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Amphibian-killing chytrid in Brazil comprises both locally endemic and globally expanding populations

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Abstract

Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), is the emerging infectious disease implicated in recent population declines and extinctions of amphibian species worldwide. *Bd* strains from regions of disease-associated amphibian decline to date have all belonged to a single, hypervirulent clonal genotype (*Bd*-GPL). However, earlier studies in the Atlantic Forest of southeastern Brazil detected a novel, putatively enzootic lineage (*Bd*-Brazil), and indicated hybridization between *Bd*-GPL and *Bd*-Brazil. Here, we characterize the spatial distribution and population history of these sympatric lineages in the Brazilian Atlantic Forest. To investigate the genetic structure of *Bd* in this region, we collected and genotyped *Bd* strains along a 2400-km transect of the Atlantic Forest. *Bd*-Brazil genotypes were restricted to a narrow geographic range in the southern Atlantic Forest, while *Bd*-GPL strains were widespread and largely geographically unstructured. *Bd* population genetics in this region support the hypothesis that the recently discovered Brazilian lineage is enzootic in the Atlantic Forest of Brazil and that *Bd*-GPL is a more recently expanded invasive. We collected additional hybrid isolates that demonstrate the recurrence of hybridization between panzootic and enzootic lineages, thereby confirming the existence of a hybrid zone in the Serra da Graciosa mountain range of Paraná State. Our field observations suggest that *Bd*-GPL may be more infective towards native Brazilian amphibians, and potentially more effective at dispersing across a fragmented landscape. We also provide further evidence of pathogen translocations mediated by the Brazilian ranaculture industry with implications for regulations and policies on global amphibian trade.

Keywords: *Batrachochytrium dendrobatidis*, chytridiomycosis, emerging infectious disease, multilocus genotyping, population genetics

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Introduction

Novel fungal diseases are on the rise worldwide (Fisher *et al.* 2012). Highly destructive wildlife and human mycoses continue to emerge including white nose

syndrome of bats (*Pseudogymnoascus destructans*; Blehert *et al.* 2009; Gargas *et al.* 2009; Minnis & Lindner 2013), fungal meningitis (*Cryptococcus* species; Kidd *et al.* 2004; Bartlett *et al.* 2008) and valley fever (*Coccidioides* species; Kirkland & Fierer 1996; Burt *et al.* 1997; Fisher *et al.* 2000). Chytridiomycosis may be the most notorious of these emerging mycoses due to its contributions to dramatic amphibian declines worldwide and its potential to lead to massive biodiversity loss (Berger *et al.* 1998; Rachowicz *et al.* 2006; Skerratt *et al.* 2007). Although the current distributions of these mycoses are often well documented, the factors contributing to their emergence and spread remain largely unknown (Fisher *et al.* 2012). An accurate reconstruction of past disease expansion—including the timing and geography of emergence, as well as the selective environment underlying virulence evolution—is necessary if we are to successfully mitigate the emergence of these pathogens. Understanding pathogen geographic and genetic history is also critical to the prediction of future emergences, new host affiliations and disease outcomes under different environmental scenarios (Burt *et al.* 1996; Fisher *et al.* 2001; Wood *et al.* 2012).

Chytridiomycosis is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (hereafter *Bd*; Longcore *et al.* 1999), which now occurs on all continents except Antarctica (Olson *et al.* 2013). A number of recent studies have explored the genetics of *Bd* associated with amphibian communities in regions experiencing declines. In the best-studied regions of chytridiomycosis outbreaks (California: Morgan *et al.* 2007; Vredenburg *et al.* 2010; Central America: Lips *et al.* 2006; Cheng *et al.* 2011; the Pyrenees: Walker *et al.* 2010; and Australia: Berger *et al.* 1998; Murray *et al.* 2010), *Bd* has recently arrived and in some cases is still spreading. Outbreak-associated pathogen strains in these regions all belong to a single, rapidly expanding clonal lineage (James *et al.* 2009). This globally distributed clone, termed *Bd*-GPL (for *Global Panzootic Lineage*), shows a pattern of low genetic polymorphism without obvious geographic structure (Farrer *et al.* 2011). Recent surveys, however, have revealed the existence of novel *Bd* genotypes that are deeply divergent from potentially the hypervirulent *Bd*-GPL (Farrer *et al.* 2011; Schloegel *et al.* 2012; Bataille *et al.* 2013). These newly discovered genotypes are described from geographic localities (Korea, South Africa, Switzerland and Brazil) that typically are not experiencing disease-associated amphibian declines, demonstrating that the evolutionary history of *Bd* is substantially more complex than previously realized. We now understand that the *Bd* evolutionary tree is composed of multiple anciently diverged lineages (Rosenblum *et al.* 2013), probably with more novel branches that have yet to be discovered.

Our study focuses on the Atlantic Forest (AF) of southeastern Brazil, where one recently discovered novel lineage, *Bd*-Brazil, is hypothesized to be enzootic (Schloegel *et al.* 2012). We chose to investigate the regional population genetics of *Bd* in this zone of deep ancestral variation because the pathogen dynamics in the AF remain enigmatic. *Bd* is widespread in southeastern Brazil (Toledo *et al.* 2006; Lisboa *et al.* 2013; Valencia-Aguilar *et al.* 2015); however, the dramatic, rapid declines of amphibian species, well documented in other Neotropical regions (Lips *et al.* 2006; Cheng *et al.* 2011), have not been observed here. The few modern reports of amphibian declines and local extinctions in this area have not been directly attributed to the emergence of *Bd*, although their timing is contemporaneous with those in the rest of Latin America (Heyer *et al.* 1988; Eterovick *et al.* 2005; Silvano & Segalla 2005).

Retrospective studies of museum-preserved amphibians in Brazil suggest that *Bd* infection prevalence has remained constant in the coastal AF for over a century (approximately 24% prevalence since 1894; Rodriguez *et al.* 2014). Furthermore, highly divergent lineages (*Bd*-GPL and *Bd*-Brazil) that separated from a common ancestor up to 105 000 years ago coexist there (Rosenblum *et al.* 2013) and are capable of hybridizing (Schloegel *et al.* 2012). This was the first report of outcrossing in *Bd*, a pathogen initially thought to only reproduce asexually (Morehouse *et al.* 2003; James *et al.* 2009). Evidence that *Bd* is capable of a sexual cycle in this part of its range is of significant consequence to *Bd* pathogen dynamics, because this creates the possibility that the evolution of virulence in this region, and elsewhere, may be accelerated by sexual recombination.

The Brazilian AF is a major global biodiversity hotspot (Myers *et al.* 2000). Although the biome is highly fragmented and deforested with over 84% of its original range lost (Ribeiro *et al.* 2009), Brazil boasts the highest diversity of amphibian species of any nation (Wake & Vredenburg 2008), and around 60% of these amphibian species are endemic to the AF (Haddad *et al.* 2013). Brazil is also home to the greatest number of North American bullfrog (*Lithobates catesbeianus*) farms in the Western Hemisphere (Schloegel *et al.* 2010). Bullfrogs are highly tolerant to *Bd* infection, show limited disease symptoms (Garner *et al.* 2006), and have become established throughout southeastern Brazil (Both *et al.* 2011), making them a potential vector species (Rödder *et al.* 2013). The ranaculture export industry in Brazil introduces the additional dynamic of non-native amphibians with the capacity to transmit *Bd* asymptomatically and presents a plausible mechanism for the intercontinental movement of *Bd* genotypes (James *et al.* 2015).

Here, we report on a large-scale regional sampling of field-isolated *Bd* strains from the Brazilian AF, with the

goal of characterizing the spatial distribution and population genetic structure of Brazilian *Bd* lineages relative to the globally distributed *Bd*-GPL. These sympatric populations of divergent lineages are an excellent system with which to explore the roles of genetic structure, sexual recombination and local adaptation in shaping the evolution of hypervirulent pathogens. Specifically, our aims were to elucidate the geographic distribution of divergent *Bd* genotypes across the AF, to quantify genetic diversity within and among *Bd* populations occurring in Brazil, to determine whether strains are long-term enzootics or recently introduced, and to identify the extent of sexual recombination and hybridization in the region. We also assessed the relationship of *Bd* genotypes recovered from the AF to a global pool of previously described *Bd* strains. Combined, our results provide insight into the history of chytridiomycosis in a crucial region of amphibian biodiversity and relate the genetics of *Bd* in this region with that of the ongoing global panzootic.

Materials and methods

Field sampling and pathogen isolation

During peak rainfall months (January through February) of 2013 and 2014, we collected native larval anurans at 10 collection sites across six Brazilian states. The infection patterns in amphibian larvae provide a reasonable proxy for infection patterns in the amphibian community across developmental stages. Larvae have been shown to maintain infection throughout metamorphosis (McMahon & Rohr 2015) and are readily infected with *Bd* strains carried by adults sharing the same environment (Greenspan *et al.* 2012; Bataille *et al.* 2013) as most amphibian species do in the Brazilian AF (Haddad *et al.* 2013). Our north–south transect spanned 2400 km of the AF from the northeastern state of Bahia to the southeastern state of Santa Catarina (39.55°W, 15.42°S to 49.9°W, 27.67°S; Fig. 1). We represented collection points less than 10 km apart by a central coordinate for geographic analyses.

