



## Variation in phenotype and virulence among enzootic and panzootic amphibian chytrid lineages



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### ABSTRACT

The Global Panzootic Lineage of the fungus *Batrachochytrium dendrobatidis* (*Bd*-GPL) is threatening amphibians worldwide. In contrast, four lineages (*Bd*-Brazil, *Bd*-CH, *Bd*-Cape, and *Bd*-Korea) that diverged early in the history of *Bd* have not yet been directly linked to amphibian declines. *Bd* likely evolves in response to strong selective pressure imposed by hosts and the environment, leading to differences among pathogen phenotypes and genotypes that may directly affect virulence. Here, we report on variation in phenotype, genotype, and virulence of *Bd*-Brazil and *Bd*-GPL. Specifically, we (i) used a controlled infection experiment to compare virulence between one *Bd*-Brazil and three *Bd*-GPL isolates on a North American amphibian host (*Lithobates sylvaticus*), (ii) tested for relative phenotypic and genotypic differentiation among *Bd* isolates from Brazil, and (iii) tested for possible correlations between environmental variables and *Bd* phenotypes. We found substantial variation in virulence among *Bd*-GPL isolates and found that our *Bd*-Brazil isolate showed virulence comparable to an average North American *Bd*-GPL. North American hosts infected with a *Bd*-GPL isolate from Panama did not show significant mortality. *Bd* phenotypes varied significantly across sampling locations; these phenotypes were neither spatially clustered nor correlated with any environmental variables. Additionally, we found a surprising lack of correlation between genotypic divergence and zoospore and zoosporangium sizes in our sample. Although *Bd*-Brazil was less virulent infecting *L. sylvaticus* than one *Bd*-GPL isolate, this endemic lineage still caused ~50% mortality in our experimental North American hosts. This indicates that *Bd*-Brazil has the potential to kill amphibians if introduced to naïve wild populations. Our findings underscore that characterizing virulence of multiple *Bd* isolates and lineages is important for understanding the evolutionary history and diversity of *Bd*.

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### 1. Introduction

Emerging infectious diseases have potentially destructive effects on wildlife, often causing severe population declines and extinctions (Daszak et al., 2000). Many pathogens are highly sensitive to variation in temperature, precipitation, and humidity (Colhoun,

1973; Altizer et al., 2006), and thus their distributions and dynamics can be limited by macro- and micro-environmental conditions in their invasive ranges (Lomolino et al., 2009). Several recent epizootic outbreaks have been attributed to changes in environmental stressors that presumably trigger rapid pathogen proliferation and expansion (Epstein, 2001; Harvell et al., 2002; Pounds et al., 2006). Environment alone, however, does not explain variation in pathogen virulence and disease outbreaks. Genotypic differences among pathogen isolates can directly affect virulence (Fisher et al., 2009; Yap et al., 2014), and many pathogens show high adaptive potential, modifying both their phenotypes and

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genomes (Farrer et al., 2013) to increase transmission and fitness when encountering new hosts and environments (Reece et al., 2009; Searle et al., 2015). Thus, our ability to understand mechanisms underlying pathogen virulence depends upon characterizing both phenotypic and genetic diversity that is present in pathogen populations, and examining how those factors interact with hosts and the environment to produce different disease outcomes (Fisher et al., 2009; James et al., 2015).

