This is a medium size species (Snout-Vent Length 23.9 – 27.9 mm). Characterized by a marbled dorsal pattern on the body and limbs; a well-developed nuptial pad at the base of the first finger; a hidden tympanum; a red cast on the

concealed areas of thigh, tibia, inner border of foot, and inguinal region; and a morphologically specialized tadpole with expanded labia (PEIXOTO et al., 2003). Baseline data on ecology of this species is absent. *Phyllodytes gyrinaethes* major threats are related to habitat destruction due to agricultural development, wood extraction, human settlement and collection of bromeliads (FREIRE et al., 2004).

Figure 5 - *Phyllodytes gyrinaethes* adult.



Photo Author, 2014

2.3.1.2 *Phyllodytes edelmoi* Peixoto, Caramaschi & Freire 2003

This species was described from Mata do Catolé, Maceió municipality, Alagoas State, Brazil (paratypes in Fazenda Bananeiras, ESEC Murici) and was found in two additional localities, Mata da Salva, Rio Largo. Alagoas State, and in Jaqueira municipality, Pernambuco State, Brazil (IUCN, 2014). It occurs from sea level up to 650 m.a.s.l. Its population status is unknown and is considered as data deficient species in the IUCN red list (IUCN, 2014).

This is a medium sized species allocated in *P. luteolus* group (Snout-Vent Length 23.5 – 28.8 mm) and is characterized by a dark brown to black dorsum with many pearl white spots in post-metamorphic and young specimens; a conspicuous pearl white spot

on the dorsum of the tadpole; in life, adults deep golden yellow, no distinct tubercle on the tip of the snout; and nares on slightly elevated prominences (PEIXOTO; CARAMASCHI; FREIRE, 2003).

The major threats to *P. edelmoi* are habitat loss due to agricultural development, wood extraction, human settlement and collection of bromeliads. The area where the species is found is suffering severe fragmentation and reduction in extent of forest patches, and some populations in Alagoas appear to be at severe risk as a consequence (FREIRE et al., 2004).

Figure 6 - *Phyllodytes edelmoi* adult.



Photo: Lisboa, B. 2014

2.3.1.3 *Phyllodytes acuminatus* Bokermann, 1966

This species is known from Alagoas State (its type-locality at Mangabeiras municipality) and Pernambuco State (Buíque municipality), Brazil (FROST, 2014). *Phyllodytes acuminatus* is a moderate-sized species (Snout-Vent Length 21.1 – 24.9 mm) and is characterized by a having acuminate snout in a dorsal view and subacute snout in lateral view (BOKERMANN, 1966). *Phyllodytes acuminatus* tadpole is similar to most species of the genus. However, the presence of a double row of marginal papillae

surrounding all the oral apparatus (except on most of the upper labium) is their main difference (CAMPOS et al., 2014). The major threats to *P. acuminatus* are probably related to habitat loss due to agriculture, livestock grazing, logging, human settlement and collection of bromeliads (PEIXOTO; PIMENTA, 2004).

Figure 7 - *Phyllodytes acuminatus* adult.



Photo: Lisboa, B. 2014

2.3.2 Bromeliad anurans and *Batrachochytrium dendrobatidis*

Amphibians have different degrees of associations with tank bromeliads (HOORN et al., 2010; HORRES et al., 2007; LEHTINEN, 2004; SCHULTE; BARFUSS; ZIZKA, 2009). Amphibians infected with *Bd* in arboreal bromeliads have shown high prevalence especially in lowland rainforests compared to other microhabitats (MCCRACKEN et al., 2009). Furthermore, the presence of *Bd* has been detected in water of bromeliads suggesting that these sites may act as environmental reservoirs (COSSEL; LINDQUIST, 2009). This provides the idea of the importance to study anurans associated with bromeliads regarding *Bd* infection.

Anurans infected with *Bd* in bromeliads may shed light on the dynamic of this disease for many reasons. Firstly, bromeliad tanks have been considered as perfect microhabitat reservoirs for *Bd* throughout its Neotropical distribution, specially for the South Atlantic forest anurans (MCCRACKEN et al., 2009; RODRIGUEZ et al., 2014; STUCKERT et al., 2009). Secondly, bromeliad morphology and humid reservoir conditions could allow both (amphibians and *Bd*) to survive throughout temporal constraints of precipitation and heat, as has been predicted for Jamaican phytotelmata amphibians (HOLMES; MCLAREN; WILSON, 2014). Finally, tank bromeliads are an interesting natural microcosms with replicated scenario for modeling ecological studies (KITCHING, 2001).

3 CHAPTER 1

The chytrid fungus acts as a generalist pathogen that infects species-rich amphibian families in Brazilian rainforests

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Running page head: *Bd* as generalist pathogen in Brazil

ABSTRACT

The fungus *Batrachochytrium dendrobatidis* (*Bd*) is one of the main causes of declines in amphibian populations. This fungus is 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 have reported 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* positive in an anuran caught in the wild in Amazonia. In total, we screened 27 anuran species (90 individuals) for *Bd*, 81.5% (22 species, 39 individuals) of which showed positive results. All samples collected in Bahia (two individuals), Pernambuco (three individuals), Pará (one individual), and Minas Gerais (one 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 may partly explain the pandemic and widespread nature of *Bd* infection in amphibians.

Keywords: Pathogen, *Batrachochytrium dendrobatidis*, 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 considered one of the main causes of these population declines (Baillie et al. 2004, Fisher et al. 2012). Particularly, the aquatic chytrid fungus *Batrachochytrium dendrobatidis* (hereafter referred to as *Bd*) has led to the decline of several amphibian populations and species worldwide (Stuart et al. 2004, Lips et al. 2005, La Marca et al. 2005, Pounds et al. 2006, Skerratt et al. 2007, Fisher et al. 2009, 2012), and it has mostly affected species with a high dependence on water (Bielby et al. 2008, Catenazzi et al. 2010). Previous studies have suggested that *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 environmental tolerance, 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 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 impacts 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): two 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, two anuran species in Cerrado (Ramalho et al. 2013) and 110 species in several habitats of the Atlantic Forest (Carnaval et al. 2005, 2006, Toledo et al. 2006a, 2006b, Sluys et al. 2007, Ferreira et al. 2008, Brito-Gitirana et al. 2009, Becker & Zamudio 2011, De Paula 2011, De Paula & Catão-Dias 2011, Gründler et al. 2012, Vieira et al. 2012, 2013, Lisboa et al. 2013, Rodriguez et al. 2014) have been recorded as *Bd*-positive (Table S1), with records dating back to more than 100 years 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 have provided 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.

MATERIAL AND METHODS

We conducted fieldwork in five Brazilian localities: four 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 one 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 five 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 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 (Carnaval et al. 2005, 2006, Toledo et al. 2006a, 2006b, Sluys et al. 2007, Ferreira et al. 2008, Brito-Gitirana et al. 2009, Becker & Zamudio 2011, De Paula 2011, De Paula & Catão-Dias 2011, Gründler et al. 2012,

Vieira et al. 2012, 2013, Lisboa et al. 2013, Rodriguez et al. 2014) 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 statistical computing environment (R Development Core Team 2013) after normalizing data through a log transformation.

RESULTS

Out of the 27 sampled species from eight anuran families, 22 (81.5%) tested positive for *Bd* (Table 1). Out of the 90 sampled individuals from five localities (Figure 1), 39 (43.3%) tested positive for *Bd* infection. All the 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 was lower in Leptodactylidae (38%; 3 species; 8 individuals) and Bufonidae (25%; 3 species; 8 individuals). *Bd* prevalence among families did not differ ($F = 0.80$, $df = 3$, $p = 0.50$). Infection load was the highest in Craugastoridae ($\log 1.39GE \pm 1.51$, $n = 9$ individuals) and Hylidae ($\log 1.05GE \pm 0.87$, $n = 21$ individuals), although loads did not differ between families ($F = 0.92$, $df = 5$, $p = 0.47$). The highest intensity of infection ($\log 1.79 - 5.20$) was detected in *Crossodactylus 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* ($\log 0 - 1.40$). *Bd* was not detected in *Rhinella hoogmoedii*, *Gastrotheca* sp., *Aplastodiscus sibilatus*, *Hypsiboas semilineatus*, and

Leptodactylus natalensis (only one individual was tested for all these species). The sampled individual of *Adelphobates galactonotus* collected from Pará, Amazonia, was infected.

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

DISCUSSION

Among the six main Brazilian biomes, three 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. 2009). Although bullfrogs have been suggested as potential reservoirs and vectors of *Bd* (Schloegel et al. 2009), *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); majority of these reports are from the southern region, where two divergent strains of *Bd* (*Bd*-GPL and *Bd*-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 1,600 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 (Rödger 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 (Rodríguez 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 et al. 1999), Spain (Bosch et al. 2001), United States (Vredenburg et al. 2010), and Venezuela (La Marca et al. 2005), where high prevalence (Lips et al. 2005) and high *Bd* infection loads (Vredenburg et al. 2010) have been associated with local mass extinctions and fatal declines.

The Atlantic forest is one of the most threatened forests in the world, where more than 80% of the remaining fragments are smaller than 0.5 km² (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 (Figure 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 types of host species (Walker et al. 2010, Crawford et al. 2010). In contrast, *Bd* infections in Europe and the United States are associated with amphibian families that display similar life-history traits (Corey & Waite 2008, Bancroft et al. 2011, Baláž et al. 2013). 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). 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 south and southeast 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°S to 31°S and 35°W 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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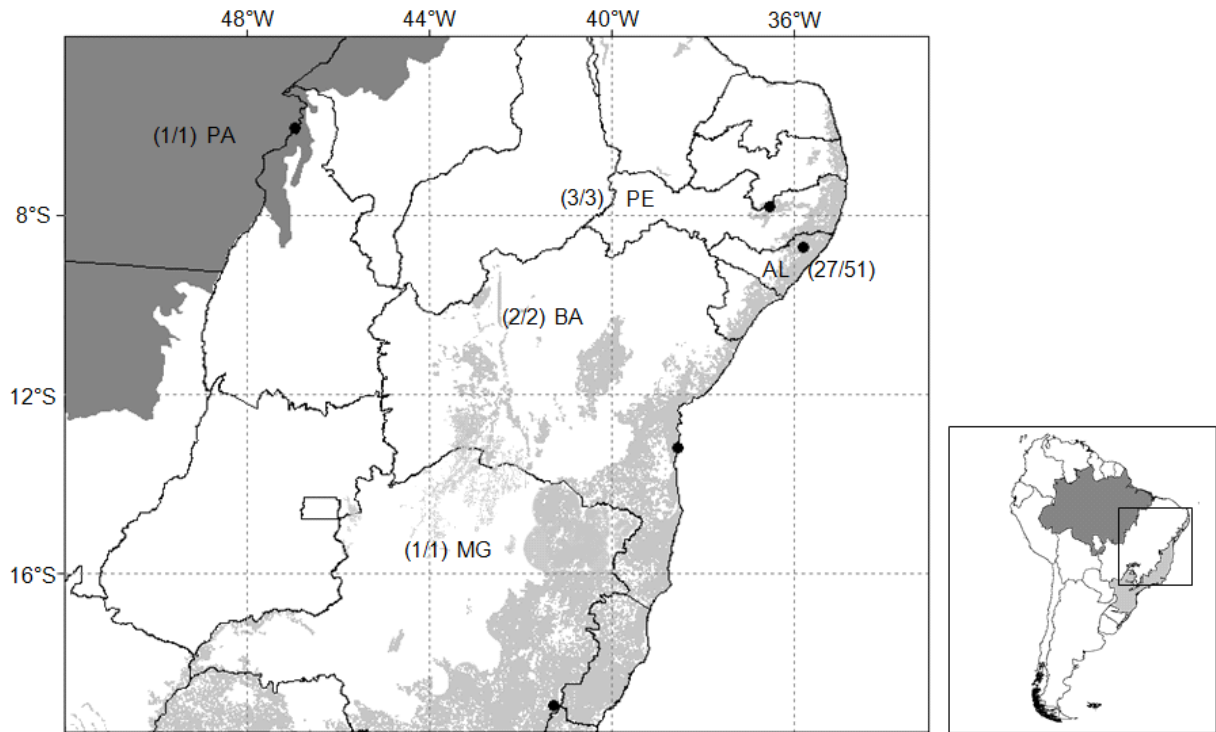