We used a 10X hand lens to screen larvae in the field for signs of chytridiomycosis by assessing the level of oral tissue dekeratinization (Knapp & Morgan 2006). We euthanized animals with signs of *Bd* infection by pithing the brain and spinal cord immediately before confirming the infection with a compound microscope. We dissected infected oral tissues for pathogen isolation on 1% tryptone agar with 0.2 mg/mL penicillin-G and 0.4 mg/mL streptomycin sulphate (Longcore 2000). Isolates of *Bd* were maintained on 1% tryptone agar at 20–21 °C until sufficient growth had occurred for DNA extraction. Finally, we cryopreserved replicate cultures of all isolates at −80 °C in 1% tryptone broth with cryoprotectant solution (Boyle *et al.* 2003) and deposited them in the University of Maine chytrid culture collection (JEL) and the Universidade Estadual de Campinas *Bd* culture collection (CLFT).

Multilocus sequence typing

Due to increasing awareness that the genomes of *Bd* isolates change through prolonged laboratory culture (Langhammer *et al.* 2013; Voyles *et al.* 2014), we only passaged new isolates two to three times as necessary before DNA extraction. We harvested mature zoospores and sporangia from ~ 7-day-old culture transfers by aseptically scraping fungal tissue from the surface of the agar medium. We used a standard CTAB miniprep protocol with chloroform and isoamyl alcohol to extract DNA from *Bd* isolates (Zolan & Pukkila 1986). We then amplified DNA extracts with ExTaq DNA polymerase (TaKaRa) and purified the PCR products using ExoSAP-IT (Affymetrix). We Sanger sequenced 12 polymorphic multilocus sequence typing (MLST) loci on an ABI 3730 DNA analyser (Applied Biosystems) at the University of Michigan DNA Sequencing Core. Seven of these MLST markers were previously described (*8009X2*, *BDC5*, *BdSC3.1*, *BdSC4.16*, *BdSC6.15*, *BdSC7.6*, *R6064*; Morehouse *et al.* 2003; Morgan *et al.* 2007; James *et al.* 2009; Schloegel *et al.* 2012). Because previously published markers were designed before the discovery of the *Bd*-Brazil lineage, and may be biased towards capturing variation in *Bd*-GPL, we designed five new markers for this study (*BdSC2.0*, *BdSC6.8*, *BdSC9.1*, *BdSC11.5*, *BdSc16.2*; Table S1, Supporting information). For a subset of our samples, we also sequenced markers *BDC24* (James *et al.* 2009), *BdSC4.3* and *BdSC8.10* (Schloegel *et al.* 2012) to compare with previously published global *Bd* genotypes; however, we discontinued sequencing of these markers when they were observed to be monomorphic within each major lineage in our transect.

To develop the additional markers, we explored a data set of published *Bd* genomes, including representatives of the *Bd*-GPL, *Bd*-Cape and *Bd*-Brazil lineages (Farrer *et al.* 2011; Rosenblum *et al.* 2013), and searched for regions of high potential heterozygosity with a custom-designed, sliding-window PERL script. We also found protein-coding regions containing trinucleotide repeat expansions, which are known to be of potential utility as population informative markers (Di Rienzo *et al.* 1994; Orr & Zoghbi 2007), by BLASTN of the reference genome of *Bd* (JEL423; Broad Institute version 17-Jan-2007). We then screened regions of high relative heterozygosity and variable repeating sequence for polymorphic sites by designing primers in flanking regions using PRIMER 3 (Rozen & Skaletsky 1999).

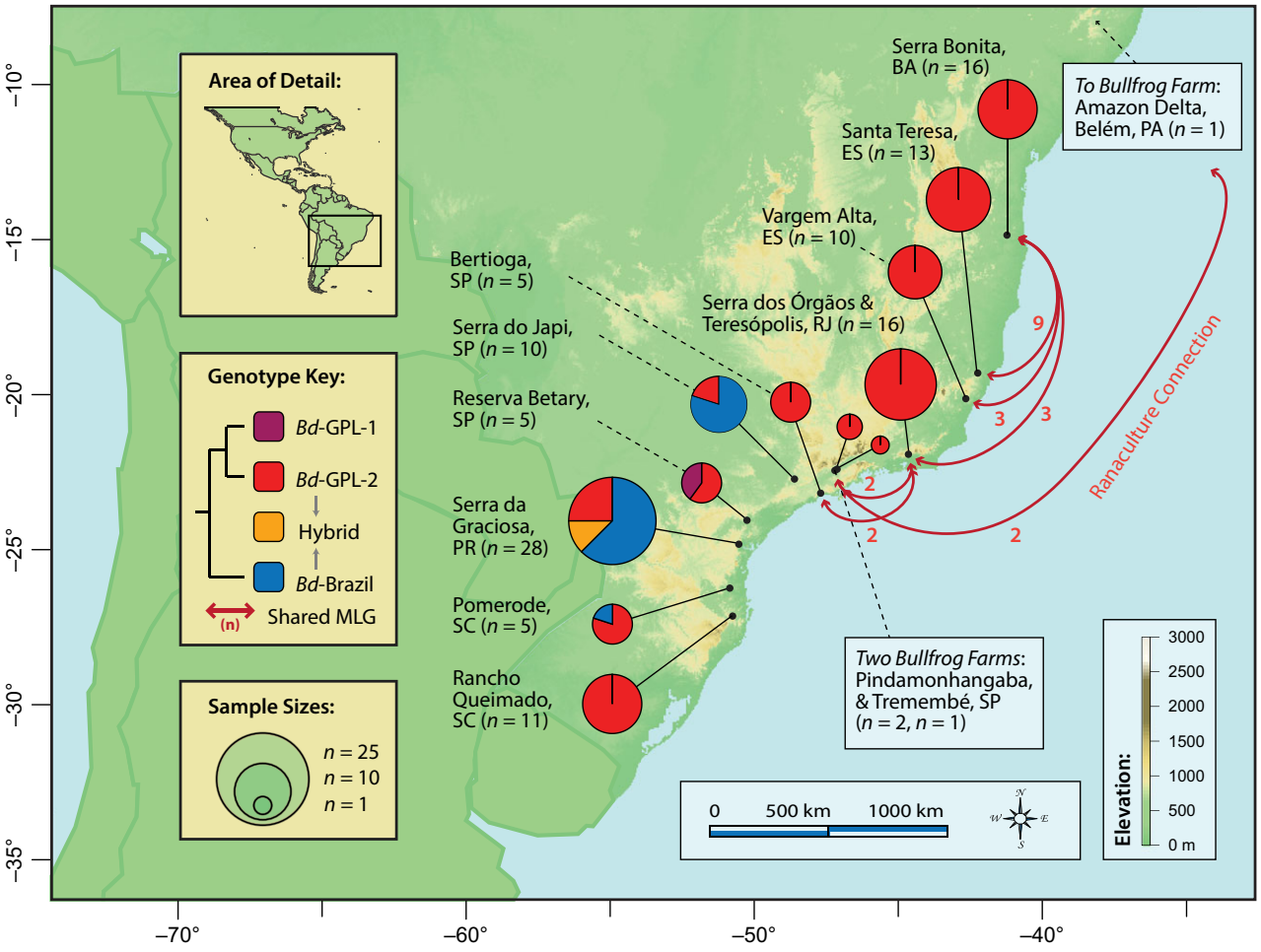

Fig. 1 Spatial distribution of *Bd*-GPL-1, *Bd*-GPL-2, hybrid and *Bd*-Brazil genotypes at collection sites along a 2400-km transect of the Atlantic Forest of Brazil. Diameters of pie graphs represent sample sizes. A hybrid zone is evident in Serra da Graciosa, Paraná. One site; Serra do Japi, São Paulo supports a higher frequency of Brazilian endemic genotypes than any other sample site in the Atlantic Forest. Red arrows indicate shared multilocus genotypes inferred from 12 markers and the total number of clonal isolates observed.

Data analyses

We assigned genotypes to each *Bd* isolate by comparing nucleotide sequences to reference sequences with SEQUENCHER v4.10.1 (GeneCodes). We calculated descriptive indices of molecular diversity including observed heterozygosity (H_O), and average gene diversity (expected heterozygosity, H_E; Nei 1987) with ARLEQUIN v3.5.1.3 (Excoffier & Lischer 2010). To quantify the degree of genetic similarity between geographic populations, we calculated pairwise F_{ST} values between populations with ARLEQUIN and constructed a population level neighbour-joining dendrogram from the resulting F_{ST} matrix with the R package GPLOTS.

In the absence of sexual reproduction, clonal diploid lineages are predicted to accumulate heterozygosity through mutation leading to highly negative F_{IS} values (De Meeûs *et al.* 2006). To test for evidence of historical recombination within lineages, we calculated global and locus-specific F_{IS} values for individual populations using Weir & Cockerham's (1984) method implemented in GENEPOP v4.0.10 (Rousset 2008). We also conducted Hardy–Weinberg (HW) exact tests for deviations from expectation under a random mating model for each locus with GENEPOP. As an alternative test for recombination utilizing disequilibrium among loci, we determined the index of association (I_A; Smith *et al.* 1993; Agapow & Burt 2001) for each geographic population. The index of association (I_A) describes the degree of disequilibrium between genotypes and has been useful in inferring the occurrence of cryptic recombination in putatively asexual populations (Burt *et al.* 1996). We tested for significant deviation from 1000 random multilocus permutations of genotypes under a random mating model with POPPR v1.1.2 (Kamvar *et al.* 2014).