The emergence of the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) is a leading cause of global amphibian declines, especially in highly diverse tropical amphibian communities (Berger et al., 1998; Longcore et al., 1999; Lips et al., 2008; Cheng et al., 2011). Early comparative genetic studies of *Bd* isolates found low genetic diversity consistent with a rapid expansion of the pathogen (Morehouse et al., 2003; James et al., 2009). However, the recent discovery of highly divergent lineages from Brazil, South Africa, Switzerland, and Korea have vastly increased the known genetic diversity within this globally distributed pathogen (Farrer et al., 2011; Schloegel et al., 2012; Bataille et al., 2013). Genomic resequencing of global isolates revealed that a recently diversified and hypervirulent Global Panzootic Lineage (GPL) is associated with massive amphibian population declines in Central America, Australia, and North America (Farrer et al., 2011; Rosenblum et al., 2013). Four additional lineages, *Bd*-CH, *Bd*-Cape, *Bd*-Korea, and the narrowly distributed *Bd*-Brazil, are the earliest divergences in the evolutionary history of *Bd* populations (Bataille et al., 2013; Rosenblum et al., 2013; Jenkinson et al., 2016), and at least one of those lineages (*Bd*-Cape) showed lower virulence when compared to GPL (Farrer et al., 2011). Thus far, the phenotypic and genetic diversity and virulence of other non-GPL isolates have not been characterized.

Brazil harbors a large diversity of amphibian hosts (Toledo and Batista, 2012; Haddad et al., 2013) and is also home to two *Bd* lineages (Schloegel et al., 2012; Rodriguez et al., 2014; James et al., 2015). *Bd*-Brazil, a highly divergent lineage from *Bd*-GPL, was isolated from field localities in southeastern Brazil (Schloegel et al., 2012). In contrast, *Bd*-GPL is widespread throughout the whole Atlantic Forest range (Rodriguez et al., 2014; Jenkinson et al., 2016). Extensive field collections have now demonstrated that *Bd*-Brazil coexists (in some cases syntopically) with *Bd*-GPL in the southern part of the *Bd*-GPL range (Jenkinson et al., 2016), and retrospective analyses confirm that both lineages have been endemic in the Atlantic Forest for over 100 y (Rodriguez et al., 2014). Furthermore, a hybrid isolate between *Bd*-Brazil and *Bd*-GPL was discovered within a narrow range of 10 km in southern Brazil (Jenkinson et al., 2016). Despite the high genetic diversity of *Bd* in Brazil's Atlantic Forest, amphibian populations in Brazil do not show the massive population declines that are typical of areas of GPL emergence (Toledo et al., 2006; Becker and Zamudio, 2011; Becker et al., 2014, 2016a, 2016b; Rodriguez et al., 2014). Although a few retrospective studies report the disappearance of stream-breeding frog species in some well-studied areas (Heyer et al., 1988; Weygoldt, 1989; Eterovick et al., 2005), none of the published reports have to date directly attributed these population changes to *Bd* emergence. Therefore, host-pathogen dynamics of *Bd* in Brazil might fundamentally differ from those observed in the western US, Central America, and Australia. The long term presence of *Bd*-Brazil in the Atlantic Forest, combined with the apparent absence of population declines in areas of high *Bd* prevalence has led to the suggestion that the endemic *Bd*-Brazil predates the invasion of *Bd*-GPL (Jenkinson et al., 2016), and that, perhaps, amphibian populations in Brazil are not significantly affected by *Bd* in the wild (Becker and Zamudio, 2011; Becker et al., 2016a). Lower *Bd* susceptibility in Brazilian amphibians could be due to the shared evolutionary history between *Bd*-Brazil and local amphibian hosts that may have

permitted the evolution of host defenses, especially if *Bd*-Brazil is less virulent than *Bd*-GPL. In this scenario, the evolution of host defenses developed against *Bd*-Brazil may also have provided protection against *Bd*-GPL at the time of its emergence. Additionally, *Bd*-Brazil has been recently detected in the U.S. on an individual of *Lithobates catesbeianus* imported from a Brazilian bullfrog farm (Schloegel et al., 2012). The presence of *Bd*-Brazil in the U.S. underscores the importance of testing the virulence of this lineage in North-American amphibians.

Here, we report on variation in phenotype and virulence among *Bd*-GPL and *Bd*-Brazil isolates. We used a controlled infection experiment to compare virulence among one *Bd*-Brazil and three *Bd*-GPL isolates on North American amphibians. We also genotyped and phenotypically profiled eight new isolates of *Bd* collected in Brazil, and tested for relative genotypic and phenotypic differentiation among them. Finally, we examined possible correlations between environmental variables and phenotypic differentiation among isolates. We found that *Bd* isolates, even those recently derived from a common ancestor, are phenotypically differentiated independent of environmental variables, which may have consequences for virulence and disease emergence.

