Genetic characterization of chytrids isolated from larval amphibians collected in central and east Texas

Thomas L. Marshall[12], Carlos R. Baca[1], Decio T. Correa[2], Michael R. J. Forstner[1], Dittmar Hahn[1], and David Rodriguez[1*]

[1]Department of Biology, Texas State University, 601 University Dr., San Marcos, TX 78666, USA

[2]Department of Integrative Biology, University of Texas at Austin, 2415 Speedway, Austin, TX 78712, USA

*Corresponding author: Department of Biology, Texas State University, San Marcos, Texas, 78666, United States of America.

Tel.: +01 512 245 1020

E-mail address: drdz@txstate.edu

ABSTRACT

Chytridiomycosis, an emerging infectious disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), has caused amphibian population declines worldwide. *Bd* was first described in the 1990s and there are still geographic gaps in the genetic analysis of this globally distributed pathogen. Relatively few genetic studies have focused on regions where *Bd* exhibits low virulence, potentially creating a bias in our current knowledge of the pathogen's genetic diversity. Disease-associated declines have not been recorded in Texas (USA), yet *Bd* has been detected on amphibians in the state. These strains have not been isolated and characterized genetically; therefore, we isolated, cultured, and genotyped *Bd* from central Texas and compared isolates to a panel of previously genotyped strains distributed across the Western Hemisphere. We also isolated other chytrids not known to infect amphibians from east Texas. To identify larval amphibian hosts, we sequenced part of the COI gene. Among 37 *Bd* isolates from Texas, we detected 19 unique multi-locus genotypes, but found no genetic structure associated with host species, Texas localities, or across North America. Isolates from central Texas exhibit high diversity and genetically cluster with *Bd*-GPL isolates from the western U.S. that have caused amphibian population declines. This study genetically characterizes isolates of *Bd* from the south central U.S. and adds to the global knowledge of *Bd* genotypes.

Keywords: *Batrachochytrium dendrobatidis*; pathogen dynamics; MLST; culture; genotyping; anuran host

Introduction

Emerging infectious diseases (EIDs) — those that have recently increased in incidence, impact, virulence, geographic or host range, or have recently evolved — are a growing threat to human health and global biodiversity (Daszak *et al.* 2000, 2003). Over the past two decades, an increasing proportion of EIDs have been attributed to pathogenic fungi. These include powdery mildew on important crop plants, white-nosed syndrome in bats, snake fungal disease, colony collapse disorder in bees, and sea-fan aspergillosis in corals (Fisher *et al.* 2016; Fisher *et al.* 2012). Despite their immense economic and ecological impact, the genetic diversity of many of these fungi is still poorly understood (Fisher *et al.* 2016). None of these pathogens have been as devastating in their impact as *Batrachochytrium dendrobatidis* (*Bd*), the aquatic chytrid that induces chytridiomycosis, an epidermal infection in amphibians (Lips 2016).

Bd, a member of the Rhizophydiales order of fungi, causes infections when its flagellated zoospores encyst in a host's skin and develop into zoosporangia (Letcher *et al.* 2006; Longcore *et al.* 1999). It was first described and attributed to disease outbreaks in the late 1990s (Longcore *et al.* 1999) and is now believed to be responsible for declines in amphibian populations dating back at least to the 1970s (Lips *et al.* 2004). *Bd* is found on every continent except Antarctica, is known to have infected over 700 species of amphibians, and has caused mass mortality events including species extinctions in Central and northern South America, the western U.S., Australia, and Spain (Berger *et al.* 1998; Bosch *et al.* 2001; Lips *et al.* 2006; Vredenburg *et al.* 2010). However, certain amphibian species and geographic regions have seemingly been unaffected by chytridiomycosis, even where *Bd* is present (Lips 2016).

Recent phylogenomic analyses of *Bd* have revealed multiple lineages that include the widespread global panzootic lineage (GPL), which is implicated in most disease outbreaks and

can be further divided into a temperate clade (*Bd*-GPL1) and a tropical clade (*Bd*-GPL2). Other lineages include the putatively endemic and less virulent *Bd*-Cape (found in South Africa and Spain), *Bd*-CH from Switzerland (Farrer *et al.* 2011), and *Bd*-Korea (Bataille *et al.* 2013). Sampling efforts have also led to the discovery of a strain in Japan that may be host specific (Goka *et al.* 2009) and *Bd*-Brazil (Jenkinson *et al.* 2016; Rodriguez *et al.* 2014; Schloegel *et al.* 2012), which genomic analyses have indicated is the earliest diverged *Bd* lineage currently known (Rosenblum *et al.* 2013).