We used PAUP* v4.0b10 (Swofford 2002) to construct a neighbour-joining dendrogram of newly collected isolates and previously published genotypes after clone correction (removal of identical genotypes within a geographic population to account for nonindependent sampling). We estimated genetic distance between genotypes for this analysis with a *hetequal* coding strategy, which assumes heterozygous polymorphisms in each marker to be one step from the nearest heterozygote and two steps from other heterozygotes (Mountain & Cavalli-Sforza 1997; James *et al.* 2009). Support values for clades in the neighbour-joining dendrogram were inferred by bootstrapping over 1000 replicates. We visualized genotype clustering of our samples within a globally sampled panel of previously published *Bd* genotypes with a principal components analysis (PCA) conducted using R packages ADE4 (Dray & Dufour 2007) and ADEGENET (Jombart 2008). For this analysis, we were constrained to a set of markers overlapping with those sequenced in prior studies. Because of this, we used a subset of our isolates for which the monomorphic markers *BDC24*, *BdSC4.3* and *BdSC8.10* were sequenced. Finally, we constructed a summary map of genotype distributions in southeastern Brazil using the R packages MAPTOOLS and PLOTRIX.

Ethics statement

We performed all investigations involving live animals and the international export of pathogen cultures following protocols approved by the University of Michigan's Institutional Animal Care and Use Committee (protocols: PRO00000009 and PRO00005605), and the Brazilian Ministry of the Environment's Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio permits: 27745-8 and 35779-4).

Results

Heterogeneous distribution of enzootic and hybrid lineages

We successfully isolated 111 new strains of *Bd* from infected anurans across our sampling transect (Table 1) and analysed them along with eleven previously published Brazilian isolates, including five isolates from Brazilian farmed *L. catesbeianus* (Schloegel *et al.* 2012). We recovered 77 unique multilocus genotypes (MLG) after clone-correcting our data set; 61 were *Bd*-GPL and 14 were *Bd*-Brazil (Table 2). We collected two new hybrid strains represented by a single clonal MLG, which was distinct from that of the hybrid strain originally reported by Schloegel *et al.* (2012). For our seven lineage-informative markers (*8009X2*, *BDC5*, *BdSC2.0*, *BdSC4.16*, *BdSC6.15*, *BdSC6.8*, *BdSC9.1*), there were no shared alleles between the *Bd*-GPL and *Bd*-Brazil lineages. Our hybrid strains were always heterozygous with one allele from each parental lineage at each of these informative markers. Additionally, no more than two alleles were ever observed at any of the 12 markers, confirming that these were hybrid strains, and not cases of coinfection by *Bd*-Brazil and *Bd*-GPL.

Bd-Brazil and hybrid genotypes were confined to a narrow coastal zone between 23°S and 27°S in the southeastern AF (Fig. 1). Representatives of the globally distributed *Bd*-GPL lineage were found at all 10 of our sampling sites and were the only genotypes present on non-native amphibians (Table 1). The PCA with a global pool of published *Bd* genotypes showed that the Brazilian AF harbours a high level of overall genetic diversity when compared to the global panel of *Bd*-GPL strains (Fig. 2). The diagnostic marker *R6046* (Morehouse *et al.* 2003), which differentiates the mostly temperate North American/European *Bd*-GPL-1 clade of Schloegel *et al.* (2012) from the globally distributed *Bd*-GPL-2, showed that all of our *Bd*-GPL representatives belonged to the globally distributed *Bd*-GPL-2 group except for two isolates from Reserva Betary in São Paulo State which belonged to *Bd*-GPL-1 (Fig. 3).

The proportion of enzootic and hybrid genotypes across all sampled sites in our transect was 23.9% (21.4% *Bd*-Brazil; 2.5% hybrids). However, the prevalence of non-*Bd*-GPL genotypes, in sites where present, ranged from 80.0% (8/10) in Serra do Japi, São Paulo; and 73.1% (19/26) in Serra da Graciosa, Paraná; to 20.0% (1/5) in Pomerode, Santa Catarina. *Bd*-Brazil and hybrid genotypes were not found at the northern or southern extremes of the transect. Where present, these genotypes were restricted to hosts in the genera *Hylodes* and *Bokermannohyla*. Our two newly isolated hybrid strains were from *Bokermannohyla hylax* hosts, both from the Serra da Graciosa hybrid site in the state of Paraná where a previous hybrid strain was reported (Schloegel *et al.* 2012).

Patterns of genetic diversity of Bd lineages in the Atlantic Forest

Global heterozygosity (H_O) across all AF isolates was 0.473, gene diversity (H_E) was 0.511 (Table 2), and the inbreeding coefficient (F_{IS}) was 0.074 after clone correction. When analysed independently, all major lineages had negative F_{IS} values, indicating an excess of heterozygotes relative to HW equilibrium expectations (Table 3). The *Bd*-GPL lineage had slightly higher overall H_O across all alleles compared to the global mean (0.475), while *Bd*-Brazil was slightly less heterozygous (0.423), but this difference in heterozygosity was not

Table 1 Atlantic Forest *Bd* isolates analysed in this study with associated collection dates, geographic origins, host species and collectors

Isolate	Lineage	Year	Geographic origin, Municipality	State	Host species	Collector
CLFT021	*Bd*-GPL	2010	Serra do Japi, Cabreúva	SP	Unidentified sp.	L. F. Toledo & C. A. Vieira
CLFT024/02	Hybrid	2011	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	L. F. Toledo & C. A. Vieira
CLFT026	*Bd*-GPL	2011	Reserva Betary, Iporanga	SP	*Hypsiboas faber*	C. Lambertini
CLFT029	*Bd*-GPL	2011	Serra do Japi, Jundiaí	SP	*Hypsiboas albopunctatus*	C. Lambertini
CLFT030	*Bd*-GPL	2012	Bertioga	SP	*Hylodes phyllodes*	C. Lambertini
CLFT031	*Bd*-GPL	2012	Bertioga	SP	*Hylodes phyllodes*	C. Lambertini
CLFT032	*Bd*-GPL	2012	Bertioga	SP	*Hylodes phyllodes*	C. Lambertini
CLFT033	*Bd*-GPL	2012	Bertioga	SP	*Hylodes phyllodes*	C. Lambertini
CLFT034	*Bd*-GPL	2013	Bertioga	SP	*Hylodes phyllodes*	T. S. Jenkinson
CLFT035	*Bd*-GPL	2013	Reserva Betary, Iporanga	SP	*Hypsiboas faber*	K. R. Zamudio
CLFT036	*Bd*-GPL	2013	Reserva Betary, Iporanga	SP	*Hypsiboas faber*	D. Rodriguez
CLFT037	*Bd*-GPL	2013	Reserva Betary, Iporanga	SP	*Hypsiboas faber*	K. R. Zamudio
CLFT038	Hybrid	2013	Serra da Graciosa, Morretes	PR	*Bokermannohyla hylax*	T. S. Jenkinson
CLFT039	Hybrid	2013	Serra da Graciosa, Morretes	PR	*Bokermannohyla hylax*	T. S. Jenkinson
CLFT040	*Bd*-Brazil	2013	Serra da Graciosa, Morretes	PR	*Bokermannohyla hylax*	L. F. Toledo
CLFT041	*Bd*-Brazil	2013	Serra da Graciosa, Morretes	PR	*Bokermannohyla hylax*	D. Rodriguez
CLFT042	*Bd*-GPL	2013	Reserva Betary, Iporanga	SP	*Hypsiboas faber*	C. M. Betancourt
CLFT043	*Bd*-GPL	2013	Serra da Graciosa, Morretes	PR	*Bokermannohyla hylax*	T. S. Jenkinson
CLFT044	*Bd*-Brazil	2013	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	C. M. Betancourt
CLFT045	*Bd*-GPL	2013	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson
CLFT046	*Bd*-GPL	2013	Serra da Graciosa, Morretes	PR	*Bokermannohyla hylax*	C. M. Betancourt
CLFT047	*Bd*-GPL	2013	Serra da Graciosa, Morretes	PR	*Bokermannohyla hylax*	C. M. Betancourt
CLFT048	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	C. M. Betancourt
CLFT049	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	T. S. Jenkinson
CLFT050	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	C. M. Betancourt
CLFT051	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	T. S. Jenkinson
CLFT052	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	C. M. Betancourt
CLFT053	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	K. R. Zamudio
CLFT054	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	D. Rodriguez
CLFT055	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	T. Y. James
CLFT056	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	T. S. Jenkinson
CLFT057	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	C. M. Betancourt
CLFT058	*Bd*-GPL	2013	Rancho Queimado	SC	*Hylodes meridionalis*	T. S. Jenkinson
CLFT060	*Bd*-GPL	2013	Pomerode	SC	*Hylodes meridionalis*	T. S. Jenkinson
CLFT061	*Bd*-Brazil	2013	Pomerode	SC	*Hylodes meridionalis*	C. M. Betancourt
CLFT062	*Bd*-GPL	2013	Pomerode	SC	*Hylodes meridionalis*	C. M. Betancourt
CLFT063	*Bd*-GPL	2013	Pomerode	SC	*Hylodes meridionalis*	C. M. Betancourt
CLFT064	*Bd*-GPL	2013	Pomerode	SC	*Hylodes meridionalis*	C. M. Betancourt
CLFT065	*Bd*-Brazil	2013	Serra do Japi, Jundiaí	SP	*Hylodes japi*	C. M. Betancourt
CLFT066	*Bd*-Brazil	2013	Serra do Japi, Jundiaí	SP	*Hylodes japi*	J. E. Longcore
CLFT067	*Bd*-Brazil	2013	Serra do Japi, Jundiaí	SP	*Hylodes japi*	C. M. Betancourt
CLFT068	*Bd*-Brazil	2013	Serra do Japi, Jundiaí	SP	*Hylodes japi*	C. M. Betancourt
CLFT070	*Bd*-Brazil	2013	Serra do Japi, Jundiaí	SP	*Hylodes japi*	J. E. Longcore
CLFT071	*Bd*-Brazil	2013	Serra do Japi, Jundiaí	SP	*Hylodes japi*	C. M. Betancourt
CLFT073	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	*Aplastodiscus* sp.	C. M. Betancourt
CLFT074	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	Unidentified sp.	C. M. Betancourt
CLFT075	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	Unidentified sp.	T. Y. James
CLFT076	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	*Bokermannohyla* sp.	C. M. Betancourt
CLFT077	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	*Bokermannohyla* sp.	C. M. Betancourt
CLFT078	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	*Bokermannohyla* sp.	T. Y. James
CLFT079	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	*Bokermannohyla* sp.	T. Y. James
CLFT080	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	*Bokermannohyla* sp.	C. M. Betancourt
CLFT081	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	Unidentified sp.	C. M. Betancourt
CLFT082	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	*Bokermannohyla* sp.	C. M. Betancourt