Figure 1. Positive records of *Batrachochytrium dendrobatidis* infection reported in the present study for the Atlantic forest, in the states of Pernambuco (PE), Alagoas (AL), Bahia (BA) and Minas Gerais (MG), and for the Amazon forest, in the state of Pará (PA). In parentheses the number of individuals tested positive out of total sampled. Source for remaining forest cover: SOS Mata Atlântica/INPE 2012.

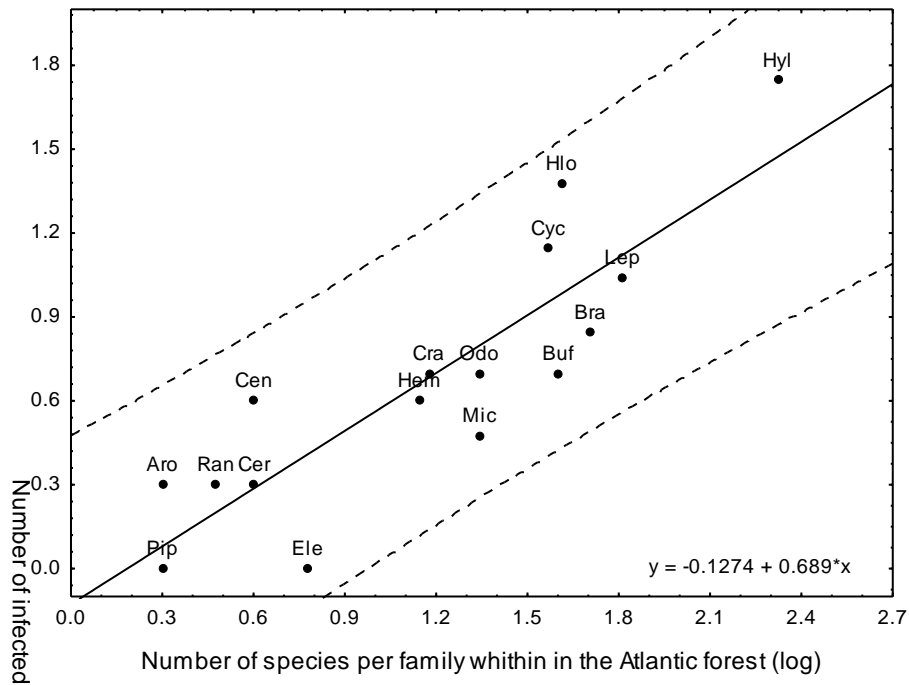


Figure 2. Linear regression of the number of species per family reported in the Atlantic forest on the number of infected species ($r^2 = 0.753$, $p < 0.001$, $df = 14$). Dotted lines represent upper and lower 95 % confidence intervals. Aro: Aromobatidae, Bra: Brachycephalidae, Buf: Bufonidae, Cen: Centrolenidae,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

Table 1. Anuran species sampled for *Batrachochytrium dendrobatidis* presence in the Amazonia and in the Atlantic forest. Zoospore equivalents (mean and standard deviation [SD] was calculated only for positive individuals).

Taxa	Locality/State	Zoospore equivalents			Prevalence (Infected/Analyzed individuals)
		Mean	Min-Max	SD	
Bufonidae					
<i>Frostius pernambucensis</i>	Murici/Alagoas	62.17	-	-	1/2
<i>Rhinella crucifer</i>	Murici/Alagoas	4.79	-	-	1/3
<i>Rhinella hoogmoedi</i>	Murici/Alagoas	-	-	-	0/3
Craugastoridae					
<i>Pristimantis vinhai</i>	Itacaré/Bahia	2.37	-	-	1/1
<i>Pristimantis ramagii</i>	Murici/Alagoas	25.51	3.53-135.72	48.71	7/14
Dendrobatidae					
<i>Adelphobates galactonotus</i>	Santa Cruz Dos Martírios/Pará	1.56	-	-	1/1
Hemiphractidae					
<i>Gastrotheca</i> sp.	Murici/Alagoas	-	-	-	0/1
Hylidae					
<i>Aplastodiscus sibilatus</i>	Murici/Alagoas	-	-	-	0/1
<i>Dendropsophus minutus</i>	Murici/Alagoas	12.4	-	-	1/2
<i>Hypsiboas albomarginatus</i>	Murici/Alagoas	1.05	-	-	1/1
<i>Hypsiboas crepitans</i>	Poçoão/Pernambuco	34.62	2.2-67.05	45.85	2/2
<i>Hypsiboas exastis</i>	Murici/Alagoas	7.88	-	-	1/1
<i>Hypsiboas semilineatus</i>	Murici/Alagoas	-	-	-	0/1
<i>Phyllodytes</i> cf. <i>acuminatus</i>	Murici/Alagoas	16.33	-	-	1/1
<i>Phyllodytes</i> cf. <i>edelmoi</i>	Murici/Alagoas	25.56	-	-	1/1
<i>Phyllodytes edelmoi</i>	Murici/Alagoas	60.22	2.95-227.27	111.37	4/12
<i>Phyllodytes gyrinaethes</i>	Murici/Alagoas	359.16	1.72-1276.49	555.53	5/24
<i>Phyllodytes tuberculosus</i>	Itacaré/Bahia	2.93	-	-	1/1
<i>Phyllomedusa nordestina</i>	Murici/Alagoas	72.63	3.30-141.95	98.04	2/2
<i>Scinax</i> cf. <i>eurydice</i>	Carangola/Minas Gerais	1.64	-	-	1/1
<i>Scinax nebulosus</i>	Murici/Alagoas	4.32	-	-	1/2
<i>Scinax pachyrcus</i>	Poçoão/Pernambuco	1.88	-	-	1/1
Hylodidae					
<i>Crossodactylus dantei</i>	Murici/Alagoas	2793.2	264.98-5321.50	3575.55	2/2
Leptodactylidae					
<i>Physalaemus</i> cf. <i>cuvieri</i>	Murici/Alagoas	7.91	4.27-11.55	5.14	2/6
<i>Leptodactylus natalensis</i>	Murici/Alagoas	-	-	-	0/1
<i>Leptodactylus</i> cf. <i>mystaceus</i>	Murici/Alagoas	5.1	-	-	1/1
Odontophrynidae					
<i>Proceratophrys renalis</i>	Murici/Alagoas	6.01	-	-	1/2

4 CHAPTER 2

Jumping into a trap: bromeligenous anuran selects optimum microhabitat for the chytrid fungus

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Running page head: Bromeligenous selects optimum microhabitat for *Bd*

ABSTRACT

The chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has been identified as a major threat to several amphibian populations inhabiting tropical forests. Amphibians that inhabit the phytotelmata (water tanks) of bromeliads may be especially at risk since the humid, environmentally buffered microhabitat also seems an ideal microhabitat for *Bd* presence. To test this hypothesis we sampled adults and tadpoles of the bromeligenous anuran *Phyllodytes edelmoi* (endemic to the northern Brazilian Atlantic forest) from the bromeliad *Portea leptantha*. We used a qPCR assay for detecting *Bd* in anurans. We analyzed eight bromeliad characteristics (phytotelm water temperature and pH, canopy closure, phytotelm diameter, leaves number, bromeliad maximum column depth to store water, bromeliad relative volume, and season). Adult frogs tended to select bromeliads with smaller diameter, more leaves and a relatively higher volume of water. Moreover, *Bd* was more prevalent in frogs inhabiting bromeliad phytotelmata with smaller diameters. Our findings indicate that the selection of smaller diameter bromeliads by *P. edelmoi* leads to higher probability of *Bd* presence, suggesting a microhabitat trait with higher number of *P. edelmoi* individuals that could amplify *Bd* presence in the environment. More generally, the nonrandom choice of bromeliad by this species demonstrates the importance of fine scale ecological knowledge of both anuran and fungus for predicting and countering the spread of this potentially lethal pathogen.

KEY WORDS: Pathogen, *Batrachochytrium dendrobatidis*, *Phyllodytes*, Atlantic forest, Bromeliad

INTRODUCTION

The amphibian-killing chytrid fungus, *Batrachochytrium dendrobatidis* (hereafter *Bd*) has now been detected in over 500 species worldwide (Olson et al. 2013). Despite of its documented role in amphibian population declines (e.g. Stuart et al. 2008, Wake & Vredenburg 2008, Lips et al. 2008), the spatial and temporal dynamics of *Bd* enzootic stage populations (Lips et al. 2008, Briggs et al. 2010) are poorly understood at a smaller scale. For example, amphibians of the Brazilian Atlantic forest (AF) showed, at a larger scale, an enzootic pattern in apparently non-declining populations that suggests a historic relationship with *Bd* that dates back at least 100 years (Rodriguez et al. 2014). However, the lack of evidence for an association between amphibian population declines and *Bd* reported in AF (Eterovick et al. 2005, Lips et al. 2005) suggests a complex, long-term fine scale interaction of *Bd* an anurans in this domain (Schloegel et al. 2012, Rosenblum et al. 2013, Rodriguez et al. 2014).

The AF is characterized by a high diversity, and endemism of many microorganisms, plants, invertebrates, and vertebrates (e.g. Richardson 1999, Myers et al. 2000, Siqueira & Tabarelli 2006, Schulte et al. 2009, Stuckert et al. 2009, Ribeiro et al. 2009, 2011, Gonçalves-Souza et al. 2010, Silva et al. 2012b) probably as a consequence of historical events, topography and climatic variability. Amphibian diversity extends to a diverse repertoire of reproductive modes, morphological and behavioral adaptations (Haddad & Prado 2005). Moreover, recent discoveries of ancient lineages, high diversity, endemism of *Bd* (Rosenblum et al. 2013, Schloegel et al. 2012) and detection in more than 130 amphibian species (e.g. Toledo 2006, Carnaval et al. 2006, Vieira et al. 2012, Lisboa et al. 2013, Rosenblum et al. 2013, Valencia-Aguilar et al. *in press*) suggests that many new interactions remain unknown. For instance, climatic variation, *Bd* strain, amphibian susceptibility, associated microfauna and non-amphibian species could all shape how the disease interacts with amphibians in given sites (Fisher et al. 2009, Voyles et al. 2011, 2012, Schmeller et al. 2013).