Recently revealed diversity within the *Bd* clade, coupled with the fact that there are still several geographic gaps in our current knowledge of *Bd* genotypes (James *et al.* 2015), suggests additional diversity is yet to be discovered. However, relatively few studies have examined *Bd* genotypes from regions lacking population declines, potentially creating a bias in our knowledge of the pathogen's genetic diversity (Bataille *et al.* 2013). It is likely that *Bd*'s origin can be traced to a region in which amphibians have co-evolved with the fungus and where severe population declines and extinctions are not occurring (James *et al.* 2015). While recent work has begun to explore such regions of potential origin, genotyping in additional understudied areas is imperative and is facilitated by isolation and culturing of the fungus (Bataille *et al.* 2013; Jenkinson *et al.* 2016).

In the U.S., *Bd*-GPL has shown patterns of introduction and spread (Padgett-Flohr & Hopkins 2009) and has caused host declines in the Sierra Nevada of California (Briggs *et al.* 2010; Wake & Vredenburg 2008). On the other hand, long-term persistence and a lack of chytridiomycosis related host declines is also evident in the Midwest (Illinois) (Talley *et al.* 2015). Texas is a part of the south-central U.S. where, like the Midwest, no known chytridiomycosis-associated amphibian declines or extinctions have been detected. Studies have

confirmed the presence of *Bd* in the eastern (Saenz *et al.* 2010) and central (Gaertner *et al.* 2012; Gaertner *et al.* 2009a; Gaertner *et al.* 2009b; Gaertner *et al.* 2010) parts of the state, yet no strains from these regions have been genotyped or isolated thus far (James *et al.* 2015). While a lack of known *Bd*-related population declines or extinctions in the area could be an indication that the pathogen has a long local history, other factors, such as climate or host resistance, may be responsible for attenuating disease outbreaks in the region. Studying the genetics of the fungus in Texas and its amphibian hosts is an important first step in understanding potential disease dynamics between amphibians and *Bd* in the region. Additionally, by genetically characterizing isolates from Texas and placing them in the context of a more broadly distributed panel of strains, we can begin to gain a more complete evolutionary picture of the *Bd* group, and perhaps a better understanding of the origins of its most virulent lineages. Therefore, the goal of this study was to isolate *Bd* from larval anurans in central and east Texas, genetically identify anuran hosts, and compare genetic variation among chytrid strains present in this region to those distributed across the Western Hemisphere.

Methods and Materials

Host collection

We collected anuran larvae from ponds in Travis, Hays, Bastrop, Kaufman, and Houston counties in central and eastern Texas from December 2015 to April 2016 (collection permit: SPR-0102-191) and stored them in 50 ml Falcon tubes or new plastic containers with water from the collection site (Table 1). We transported all collected tadpoles, in Styrofoam containers kept cool with synthetic ice packs, to Texas State University where they were euthanized following animal care and use protocols IACUC2014116499 and IACUC2015121220. Each tadpole was

given a unique identifier, and a tail clipping from each tadpole was preserved in 95% ethanol for DNA extraction.

Fungal isolation, culture, and cryopreservation

We excised the keratinized jaw sheaths and tooth rows from each tadpole and transferred them to 1% typtone agar petri dishes. We cleaned the mouthparts by dragging them through fresh agar and transferring them to 1% tryptone agar petri dishes with 200 mg/ml penicillin-G and 200 mg/ml streptomycin sulfate for isolation of chytrids (Longcore 2000). We monitored the plates for growth of zoosporangia and active zoospores for up to two weeks. Once enough active zoospores were observed, we removed a section of the agar containing active zoospores to tryptone broth (16 g tryptone in 1,000 ml deionized water; autoclaved) to cultivate the isolate. Once there was a high density of active zoospores, we transferred a 1-ml aliquot of broth from each isolate to a 1.5-ml centrifuge tube. We used a cryoprotectant solution consisting of 80 ml tryptone broth, 10 ml dimethyl sulfoxide, and 10 ml fetal calf serum to preserve all chytrid isolates (Boyle *et al.* 2003). For each isolate, we briefly centrifuged (2,000 rpm) tryptone broth cultures in 1.5 ml cryotubes, discarded the supernatant, and added 600 μl of cryoprotectant. All isolates were given a serially numbered unique identifier (TXST – Texas State University) and were stored at -80°C (Appendix I).

Genetic methods

To extract DNA, we centrifuged 1 ml aliquots of live broth cultures, discarded the supernatant, and extracted DNA from the pellet using the Mammalian Tissue and Rodent Tail Genomic DNA Purification Protocol in the Thermo Scientific GeneJET Genomic DNA Purification Kit #K0722 (Thermo Fisher Scientific, Inc.). We determined extraction success with gel electrophoresis using a 1% agarose gel stained with GelRed (Biotium) in 1X TBE buffer. We

performed conventional Polymerase Chain Reaction (PCR) on DNA from each successfully

extracted isolate using primers for previously described multilocus sequence typing (MLST)

markers (8009X2, BdC5, BdSc2.0, BdSc4.16, BdSc7.6, R6046, BdSC6.15) and the 18S rRNA

gene (James *et al.* 2009; Jenkinson *et al.* 2016; Morehouse *et al.* 2003; Morgan *et al.* 2007;