Table 1 *Continued*

Isolate	Lineage	Year	Geographic origin, Municipality	State	Host species	Collector
CLFT083	*Bd*-GPL	2013	Lago Iacy, Teresópolis	RJ	*Scinax hayii*	C. M. Betancourt
CLFT084	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	*Bokermannohyla* sp.	C. M. Betancourt
CLFT085	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	Unidentified sp.	C. M. Betancourt
CLFT086	*Bd*-GPL	2013	Serra dos Órgãos National Park	RJ	Unidentified sp.	C. M. Betancourt
CLFT087	*Bd*-GPL	2013	Lago Iacy, Teresópolis	RJ	*Scinax hayii*	C. M. Betancourt
CLFT088	*Bd*-GPL	2013	Lago Iacy, Teresópolis	RJ	*Scinax hayii*	C. M. Betancourt & T. S. Jenkinson
CLFT095	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Aplastodiscus* sp.	T. S. Jenkinson
CLFT096	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Aplastodiscus* sp.	C. Lambertini
CLFT097	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Aplastodiscus* sp.	A. V. Aguilar
CLFT098	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Aplastodiscus* sp.	C. Lambertini
CLFT099	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Aplastodiscus* sp.	T. S. Jenkinson
CLFT100	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	C. Lambertini
CLFT101	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Aplastodiscus* sp.	A. V. Aguilar & T. S. Jenkinson
CLFT102	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	T. S. Jenkinson
CLFT103	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	A. V. Aguilar
CLFT104	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	A. V. Aguilar
CLFT105	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	T. S. Jenkinson
CLFT106	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	T. S. Jenkinson
CLFT107	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	C. Lambertini
CLFT108	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	T. S. Jenkinson
CLFT109	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	T. S. Jenkinson
CLFT110	*Bd*-GPL	2014	Serra Bonita, Camacan	BA	*Bokermannohyla* sp.	A. V. Aguilar
CLFT111	*Bd*-GPL	2014	Santa Teresa	ES	*Aplastodiscus* sp.	T. S. Jenkinson
CLFT113	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	T. S. Jenkinson
CLFT114	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	A. V. Aguilar
CLFT115	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	A. V. Aguilar
CLFT116	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	T. S. Jenkinson
CLFT117	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	C. Lambertini
CLFT118	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	A. V. Aguilar
CLFT119	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	A. V. Aguilar
CLFT120	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	A. V. Aguilar
CLFT121	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	A. V. Aguilar
CLFT122	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	T. S. Jenkinson
CLFT123	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	C. Lambertini
CLFT124	*Bd*-GPL	2014	Santa Teresa	ES	*Bokermannohyla* sp.	A. V. Aguilar
CLFT126	*Bd*-GPL	2014	Vargem Alta	ES	*Phyllomedusa* sp.	A. V. Aguilar
CLFT127	*Bd*-GPL	2014	Vargem Alta	ES	*Dendropsophus minutus*	T. Y. James
CLFT128	*Bd*-GPL	2014	Vargem Alta	ES	*Aplastodiscus* sp.	T. Y. James
CLFT129	*Bd*-GPL	2014	Vargem Alta	ES	*Aplastodiscus* sp.	A. V. Aguilar
CLFT130	*Bd*-GPL	2014	Vargem Alta	ES	*Scinax fuscovarius*	A. V. Aguilar
CLFT131	*Bd*-GPL	2014	Vargem Alta	ES	*Lithobates catesbeianus*	T. S. Jenkinson
CLFT132	*Bd*-GPL	2014	Vargem Alta	ES	*Dendropsophus minutus*	A. V. Aguilar
CLFT133	*Bd*-GPL	2014	Vargem Alta	ES	*Phyllomedusa* sp.	A. V. Aguilar
CLFT134	*Bd*-GPL	2014	Vargem Alta	ES	*Phyllomedusa* sp.	T. S. Jenkinson
CLFT135	*Bd*-GPL	2014	Vargem Alta	ES	*Scinax fuscovarius*	K. R. Zamudio
CLFT136	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Bokermannohyla hylax*	T. S. Jenkinson
CLFT137	*Bd*-GPL	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson
CLFT138	*Bd*-GPL	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	C. Lambertini
CLFT139	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson
CLFT141	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	L. F. Moreno de Lima
CLFT142	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Crossodactylus schmidti*	P. P. Morão
CLFT143	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson
CLFT144	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson
CLFT145	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	P. P. Morão
CLFT146	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson

Table 1 *Continued*

Isolate	Lineage	Year	Geographic origin, Municipality	State	Host species	Collector
CLFT148	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson
CLFT149	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson
CLFT150	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	P. P. Morão
CLFT151	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	P. P. Morão
CLFT152	*Bd*-GPL	2014	Serra da Graciosa, Morretes	PR	*Crossodactylus schmidti*	T. S. Jenkinson
CLFT153	*Bd*-Brazil	2014	Serra da Graciosa, Morretes	PR	*Hylodes cardosoi*	T. S. Jenkinson
JEL648	*Bd*-Brazil	2010	Serra do Japi, Jundiaí	SP	*Hylodes japi*	J. E. Longcore
JEL649	*Bd*-Brazil	2010	Serra do Japi, Jundiaí	SP	*Hylodes japi*	J. E. Longcore
LMS902	*Bd*-GPL	2008	Pindamonhangaba (farm)	SP	*Lithobates catesbeianus*	L. M. Schloegel
LMS925	*Bd*-GPL	2008	Pindamonhangaba (farm)	SP	*Lithobates catesbeianus*	L. M. Schloegel
LMS929	*Bd*-GPL	2008	Belém (farm)	PA	*Lithobates catesbeianus*	L. M. Schloegel
LMS931	*Bd*-GPL	2009	Tremembé (farm)	SP	*Lithobates catesbeianus*	L. M. Schloegel
UM142	*Bd*-Brazil	2009	Ypsilanti, U.S.A. (market)	MI	*Lithobates catesbeianus*	T. Y. James

Brazilian state abbreviations are as follows: Bahia (BA), Espírito Santo (ES), Rio de Janeiro (RJ), São Paulo (SP), Paraná (PR) and Santa Catarina (SC).

significant (Wilcoxon rank-sum test, $P = 0.885$). As expected, the hybrid isolates displayed significantly higher levels of observed heterozygosity than the other lineages ($H_O = 0.750$; Wilcoxon rank-sum test, $P = 0.012$).

Mean gene diversity (H_E) across populations differed significantly between lineages, 0.374 in *Bd*-GPL and 0.287 in *Bd*-Brazil (Wilcoxon rank-sum test, $P = 0.041$). Evidence of marker ascertainment bias was observed, however, when our newly developed markers were analysed separately. The significant difference in average gene diversity was not evident when mean H_E was calculated using only our new markers designed from genome sequences of *Bd*-Brazil (Fig. 4; Wilcoxon rank-sum test, $P = 0.909$), whereas previously published markers analysed separately differed in mean H_E (Wilcoxon rank-sum test, $P = 0.030$). Average allele richness over all loci ranged from 1.725 alleles in *Bd*-GPL to 1.667 in *Bd*-Brazil. The hybrid population had significantly higher gene diversity than the other lineages ($H_E = 0.569$; Wilcoxon rank-sum test, $P = 0.004$), and elevated mean allele richness (2.083 alleles). Genotypic diversity (defined as the proportion of unique MLGs per sample) of the entire AF data set was 0.658. Genotypic diversity did not differ between lineages, with average genotypic diversities of 0.583 in *Bd*-Brazil vs. 0.685 in *Bd*-GPL (Wilcoxon rank-sum test, $P = 0.731$). One of our twelve sampled loci (*BdSC16.2*) was monomorphic in *Bd*-GPL, whereas three loci (*8009X2*, *BDC5*, *BdSC4.16*) were monomorphic in *Bd*-Brazil.

Population genetic structure of Atlantic Forest Bd isolates

Both lineages were subdivided by geography (Fisher's exact test; both lineages: $P < 0.001$). A clustering dendrogram constructed from pairwise F_{ST} values between *Bd*-GPL populations with more than three sequenced isolates grouped geographic populations into two major groups with high bootstrap support (Fig. 5). These groups are unexpectedly structured in that three populations from the extreme northern transect (Serra Bonita, Bahia; Vargem Alta, Espírito Santo; and Santa Teresa, Espírito Santo) cluster with the extreme southern population of Rancho Queimado, Santa Catarina. Geographic subpopulations of *Bd*-GPL were weakly isolated by distance ($r = 0.012$; Mantel test $P = 0.037$). We did not test for significant isolation by distance in *Bd*-Brazil populations due to the limited sample size of populations.