## 2. Materials and methods

### 2.1. Larval infections

We estimated virulence of a *Bd*-Brazil isolate (UM142) and three *Bd*-GPL isolates (JEL258 and JEL404 isolated in Maine, U.S.A., and JEL423 isolated in Panama) using North American wood frogs (*Lithobates sylvaticus*) as the experimental host. We collected eight egg masses from a pond in Orono, Maine, U.S.A., and once tadpoles reached Gosner stage 26–30 (limb buds just beginning to emerge) we randomly assigned 10 tadpoles each to 20 tanks containing 8 L of water. Four tanks (40 tadpoles total) were then assigned to each of five treatments (four different *Bd* isolate treatments and one negative control). Experimental tanks were maintained at 22 °C with a 12 hr-light/12 hr-dark cycle. *Bd* inoculations were performed on day 0 and on day 17 using  $10^6$  zoospores per tank. Metamorphosis began on day 21, and metamorphs were moved to different containers in groups, with a small pool of water and wet paper towels. Survival was monitored daily, dead animals were noted, and dying animals were euthanized with an overdose of MS-222 if they showed lack of righting response, which is a typical sign of advanced stages of chytridiomycosis (Voyles et al., 2009). The experiment concluded on day 70, at which point all remaining animals were euthanized.

### 2.2. *Bd* isolates, phenotypes, and genetic characterization

Eight isolates of *Bd* were collected from May 2010 to February 2013 from infected tadpoles sampled at various Atlantic Forest localities (Table 1). Anuran larvae were screened in the field with a 10× hand lens for signs of chytridiomycosis by assessing the level of oral tissue dekeratinization (Knapp and Morgan, 2006). Animals with signs of *Bd* infection were euthanized, and oral tissues excised. Infected tissues were prepared for pathogen isolation on 1% tryptone agar with 0.2 mg mL<sup>-1</sup> penicillin-G and 0.4 mg mL<sup>-1</sup> streptomycin sulfate. Isolates of *Bd* were maintained on 1% tryptone agar at 20–21 °C until sufficient growth had occurred for DNA extraction.

We measured phenotypes for the same eight *Bd* isolates (Table 1) following methods described in previous studies (Fisher et al., 2009; Flechas et al., 2013; Lambertini et al., 2016). Each *Bd* isolate was inoculated into 5 mL of liquid medium composed of 1% tryptone and incubated at 21 °C for 7 d. At the end of that period we

**Table 1**

*Batrachochytrium dendrobatidis* isolates (voucher code and lineage), zoospore and zoosporangium diameters ( $\mu\text{m}$ ) [mean  $\pm$  standard deviation (range)], host species, and collecting sites: latitude/longitude, elevation (above sea level), municipality, state (MG = Minas Gerais; SP = São Paulo; PR = Paraná).