Bd could be considered as a generalist pathogen of anurans in the AF (Valencia-Aguilar et al. *in press*). Nevertheless, certain pond breeders and/or terrestrial developers in natural closed canopies may be at greater risk to chytrid infections in the AF (Becker & Zamudio 2011, Gründler et al. 2012). Conversely, open or degraded areas might also function as reservoir areas (Sluys & Hero 2009, Raffel et al. 2010, Becker et al. 2012). Moreover, the natural history or behavioral traits (e.g. microhabitat choice) of some amphibians (Silva et al. 2012a) may permit *Bd* to persist in suboptimal environments (Becker & Zamudio 2011, Forrest & Schlaepfer 2011). In other words, to understand *Bd*-amphibian dynamics within a habitat we need to identify the factors that influence their fine scale distribution. Significantly, interactions between bromeliad anurans and *Bd* have not been studied at a fine scale and it is likely that bromeliad traits that are favored by anurans survival may also be those that promote the survival of *Bd* for specific reasons. Firstly, bromeliad phytotelm (plural phytotelmata = “plants that hold water”) have been identified as perfect microhabitat reservoirs for *Bd* throughout its distribution (McCracken et al. 2009, Stuckert et al. 2009, Rodriguez et al. 2014). Secondly, the morphology and humidity within phytotelmata could allow both *Bd* and amphibians to survive adverse environmental conditions, as has been demonstrated for phytotelmata amphibians (Holmes et al. 2014).

Bromeliad importance for amphibians is principally associated with the presence of phytotelm which provide protection to amphibians avoiding desiccation, offering shelter, and food resource (Kitching 2000, Lehtinen 2004). Moreover, amphibians have different degrees of association with phytotelm bromeliads (Bromeliaceae) - a plant family mostly distributed in the Neotropics (Lehtinen 2004, Horres et al. 2007, Schulte et al. 2009). Specifically, anurans in bromeliads can be functionally split into bromelicolous (when use bromeliads sporadically as shelter) (Peixoto 1995) and bromeligenous/bromeligenes (when they complete their entire life cycle within bromeliads) (e.g. Lehtinen 2004, Pederassi et al. 2012, Sabagh et al. 2012). Moreover,

bromeliad morphological traits are generally chosen by anurans (e.g. Bandoni & Navas 2004, Pederassi et al. 2012) but other types of selection criteria may be triggered by reproductive ecological traits, such as behavior, parental care, food availability, avoidance of predators, avoidance of competition with phylogenetically related species among others (e.g. Brown et al. 2008, Ryan & Barry 2011, Poelman et al. 2013). These traits may be ideal for exploring *Bd*-amphibian interactions in the wild.

Here, we use bromeliads to model anuran associations with *Bd* at finer spatial scales. Specifically, we use one species of the bromeligenous genus *Phyllodytes* to explore traits associated with *Bd* in a rocky outcrop habitat. Although the species' systematic status is not clear (Wiens et al. 2005, Jowers et al. 2008, Pyron & Wiens 2011), this genus spawn its eggs and the exotrophic tadpoles develop in bromeliads (reproductive mode 6) (Haddad & Prado 2005), and it is possible that they also show parental care and territorial behavior (Pers. obs.). We evaluate: 1) whether the presence of *Phyllodytes edelmoi* is associated with larger phytotelmata due to its greater environmental buffer capacity; 2) whether the presence of *Bd* in *Phyllodytes edelmoi* are associated with bromeliad characteristics; and 3) if there are similar phytotelmata characteristics related to both *Phyllodytes edelmoi* and *Bd* presence.

MATERIAL AND METHODS

Estação Ecológica de Murici (ESEC de Murici) is located in the Murici, Flexeiras e Messias municipalities, Alagoas State, Brazil (Figure S1). Samples were taken in two rocky outcrops (between 500 to 600 masl) in an open area inside the *Mata da Bananeira* forest fragment (-35.8667°, -9.2000°; and -35.8583°, -9.21305°, Datum WGS84). The climate is defined as subtropical with dry summer (Köppen's climate classification), being hot and humid, with a rainy season between April and August, and most drought period between November and January (Alvares et al. 2013). This forest fragment has an extension of 26.28 km² and is located at North biogeographic region of the AF, and is defined as Pernambuco interior (semideciduous) forests

eco-region (Vasconcelos et al. 2014). Forests remains in the North AF area represent near 11.5% (56,400 km²) of the original forest (Ribeiro et al. 2009). Certainly, deforestation has been a major threat in this area (Ribeiro et al. 2009).

We focused our sampling in tank bromeliad species *Portea leptantha* Harms (Bromeliaceae, Figure S2 B). It is a medium-sized to large bromeliad, epiphytic or terrestrial, that occurs in rocky patches in northeastern Brazil (Gomes & Alves 2010). We recorded the presence of an endemic hylid bromeligenous species, *Phyllodytes edelmoi* Caramaschi & Freire 2003 (Figure S2 C, D). It is known from the states of Alagoas and Pernambuco (from sea level up to 650 masl). It is taxonomically allocated based on coloration in the *P. luteolus* group (Faivovich et al. 2005). It is a medium-sized species (Snout Vent Length 23.5–28.8 mm), with adults characterized by a deep golden yellow color and a conspicuous pearl white spot on the dorsum on the tadpole (Peixoto et al. 2003).

Two field trips of seven days each (dry and rainy season) were conducted from 20th - 27th September 2013 (rainy season), and 14th - 21th March, 2014 (dry season). Floor bromeliad patches were sampled, respectably of 2,323 m² and 4,878 m², on open forest rock outcrops (Figure S2 A). To gather data for anuran selection of bromeliads a "case-control design" was used (Keating & Cherry 2004). In other words, detecting presence of anurans with random samples design required a larger sample for meaningful analysis (Keating & Cherry 2004). In such case, floor bromeliad with phytotelmata were stratified by drawing with replacement a random sample of 19 used locations, and a second random sample of 37 unused locations. We considered a functional sampling unit *Portea leptantha* bromeliads due to its water reservoir characteristics, and focus on the more abundant inhabitant *Bd* host *Phyllodytes edelmoi*. In each bromeliads path, bromeliads were visited only once and measured for microhabitat characteristics at least 8 m apart of each other. In total, 56 bromeliads were sampled across two sites in both seasons (Figure S3).

First, we conducted a visual encounter survey -VES - for the presence of *Phyllodytes edelmoi* in bromeliads during their nocturnal activity with a total of 24 hour/person effort (between 19:00–22:00 h). Second, we used a random table to select bromeliads in the day, and marked them as unused bromeliads samples. Afterwards we measured environmental data from both used and unused samples. Specifically, we recorded seven bromeliad morphological characteristics: tank diameter (tank size along the second leaf of the bromeliad), size of the largest leaf, relative volume of water at bromeliad center calculated as $V=1/3\pi r^2 h$ where V is the volume of a conic solid (L) with a distance base “ b ” and the height “ h ” (the perpendicular distance from the base to the apex), maximum column capacity to store water (using a steel of 5 meters), number of leaves, pH inside bromeliads’ water with a pH tester Model PH-107, water microhabitat temperature in the center of the bromeliad [T_m] with an infrared thermometer Benetech GM 300 (degrees Celsius°). Relative humidity [HR] and ambient air temperature [T_a] were measured with a Digital Hygrothermometer HC-520. Canopy closure was measured with a Nikon Coolpix 950 automatic mode photos on a tripod at 1.45 m above the ground with an angle lens $>30^\circ$ (Paletto & Tosi 2009). The camera LCD was always facing north. Seasons were defined by the total amount of precipitation (mm) of each month of survey. Bromeliads spatial distributions were taken with a Garmin GPS e-trex. Bromeliad characteristics were taken between 10:00–16:30 h and we post-checked for anuran absence in marked as unused bromeliads (night). Finally, adults of *Phyllodytes edelmoi* individuals of were swabbed (Table S1) following the protocol proposed by Hyatt et al. 2007 with some modifications (Lambertini et al. 2013) and tadpoles were collected as swabs (Lambertini et al. 2013).

Molecular Analyses

DNA extraction of each sample was performed according to Boyle et al. 2004 with some modifications (Lambertini et al. 2013). ITS-1 region of *Bd* strain CLFT 023 (from Monte Verde, Camanducaia, Minas Gerais State, Brazil) was used to build the standard curve for infection

intensity quantification (Longo et al. 2013). Results were expressed as zoospore genomic equivalents (GE) per individual.

Data analyses

Images of the canopy were converted to an 8 bit format with a binary (Black/white pixels format), and the percentage of pixels that were black were counted with IMAGEJ analysis software (Schneider et al. 2012). Spatial autocorrelation was assessed by performing Moran's I test for each variable. Also a Pearson's correlation between variables with normal distribution was performed. Consequently, variables without high correlated coefficient ($R^2 < 0.7$) were selected. Variables were model with a logistic regression (GLM) with a "backward-forward stepwise" procedure to suggest which variables had influence in *Bd* presence, *Phyllodytes edelmoi* adult selection, and tadpole presence. Significant likelihood-ratio explained the variation in the dependent variable (presence). Significance was evaluated with a Wald statistic X^2 , based on a predictive model compared to a null model, with a Hosmer-Lemeshow (HL) goodness of fit test (Peng et al. 2002). Because we use a case-control sampling design, close attention is required to interpret patterns correctly. In the same way, a multiple logistic regression (GLM) with a step-by-step procedure was conducted to test for *Bd* preference on anurans found in bromeliad, and to determine which variables are most influential in predicting the presence of *Bd*. All analysis were performed in R-Software Package (Team 2014).

RESULTS

Microclimatic conditions and phytotelm characteristics, for a total of 56 *Portea leptantha* bromeliads, show that microhabitat characteristics are apparently similar in the same season (Table S2). In the same way, presence of *Phyllodytes edelmoi* in 19 bromeliads (Dry season n=7; Rainy season n=12) was observed with similar frequency. It should be noted that significant difference shown for canopy closure in rainy season (Table S2) could be explained by its spatial autocorrelated nature (Table S3). On the other hand, *P. edelmoi* presence and absence in

bromeliad in dry season (Table 1) has significantly higher pH [$5 (\pm 0.67 \text{ s.d.})$] than bromeliads in rainy season (Mann-Whitney U test, $z = 14$, $P = 0.02$). Moreover, bromeliads with absence of frogs shown variability in microhabitat temperature (t-test, $t = -3.83$ $P = 0.001$) and ambient air temperature (t-test, $t = -2.38$ $P = 0.02$) for sampled seasons (Table 1).