Despite the significant subdivision among populations, four *Bd*-GPL MLGs were shared among sample sites in our transect indicating gene flow, or recent, rapid expansion (Figs 1 and 3). The population of Serra Bonita, Bahia, shared one MLG each with the adjacent northeastern sample sites of Santa Teresa and Vargem Alta, both in Espírito Santo State (maximum distance = 521 km). Serra dos Órgãos, Rio de Janeiro State and Bertioga in São Paulo State (distance = 342 km) shared one MLG. The greatest distance between shared MLGs from native amphibians was between Serra Bonita and Serra dos Órgãos (891 km). We also found shared MLGs associated with the ranaculture industry. Isolates from one bullfrog farm in Tremembé, São Paulo, shared MLGs with those from both native and farmed amphibians. This bullfrog farm isolate (LMS931) shared a clonal genotype with an isolate collected in Serra dos Órgãos, a protected national park. The Tremembé farm isolate also shared a genotype with an isolate from a bullfrog farm in Belém, Pará (Schloegel *et al.* 2012), in the Amazon River delta separated by over 2600 km. No shared *Bd*-GPL MLGs were observed in

Table 2 Atlantic Forest *Bd* populations sampled for this study with respective sample sizes (*N*) and indices of genetic diversity

					Clone-corrected data	
Populations	*N*	MLGs	Mean allele richness	Genotypic diversity	Observed heterozygosity (H_O)	Expected heterozygosity (gene diversity, H_E)
1. Serra Bonita, BA	16	7	1.667	0.438	0.536	0.330
2. Santa Teresa, ES	13	7	1.667	0.539	0.429	0.306
3. Vargem Alta, ES	10	7	1.750	0.700	0.500	0.350
4. Serra dos Órgãos & Teresópolis, RJ	16	14	1.750	0.875	0.512	0.369
5. Serra do Japi, SP	10					
Bd-GPL	2	2	1.667	1.000	0.500	0.417
Bd-Brazil	8	6	1.583	0.750	0.403	0.292
6. Bertioga, SP	5	5	1.667	1.000	0.417	0.328
7. Reserva Betary, SP	5	4	1.833	0.800	0.458	0.417
8. Serra da Graciosa, PR	26					
Bd-GPL	7	7	1.917	1.000	0.488	0.406
Bd-Brazil	16	7	1.750	0.438	0.440	0.293
Hybrids	3	2	2.083	0.667	0.750	0.569
9. Pomerode, SC	5					
Bd-GPL	4	3	1.667	0.750	0.417	0.350
Bd-Brazil	1	1	1.417	1.000	0.417	0.417
10. Rancho Queimado, SC	11	9	1.667	0.818	0.444	0.304
All *Bd*-GPL	89	61		0.685	0.475	0.374
All *Bd*-Brazil	25	14		0.583	0.423	0.287
Global	117	77		0.658	0.473	0.511

Populations of enzootic and hybrid lineages are shaded grey.

the southwestern sample sites of the collection transect, and no MLGs were shared between *Bd*-Brazil populations (maximum distance = 320 km).

The neighbour-joining dendrogram (Fig. 3) revealed a lack of geographic structure in the *Bd*-GPL lineage. Instead, clades were composed of isolates from disparate geographic populations and several clades included *Bd*-GPL populations from extremes of the AF transect. Conversely, geographic populations of *Bd*-Brazil form site-specific clades with the exception of the isolate CLFT071 from Serra do Japi, São Paulo State, which forms a clade with the *Bd*-Brazil isolate UM142, originally cultured from a captive *L. catesbeianus* for sale in a United States food market (Schloegel *et al.* 2012). The PCA of AF isolates with a global pool of previously sequenced isolates showed significant clusters representing all genotypic lineages known to occur in the Western Hemisphere (Fig. 2), with a total of 21.7% of genetic variation explained by the first three principal components. Brazilian AF MLGs of *Bd* are represented in each cluster. The PCA also shows the *Bd*-GPL clade forming two clusters representing the *Bd*-GPL-1 and *Bd*-GPL-2 split. Our AF *Bd*-GPL-1 representative, for which we sequenced sufficient overlapping loci with previously sequenced isolates, was separated from the rest of the *Bd*-GPL-1 cluster. The two Brazilian hybrid MLGs are separated across all three axes of our PCA, indicating an appreciable degree of genetic distance between hybrid MLGs.

Signatures of recombination in Atlantic Forest Bd populations

Both *Bd*-GPL and *Bd*-Brazil lineages had highly negative F_{IS} estimates, as predicted for a predominantly asexual population (De Meeûs *et al.* 2006). We calculated F_{IS} values from clone-corrected data to control for nonindependent clonal samples (Table 3). The *Bd*-GPL displayed an F_{IS} closer to zero (−0.245) than the *Bd*-Brazil lineage (−0.416). Both the *Bd*-GPL and *Bd*-Brazil lineages deviated from expected heterozygosities under HW equilibrium expectations ($P < 0.001$ and $P = 0.0012$, respectively). However, not all loci matched these trends. When analysed by lineage using HW exact tests, we failed to reject the null expectation for 36.4% (4/11) of the informative markers in the *Bd*-GPL group (significance cut-off $\alpha = 0.05$). Within the *Bd*-Brazil lineage, we failed to reject the null expectation in 42.9% (3/7) of the

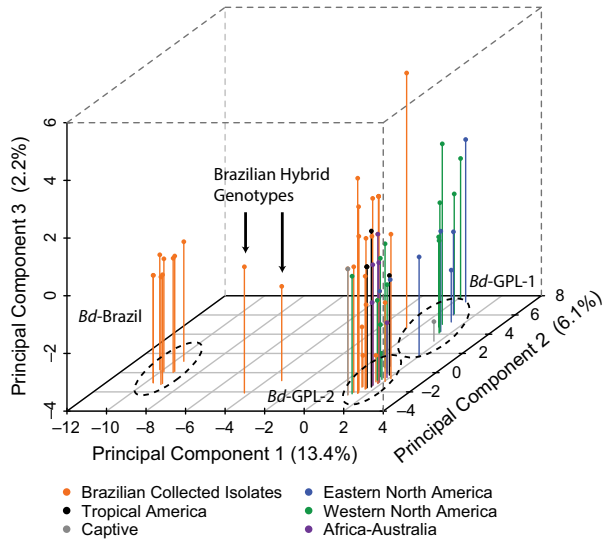

Fig. 2 Principal components ordination plot of a global panel of *Bd* representatives from this and previously published studies, for which 10 multilocus sequence typing markers have been sequenced. Brazilian Atlantic Forest multilocus genotypes are highlighted in orange and show the greatest degree of genetic diversity of any sampled global region. The major lineages *Bd*-Brazil, *Bd*-GPL-1, and *Bd*-GPL-2 are outlined. The three major principal components explain 21.7% of currently sampled genetic variation in *Bd*.

informative markers. To eliminate the potential artefact of reduced heterozygosity in pooled populations that are significantly subdivided (the Wahlund effect), we also performed HW exact tests on each geographic population with more than three sequenced MLGs (Table S2, Supporting information). Among the nine *Bd*-GPL populations with adequate sampling, 81.3% (61/75) of the informative markers did not differ from null HW expectations, and 76.9% (10/13) of the informative loci did not significantly differ from null expectations in the *Bd*-Brazil populations.

In a separate test for historical recombination, genotype data from both the *Bd*-GPL and *Bd*-Brazil lineages were randomly shuffled over 1000 permutations using a nonparametric bootstrap resampling approach to generate a null distribution of I_A values under a random recombination model. The observed index of association estimated for *Bd*-GPL significantly differed from the randomized distribution (Table 3 and Fig. 6A; $P = 0.009$), whereas the I_A of our *Bd*-Brazil data set did not (Fig. 6B; $P = 0.465$).

Discussion

Emerging fungal pathogens are a growing threat to global biodiversity and have already disrupted host populations throughout a range of habitats (Fisher *et al.* 2012). Despite the urgent need to comprehend the causes and consequences of disease emergence, our understanding of fungal pathogen biology lags behind that of other taxonomic groups (Giraud *et al.* 2008), which in turn hinders an informed response to their outbreaks. Prior population studies of fungal pathogen systems have revealed that divergent host adaptation (Fisher *et al.* 2005; Gladieux *et al.* 2011), recombination (Stukenbrock *et al.* 2012) and pathogen translocation to new environments (Gladieux *et al.* 2015) may all play important roles in emergence. Our study presents a large-scale regional sample of genotyped *Bd* isolates from the Brazilian AF. The AF is the only global region where all of the aforementioned forces appear to have contributed to local *Bd* population dynamics. As such, the examination of these populations provides a valuable opportunity to better understand the evolutionary history of *Bd*, and to predict the consequences of lineage divergence, hybridization, and strain translocation on disease outcomes as chytridiomycosis continues to spread to new environments.

Long-term population history of Bd in the Atlantic Forest

The only extensive prior study of *Bd* in the Brazilian AF focused on a temporal sampling of museum-preserved amphibian specimens dating back to 1894 (Rodriguez *et al.* 2014). In that study, the authors genotyped 52 *Bd* infections from skin swabs using a single ribosomal marker (ITS1) and concluded that *Bd* had not been introduced to Brazil over their 116-year sampling period. Based on those results, the authors hypothesized that both the *Bd*-GPL and *Bd*-Brazil lineages may have been endemic to the AF. However, based on a single hypervariable marker (Nilsson *et al.* 2008; Bataille *et al.* 2013), the Rodriguez *et al.* (2014) study was not able to address the history of *Bd* in Brazil before their earliest sample. On the other hand, our multilocus data set provided a more robust opportunity to make inferences about population history before 1894. Both of our studies conclude that *Bd*-Brazil is an endemic lineage to the AF, but our study calls into question the hypothesis that *Bd*-GPL originated in Brazil. The combined evidence between our two studies agrees that *Bd*-GPL was already in Brazil before the import, and subsequent escape, of the North American bullfrog for trade in the early 20th century, but the question to be resolved is whether *Bd*-GPL has been present in the AF as a long-term endemic.