Schloegel *et al.* 2012) (Table S1). Amplifications were performed in 25 µl volumes consisting of

12.5 µl DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific, Inc.), 11.5 µl nuclease free

water, 0.25 µl forward primer (10 µM), 0.25 µl (10 µM) reverse primer, and 0.5 µl template

DNA. Thermocycling conditions consisted of an initial denaturing step of 2 min at 95°C, then 32

cycles of 30 s at 95°C, 30 s at 52°C to 60°C depending on the primer pair (Table S1), 45 s at

72°C, with a final extension of 10 min at 72°C. We treated 5 µl aliquots of PCR product with 2

µl ExoSAP-IT (Affymetrix Inc.) and incubated the total volume at 37°C for 15 min and then

80°C for 15 min. Using each of the MLST primers, we performed cycle sequencing reactions

using Big Dye v3.1 dye terminator (Applied Biosystems, Inc.). We incubated hydrated G-50

Sephadex (2.6 g/45 ml H_2O) at room temperature for 30 min, pipetted 400-µl aliquots into

individual wells in a filter plate, and centrifuged the plate at 3000 rpm for 2 minutes to create a

matrix through which cycle sequenced products were passed for purification. We dehydrated the

purified cycle sequenced products, added 12 µl of formamide, and incubated them for 3 min at

94°C and immediately cooled them to 4°C. We electrophoresed the cycle sequenced products on

an ABI 3100-Avant genetic analyzer (Applied Biosystems, Inc.) and trimmed and edited the

resulting chromatograms using Geneious v9.1.5 (Kearse *et al.* 2012).

Data Analysis

We genotyped strains using reference sequences from Jenkinson *et al.* (2016). We then

used a set of genotyped strains from across North and South America to perform comparative

analyses and test for genetic structure across the Western Hemisphere. We limited our analyses to the Western Hemisphere owing to the relatively few number of strains sequenced at these same markers in the Eastern Hemisphere. These strains were selected from two previous studies that used at least six of the same genetic markers used in our study (Jenkinson *et al.* 2016; Schloegel *et al.* 2012). We excluded any strains that had been isolated from amphibians not found in the wild (this included animals from zoos, farms, and markets) for our analyses. The resulting dataset consisted of 177 strains, including the 37 Texas strains isolated in our study. Before calculating measures of genetic diversity, we divided our dataset into populations and subpopulations. These populations (and subpopulations) included temperate America (subpopulations: eastern North America, Texas, and western North America), tropical America (subpopulations: Panama, Brazil-GPL), *Bd*-Brazil, and *Bd*-Brazil/GPL hybrids. For each population and subpopulation, we calculated genotypic diversity and allelic richness using the R package POPGENREPORT (Adamack & Gruber 2014). Next, we clone-corrected our datasets by removing identical genotypes from the same region. This resulted in a dataset that included the genotypes of 68 isolates, including 15 unique Texas isolates, at six MLST markers (Appendix 2). We then calculated gene diversity, or expected heterozygosity (H_E), and observed heterozygosity (H_O) using the R package ADEGENET (Jombart 2008) and calculated pairwise F_{ST} values using GENEPOP (Rousset 2008).

To test for genetic structure among *Bd* isolates, we used STRUCTURE (Pritchard *et al.* 2000) with values of *K* (number of genetic demes defined *a priori*) ranging from 1 to 12 and 5 iterations per *K*, using 500,000 Markov Chain Monte Carlo repetitions with a burn-in of 100,000. We determined the best number of populations by calculating ΔK (Evanno *et al.* 2005) using STRUCTURE HARVESTER (Earl & Vonholdt 2012) and obtained average membership

probabilities across all iterations per *K* using the program CLUMPP (Jakobsson & Rosenberg 2007). We performed an initial analysis with only our Texas isolates and their genotypes at all seven loci (8009x2, BdC5, BdSC4.16, BdSC7.6, R6046, BdSC6.15, BdSC2.0) to test for genetic structure within the state, followed by a second analysis combining the Texas isolates with the set of isolates from North and South America that had been sequenced and genotyped using at least six of the same markers (8009x2, BdC5, BdSC4.16, BdSC7.6, R6046, BdSC6.15). Both STRUCTURE analyses were performed using clone-corrected data.

Host Sequencing and Identification

We extracted DNA from tail muscle of collected tadpoles and toes of seven adult *Rana berlandieri* using the same extraction protocol described for *Bd*. Conventional PCR was performed to amplify the cytochrome c oxidase I (COX1) gene (Table S1), and PCR products were purified and sequenced using the same methods described for *Bd* sequencing. Chromatograms were trimmed, edited and aligned in Geneious v 9.1.5 (Kearse *et al.* 2012). Using Clustal W, implemented through Geneious, we carried out a multiple sequence alignment of host sequence data (cost matrix = clustalw, gap open cost = 15, gap extend cost = 6.66). We used sequence similarity to assign anuran larvae to species and visualized the distance matrix (HKY) using a neighbor-joining topology. We included reference sequences from *Pseudacris streckeri* (GenBank accession no. **KJ536156**), *Rana muscosa* (GenBank accession no. **KU985709**), *R. clamitans* (GenBank accession no. **KY587195**), and *R. sphenocephala* (GenBank accession no. **KT388406**) and newly generated reference sequences for adult *R. berlandieri* (GenBank accession no. **MG969220 - MG969226**). We suspected that some of the larvae belonged to these species based on geographic distribution and general morphology. *Rana muscosa* was chosen as an outgroup to Texas ranids.