Binary GLM analysis for each *Phyllodytes edelmoi* stage (males and tadpoles) in bromeliads took into account eight uncorrelated variables (Table S5, Table S6). In the Moran's I analysis, variables associated with bromeliads samples show no significant autocorrelation (except canopy closure and relative humidity but they were dismissed by stepwise AIC), at least 15 meters apart for both seasons ($p < 0.05$; Table S3, Table S4, Figure S3). Variables that influenced *P. edelmoi* adult selection and tadpole presence in bromeliads included diameter, number of leaves, and relative bromeliad volume of water (Table 2). Patterns of tadpole presence and adult selection of bromeliad with variable effects were plotted (Figure 1 A- F). Models fit also were obtained by HL goodness of fit, and no significance ($p < 0.05$) was found for adult selection ($\chi^2 = 6.0777$, $df = 8$, $p = 0.64$), and tadpole presence ($\chi^2 = 6.2432$, $df = 8$, $p = 0.62$) showing that both models seemed appropriate.

Molecular analyses (qPCR) of *Phyllodytes edelmoi* adults ($n = 22$) and tadpoles ($n = 18$) showed presence of *Bd*. In general, bromeliads with positive adult amphibians also showed positive tadpole samples ($n = 3$), and aggregate tadpoles in bromeliad were positive as well. Similarly, negative samples of bromeligenous adults correspond to negative samples of tadpoles ($n = 4$). *Phyllodytes edelmoi* adults exhibited an average GE load of $40.6 (\pm 82.7 \text{ s.d.})$, and prevalence was $31.8 \% (7/22)$. In the same way, *P. edelmoi* tadpoles showed a prevalence of $41.1 \% (7/17)$. Eight variables were used for a binary logistic regression (GLM) (microhabitat temperature, pH, diameter, volume, leaves number, column total capacity, canopy closure, and season). Overall variables associations with *Bd* were selected by stepwise AIC (Table 3). Although canopy closure was spatially correlated ($p = 0.003$), it was not selected by AIC criterion for the model.

Bd presence was associated negatively with phytotelm diameter, and positively with maximum column capacity for store water (Table 3). Nevertheless, only phytotelm diameter was significant for the model ($p = 0.039$). Variables on the model interaction were plotted for presence of *Bd* from anurans in bromeliads (Figure 2). Hosmer and Lemeshow (HL) goodness of fit test showed no significance ($p > 0.05$), so the model fit well for our *Bd* presence data (*P. edelmoi* infected with *Bd* $\chi^2 = 9.4577$, d.f. = 8, $p = 0.305$).

DISCUSSION

Phyllodytes edelmoi does not choose bromeliads randomly. This species was more frequent in bromeliads with smaller diameter, more number of leaves, and relative more volume of water. Furthermore, the chytrid fungus in *P. edelmoi* was found in individuals that inhabit smaller bromeliad diameter. In other words, this bromeligenous anuran jumps into the trap by selecting the optimum microhabitat for *Bd* presence, probably because is more likely to found more individuals in this bromeliad trait. In density-dependent transmission, contact rate between susceptible and infected individuals could be amplified by the higher number of *P. edelmoi* individuals. For instance, evidence of frog host density affecting the transmission rates of *Bd* were observed in temperate regions and microcosmos studies (e.g. Briggs et al. 2010; Venesky et al. 2014). Alternatively, the selection of this bromeliad trait may reflect that less rich aquatic microfauna in smaller bromeliads may give an advantage for *Bd* presence. In fact, microorganisms such as daphnia, ciliates and rotifers are effective for regulating *Bd* infection because they consume *Bd* zoospores in aquatic environments (e.g. Buck et al. 2011, Schmeller et al. 2013). Also, in our model *P. edelmoi* choice was influenced by water availability, and the number of leaves (that capture water), these traits are maximized in a smaller bromeliad.

Phyllodytes edelmoi individuals were positive for *Bd* in the *Mata Bananeira*, ESEC Murici. Furthermore, the chytrid fungus shows high prevalence on this bromeligenous species, but

relative low infection intensity. In fact, high prevalence patterns had been reported for south AF including phytotelm microhabitats (Rodriguez et al. 2014). Specifically, anurans that inhabit phytotelm appear with a high prevalence of 26.8 % (95% CI 0.21.9–32.4) (Rodriguez et al. 2014). Similarly, prevalence reported in our study fits well within the parameters of prevalence in the south of the AF. We found relative fewer *Bd*-infected individuals (tadpoles and adults) during the dry season, and it seems like that there is less infection intensity in this season as well. These open patches of bromeliads are areas exposed to high temperatures, and it is plausible that *Bd* infection has been regulated by a “hot bath” (Forrest & Schlaepfer 2011). Presence of *Bd* was detected despite the temperature constrain in this habitat. Also our observations suggest that vertical transmission of *Bd* may occur between parents and tadpoles. Although our sample is very small, similar research direction was suggested by Holmes et al. (2014) with Jamaican bromeliad anurans with parental care. Alternately, it is possible that adults transmit innate immune defenses for *Bd* resistance (e.g. Walke et al. 2011).

In a region with high temperatures and less precipitation as found in the North AF (Alvares et al. 2013, Vasconcelos et al. 2014), we expected that *Bd* might experiment a limited zoospore growth (Becker & Zamudio 2011), although not its exclusion within bromeliads. Alternately, different *Bd* strains (Rosenblum et al. 2013) may have different precipitation tolerance and temperature constraints (Stevenson et al. 2013), and our pattern could be more influenced by the type of strain (which is unknown for bromeliad species) or by local adaptation that make possible *Bd* persists in this harsh climatic condition. However, the presence of *Bd* in certain bromeliads in rocky outcrops may be explained by other factors. First, Cossel & Lindquist (2009) found *Bd* inside phytotelm water of bromeliads which may be responsible to infect individuals. Second, other host species (bromeliculous anurans) might carry *Bd* inside bromeliads and may alter behaviors, such as schooling or foraging, changing the likelihood of encountering *Bd* or transmitting it to other individuals (Searle et al. 2011). For instance, in the outcrops patches in

the ESEC Murici, *Pristimatis ramagii* (Craugastoridae) was sporadically found in bromeliads. Finally, species that have tolerance to open rocky outcrops may be less susceptible to *Bd* (Becker & Zamudio 2011). Therefore, it may be possible that the presence of *Bd* in open areas might be linked to an interspecific host dynamic (Becker et al. 2014).

Bromeliad morphological traits that are related to size as volume of water, number of leaves, size of leaves, and size of bromeliad have been related to anurans presence in the neotropical region (e.g. Bandoni & Navas 2004, Stuckert et al. 2009, Pederassi et al. 2012, Poelman et al. 2013). Specifically, presence of a bromeligenous hylid species of the genus *Scinax* in the AF has been related to a larger bromeliad size (Bandoni & Navas 2004, Pederassi et al. 2012). In contrast, our study shows that selection was not random but differs from this bromeligenous anuran. Smaller phytotelm were the preferred choice by *P. edelmoi* males. This selection can be explained by its reproductive mode (Haddad & Prado 2005) that might show parental care and territorial behavior in tree holes or bromeliads (pers. obs.). For instance, species of poison frogs (family Dendrobatidae) from Costa Rica and Ecuador that display some type of parental care have selected smaller phytotelm (Brown et al. 2008, Ryan & Barry 2011). Moreover, this preference was observed in closer related species, and has evolved at least for this group of dendrobatids (Brown et al. 2008). In other words, breeding habitat selection seems to have evolved together with changes in parental care for niche partitioning. Smaller phytotelm appears to have less microorganism diversity (Kitching 2001, Jocque & Field 2014) and suggests advantages of a less microorganism competitive environment for the tadpoles, predation by other tadpoles or invertebrates (Brown et al. 2008). Furthermore, the choice of *P. edelmoi* individuals for less rich microhabitat may also be an advantage for both survival of tadpoles and maybe *Bd* infection.

The association between *Bd* and bromeligenous anurans suggests that individual behavior variation might help *Bd* to persist during periods of unfavorable abiotic conditions. Specifically,

the limits for *Bd* survival in open rocky outcrop are apparently restricted by desiccation, as in other regions (Puschendorf et al. 2009, Holmes et al. 2014). Similarly, *Bd* cannot survive for longer periods of time without water availability (Johnson & Speare 2005) and consequently, it also needs the largest water reservoir inside the small bromeliad spectrum. *Bd* model found in *P. edelmoi* may be useful for other bromeligenous or bromeliculas species that depend on bromeliad for attain humidity, and temperature environmental constraints. As for many ecological studies (Kitching 2001) it seems like the tank of bromeliads is a widespread, interesting and replicated scenario for the study of *Bd* dynamics.

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Table 1. Microclimatic conditions and phytotelm characteristics of bromeliads with (presence) and without (absence) *Phyllodytes edelmoi* during dry and wet seasons. Phytotelm diameter = Bromeliad diameter, bromeliad length of biggest leaf = Length, bromeliad leaves number = Leaves, Bromeliad maximum column size to store water = Column, bromeliad relative volume = Volume, pH = pH, microhabitat temperature = Tm, ambient air temperature = Ta, ambient Relative humidity = HR %, proportion of obscured sky by vegetation in a single point = Canopy closure (%). All data given as mean \pm standard deviation (s.d); (*) Significance level for (W) Mann-Whitney pairwise comparisons or (t) Welch t-test ($p < 0.05$); (.) Significance level ($p < 0.1$).

Variable	Presence				Absence			
	Dry Mean (s.d.)	Rainy Mean(s.d.)	Test	P	Dry Mean (s.d.)	Rainy Mean (s.d.)	Test	P
Season								
Diameter (cm)	15.24 (\pm 4.26)	17.3 (\pm 3.15)	t = -1.13	0.28	18.3 (\pm 3.05)	17.7(\pm 4.24)	t = -0.50	0.62
Length (cm)	64.5 (\pm 24.7)	78.1 (\pm 14.64)	t = -1.31	0.22	77.3 (\pm 14.9)	67.6 (\pm 17.2)	t = -1.92	0.06
Leaves (number)	15 (\pm 2.38)	16.1 (\pm 1.87)	W = 28	0.34	14.1 (\pm 2.6)	14.8 (\pm 3.05)	W = 187	0.55
Column depth (cm)	27.6 (\pm 6.9)	30.4 (\pm 3.76)	t = -0.99	0.35	26.8 (\pm 5.2)	31.3 (\pm 4.63)	t = 2.92	0.005*
Volume (L)	0.17 (\pm 0.13)	0.21(\pm 0.09)	t = -0.69	0.50	0.14 (\pm 0.07)	0.17(\pm 0.09)	t = 0.96	0.34
pH	5 (\pm 0.67)	4.3 (\pm 0.72)	W = 14	0.03*	5.25 (\pm 0.46)	4.25 (\pm 0.52)	W = 39	0.001*
Tm (°C)	27.5 (\pm 3.25)	26.4 (\pm 4.57)	W = 29	0.42	27.8 (\pm 3.01)	24.1 (\pm 3.06)	t = -3.83	0.001*
Ta (°C)	30.53 (\pm 2.38)	29.3 (\pm 4.93)	t = = 0.7	0.49	31.57 (\pm 2.94)	28.2 (\pm 5.52)	t = -2.38	0.02*
HR (%)	66.29 (\pm 6.47)	68.8 (\pm 14.58)	t = -0.50	0.62	64.24 (\pm 8.30)	66.2(\pm 15.39)	t = 0.51	0.61
Canopy closure (%)	29.02 (\pm 22.2)	5.9 (\pm 13.03)	W = 13	0.01*	20.6 (\pm 20.44)	9.81 (\pm 12.49)	W = 155	0.15

Table 2. Logistic regressions of *Phyllodytes edelmoi* adult males and tadpoles presence in bromeliads selected by stepwise procedure using Akaike's information criterion. Phytotelm diameter = Bromeliad diameter, bromeliad leaves number = Leaves, bromeliad relative volume = Volume. Adult (n=50); Tadpole (n=56). (*) Significance level ($P < 0.05$) for Wald z-statistic test.