Population genetic theory predicts lineages that have been stable in a given locality should have proportionally greater genetic diversity than recently translocated lineages due to the founder effect (Hartl & Clark 1997).

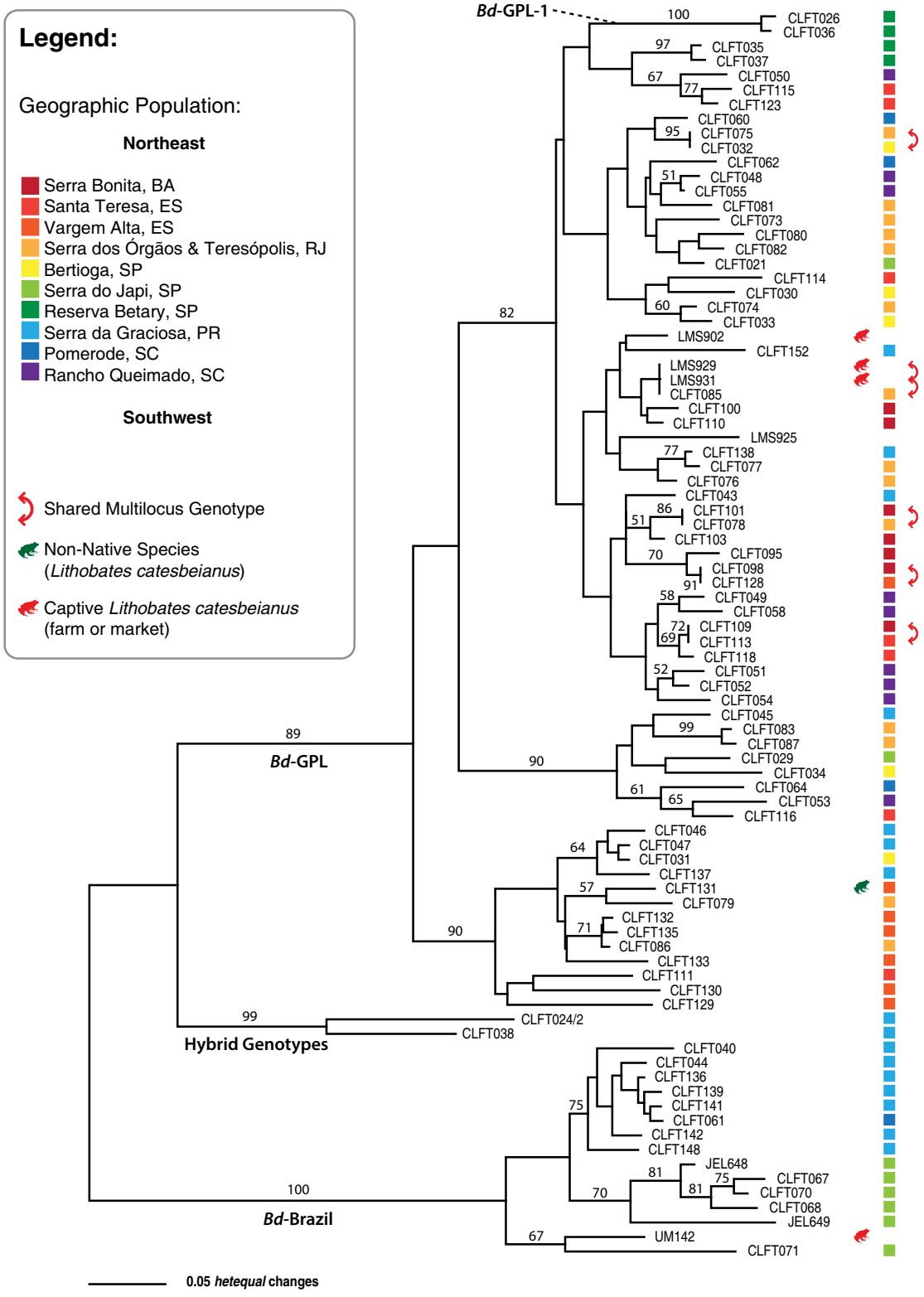

Fig. 3 Neighbour-joining dendrogram of Brazilian Atlantic Forest multilocus genotypes based on 12 multilocus sequence typing markers, using a *hetequal* distance matrix. Genotypes are labelled with a representative isolate. Nodes leading to major lineages indicated (*Bd*-Brazil, *Bd*-GPL-1 and *Bd*-GPL-2). Collection localities are indicated by a colour scale. Shared multilocus genotypes are indicated by curved arrows, and isolates from non-native and captive hosts are marked with green and red icons, respectively. Nodes with bootstrap support greater than 50% across 1000 bootstrap replicates are indicated.

Table 3 Population-specific inbreeding coefficients and indices of association after clone correction, with associated *P*-values from the results of Hardy–Weinberg exact tests and random permutation tests under a model of random recombination

	Hardy–Weinberg exact test		Index of association permutation test	
Population	F_{IS}	*P*-value	I_A	*P*-value
1. Serra Bonita, BA	−0.709	**<0.0001**	−0.291	0.8111
2. Santa Teresa, ES	−0.450	**0.0016**	0.346	0.1728
3. Vargem Alta, ES	−0.482	**0.0003**	0.086	0.3287
4. Serra dos Órgãos & Teresópolis, RJ	−0.406	**<0.0001**	0.281	0.0629
5. Serra do Japi, SP				
Bd-GPL	−0.333	0.3500	*Permutation test not conducted* ($N < 3$)	
Bd-Brazil	−0.436	**0.0068**	0.179	0.2577
6. Bertioga, SP	−0.316	0.0547	−0.019	0.4595
7. Reserva Betary, SP	−0.119	0.2857	3.38	**0.0020**
8. Serra da Graciosa, PR				
Bd-GPL	−0.224	0.1534	1.063	**0.0050**
Bd-Brazil	−0.569	**0.0001**	−0.009	0.4386
Hybrids	−0.565	**0.0382**	*Permutation test not conducted* ($N < 3$)	
9. Pomerode, SC				
Bd-GPL	−0.250	0.2726	−0.556	0.6623
Bd-Brazil	*Exact test not conducted* ($N = 1$)		*Permutation test not conducted* ($N < 3$)	
10. Rancho Queimado, SC	−0.506	**<0.0001**	0.517	**0.0290**
All *Bd*-GPL	−0.245	**<0.0001**	0.226	**0.0099**
All *Bd*-Brazil	−0.416	**0.0012**	−0.013	0.465
All Populations	0.074	**<0.0001**		

P-values in bold indicate significant deviations from null expectations under Hardy–Weinberg equilibrium or an association model of random recombination. Populations of enzootic and hybrid lineages are shaded grey.

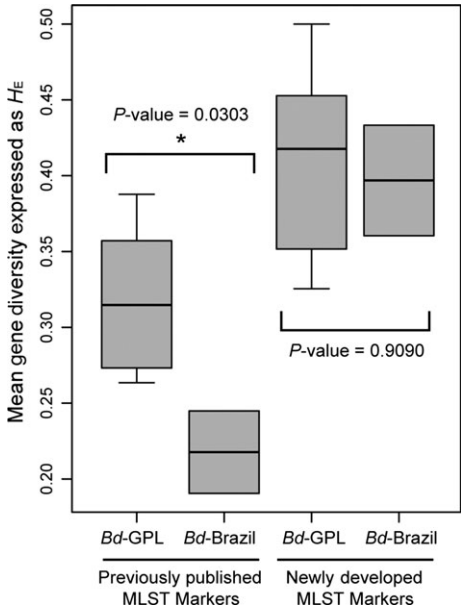

Fig. 4 Comparison of previously published MLST markers and newly developed markers accounting for genomic variation in *Bd*-Brazil. Previously published markers show bias towards capturing variation in the *Bd*-GPL lineage. Mean gene diversity differs significantly among lineages (*) only when calculated separately using previously published markers (Wilcoxon rank-sum test), but significant differences are not observed when newly developed markers are analysed separately.

Based on our analysed set of marker loci, it would initially appear that *Bd*-GPL is as genetically diverse as *Bd*-Brazil. Upon further investigation, however, multiple lines of evidence from our study support the hypothesis that the *Bd*-Brazil lineage may have been present in the Brazilian AF longer than *Bd*-GPL.

First, estimates of genetic diversity based on population markers designed before the discovery of novel Brazilian lineages are confounded by an inherent bias towards capturing variation in *Bd*-GPL and not in *Bd*-Brazil. When our newly developed markers are analysed independently, a difference in gene diversity (H_E) between the two lineages is no longer observed (Fig. 4). In a recent study of comparative genomic diversity which included two *Bd*-Brazil isolates and a global panel of *Bd*-GPL isolates (Rosenblum *et al*. 2013), higher heterozygosity was observed within *Bd*-Brazil strains lending support to our hypothesis at the genomic level. Within our data set, other historical factors specific to Brazil may have also had an effect on current day diversity estimates. Multiple successive introductions of *Bd*-GPL—which we infer must have occurred at least twice based on the co-occurrence of both major GPL genotypes (*Bd*-GPL1 and *Bd*-GPL2) in the Reserva Betary population—would increase diversity in the *Bd*-GPL obscuring the expected differences in diversity between the *Bd*-GPL and *Bd*-Brazil lineages. Because of these variable factors influencing our observed genetic diversity, we chose not to base our conclusions on this line of evidence, opting instead for stronger infralineage based comparisons.