Isolate	Lineage	Zoospore diameter ( $\mu\text{m}$ )	Zoosporangium diameter ( $\mu\text{m}$ )	Amphibian host	Latitude/Longitude	Elevation (m)	Locality
CLFT 001	<i>Bd-Brazil</i>	3.81 $\pm$ 0.61 (2.70–4.99)	29.29 $\pm$ 7.88 (18.26–48.43)	<i>Hylodes japi</i>	–23.249292/ –46.949915	1100	Jundiá, SP
CLFT 021	<i>Bd-GPL</i>	4.29 $\pm$ 0.56 (2.99–5.37)	31.40 $\pm$ 6.51 (18.86–49.99)	Unidentified	–23.314360/ –47.098605	705	Cabreúva, SP
CLFT 023	<i>Bd-GPL</i>	3.79 $\pm$ 0.65 (2.47–5.52)	33.17 $\pm$ 6.48 (23.56–49.67)	<i>Hypsiboas</i> sp.	–22.866361/ –46.033068	1540	Camanducaia, MG
CLFT 024.1	<i>Bd-GPL</i>	4.49 $\pm$ 0.55 (3.55–6.07)	36.35 $\pm$ 5.73 (26.93–58.84)	<i>Hylodes cardosoi</i>	–25.358990/ –48.878570	250	Morretes, PR
CLFT 026	<i>Bd-GPL</i>	3.90 $\pm$ 0.76 (2.41–6.20)	24.49 $\pm$ 3.96 (17.24–40.90)	<i>Hypsiboas faber</i>	–24.590532/ –48.626003	95	Iporanga, SP
CLFT 029-00	<i>Bd-GPL</i>	3.66 $\pm$ 0.54 (2.68–5.37)	21.45 $\pm$ 3.75 (13.22–33.85)	<i>Hypsiboas</i> cf. <i>albopunctatus</i>	–23.241410/ –46.937760	1120	Jundiá, SP
CLFT 029-01	<i>Bd-GPL</i>	4.21 $\pm$ 0.69 (2.70–5.79)	23.69 $\pm$ 4.56 (16.59–38.09)	<i>Scinax hiemalis</i>	–23.241410/ –46.937760	1120	Jundiá, SP
CLFT 034	<i>Bd-GPL</i>	3.87 $\pm$ 0.63 (2.90–5.24)	21.54 $\pm$ 6.06 (10.80–35.76)	<i>Hylodes phyllodes</i>	–23.707690/ –46.035859	271	Biritiba-Mirim, SP

aliquoted cultures of each *Bd* isolate in duplicate onto slides and took 30 photographs of random quadrats of each slide at 1000 $\times$  magnification. We measured the diameter of the 60 largest zoospores and the 60 largest zoosporangia from each isolate using TSVIEW<sup>®</sup> software (see Lambertini et al., 2016).

To characterize genotypes, we harvested mature zoospores and sporangia from 7 d-old cultures by scraping fungal tissue from agar plates. We extracted DNA using a standard CTAB miniprep protocol with chloroform and isoamyl alcohol (Zolan and Pukkila, 1986) and amplified each culture at 10 polymorphic loci. We used markers 8009X2, BdC24, BdC5, BdSC4.3, BdSC4.16, BdSC6.15, BdSC8.10, BdSC3.1, BdSC7.6, and R6064 because they distinguish *Bd-Brazil* from *Bd-GPL* and are useful for studying Atlantic Forest populations (Jenkinson et al., 2016; Lambertini et al., 2016). Successful amplicons were purified using ExoSAP-IT (Affymetrix), and PCR products cycle-sequenced with fluorescently labeled terminator nucleotides using the BigDye reaction kit (Applied Biosystems).

### 2.3. Environmental correlates of *Bd* phenotypes

For each source locality for our *Bd* isolates (Table 1) we recorded spatial information on latitude, and quantified elevation, mean annual temperature, and mean annual rainfall using WorldClim/Bioclim layers (1000 m resolution), available at <http://www.worldclim.org/bioclim> (Hijmans, 2005). These metrics of temperature and precipitation are averaged from 50 y records (1950–2000) from a network of climatic stations throughout the globe.

### 2.4. Statistical analyses

We quantified mortality for each of the five experimental groups of wood frogs (four groups, each infected as tadpoles with one of the four experimental *Bd* isolates and one uninfected control group). We used Analysis of Variance (ANOVA) to compare mean mortality at the end of the experiment in the five treatments, using tanks as replicates, and made pairwise comparisons between treatment means with the Fisher LSD test.