Stage	Variable	Estimate	Std. Error	Wald z-statistic	Pr(> z)
Adult Male	(Intercept)	-2.0043	2.6203	-0.765	0.4443
	Bromeliad diameter	-45.6142	18.3325	-2.488	0.0128*
	Leaves	0.411	0.2046	2.009	0.0446*
	Volume	11.1298	5.3394	2.084	0.0371*
Tadpole	(Intercept)	-2.3674	1.9885	-1.191	0.2338
	Bromeliad diameter	-28.2119	12.4228	-2.271	0.0231*
	Leaves	0.3539	0.1489	2.377	0.0174*
	Volume	5.891	4.0026	1.472	0.1411

Table 3. Logistic regressions for the presence of *Batrachochytrium dendrobatidis* in bromeliads in habitat by *Phyllodytes edelmoi* selected by stepwise procedure using Akaike's information criterion. Phytotelm diameter = Bromeliad diameter, bromeliad maximum Column capacity to store water = Column depth. (n = 20). (*) Significance level (P <0.05) for Wald z-statistic test. (.) Significance level (P <0.1).

Species	Variable	Estimate	Std. Error	Wald z	Pr(> z)
<i>Batrachochytrium dendrobatidis</i>	(Intercept)	1.226	3.16	0.388	0.6979
	Bromeliad diameter	-108.328	52.388	-2.068	0.0387*
	Column depth	52.854	29.592	1.786	0.0741.

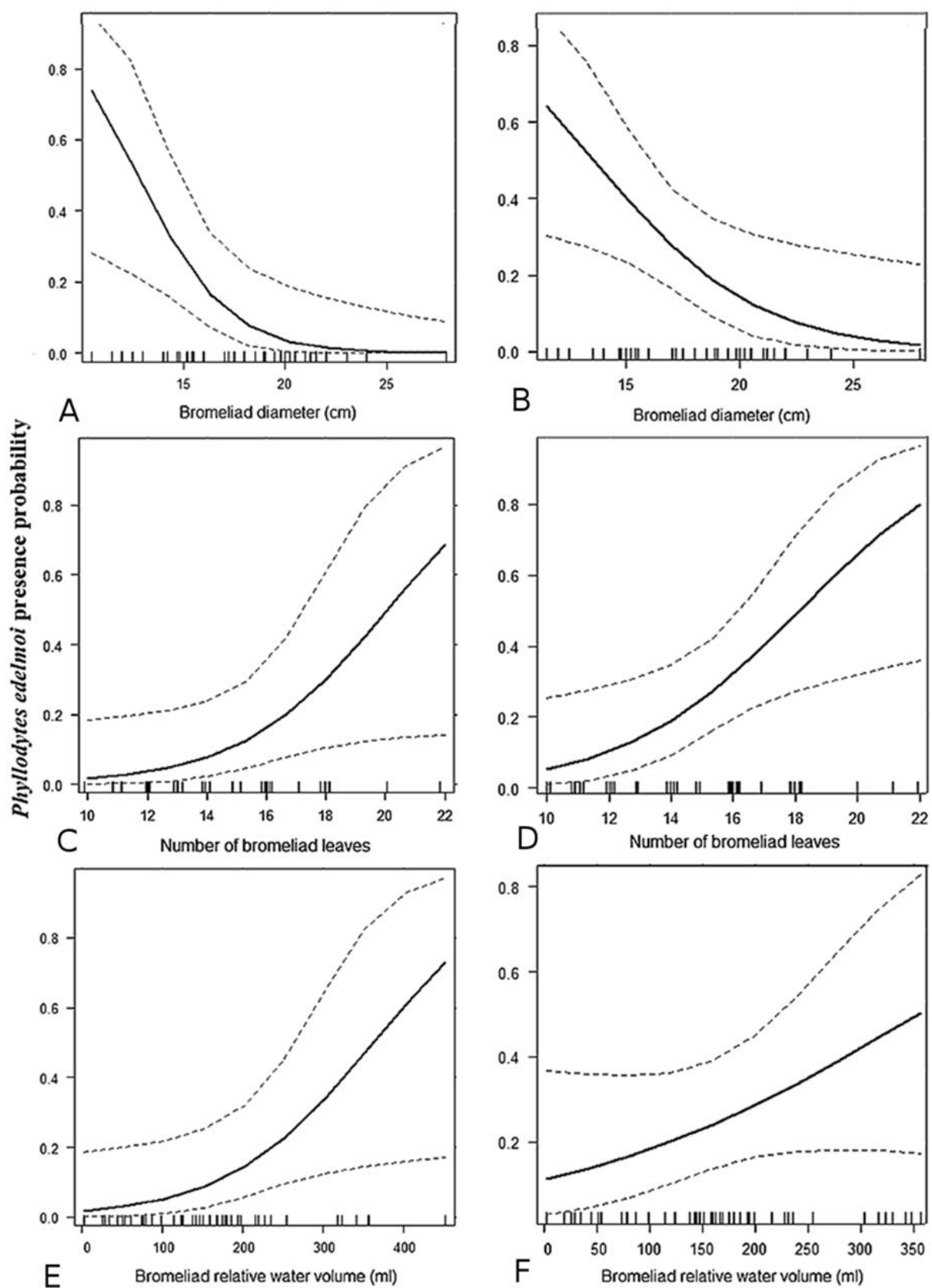


Figure 1. Interaction of bromeliad diameter (cm), number of leaves, and relative volume of water (L) in the logit model fit to *Phyllodytes edelmoi* adult males presence data in *Portea leptantha* bromeliad. Vertical axis is labeled on the probability scale, and a 95-percent pointwise confidence interval is drawn around the estimated effect. Horizontal axis shows observe values. Phytotelm diameter = Bromeliad diameter, bromeliad relative volume of tank = Volume. (A, C, E) Adult and (B, D, F) Tadpole.

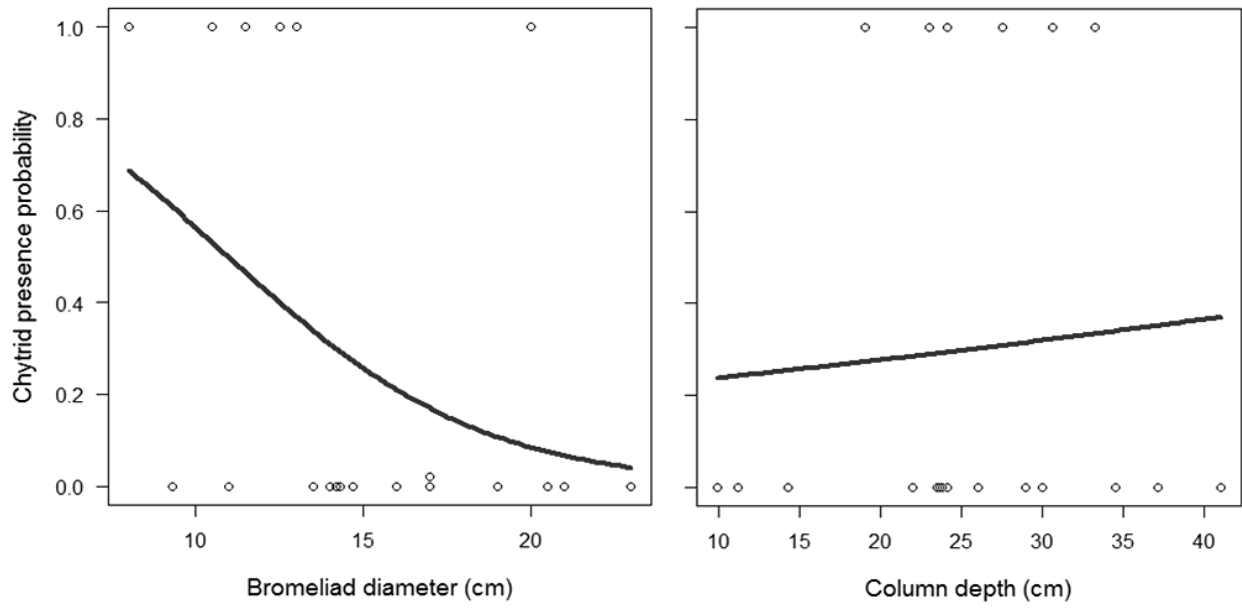


Figure 2. Interaction of bromeliad diameter (cm) and bromeliad maximum column depth to store water (ml) in the logit model fit to *Bd* presence data in *Portea leptantha* bromeliad. (A) Phytotelm diameter = Bromeliad diameter, (B) Bromeliad maximum Column capacity to store water = Column depth

5 GENERAL DISCUSSION

Survey data in the ESEC of Murici, Alagoas showed that *Bd* is a common pathogen in amphibians on this area. The chytrid fungus was found on 6 of 7 screened families and 71.2% (15/21) of those species were positive for *Bd*. Nevertheless, amphibian die-offs or mortality were neither observed nor evidence of adult clinical signals for *Bd* was detected. This supports the idea that for the Atlantic forest adults are asymptomatic to *Bd* and apparently it is not the main factor of amphibian decline in the Atlantic forest (BECKER et al., 2007; RODRIGUEZ et al., 2014; TOLEDO, 2006).

Batrachochytrium dendrobatidis was found in three bromeligenous species (*Phyllodytes acuminatus*, *P. edelmoi*, and *P. gyrinaethes*) in the ESEC Murici, north Atlantic forest. Furthermore, *Bd* had high prevalence on these bromeligenous species (e.g. *P. edelmoi* 31.8 % (7/22); *P. gyrinaethes* 20.8 % (5/24); excluding *P. acuminatus* $n=1$), but relative low infection intensity. Specifically, *P. edelmoi* adults exhibited an average GE load of 40.6 (\pm 82.7 s.d.) and *P. gyrinaethes* have higher average of GE load 359.16(\pm 555.53 s.d.). In fact, high prevalence patterns had been reported for South Atlantic forest phytotelm microhabitat samples (RODRIGUEZ *et al.*, 2014). Similarly, prevalence reported in our study fits well within the prevalence parameters (95% CI 0.21.9 – 32.4) in the south of the Atlantic forest (RODRIGUEZ *et al.*, 2014). Until now, the strain found in the ESEC de Murici was *Bd*-GPL but the sample was extracted from another hylid frog *Aplastodiscus sibilatus*. Specific survey for *Bd* strain is needed for *Phyllodytes*.