Results

We attempted to isolate *Bd* from a total of 140 tadpoles collected from sites in central, east, and north-central Texas (Fig. 1). From this total, we successfully cultured 41 chytrid isolates from 40 individual tadpoles comprising three host species. Of these 41 isolates, 37 were identified as *Bd* (Appendix 1). A total of 28 isolates have been characterized using all seven MLST markers, seven have been characterized at six loci, and two were characterized at five loci (Supplemental data). We were unable to amplify DNA of four isolates from two *R. clamitans* larvae using these markers but did amplify and sequence part of the 18S rRNA gene (Appendix 1). Using the NCBI BLAST search tool (Clark *et al.* 2016), we matched isolates TXST038 and TXST039 to *Rhizophlyctis harderi* (GenBank accession no. **AF164272**; match = 98%, coverage = 100%, E value = 0.0), which has recently been re-named *Uebelmesseromyces harderi* (Powell *et al.* 2015), TXST041 to *Chytriomyces* sp. (GenBank accession no. **DQ536486**; match = 98%, coverage = 100%, E value = 0.0), and isolate TXST038 to *Hyaloraphidium curvatum* (GenBank accession no. **NG_017172**; match = 99%, coverage = 100%, E value = 0.0), all of which are species of chytrids not known to infect vertebrate hosts. Among the 37 *Bd* isolates, there were 19 unique multi-locus genotypes (MLGs), and no MLGs were found at more than one collection site (Supplemental data).

A neighbor-joining topology (Fig. S1) revealed four distinct clades among potential hosts. Three clades were associated with *R. clamitans, R. sphenocephala,* and *Pseudacris streckeri* reference sequences. We suspected that some of our tadpole specimens were *Rana berlandieri*, although a COI reference sequence for this species was unavailable in GenBank. Thus, we sequenced seven *R. berlandieri* metamorphs from central, south, western parts of

Texas (Appendix 1) to verify species assignments. All newly generated sequences were accessioned into GenBank (chytrid 18S, **MG979804–MG979842**; amphibian COI, **MG969220– MG969347**).

Measures of Genetic Diversity

Gene diversity (H_E) among the clone corrected Western Hemisphere dataset was 0.506 and observed heterozygosity (H_O) was 0.370. When all *Bd*-GPL strains were divided into temperate and tropical groups, the temperate group had both higher H_E and H_O (0.402 and 0.394, respectively) than the tropical group (0.358 and 0.340, respectively). Both groups had higher H_E and H_O than *Bd*-Brazil (0.189 and 0.214, respectively). While larger pooled populations had higher expected than observed heterozygosity, the subpopulations in our dataset tended to show the reverse trend (except for the GPL in western North America and southeastern Brazil). While Texas strains had lower H_E (0.348) than both eastern and western North American subpopulations, H_O (0.387) was intermediate between the two other North American regions. All North American subpopulations, including Texas, had higher H_O than Panama and southeastern Brazil, while Texas was the only North American subpopulation to have lower H_E than the GPL in southeastern Brazil. Panama had the lowest H_E and H_O of all GPL subpopulations. The temperate GPL had higher genotypic diversity (0.491) and allelic richness (1.81) compared to the tropical GPL (0.250 and 1.68, respectively) and *Bd*-Brazil (0.280 and 1.34, respectively). Texas strains had lower genotypic diversity (0.405) and allelic richness (1.72) than eastern and western North American strains but displayed greater diversity in these measures than both tropical groups. Not surprisingly, the GPL/*Bd*-Brazil hybrid strains had higher measures of genetic diversity than all other groups (Table 2).

Cluster Analyses

We performed a STRUCTURE analysis using seven loci on the 19 unique MLGs from Texas and did not detect population structure among these strains. We then conducted a STRUCTURE analysis on the combined dataset that included our Texas isolates, as well as additional Western Hemisphere genotypes from two previous studies (Jenkinson *et al.* 2016; Schloegel *et al.* 2012). For these analyses, we chose six MLST markers (r6046, BdSc6.15, 8009x2, BdC5, BdSC4.16, BdSC7.6) that had been sequenced in all three studies, thus we used 15 unique MLGs from Texas to compare against other New World isolates (Appendix 2).