Second, if *Bd*-GPL had been present as a long-term endemic in the AF, geographic structuring should be evident, especially after more than four centuries of anthropogenic habitat fragmentation introducing barriers to dispersal. Three geographic analyses independently show that *Bd*-GPL has not been present in the AF long enough for the establishment of geographic structuring. In contrast, *Bd*-Brazil is geographically structured, most likely as a result of long-term endemism. Our genotype dendrogram (Fig. 3) shows a

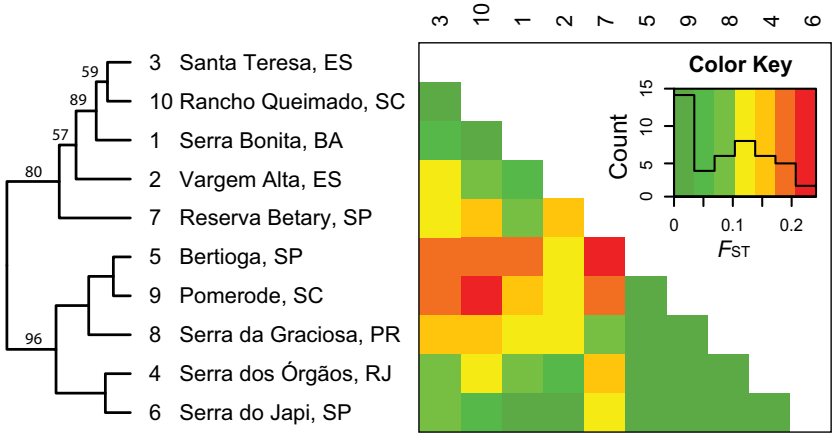

Fig. 5 Heatmap of the pairwise F_{ST} matrix between *Bd*-GPL populations and the neighbour-joining dendrogram showing inferred relationships between sample populations based on genetic differentiation. Population labels are numbered from northernmost (1) to southernmost (10) localities. Greener colours indicate low population differentiation (F_{ST} closer to zero), increasing to red to indicate greater population differentiation. Dendrogram nodes with bootstrap support greater than 50% across 1000 bootstrap replicates are indicated.

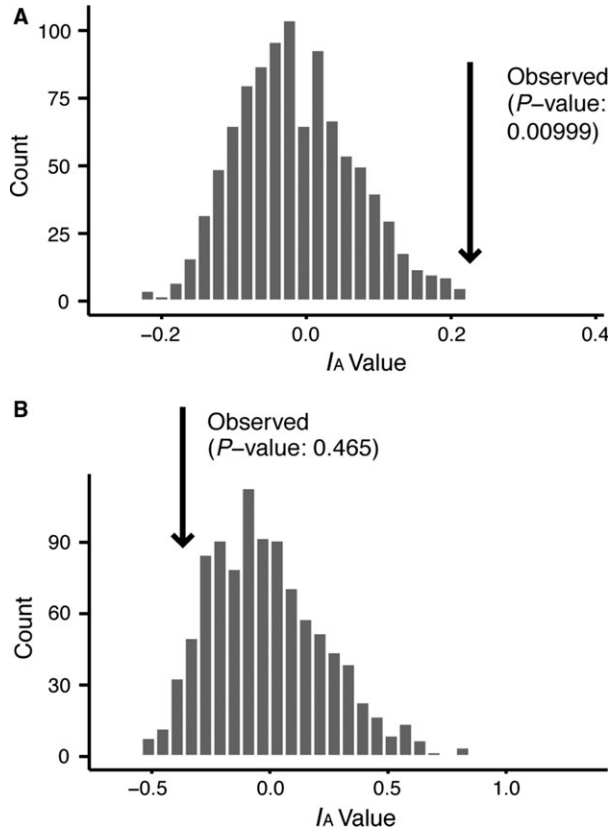

Fig. 6 Histograms of simulated index of association from 1000 permutations of randomization tests under a null model of allelic recombination, and observed values of I_A (indicated by arrows) for the *Bd*-GPL (A) and *Bd*-Brazil lineages (B). *P*-values correspond to the results from the random permutation test comparing observed indices to the distribution of simulation results.

distinct lack of geographic structure in the *Bd*-GPL clade, whereas our *Bd*-Brazil genotypes form clades corresponding with geographic origin. Likewise, when we cluster our *Bd*-GPL populations by pairwise F_{ST}, we observe only a minor relationship between genetic divergence and geography (Fig. 5). The pairwise F_{ST} analysis indicates that geographically distant populations of *Bd*-GPL are often less differentiated from one another than they are to their adjacent counterparts, suggesting a rapid and recent expansion (Excoffier *et al.* 2009). While the possibility exists that historical geographic structure in *Bd*-GPL could be masked by recent long-range movement of *Bd*-GPL through the bullfrog trade, this scenario is unlikely given the lack of such long-range movement in *Bd*-Brazil, which is also known to infect bullfrogs in the ranaculture industry (Schloegel *et al.* 2012), and whose range overlaps with the potential invasive range of bullfrogs in the AF. It is unlikely that bullfrogs would differentially transmit *Bd*-GPL to produce the pattern we observe.

Third, only *Bd*-GPL populations share MLG clones, probably due to a recent spread of *Bd*-GPL. Even at short geographic distances, MLGs were never shared among *Bd*-Brazil populations, suggesting that these populations have been separated for longer periods of time without migration. Our observation of shared MLGs concentrated to northern *Bd*-GPL populations indicates that this lineage may have recently expanded northward. If, as we suspect, this pattern were produced by rapid expansion of a recently introduced *Bd*-GPL founding population, *Bd*-GPL populations should show little isolation by distance. Indeed, a Mantel test resulted in a weak correlation between genetic dissimilarity and geo-

graphic distance. Together, these analyses imply a scenario of *Bd*-GPL introduction within the last few centuries and reflect a relatively short period of time for the accumulation of variation between populations. Again, it is difficult to discern between our hypothesis of historical expansion and a recent increase in gene flow between current populations as the cause of this pattern. Given the highly fragmented nature of the AF, we believe that the former scenario is more plausible. The significant isolation by distance we observe between *Bd*-GPL populations, albeit weak, indicates that any recent gene flow between populations would have been minimal. We cannot, however, discount the possibility that anthropogenic movement of amphibians may have played a role in shaping the population structure of *Bd*-GPL in these native amphibian hosts.

Finally, differences in the significant association of alleles from the randomly permutated data sets may indicate major differences in the population histories of the two divergent AF lineages (Fig. 6). Under random recombination over sufficient time, the index of association between alleles in a population is predicted to approach zero (Smith *et al.* 1993). In clonal populations —where recombination has been rare or absent—alleles are passed on to asexual daughters in complete disequilibrium, resulting in significantly nonzero I_A values as seen in *Bd*-GPL populations. In contrast, our results indicated that the *Bd*-Brazil lineage has been present in the AF long enough to display genotypic equilibrium through rare recombination. The same tests repeated within our subdivided populations show that the significant association of alleles in the *Bd*-GPL is not solely due to population subdivision. There may be several possible explanations for the disparity in the association of alleles between lineages. One possibility is that recombination rates differ between the two lineages. A study of the recently discovered, divergent Swiss (*Bd*-CH) and African (*Bd*-Cape) lineages suggested that representatives of the divergent lineages might have elevated rates of mitotic recombination relative to *Bd*-GPL (Farrer *et al.* 2013). Another possibility may be that the observed index of association in *Bd*-Brazil is a product of long-term demographic stability. Differential rates of recombination between *Bd*-GPL and *Bd*-Brazil have never been examined, and while out of the scope of this study, should be a priority for future research.

Implications of current lineage distributions

One of the most striking aspects of our field data was the restriction of enzootic lineages (*Bd*-Brazil and hybrids) to a narrow portion of the AF. One explanation for this pattern may be that enzootic lineages require a higher degree of environmental or host specificity than the *Bd*-GPL lineage. Temperature and humidity are probable abiotic factors restricting the spread of enzootic lineages through the AF given that the latitudinal range in which we found *Bd*-GPL is much greater than that of *Bd*-Brazil. Whether *Bd*-GPL populations are better able to tolerate extremes in temperature and moisture, however, remains to be tested experimentally. Our results indicate that *Bd*-GPL arrived more recently to the AF than *Bd*-Brazil and that it shows signatures of a recent demographic expansion. Taken together, these findings support the hypothesis that *Bd*-GPL may be a better disperser across fragmented landscapes. The southern range of the AF in the states of São Paulo, Paraná and Santa Catarina contain the most intact remnant patches of forested terrain in coastal Brazil, whereas the northern transect in our study has experienced a history of greater deforestation (Pinto *et al.* 2014). Studies in this region have shown that *Bd* infection is more prevalent in pristine vs. disturbed habitats (Becker & Zamudio 2011). A fruitful avenue for future research will be to determine whether certain *Bd* strains themselves are better able to tolerate extreme or degraded habitats, or whether they are better able to disperse through other mechanisms such as infective differences on specific host species.