The choice of *P. edelmoi* individuals for less rich microhabitat may also be an advantage for both survival of tadpoles and maybe *Bd* infection. Smaller phytotelm appears to have less microorganism diversity (KITCHING, 2001; JOCQUE; FIELD, 2014) and proposes advantages of a less microorganism competitive environment for the tadpoles, or predation by other tadpoles or invertebrates (BROWN; MORALES; SUMMERS, 2008). Also microorganisms such as daphnia, ciliates and rotifers consume *Bd* zoospores in aquatic environments (BUCK; TRUONG; BLAUSTEIN, 2011; SCHMELLER et al., 2013) suggesting a less diverse microcosms for *Bd* predators in smaller bromeliads.

Phyllodytes edelmoi does not select bromeliad tanks randomly. The species was more frequent in bromeliads with smaller diameter, more number of leaves, and relative more volume of water. These bromeliad traits possibly were maximized in a smaller phytotelm. This selection can be explained in parts by its reproductive mode (HADDAD; PRADO, 2005) that show some kind of parental care and territorial behavior (Pers. obs.) in tree holes or bromeliads. In other words, breeding habitat selection may evolved together with changes in parental care for niche partitioning and for a successful survival (BROWN; MORALES; SUMMERS, 2008). The North Atlantic forest which has high temperature regimes (ALVARES et al., 2013) could be a good example of how constraints may produce a careful pick of the type of bromeliad by bromeligenous species.

Phyllodytes edelmoi model may be useful for other bromeligenous species that depend on bromeliad for attain humidity, and surmount temperature environmental constraints. Also this association between *Bd* and bromeligenous anurans suggests that individual behavior variation might help *Bd* to persist during periods of unfavorable abiotic conditions and possibly amplifying the infecting to more individuals. Specifically, the limits for *Bd* survival in open rocky outcrop are apparently restricted by desiccation, as in other regions (HOLMES; MCLAREN; WILSON, 2014; PUSCHENDORF et al., 2011). Furthermore, discovering which traits are important for *Bd* presence may lead to effective *in situ* mitigation procedures in a small scale context. As for many ecological studies (KITCHING, 2001) it seems like the tank of bromeliads is a widespread, interesting and replicated scenario for the study of *Bd* dynamics.

6 CONCLUSIONS

Batrachochytrium dendrobatidis is a common pathogen of anurans species in the Brazilian Atlantic forest domain.

The chytrid fungus was found positive for three anurans species, *Phyllodytes acuminatus*, *P. edelmoi* and *P. gyrinaethes*, in the ESEC de Murici, Alagoas State, Brazil, suggesting that is a common disease for bromeligenous anurans in this region.

The chytrid fungus in two bromeligenous species, *Phyllodytes edelmoi* and *P. gyrinaethes*, has high prevalence but relative low infection intensity in the ESEC de Murici, Alagoas State, Brazil. This high prevalence is similar to southern Atlantic forest *Bd* dynamic.

The chytrid fungus had higher probability of presence in *Phyllodytes edelmoi* individuals that inhabit smaller bromeliads with maximum capacity to store water.

Phyllodytes edelmoi individuals selected smaller *Portea leptantha* bromeliads with more leaves and relative more volume of water. These traits could be maximized in smaller bromeliads for its dependence on bromeliad for attain humidity, and surmount temperature environmental constraints.

Phyllodytes edelmoi preference of smaller *Portea leptantha* bromeliads is associated with more infected individuals of *Bd* and thus amplifying *Batrachochytrium dendrobatidis* presence in the environment. In other words, bromeliad traits chosen by this bromeligenous species showed that frog optimum microhabitat may be related to the chytrid fungus probability of presence due to the density of individuals that choose this microhabitat.

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APPENDIX A

CHAPTER 1 ELECTRONIC SUPPLEMENTARY MATERIAL

Table S1. Anuran species infected by *Batrachochytrium dendrobatidis* in the Atlantic forest (AF), Amazonia (AM) and Cerrado (CE) in the states of Pernambuco (PE), Alagoas (AL), Bahia (BA), Minas Gerais (MG), Espírito Santo (ES), Goiás (G), Rio de Janeiro (RJ), São Paulo (SP), Paraná (PR), Santa Catarina (SC), and Rio Grande do Sul (RS).

Family / Species	State	Biome	Source
Aromobatidae			
<i>Allobates olfersioides</i>	RJ	AF	Carnaval et al. 2006
Bufo			
<i>Rhinella crucifer</i>	AL	AF	Present study
<i>Frostius pernambucensis</i>	AL	AF	Present study
<i>Melanophryniscus dorsalis</i>	SC	AF	Rodriguez et al. 2014
<i>Melanophryniscus moreirae</i>	RJ, MG	AF	Sluys et al. 2007, Ferreira et al. 2008, Rodriguez et al. 2014
Brachycephalidae			
<i>Brachycephalus didactylus</i>	RJ	AF	Rodriguez et al. 2014
<i>Ischnocnema guentheri</i>	SP	AF	De Paula 2011, Gründler et al. 2012, Rodriguez et al. 2014
<i>Ischnocnema henselii</i>	SC	AF	Rodriguez et al. 2014
<i>Ischnocnema nasuta</i>	SC	AF	Rodriguez et al. 2014
<i>Ischnocnema parva</i>	SP	AF	De Paula 2011, Rodriguez et al. 2014
<i>Ischnocnema radorum</i>	SP	AF	De Paula 2011
Centrolenidae			
<i>Vitreorana eurygnatha</i>	RJ, SP, SC	AF	Rodriguez et al. 2014
<i>Vitreorana</i> sp.	MG	AF	Rodriguez et al. 2014
<i>Vitreorana uranoscopa</i>	RJ, SP, SC	AF	Rodriguez et al. 2014
Ceratophryidae			
<i>Ceratophrys aurita</i>	ES, SP, SC	AF	Rodriguez et al. 2014
Craugastoridae			
<i>Haddadus binotatus</i>	SP	AF	De Paula 2011, Gründler et al. 2012, Rodriguez et al. 2014
<i>Holoaden luederwaldti</i>	SP	AF	Rodriguez et al. 2014
<i>Pristimantis ramagii</i>	AL	AF	Present study
<i>Pristimantis vinhai</i>	BA	AF	Present study
Cycloramphidae			
<i>Cycloramphus asper</i>	SP	AF	Rodriguez et al. 2014
<i>Cycloramphus boraceiensis</i>	SP	AF	Gründler et al. 2012, Rodriguez et al. 2014
<i>Cycloramphus brasiliensis</i>	RJ	AF	Rodriguez et al. 2014
<i>Cycloramphus eleutherodactylus</i>	SP	AF	Rodriguez et al. 2014
<i>Cycloramphus fuliginosus</i>	RJ	AF	Rodriguez et al. 2014
<i>Cycloramphus izecksohni</i>	SC	AF	Rodriguez et al. 2014
<i>Cycloramphus ohausi</i>	RJ	AF	Rodriguez et al. 2014
<i>Cycloramphus semipalmatus</i>	SP	AF	Rodriguez et al. 2014
<i>Cycloramphus stejnegeri</i>	RJ	AF	Rodriguez et al. 2014
<i>Cycloramphus valae</i>	RS	AF	Rodriguez et al. 2014
<i>Thoropa miliaris</i>	SP	AF	Carnaval et al. 2006, Rodriguez et al. 2014
<i>Thoropa taophora</i>	SP	AF	Toledo et al. 2006a, Gründler et al. 2012, Rodriguez et al. 2014
<i>Thoropa petropolitana</i>	RJ	AF	Rodriguez et al. 2014
Dendrobatidae			
<i>Adelphobates galactonotus</i>	PA	AM	Present study
Hemiphraetidae			
<i>Fritziana fiissilis</i>	MG, RJ, SP, SC	AF	Rodriguez et al. 2014
<i>Fritziana goeldii</i>	RJ, SP	AF	Rodriguez et al. 2014
<i>Fritziana ohausi</i>	SP	AF	Gründler et al. 2012, Rodriguez et al. 2014
Hylidae			
<i>Aparasphenodon brunoi</i>	SP	AF	Rodriguez et al. 2014