Our STRUCTURE analysis indicated two to three genetically distinct demes in our dataset, with the highest ΔK value occurring at $K = 2$ and second highest value at $K = 3$ (Fig. S2). By using the already genetically identified isolates from the two previous studies as references, we determined that at $K = 2$, our clusters corresponded to *Bd*-GPL and *Bd*-Brazil, while at $K = 3$ substructure within the GPL corresponded to the two major clades within this group; namely *Bd*-GPL1 and *Bd*-GPL2 (Fig. 2A). However, we did not detect the presence of any *Bd*-Brazil strains in Texas. We set an arbitrary threshold of $q = 0.80$ for assigning strains to a particular cluster. Ambiguous strains that did not show strong support for a cluster included the putative GPL/*Bd*-Brazil hybrids, and two strains from Texas and three from Brazil that exhibited mixed support for the two GPL lineages. Pairwise F_{ST} values indicate much greater differentiation between *Bd*-Brazil and all other populations, while Texas isolates show no difference from eastern North American isolates and relatively low differentiation (0.040) from isolates in western North America (Fig. 2B, Table S2).

Discussion

Bd has shown contrasting pathogen dynamics in North America (Briggs *et al.* 2010; Padgett-Flohr & Hopkins 2009; Talley *et al.* 2015; Wake & Vredenburg 2008), yet genetic information from cultured isolates is mainly restricted to the western and eastern U.S., leaving a large swath of the central U.S. without data on *Bd* genotypes, and thus information on strain types. We successfully isolated 37 chytrids identified as *Bd* and four other chytrids (three species) from three different amphibian host species native to central and east Texas. As a population, *Bd* isolates in this region do not show significant genetic differentiation from other North American isolates. Further, all *Bd* isolates collected in this study belong to the globally distributed *Bd*-GPL, and none show genetic affinities to other major lineages (e.g. *Bd*-Brazil, *Bd*-CH, *Bd*-CAPE, or *Bd*-Korea).

Our measures of genetic diversity across geographic regions in the Western Hemisphere are congruent with previous studies. James *et al.* (2009) found lower H_E and H_O among tropical GPL strains compared to temperate GPL strains using MLST markers. The latitudinal gradient in genetic diversity observed by Velo-Anton *et al.* (2012) from California to Panama is also congruent with our estimates. While North American strains have the highest measures of genetic diversity, Texas strains, which represent the southernmost group of North American strains in our dataset, tend to have slightly lower measures of diversity compared to the rest of the continent. Meanwhile, strains from Panama, the region closest to the equator in our dataset, have the lowest measures of diversity among the GPL. These trends could be reflective of a longer history of *Bd* in North America compared to the tropics, but we stress the need for further isolations from North America and genomic sequencing of these strains to draw stronger conclusions about the demographic history of *Bd* in the Western Hemisphere.

The STRUCTURE results show support for two genetic demes within the GPL in Texas and throughout North America. These clusters, which are congruent to *Bd*-GPL1 and *Bd*-GPL2 (Rosenblum *et al.* 2013), are not structured by geography or host species. Of the 37 cultured Texas isolates, 32 appear to cluster with *Bd*-GPL1 isolates. Three Texas isolates (TXST021, TXST026, and TXST035) cluster with *Bd*-GPL2, and two isolates (TXST012 and TXST033) exhibit some degree of assignment uncertainty between both *Bd*-GPL groups (Fig. 2A). Evidence for hybridization between *Bd* lineages has been detected (Jenkinson *et al.* 2016; Schloegel *et al.* 2012), thus it is possible that these ambiguous strains could be the result of hybridization between the two *Bd*-GPL lineages. The presence of the *Bd*-GPL2, a mainly tropical clade (Rosenblum *et al.* 2013), in Texas is not a surprising discovery given that *Bd*-GPL2 isolates have also been documented in isolated locations across North America, but its apparent rarity relative to the *Bd*-GPL1 is consistent with the pattern seen across the continent (James *et al.* 2015). We should point out, however, that there is some ambiguity in assigning membership to these two sub-lineages within the GPL.

At the continental scale, *Bd* isolates in Texas genetically cluster with isolates responsible for severe outbreaks of chytridiomycosis in the western United States (Fig. 2B). This finding leaves us with a still unanswered question—why do these strains, which are genetically similar, cause disease and mortalities in some regions but not others? If the pathogen is genetically similar in these different regions, the other two points of the epidemiological triangle—host and environment—should be explored further. One intuitive explanation for the lack of known chytridiomycosis outbreaks in Texas is the influence of climate. Piotrowski *et al.* (2004) reported *Bd*'s optimal temperature range to be 17-25°C with a maximum threshold of 28°C. In Texas, amphibians may be able to clear or reduce *Bd* infections before they become lethal coincident to