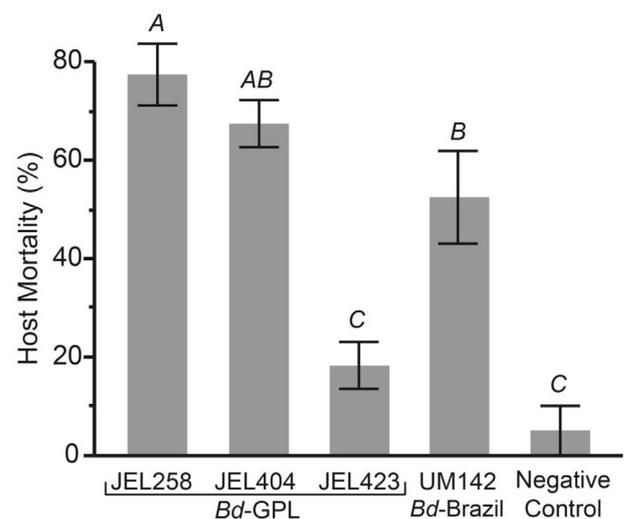
We used ANOVAs and the same *post-hoc* LSD tests for pairwise multiple comparisons to compare mean zoospore and zoosporangium size among the eight focal *Bd* isolates from Brazil. We computed genotypic distance among the eight isolates using the hetequal method (James et al., 2009), and then standardized distance values by the greatest distance measured (D/Dmax) (Fisher et al., 2009). D/Dmax values were compared to phenotypic

distances [scaled zoospore size (DZSP/DZSPmax) and scaled zoosporangium size (DSPOR/DSPORmax)] with statistical significance assessed by Mantel testing with 1000 permutations (Fisher et al., 2009). Finally, we used simple linear regressions to test for associations between our four measured environmental variables and *Bd* zoospore size and zoosporangium size.

## 3. Results

### 3.1. Assessing virulence of *Bd-Brazil* and *Bd-GPL* isolates to wood frogs infected as larvae

The ranked mortality (from highest to lowest) for frogs exposed to the four *Bd* isolates was: JEL258 (*Bd-GPL*), JEL404 (*Bd-GPL*), UM142 (*Bd-Brazil*), and JEL423 (*Bd-GPL*) (Fig. 1). JEL423 (*Bd-GPL*) was the only isolate that did not cause significant mortality of *L. sylvaticus* compared to the control treatment (ANOVA:  $F_{[4,15]} = 24.812$ ,  $P < 0.001$ ). The *post-hoc* test showed that isolate JEL258 (*Bd-GPL*) caused significantly higher mortality than JEL423, UM142, and negative controls.



**Fig. 1.** Comparison of post-metamorph mortality in experimental infections with four *Bd* isolates: three GPL isolates (JEL404 and JEL 258 from Maine; JEL423 from Panama) and one *Bd-Brazil* isolate (UM142). *Bd-Brazil* and two *Bd-GPL* isolates resulted in significantly higher mortality of *L. sylvaticus* metamorphs compared to the control treatment. Different letters indicate statistically different groups in Fisher LSD test.

### 3.2. *Bd* isolates, phenotypes, and genetic characterization

Eight *Bd* isolates from seven different localities in Brazil varied significantly in the diameter of zoospores (ANOVA:  $F_{[7,472]} = 12.854$ ,  $P < 0.001$ ) and zoosporangia ( $F_{[7,472]} = 56.943$ ,  $P < 0.001$ ). *Bd*-Brazil zoospore diameter averaged less than most *Bd*-GPL isolates, and was among the smallest of all measured isolates (Table S1, S2). Although isolates differed significantly, we found no geographical clustering of similar phenotypes. Finally, neither zoospore ( $r = -0.392$ ,  $P = 0.388$ ) nor zoosporangium size ( $r = -0.236$ ,  $P = 0.115$ ) was significantly correlated with genotype (Fig. 2).