<i>Aplastodiscus arilidae</i>	SP	AF	De Paula 2011
<i>Aplastodiscus callipygius</i>	MG	AF	Toledo et al., 2006a, Gründler et al. 2012
<i>Aplastodiscus leucopygius</i>	SP	AF	De Paula 2011, Gründler et al. 2012
<i>Aplastodiscus cf. leucopygius</i>	SP	AF	Toledo et al. 2006a
<i>Aplastodiscus perviridis</i>	MG	AF	Gründler et al. 2012, Rodriguez et al. 2014
<i>Aplastodiscus sibilatus</i>	AL	AF	Lisboa et al. 2013
<i>Dendropsophus anceps</i>	RJ	AF	Rodriguez et al. 2014
<i>Dendropsophus minutus</i>	AL	AF	Becker & Zamudio 2011, De Paula 2011, Gründler et al. 2012, Rodriguez et al. 2014, Present study
<i>Dendropsophus microps</i>	MG	AF	De Paula 2011, Gründler et al. 2012, Rodriguez et al. 2014
<i>Dendropsophus seniculus</i>	RJ	AF	Rodriguez et al. 2014
<i>Bokermannohyla astarteae</i>	SP	AF	De Paula 2011
<i>Bokermannohyla circumdata</i>	SP	AF	Toledo et al. 2006a, De Paula 2011, Gründler et al. 2012, Rodriguez et al. 2014
<i>Bokermannohyla gouveai</i>	MG	AF	Carnaval et al. 2006
<i>Bokermannohyla hylax</i>	SP	AF	Toledo et al. 2006a, Gründler et al. 2012
<i>Bokermannohyla luctosa</i>	SP	AF	Gründler et al. 2012, Rodriguez et al. 2014
<i>Bokermannohyla pseudopseudis</i>	GO	CE	Ramalho et al. 2013
<i>Bokermannohyla sapiranga</i>	GO	CE	Ramalho et al. 2013
<i>Hypsiboas albomarginatus</i>	AL	AF	De Paula 2011, Rodriguez et al. 2014, Present study
<i>Hypsiboas albopunctatus</i>	SP	AF	Toledo et al. 2006a
<i>Hypsiboas bischoffii</i>	SP	AF	De Paula 2011, Rodriguez et al. 2014
<i>Hypsiboas crepitans</i>	PE	AF	Present study
<i>Hypsobas exastis</i>	AL	AF	Present study
<i>Hypsiboas faber</i>	SP	AF	De Paula 2011, Vieira et al. 2012, Gründler et al. 2012, Rodriguez et al. 2014
<i>Hypsiboas freicanecae</i>	AL	AF	Carnaval et al. 2006, Lisboa et al. 2013
<i>Hypsiboas latistriatus</i>	MG	AF	Gründler et al. 2012
<i>Hypsiboas semilineatus</i>	MG	AF	Toledo et al. 2006a
<i>Hypsiboas pardalis</i>	SP	AF	De Paula 2011, Gründler et al. 2012, Rodriguez et al. 2014
<i>Hypsiboas polytaenius</i>	SP	AF	Rodriguez et al. 2014
<i>Hypsiboas pulchellus</i>	RS	AF	Rodriguez et al. 2014
<i>Hypsiboas prasinus</i>	SP, SC, PR	AF	Gründler et al. 2012, Rodriguez et al. 2014
<i>Itapotihyla langsdorffii</i>	SP	AF	Rodriguez et al. 2014
<i>Phrynomedusa cf. marginata</i>	SP	AF	Toledo et al. 2006a
<i>Phyllodytes cf. acuminatus</i>	AL	AF	Present study
<i>Phyllodytes cf. edelmoi</i>	AL	AF	Present study
<i>Phyllodytes edelmoi</i>	AL	AF	Present study
<i>Phyllodytes gyrinaethes</i>	AL	AF	Present study
<i>Phyllodytes tuberculosus</i>	BA	AF	Present study
<i>Phyllomedusa burmeisteri</i>	MG, ES	AF	Rodriguez et al. 2014
<i>Phyllomedusa distincta</i>	SP, PR, SC, RS	AF	Rodriguez et al. 2014
<i>Phyllomedusa nordestina</i>	AL	AF	Present study
<i>Phyllomedusa tetraploidea</i>	SC	AF	Rodriguez et al. 2014
<i>Scinax albicans</i>	RJ	AF	Toledo et al. 2006a
<i>Scinax alter</i>	SP	AF	De Paula 2011
<i>Scinax ariadne</i>	SP	AF	Rodriguez et al. 2014
<i>Scinax cf. eurydice</i>	MG	AF	Present study
<i>Scinax fuscovarius</i>	RS	AF	Rodriguez et al. 2014
<i>Scinax granulatus</i>	RS	AF	Rodriguez et al. 2014
<i>Scinax hayii</i>	SP	AF	Gründler et al. 2012, Rodriguez et al. 2014
<i>Scinax machadoi</i>	MG	AF	Rodriguez et al. 2014
<i>Scinax nebulosus</i>	AL	AF	Present study
<i>Scinax pachycrus</i>	PE	AF	Present study
<i>Scinax perpusillus</i>	RJ	AF	Rodriguez et al. 2014
<i>Scinax ranki</i>	MG	AF	Rodriguez et al. 2014
<i>Scinax trapicheiroi</i>	RJ	AF	Rodriguez et al. 2014
<i>Trachycephalus mesophaeus</i>	SC	AF	Rodriguez et al. 2014

Hylodidae

<i>Crossodactylus bokermanni</i>	MG, SP	AF	Rodriguez et al. 2014
<i>Crossodactylus caramaschii</i>	SP	AF	Carnaval et al. 2006, Rodriguez et al. 2014
<i>Crossodactylus gaudichaudii</i>	RJ, SP	AF	Rodriguez et al. 2014
<i>Crossodactylus</i> sp.	RJ	AF	Rodriguez et al. 2014
<i>Hylodes amnicola</i>	MG	AF	Rodriguez et al. 2014
<i>Hylodes asper</i>	SP	AF	De Paula 2011, Gründler et al. 2012, Rodriguez et al. 2014
<i>Hylodes cardosi</i>	PR	AF	Vieira et al. 2012
<i>Hylodes dactylocinus</i>	SP	AF	Toledo et al. 2006a
<i>Hylodes glaber</i>	RJ	AF	Rodriguez et al. 2014
<i>Hylodes heyeri</i>	SP	AF	Rodriguez et al. 2014
<i>Hylodes lateristrigatus</i>	RJ, SP	AF	Rodriguez et al. 2014
<i>Hylodes magalhaesi</i>	MG	AF	Carnaval et al. 2005, Toledo et al. 2006b, Gründler et al. 2012
<i>Hylodes meridionalis</i>	RS	AF	Toledo et al. 2006a, Rodriguez et al. 2014
<i>Hylodes nasus</i>	RJ	AF	Rodriguez et al. 2014
<i>Hylodes ornatus</i>	RJ	AF	Vieira et al. 2013, Rodriguez et al. 2014
<i>Hylodes perere</i>	MG	AF	Rodriguez et al. 2014
<i>Hylodes perplicatus</i>	SC	AF	Toledo et al. 2006a, Rodriguez et al. 2014
<i>Hylodes phyllodes</i>	SP	AF	Toledo et al. 2006a, De Paula 2011, Gründler et al. 2012
<i>Hylodes</i> sp. (aff. <i>sazimai</i>)	SP	AF	Toledo et al. 2006a
<i>Hylodes</i> sp.	SC	AF	Rodriguez et al. 2014
<i>Megaelosia</i> cf. <i>boticariana</i>	SP	AF	Toledo et al. 2006a
<i>Megaelosia massarti</i>	SP	AF	Toledo et al. 2006a
<i>Megaelosia</i> sp.	RJ, SP	AF	Rodriguez et al. 2014
Leptodactylidae			
<i>Adenomera marmorata</i>	SP	AF	Rodriguez et al. 2014
<i>Leptodactylus labyrinthicus</i>	SP	AF	Rodriguez et al. 2014
<i>Leptodactylus marmoratus</i>	SP	AF	Gründler et al. 2012
<i>Leptodactylus</i> cf. <i>mystaceus</i>	AL	AF	Present study
<i>Leptodactylus notoaktites</i>	SP, PR	AF	Rodriguez et al. 2014
<i>Leptodactylus podicipinus</i>	RS	AF	Rodriguez et al. 2014
<i>Paratelmatoobius lutzii</i>	RJ	AF	Rodriguez et al. 2014
<i>Physalaemus</i> cf. <i>cuvieri</i>	AL	AF	Present study
<i>Physalaemus olfersii</i>	SP	AF	Rodriguez et al. 2014
<i>Physalaemus signifer</i>	RJ	AF	Brito-Gitirana et al. 2009
Microhylidae			
<i>Arcovomer passarellii</i>	RJ, SP	AF	Rodriguez et al. 2014
<i>Stereocyclops incrassatus</i>	SC	AF	Rodriguez et al. 2014
Odontophrynidae			
<i>Macrogenioglottus alipioi</i>	SP	AF	Rodriguez et al. 2014
<i>Proceratophrys boiei</i>	MG, RJ, SP, PR, SC	AF	Rodriguez et al. 2014
<i>Proceratophrys melanopogon</i>	RJ, SP	AF	Rodriguez et al. 2014
<i>Proceratophrys renalis</i>	AL	AF	Present study
Ranidae			
<i>Lithobates catesbeianus</i>	RS	AF	Rodriguez et al. 2014

Table S2. Detection method for anuran species infected with *Batrachochytrium dendrobatidis* in different regions of the Atlantic forest. H: Histology, P: PCR, qP: qPCR, C: Cytology, I: Isolation in culture. Based on data present in the Table S1.

Family	Genera	Species	Detection method	Species infected / sampled species	Atlantic forest region
Aromobatidae	1	1	H	1/1	Southeast
Brachycephalidae	2	6	P, qP	6/50	Southeast, south
Bufo	3	4	qP	4/39	Northeast, southeast, south
Centrolenidae	1	3	qP	3/3	Southeast, south
Ceratophryidae	1	1	qP	1/3	Southeast, south
Craugastoridae	3	4	P, qP	4/14	Northeast, southeast
Cycloramphidae	2	13	H, P, qP	13/36	Southeast, south
Hemiphractidae	1	3	qP	3/13	Southeast
Hylidae	11	55	H, P, qP	55/209	Northeast, southeast, South
Hylodidae	3	23	H, P, qP, C, I	23/40	Southeast, south
Leptodactylidae	4	10	H, qP	10/64	Northeast, southeast, south
Microhylidae	2	2	qP	2/21	Southeast, south
Odontophrynidae	2	4	qP	4/21	Northeast, southeast, south
Ranidae	1	1	qP	1/2	South

APPENDIX B

CHAPTER 2 ELECTRONIC SUPPLEMENTARY MATERIAL

Table S1. Voucher information and *Bd* accession numbers for specimens of *Phyllodytes edelmoi* used in *Bd* analysis collected in the Estação Ecológica de Murici (ESEC Murici), Alagoas, Brazil.

<i>Bd</i> Voucher	Museum Voucher	<i>Bd</i> Voucher	Museum Voucher
SLFT 2988	11794	SLFT 1897	11484
SLFT 2996	11800	SLFT 1898	na
SLFT 2997	11801	SLFT 1904	11489
SLFT 2998	11804	SLFT 1906	na
SLFT 2999	11805	SLFT 1907	na
SLFT 3000	11806	MLFT 0036	11513
SLFT 3001	11807	MLFT 0037	11520
SLFT 3002	11808	MLFT 0038	11519
SLFT 3003	11811	MLFT 0039	11518
SLFT 3043	11571	MLFT 0040	11517
SLFT 3044	11570	MLFT 0041	11516
SLFT 1891	11478	MLFT 0042	11515
SLFT 1892	11479	MLFT 0043	11514
SLFT 1893	11480	MLFT 0044	11521
SLFT 1894	na	MLFT 0045	11522
SLFT 1895	11482	MLFT 0046	11523
SLFT 1896	11483	MLFT 0047	11524

Table S2. Measurements of microclimatic conditions and phytotelm characteristics in bromeliads with and without *Phyllodytes edelmoi* for dry and wet season. Phytotelm diameter = Bromeliad diameter, bromeliad length of biggest leaf = Length, bromeliad leaves number = Leaves, Bromeliad maximum column size to store water = Column, bromeliad relative volume = Volume, pH = pH, microhabitat temperature = Tm, ambient air temperature = Ta, ambient Relative humidity = HR %, proportion of obscured sky by vegetation in a single point = Canopy closure (%). All data given as mean \pm standard deviation (s.d); (+) = Presence, (-) = Absence; (*) Significance level for (W) Mann-Whitney pairwise comparisons or (t) Welch t-test ($p < 0.05$); (·) Significance level ($p < 0.1$).