hot summers, when temperatures regularly exceed 32°C (Nielsen-Gammon 2011). Indeed, *Bd* infections in *Acris crepitans* populations in central Texas have revealed a seasonal pattern in infection intensity, with peaks occurring in early spring followed by a decline throughout the summer months (Gaertner *et al.* 2012; Gaertner *et al.* 2009b). Additionally, temperatures in most parts of the state regularly exceed *Bd*'s thermal optimum for over six months of the year (U.S. climate data 2017). However, weather conditions cannot explain the lack of *Bd*-associated morbidity in central Texas salamanders of the genus *Eurycea*, which dwell in cool, thermally stable aquifers and springs. While *Bd* infections in these salamanders have been documented (Gaertner *et al.* 2009a), symptoms of chytridiomycosis have not been detected in these populations despite several years of investigation (Bendik 2017; Bowles *et al.* 2006; Pierce *et al.* 2010). Differences in host resistance among and even within species have been documented (Savage & Zamudio 2011; Woodhams *et al.* 2007), so it is possible that host species immunity plays a role in limiting the severity of disease outcomes in central Texas. The *Bd* strains isolated for this study will facilitate future studies necessary to disentangle the effects of both climate and host resistance on the dynamics of *Bd* infection in this region.

Our isolation and culturing efforts also recovered chytrids not known to infect vertebrate hosts, namely *Uebelmesseromyces harderi* (Rhizophydiales)*, Chytriomyces* sp. (Chytridiales)*, and *Hyaloraphidium curvatum* (Monoblepharidales) (Fig. 1, Appendix 1). Zoosporic fungi of the phylum Chytridiomycota are generally saprobes or parasites of algae, plants, and invertebrates (Barr 2001). It is possible that these chytrids were present in the pond water from which these tadpoles were collected, and that they somehow persisted on the tadpole mouthparts despite our following established procedures to clean them by dragging through sterile agar several times (Longcore *et al.* 1999). Although studies of the morphology and systematics of *U. harderi*,

Chytriomyces, and *H. curvatum* can be found in the literature, little appears to be known about the life history of these species (Forget *et al.* 2002; Letcher & Powell 2002; Powell *et al.* 2015; Ustinova *et al.* 2000). Many chytrids have similar, uninformative morphological features (James *et al.* 2000), and what appear to be successful isolations may not represent *Batrachochytrium* species. Yet, these isolations and cultures are still useful in diversity discovery efforts, especially among the Chytridiomycota, a relatively understudied group of fungi (James *et al.* 2000). By sequencing isolates at the 18S rRNA gene, we were able provide genetic and locality reference data for these species. Owing to the lack of data for environmental chytrids, we recommend genetic characterization of isolated cultures using general primers before—rather than in response to—unsuccessful amplifications of *Bd* specific primers. These efforts will inform genetic databases and accurate curation of cryopreserved microscopic fungal collections.

Our investigation of genetic variation among *Bd* isolates from parts of Texas is an important first step in understanding disease dynamics between amphibians and *Bd* in this understudied region. We have gained a more complete picture of the distribution of *Bd* strains in this region and have begun to fill a large sampling gap for *Bd* isolates in North America. There are also important conservation implications to our understanding of chytridiomycosis dynamics in the region as well. Texas is home to 16 endemic amphibian species, many of which are federally listed as species of concern, and all of which are found in either the Edwards Plateau or the coastal prairies of Texas (Tipton *et al.* 2012). Knowledge of the *Bd* strains present in these regions can inform assessments of potential disease risks to host species in the face of continued climate change and habitat alteration.

Acknowledgments

References

1. Adamack AT, Gruber B (2014) PopGenReport: simplifying basic population genetic analyses in R. *Methods in Ecology and Evolution* **5**, 384-387.

2. Barr DJS (2001) Chytridiomycota. In: *Systematics and Evolution* (ed. McLaughlin DS, J.W.), pp. 93-112. Springer Heidelberg, Germany.

3. Bataille A, Fong JJ, Cha M*, et al.* (2013) Genetic evidence for a high diversity and wide distribution of endemic strains of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* in wild Asian amphibians. *Molecular Ecology* **22**, 4196-4209.

4. Bendik NF (2017) Demographics, reproduction, growth, and abundance of Jollyville Plateau salamanders (*Eurycea tonkawae*). *Ecology and Evolution* **7**, 5002-5015.

5. Berger L, Speare R, Daszak P*, et al.* (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 9031-9036.

6. Bosch J, Martinez-Solano I, Garcia-Paris M (2001) Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation* **97**, 331-337.

7. Bowles BD, Sanders MS, Hansen RS (2006) Ecology of the Jollyville Plateau salamander (*Eurycea tonkawae* : Plethodontidae) with an assessment of the potential effects of urbanization. *Hydrobiologia* **553**, 111-120.

8. Boyle DG, Hyatt AD, Daszak P*, et al.* (2003) Cryo-archiving of *Batrachochytrium dendrobatidis* and other chytridiomycetes. *Diseases of Aquatic Organisms* **56**, 59-64.

9. Briggs CJ, Knapp RA, Vredenburg VT (2010) Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 9695-9700.

10. Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW (2016) GenBank. *Nucleic Acids Research* **44**, D67-72.