### 3.3. Climatic correlates of phenotype

We found no significant associations between environmental variables and *Bd* phenotypes [Zoospore size (latitude -  $F_{[1,6]} = 2.406$ ,  $\beta = -0.183$ ,  $P = 0.172$ ; elevation -  $F_{[1,6]} = 0.462$ ,  $\beta = -0.001$ ,  $P = 0.346$ ; mean annual precipitation -  $F_{[1,6]} = 0.064$ ,  $\beta = -0.001$ ,  $P = 0.808$ ; mean annual temperature -  $F_{[1,6]} = 0.475$ ,  $\beta = 0.004$ ,  $P = 0.377$ ). Zoosporangium size (latitude -  $F_{[1,6]} = 0.497$ ,  $\beta = -1.851$ ,  $P = 0.507$ ; elevation -  $F_{[1,6]} = 0.039$ ,  $\beta = 0.001$ ,  $P = 0.850$ ; mean annual precipitation -  $F_{[1,6]} = 0.297$ ,  $\beta = -0.001$ ,  $P = 0.605$ ; mean annual temperature -  $F_{[1,6]} = 0.061$ ,  $\beta = -0.020$ ,  $P = 0.813$ ].

## 4. Discussion

Host populations require sufficient genetic variation and time to evolve resistance to novel pathogens (Ewald, 1995). Therefore, newly introduced pathogen lineages often pose larger threats to host populations than locally endemic ones (Daszak et al., 2000; Duffy and Sivars-Becker, 2007; Farrer et al., 2011). We thus predicted that our North American host *L. sylvaticus* would be more susceptible to *Bd*-Brazil and the Panamanian isolate *Bd*-GPL JEL423 than to local *Bd*-GPL isolates, which have not caused known recent amphibian declines and may have been present in the northeastern U.S.A. for over a century (Schloegel et al., 2012; Talley et al., 2015; Jenkinson et al., 2016). Contrary to our predictions, however, our focal host experienced high mortality rates when infected with two local *Bd*-GPL isolates but did not experience significant mortality when infected with the Panamanian GPL isolate, despite

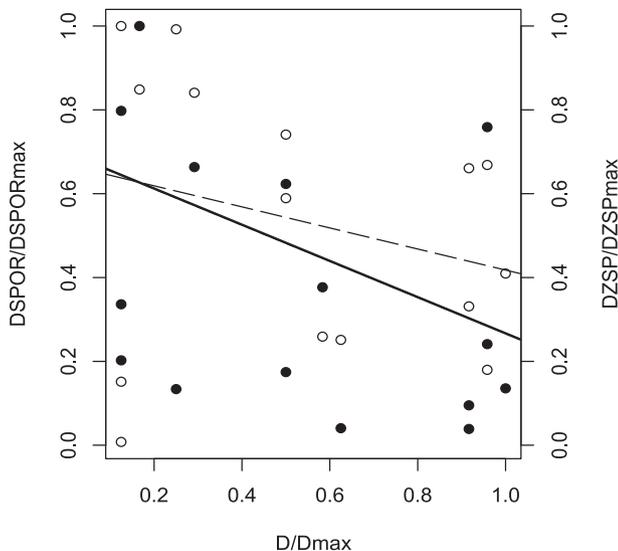
experimental studies showing high virulence of Panamanian GPL while infecting other North American amphibians (Gahl et al., 2011, 2012). Moreover, the virulence of our *Bd*-Brazil isolate fell at the high end of the distribution of virulence exhibited by GPL.

We found that *Bd*-GPL isolates from Brazil show significant phenotypic variation. Other studies have also documented high variation in zoospore and zoosporangium size of *Bd* from different regions, and sometimes at very small spatial scales (Fisher et al., 2009; Farrer et al., 2011; Schloegel et al., 2012; Flechas et al., 2013; Lambertini et al., 2016). These findings suggest that *Bd* can evolve in response to selective pressures imposed by their hosts and/or the environment (LoVerde et al., 1985; Altizer et al., 2003; Thrall and Burdon, 2003). Although *Bd* zoospore and zoosporangium sizes from different sampling localities differed significantly, these two phenotypic metrics were not spatially clustered or linked to any environmental predictor that we tested. This result indicates that environmental variation might not play a major role in shaping *Bd* phenotypic differentiation. Because *Bd* is a host-generalist pathogen among amphibians, host density, species diversity, and relative abundance could modulate host-selective pressures independent of the environment. This potential effect of host community structure in shaping *Bd* phenotypes might help explain the observed variation in susceptibility among different amphibian guilds (Lips et al., 2003; Becker et al., 2014) and the reported differences in virulence among genetically similar *Bd* isolates (Gahl et al., 2012).