Season	Dry				Rainy				
	Variable	+ Mean (s.d.)	- Mean (s.d.)	Test	P	+ Mean(s.d.)	- Mean (s.d.)	Test	P
	Diameter (cm)	15.24 (± 4.26)	18.3 (± 3.05)	t = -1.6	0.12	17.3(± 3.15)	17.7(± 4.24)	t = -0.26	0.79
	Length (cm)	64.5 (± 24.7)	77.3(± 14.9)	t = -1.3	0.23	78.1 (± 14.64)	67.6 (± 17.2)	t = -1.3	0.23
	Leaves (number)	15 (± 2.38)	14.1 (± 2.6)	t = 0.8	0.43	16.1 (± 1.87)	14.8 (± 3.05)	W = 76.5	0.16
	Column depth (cm)	27.6 (± 6.9)	26.8 (± 5.2)	t = -0.04	0.96	30.4 (± 3.76)	31.3 (± 4.63)	t = 0.28	0.79
	Volume (L)	0.17 (± 0.13)	0.14 (± 0.07)	W = 73	1.0	0.21(± 0.09)	0.17(± 0.09)	t = 1.19	0.24
	pH	5 (± 0.67)	5.25 (± 0.46)	t = -0.93	0.38	4.3 (± 0.72)	4.25 (± 0.52)	W = 106	0.88
	Tm (°C)	27.5 (± 3.25)	27.8 (± 3.01)	t = -0.20	0.84	26.4 (± 4.57)	24.1 (± 3.06)	t = 0.27	0.79
	Ta (°C)	30.53 (± 2.38)	31.57 (± 2.94)	W = 59	0.45	29.3 (± 4.93)	28.2 (± 5.52)	t = -0.95	0.36
	HR (%)	66.29 (± 6.47)	64.24 (± 8.30)	t = 0.67	0.51	68.8 (± 14.58)	66.2(± 15.39)	t = 0.67	0.51
	Canopy closure (%)	29.02 (± 22.2)	20.6 (± 20.44)	W = 55	0.33	5.9 (± 13.03)	9.81(± 12.49)	W = 55	0.02*

Table S3. Moran's I test for autocorrelation of variables in dry season in *Portea leptantha* bromeliads. Phytotelm diameter = Bromeliad diameter, bromeliad length of biggest leaf = Length, bromeliad leaves number = Leaves, Bromeliad maximum column size to store water = Column depth, bromeliad relative volume = Volume, pH = pH, microhabitat temperature = Tm, ambient air temperature = Ta, ambient Relative humidity = HR %, proportion of obscured sky by vegetation in a single point = Canopy Clousure (%), bromeligenous spatial presence = Anuro. All data given as (\pm) standard deviation (s.d). Lag= 15 meters; (*)Significance level ($p < 0.05$).

Variables	estimate	expectation	variance	s.d.	P(I) Two sided
Bromeliad diameter (cm)	0.153704	-0.038462	0.033181	1.055	0.5829
Length (cm)	-0.019088	-0.038462	0.031879	0.1085	1
Leaves (#)	-0.019088	-0.038462	0.031879	0.1085	1
Column depth (cm)	0.0578994	-0.037037	0.0099769	0.9505	0.6932
Volume (L)	-0.199274	-0.038462	0.031056	-0.9125	0.723
pH	-0.081607	-0.037037	0.010074	-0.4441	0.8119
Tm (°C)	0.0499968	-0.037037	0.0094491	0.8953	0.7796
Ta (°C)	0.089596	-0.038462	0.033137	0.7035	0.9635
HR (%)	0.107023	-0.038462	0.033158	0.799	0.8486
Canopy Clousure (%)	0.380335	-0.038462	0.032341	2.3288	0.03974 *
Anuro	0.024226	-0.038462	0.033937	0.3403	0.8628

Table S4. Mornan's I test for autocorrelation of variables in Rainy season in *Portea leptatha* bromeliads. Phytotelm diameter = Bromeliad diameter, bromeliad length of biggest leaf = Length, bromeliad leaves number = Leaves, Bromeliad maximum column size to store water = Column depth, bromeliad relative volume = Volume, pH = pH, microhabitat temperature = Tm, ambient air temperature = Ta, ambient Relative humidity = HR %, proportion of obscured sky by vegetation in a single point = Canopy closure (%), bromeligenous spatial presence = Anuran. All data given as (\pm) standard deviation (s.d). Lag= 15 meters; (*)Significance level ($p < 0.05$).

Variables	estimate	expectation	variance	s.d.	P(I) Two sided
Bromeliad diameter (cm)	0.143222	-0.038462	0.010731	1.7539	0.397
Length (cm)	0.069037	-0.038462	0.011232	1.0143	1
Leaves (number)	0.102816	-0.038462	0.011209	1.3344	0.364
Column depth (cm)	-0.0047863	-0.0384615	0.0106793	0.3259	1
Volume (L)	0.0087133	-0.0384615	0.010708	0.4559	0.648
pH	-0.194304	-0.038462	0.01026	-1.5385	0.4957
Tm (°C)	0.150922	-0.038462	0.010875	1.816	0.072.
Ta (°C)	0.117213	-0.038462	0.011158	1.4738	0.281
HR (%)	0.2087076	-0.0384615	0.011257	2.3296	0.039*
Canopy closure (%)	0.049326	-0.038462	0.010541	0.855	0.785
Anuran	0.1588671	-0.0384615	0.0115504	1.8361	0.2654

Table S5. Spearman correlations of microclimatic conditions and bromeliad characteristics in rainy season. Variables with normal data were included. Bromeliad maximum column size to store water = Column, phytotelm diameter = Diameter, bromeliad water level = H2O, ambient Relative humidity = HR %, bromeliad leaves number = Leaves, bromeliad length of biggest leaf = Length, ambient Relative humidity = HR %, microhabitat temperature = Tm, ambient air temperature = Ta, bromeliad relative volume = Volume. (*) p values with significance level of ($p < 0.05$).

	Column	Diameter	H2O	HR %	Leaves	Length	Ta	Tm	Volume
Column	1								
Diameter	0.5374	1							
H2O	0.4707	0.469	1						
HR %	-0.2115	-0.0821	-0.1	1					
Leaves	0.2037	0.6836	0.3	-0.113	1				
Length	0.5438	0.298	0.46	-0.181	0.258	1			
Ta	-0.1413	-0.0853	0.06	-0.8*	0.019	-0.028	1		
Tm	-0.1469	-0.0633	-0.1	-0.537	0.025	0.026	0.81*	1	
Volume	-0.1792	0.1675	0.24	0.159	0.273	0.126	0.14	0.225	1

Table S6. Spearman correlations of microclimatic conditions, and bromeliad characteristics in dry season. Variables with normal data were included. Bromeliad maximum column size to store water = Column, phytotelm diameter = Diameter, bromeliad water level = H2O, ambient Relative humidity = HR %, bromeliad leaves number = Leaves, bromeliad length of biggest leaf = Length, pH = pH. (*) P values with significance level of ($p < 0.05$).

	Column	Diameter	H2O	HR %	Leaves	Length	pH
Column	1.00						
Diameter	0.44	1.00					
H2O	0.72*	0.27	1.00				
HR %	-0.40	-0.06	-0.22	1.00			
Leaves	0.61	0.22	0.36	-0.17	1.00		
Length	0.46	0.46	0.28	-0.16	0.26	1.00	
pH	0.07	0.09	-0.13	0.01	0.05	0.25	1.00

Figure S1. Map of sampled localities. Brazil, Alagoas, Murici, ESEC Murici, *Mata da Bananeira*. Inset map is South America with Atlantic forest in green. Square highlights the north portion of Atlantic forest. Satelital image with yellow contourn represents Estação Ecológica de Murici (ESEC Murici) whereas A represents *Mata da Bananeira* forest fragment.

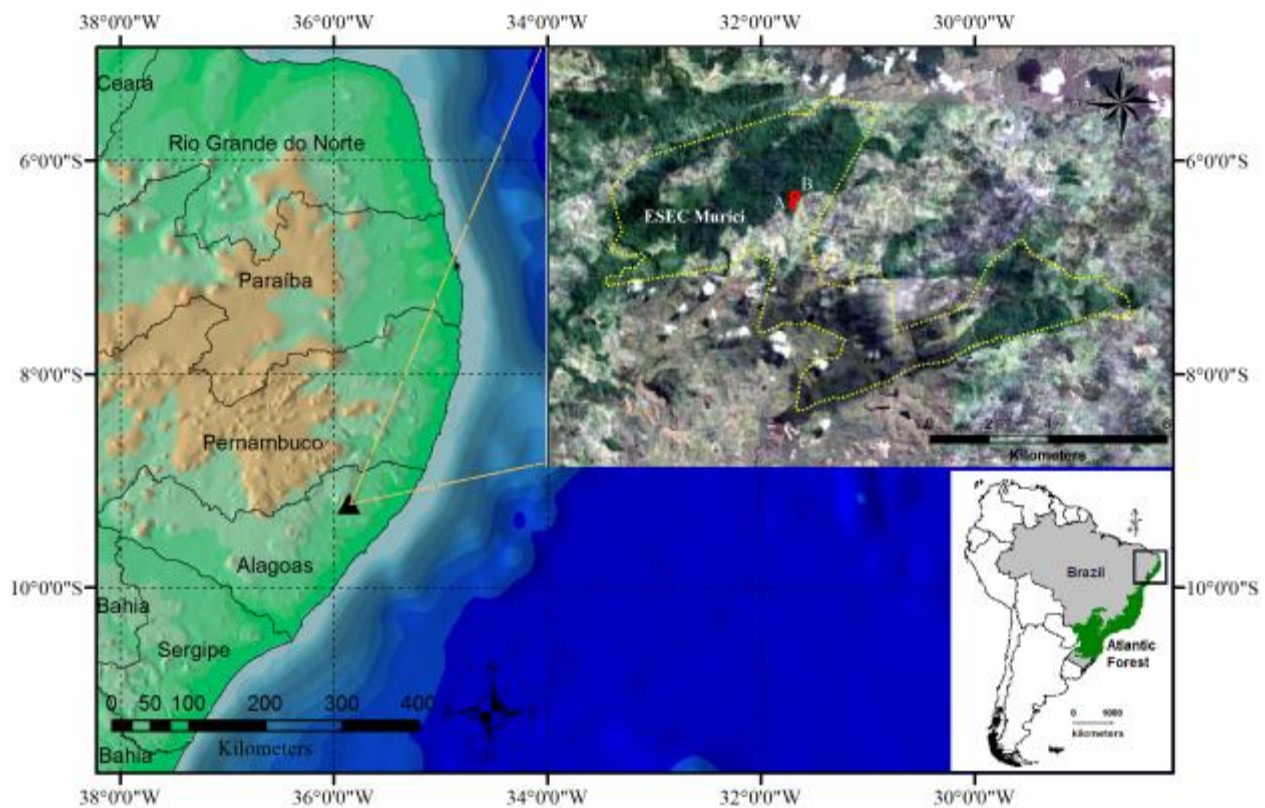


Figure S2. Site and species sampled in the (A) rocky outcrops in the ESEC Murici, Alagoas State, Brazil; (B) bromeliad *Portea leptantha*; (C) adult and (D) tadpoles of *Phyllodytes edelmoi*.



Figure S3. Linkage maps of bromeliad samples distances (m) for autocorrelation analysis. (A) Rainy season, and (B) dry season.