11. Daszak P, Cunningham AA, Hyatt AD (2000) Emerging infectious diseases of wildlife-- threats to biodiversity and human health. *Science* **287**, 443-449.

12. Daszak P, Cunningham AA, Hyatt AD (2003) Infectious disease and amphibian population declines. *Diversity and Distributions* **9**, 141-150.

13. Earl DA, Vonholdt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources* **4**, 359-361.

14. Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14**, 2611-2620.

15. Farrer RA, Weinert LA, Bielby J*, et al.* (2011) Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 18732-18736.

16. Fisher MC, Gow NA, Gurr SJ (2016) Tackling emerging fungal threats to animal health, food security and ecosystem resilience. *Philosophical Transactions of the Royal Society B: Biological Sciences* **371**.

17. Fisher MC, Henk DA, Briggs CJ*, et al.* (2012) Emerging fungal threats to animal, plant and ecosystem health. *Nature* **484**, 186-194.

18. Forget L, Ustinova J, Wang Z, Huss VAR, Lang BF (2002) *Hyaloraphidium curvatum*: A linear mitochondrial genome, tRNA editing, and an evolutionary link to lower fungi. *Molecular Biology and Evolution* **19**, 310-319.

19. Gaertner JP, Brown DJ, Mendoza JA*, et al.* (2012) Geographic variation in *Batrachochytrium dendrobatidis* occurrence among populations of *Acris crepitans blanchardi* in Texas, USA. *Herpetological Review* **43**, 274-278.

20. Gaertner JP, Forstner MR, O'Donnell L, Hahn D (2009a) Detection of *Batrachochytrium dendrobatidis* in endemic salamander species from central Texas. *Ecohealth* **6**, 20-26.

21. Gaertner JP, Gaston MA, Spontak D, Forstner MR, Hahn D (2009b) Seasonal variation in the detection of *Batrachochytrium dendrobatidis* in a Texas population of Blanchard's cricket frog

22. (*Acris crepitans blanchardi*). *Herpetological Review* **40**, 184–187.

23. Gaertner JP, McHenry D, Forstner MR, Hahn D (2010) Annual variation of *Batrachochytrium dendrobatidis* in the Houston toad (*Bufo houstonensis*) and a sympatric congener (*Bufo nebulifer*). *Herpetological Review* **41**, 456-459.

24. Goka K, Yokoyama J, Une Y*, et al.* (2009) Amphibian chytridiomycosis in Japan: distribution, haplotypes and possible route of entry into Japan. *Molecular Ecology* **18**, 4757-4774.

25. Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* **23**, 1801-1806.

26. James TY, Litvintseva AP, Vilgalys R*, et al.* (2009) Rapid global expansion of the fungal disease chytridiomycosis into declining and healthy amphibian populations. *PLoS Pathogens* **5**, e1000458.

27. James TY, Porter D, Leander CA, Vilgalys R, Longcore JE (2000) Molecular phylogenetics of the Chytridiomycota supports the utility of ultrastructural data in chytrid systematics. *Canadian Journal of Botany* **78**, 336-350.

28. James TY, Toledo LF, Rodder D*, et al.* (2015) Disentangling host, pathogen, and environmental determinants of a recently emerged wildlife disease: lessons from the first 15 years of amphibian chytridiomycosis research. *Ecology and Evolution* **5**, 4079-4097.

29. Jenkinson TS, Betancourt Roman CM, Lambertini C*, et al.* (2016) Amphibian-killing chytrid in Brazil comprises both locally endemic and globally expanding populations. *Molecular Ecology* **25**, 2978-2996.

30. Jombart T (2008) adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics* **24**, 1403-1405.

31. Kearse M, Moir R, Wilson A*, et al.* (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**, 1647-1649.

32. Letcher PM, Powell MJ (2002) A taxonomic summary of *Chytriomyces* (Chytridiomycota). *Mycotaxon* **84**, 447-487.

33. Letcher PM, Powell MJ, Churchill PF, Chambers JG (2006) Ultrastructural and molecular phylogenetic delineation of a new order, the Rhizophydiales (Chytridiomycota). *Mycological Research* **110**, 898-915.

34. Lips KR (2016) Overview of chytrid emergence and impacts on amphibians. *Philosophical Transactions of the Royal Society B: Biological Sciences* **371**, 20150465.

35. Lips KR, Brem F, Brenes R*, et al.* (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 3165-3170.

36. Lips KR, Mendelson III JR, Muñoz-Alonso A, Canseco-Márquez L, Mulcahy DG (2004) Amphibian population declines in montane southern Mexico: resurveys of historical localities. *Biological Conservation* **119**, 555-564.

37. Longcore JE (2000) Culture techniques for amphibian chytrids: recognizing, isolating, and culturing *Batrachochytrium dendrobatidis* from amphibians. *In: Getting the Jump on Amphibian Disease: Conference and Workshop Compendium, Cairns, Australia, 26–30, August 2000*, 52-54.