Previous studies indicate that smaller zoosporangium size is linked to decreased virulence, and the mechanism could be that larger zoosporangia cause greater skin damage (Fisher et al., 2009; Farrer et al., 2011; Lambertini et al., 2016). For instance, an isolate from endemic *Bd*-Cape produced both lower host infection intensities and smaller average zoosporangium size when compared to *Bd*-GPL (Farrer et al., 2011). Furthermore, *Bd*-Brazil caused lower mortality of our experimental hosts than JEL258 in addition to having smaller zoospore size (3.8 vs. 5.4  $\mu\text{m}$ ; Schloegel et al., 2012). Larger zoosporangia are expected to produce more and larger zoospores (Mitchell et al., 2008), and this variation in *Bd* phenotypes could potentially influence both transmission dynamics and virulence (Woodhams et al., 2008). Unfortunately, we lack phenotypic information for JEL404 and JEL423 to test for this relationship across all *Bd* isolates and we have not tested virulence of any of the isolates used to generate our measurements, which precludes making any further inferences about zoospore size and virulence.

A second potential mechanism to explain the observed link between phenotype and virulence among *Bd* isolates is genotypic differentiation (Farrer et al., 2013). However, we found an unexpected lack of correlation between genotypic divergence and zoospore and zoosporangium sizes in our sample. Our results differ from a similar study of mostly European *Bd* isolates, where genetic distance predicted average zoosporangium size but not zoospore production (Fisher et al., 2009). This pattern could arise from localized adaptation to varying environments, but this hypothesis is not supported by our environmental analyses. Variation could also be due to epigenetic differences among isolates or microevolution due to mitotic recombination or changes in chromosome copy number as has been observed to occur rapidly in culture (Refsnider et al., 2015). Nonetheless, the most likely explanation for the observed discordant genotypic and phenotypic variation among isolates is local adaptive divergence of *Bd* to host species, populations, or communities. Although our data cannot specifically test these mechanisms, they suggest that *Bd* virulence and phenotype will only partially be explained by genotypic differences.

In our study the narrowly distributed *Bd*-Brazil had virulence comparable to an average *Bd*-GPL isolate. These findings, along



**Fig. 2.** Scaled genotypic distance ( $D_{\text{GEN}}/GEN_{\text{max}}$ ) against scaled zoospore size ( $D_{\text{ZSP}}/DZSP_{\text{max}}$ ; filled circles and solid line) and scaled zoosporangium size ( $D_{\text{SPOR}}/DSPOR_{\text{max}}$ ; empty circles and dashed line).

with the large phenotypic and genotypic variation in *Bd*-GPL at very narrow geographic ranges independent of environmental conditions, underscore the potential for rapid diversification and flexibility of *Bd*-GPL as a globally invasive lineage (Farrer et al., 2013). Our results also indicate that if *Bd*-Brazil could spread from Brazil to the U.S.A., it could become a novel disease-causing agent in North America. Thus, further investigation on the epidemiology of *Bd*-Brazil is needed. Our *Bd*-Brazil isolate caused ~50% mortality of our experimental North-American host, indicating that lineages endemic in one region might cause negative effects if introduced to naïve populations. Future work should culture additional *Bd*-Brazil isolates to test how variable virulence is within this clade and also to test whether *Bd*-Brazil is less virulent than *Bd*-GPL in Brazilian host species. In addition, the large observed variation in zoospore and zoosporangium sizes may be linked to virulence, and future experiments might inform the role that differences in ploidy levels have in infection dynamics, even when isolates are genetically closely related. Characterizing phenotypes, genotypic diversity, and above all, virulence of *Bd* lineages and isolates has important implications for understanding the consequences of *Bd* diversity globally.

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### Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.funeco.2016.11.007>.

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