Our sampling effort was not designed to explicitly address the question of differences in host specificity between lineages, but the predominant trend in our results is that *Bd*-GPL is able to infect a wider assemblage of amphibian hosts in the AF (Table 1). Interestingly, the northernmost extent of *Bd*-Brazil's observed range coincides with a known biogeographic delimitation between northern and southern climatically adapted AF species (Carnaval *et al.* 2014). Taxonomic groups across this north/south split include many amphibians that may have diversified in separate biogeographic refugia (Carnaval *et al.* 2009; Thomé *et al.* 2010). Palaeoclimatic modelling suggests that during the Late Quaternary glacial maxima, the AF was restricted to smaller, climatically stable refugia.

The predicted refugia most relevant to our collection transect are the large northern Bahia refugium, and a series of smaller southern refugia in the coastal regions of the present-day states of São Paulo and Paraná. These refugia are centres of high host phylogenetic endemism (Carnaval & Moritz 2008), and the *Bd*-Brazil lineage has only been found within the southern centre of historical diversification corresponding to the São Paulo and Paraná refugia. The geographic restriction of *Bd*-Brazil to this centre of AF microendemism, in conjunction with our data supporting the long-term endemism of this lineage, leads us to hypothesize that *Bd*-Brazil was similarly restricted to these southern refugia, where it became locally adapted to co-occurring south-

ern host species. Subsequently, its current distribution may reflect a history of tracking hosts that remained confined to the southern AF due to a combination of habitat heterogeneity and migration barriers.

Sexual reproduction and a pathogen hybrid zone

Hybridization can be a driving force in the evolution of fungal pathogen populations (Stukenbrock *et al.* 2012). Studies of other eukaryotic pathogens show that major changes in phenotype by sexual recombination and hybridization can play a pivotal role in the emergence of virulence (Grigg *et al.* 2001; Sibley & Ajioka 2008). *Bd* genotypes that have been geographically or environmentally isolated should have diverged from each other over time as they adapted to local host defences. Sexual outcrossing adds a new dimension by which *Bd* might explore the fitness landscape, particularly through the generation of variation in pathogenic phenotype. Experimental infections show that the original Brazilian hybrid strain CLFT024/02 causes greater mortality in a non-Brazilian amphibian host (*Lithobates sylvaticus*) than representative strains from either the parent *Bd*-Brazil or *Bd*-GPL lineages (Betancourt Román *et al.* in review). If similar effects occur in local host populations, the ecological implications could be serious, and the need for more robust biosecurity measures to prevent the export of hybrid strains from Brazil will be pressing.

Our survey recovered two new isolates of hybrid genotypes from Serra da Graciosa, the hybrid locality originally reported by Schloegel *et al.* (2012). Although sexual reproduction has not been directly observed in *Bd* in vitro, sexual recombination has probably been an important influence on its genetic history (James *et al.* 2009; Rosenblum *et al.* 2013). Our two new hybrid isolates appear to be genetic clones of each other (a single MLG), but significant genetic differences exist between our hybrid isolates and the originally described hybrid CLFT024/02. The hybrid MLGs are distinctly separated across all three axes in our PCA (Fig. 2) and differ at five of our 12 sequenced markers. Of these differences, four loci show patterns inconsistent with the inheritance of alleles from the same *Bd*-GPL or *Bd*-Brazil gamete (i.e. different lineage specific parental alleles are present in either hybrid MLG). Hence, these data demonstrate the occurrence of multiple hybridization events in the Paraná hybrid zone.

An alternative explanation is that these hybrid genotypes are divergent lineages resulting from a parasexual mating (a nonmeiotic fusion of diploid parents with the subsequent loss of chromosomes back to the diploid state), which is known to occur in many groups of fungi (Buxton 1956; Caten & Jinks 1966). This would involve tetraploid intermediates and may explain the higher ploidy levels observed in CLFT024/02 (Schloegel *et al.* 2012). If hybridization was unrestricted, the expected frequency of hybrid strains should approximately equal the frequency of parental genotypes. Hybrid isolates are rarer than expected in the hybrid zone, which may be due to the incipient accumulation of Dobzhansky–Muller incompatibilities hindering the viability of hybrid offspring, or it could represent the rareness of mating opportunities. It remains to be determined whether specific ecological conditions in the Serra da Graciosa site promote the outcrossing of otherwise reproductively isolated lineages. This site may be a recent contact zone between two previously isolated mating types of *Bd* that recently came back into contact without having lost the ability to outcross.

When testing for the signature of historical sexual reproduction, we could not reject that genotypes in AF *Bd* populations were in HW equilibrium. Our analyses may have been constrained by sample size and the technical challenges involved in producing statistically powerful MLST data, but independent tests produce results inconsistent with a scenario of strict asexuality in both the *Bd*-Brazil and *Bd*-GPL lineages. Contrasting the results of our HW exact tests with our I_A permutation tests (which are more sensitive to rare recombination) indicates that there is variance among loci in heterozygosity excess, a pattern that can be explained by very rare sex (Balloux *et al.* 2003) or by mitotic recombination with variable effects across loci. Genotypic equilibrium in *Bd*-Brazil is consistent with an older lineage, in which more time has allowed recombination to break down linkage associations. Furthermore, absence of HW equilibrium may show that some loci are under selection to maintain heterozygosity, perhaps through overdominance. Deeper knowledge about the historical degree of sexual reproduction in *Bd* may hold the key to the origin of the global chytridiomycosis panzootic. Our MLST data may not be sufficient to provide satisfactory conclusions about historical recombination events, because those genetic signatures may be eroded by mitotic recombination. Alternatively, a genome resequencing approach combined with predictive population genetic models of genomic heterozygosity under differing reproductive scenarios of may provide greater utility in addressing the influence of historical sexual recombination in shaping present-day lineages of *Bd*.

Roles of anthropogenic disease translocation

Our results also provide evidence of recent genotype translocation between *Bd*-GPL populations in the northeast region of our sampling transect and the South American ranaculture industry. The incorporation of five strains recovered from captive *L. catesbeianus* at

three Brazilian bullfrog farms and one United States food market (Schloegel *et al.* 2012) provides further insight into the role of the amphibian trade in the long-distance dispersal of *Bd* strains. Most revealing was the distance between shared MLGs (based on our 12 marker data set) recovered from two geographically distant farms 2600 km apart (in São Paulo State and Pará State). This is over three times the distance of shared MLGs between natural populations of native amphibians in the AF and shows that ranaculture in Brazil is responsible for long-distance *Bd* transmission. The *Bd*-Brazil representative previously isolated from a market in the Detroit metro area, Michigan, United States, (UM142; Schloegel *et al.* 2012) forms a clade with a *Bd*-Brazil isolate from Serra do Japi, São Paulo (Fig. 3). These results, along with the demonstrated niche overlap between *Bd* and *L. catesbeianus* (Rödder *et al.* 2013), illustrate the growing problem of pathogen transport through the South American bullfrog trade.

Conclusion

We hypothesize that the divergent *Bd* lineages in Brazil have each experienced very distinct population histories, but have been brought into close contact in portions of the AF. Our findings that *Bd*-Brazil has a higher degree of geographic structure and may have experienced a greater degree of historical recombination than *Bd*-GPL support a hypothesis of long-term endemism of *Bd*-Brazil, and one or more recent introductions, followed by rapid northward expansion of *Bd*-GPL. Our study expands the known range of the recently discovered *Bd*-Brazil lineage in the AF of Brazil, and we document the existence of a hybrid zone in the state of Paraná with the collection of additional hybrid isolates.

A better understanding of how genetic diversity and phenotypic differences in heterogeneous environments underlie selection on pathogen virulence will be necessary to predict and prevent future emerging diseases like chytridiomycosis. We suggest that crucial insights may be found by disentangling the interplay between cross–strain interactions such as competition and sexual recombination. Although we still have much to learn about these interactions between the pathogen lineages detailed herein, the population genetics of *Bd* in the Brazilian AF show that both forces may be shaping disease dynamics of the region and that the long-range transport of these *Bd* genotypes will pose consequences to pathogen evolution at the global scale.

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T.S.J., T.Y.J., L.F.T. and K.R.Z. conceived of and designed the study. T.S.J., C.M.B., C.L., A.V.A., D.R., C.H.L.N., J.R.G., A.M.B., K.R.Z., J.E.L., L.F.T. and T.Y.J. performed fieldwork. T.S.J., C.M.B., C.L., J.E.L., L.F.T., D.S.L. and T.Y.J. performed laboratory work. T.S.J. and T.Y.J. analysed the data. T.S.J. and T.Y.J. wrote the article.

Data accessibility

Multilocus genotype data based on 12 markers with representative allele sequences for each locus has been provided in Genepop format as supplementary information (Appendix S1, Supporting information). Recoded genotype data for neighbour-joining dendrogram and associated distance matrix in Nexus format have been provided as supplementary information (Appendix S2, Supporting information). Multilocus genotype data based on 10 markers and a previously published panel of *Bd* isolates for PCA with representative allele sequences for each locus have been provided in Genepop format as Supporting Information (Appendix S3, Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Multilocus sequence typing marker details for loci analysed in this study.

Table S2 Locus-specific F_{IS} values of by population and lineage.

Appendix S1 Multilocus genotype data for Brazilian Atlantic Forest *Batrachochytrium dendrobatidis* based on 12 loci (Genepop format) and representative allele sequences for each locus.

Appendix S2 Multilocus genotype data based on 12 loci for Brazilian Atlantic Forest *Batrachochytrium dendrobatidis* recoded for *hetequal* distance (Nexus format) and associated distance matrix.

Appendix S3 Multilocus genotype data for a global panel of *Batrachochytrium dendrobatidis* based on 10 loci (Genepop format) and representative allele sequences for each locus.