38. Longcore JE, Pessier AP, Nichols DK (1999) *Batrachochytrium dendrobatidis* gen et sp nov, a chytrid pathogenic to amphibians. *Mycologia* **91**, 219-227.

39. Morehouse EA, James TY, Ganley ARD*, et al.* (2003) Multilocus sequence typing suggests the chytrid pathogen of amphibians is a recently emerged clone. *Molecular Ecology* **12**, 395-403.

40. Morgan JA, Vredenburg VT, Rachowicz LJ*, et al.* (2007) Population genetics of the frog-killing fungus *Batrachochytrium dendrobatidis*. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 13845-13850.

41. Nielsen-Gammon JW (2011) The changing climate of Texas. In: *The Impact of Global Warming in Texas, 2nd ed.* (ed. Schmandt JN, G.R.; Clarkson, J.). University of Texas Press, Austin, TX.

42. Padgett-Flohr GE, Hopkins RL (2009) *Batrachochytrium dendrobatidis*, a novel pathogen approaching endemism in central California. *Diseases of Aquatic Organisms* **83**, 1-9.

43. Pierce BA, Christiansen JL, Ritzer AL, Jones TA (2010) Ecology of Georgetown salamanders (*Eurycea naufragia*) within the flow of a spring. *Southwestern Naturalist* **55**, 291-297.

44. Powell MJ, Letcher PM, Chambers JG, Roychoudhury S (2015) A new genus and family for the misclassified chytrid, *Rhizophlyctis harderi*. *Mycologia* **107**, 419-431.

45. Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* **155**, 945-959.

46. Rodriguez D, Becker CG, Pupin NC, Haddad CF, Zamudio KR (2014) Long-term endemism of two highly divergent lineages of the amphibian-killing fungus in the Atlantic Forest of Brazil. *Molecular Ecology* **23**, 774-787.

47. Rosenblum EB, James TY, Zamudio KR*, et al.* (2013) Complex history of the amphibian-killing chytrid fungus revealed with genome resequencing data. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 9385-9390.

48. Rousset F (2008) Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources* **8**, 103-106.

49. Saenz D, Kavanagh BT, Kwiatkowski MA (2010) *Batrachochytrium dendrobatidis* detected in amphibians from National Forests in Eastern Texas, USA. *Herpetological Review* **41**, 47.

50. Savage AE, Zamudio KR (2011) MHC genotypes associate with resistance to a frog-killing fungus. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 16705-16710.

51. Schloegel LM, Toledo LF, Longcore JE*, et al.* (2012) Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. *Molecular Ecology* **21**, 5162-5177.

52. Talley BL, Muletz CR, Vredenburg VT, Fleischer RC, Lips KR (2015) A century of *Batrachochytrium dendrobatidis* in Illinois amphibians (1888-1989). *Biological Conservation* **182**, 254-261.

53. Tipton BL, Hibbitts TL, Hibbitts TD, Hibbitts TJ, LaDuc TJ (2012) *Texas Amphibians: A Field Guide* University of Texas Press, Austin, TX.

54. U.S. climate data (2017) *https://www.usclimatedata.com/*.

55. USDA/NRCS - National Geospatial Center of Excellence (2015) 1981-2010 Annual Average Maximum Temperature by State. *https://tnris.org/data-catalog/entry/precipitation/*.

56. Ustinova I, Krienitz L, Huss VAR (2000) *Hyaloraphidium curvatum* is not a green alga, but a lower fungus; Amoebidium parasiticum is not a fungus, but a member of the DRIPs. *Protist* **151**, 253-262.

57. Velo-Anton G, Rodriguez D, Savage AE*, et al.* (2012) Amphibian-killing fungus loses genetic diversity as it spreads across the New World. *Biological Conservation* **146**, 213-218.

58. Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ (2010) Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 9689-9694.

59. Wake DB, Vredenburg VT (2008) Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 11466-11473.

60. Woodhams DC, Ardipradja K, Alford RA*, et al.* (2007) Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. *Animal Conservation* **10**, 409-417.

Table 1. Collection sites (abbreviations) for anuran larvae, collection dates, county (Texas, USA), GPS coordinates of specific localities, number of anuran larvae collected, number of chytrid strains isolated (including non-*Batrachochytrium dendrobatidis* chytrids), and host species from which chytrids were successfully isolated.

Site	Date	County	Latitude (N)	Longitude (W)	Larvae Collected	Chytrid Isolates	Host Species
Abby's Pond (AP)	Feb 2016	Hays	29.9772	-97.8984	6	3	*R. berlandieri*
Bee Cave (BC)	Mar 2016	Travis	30.2542	-97.9392	6	4	*R. berlandieri*
Brackenridge Field Laboratory (BFL)	Mar 2016	Travis	30.2837	-97.7801	40	11[a]	*R. berlandieri*
Davy Crockett National Forest (DCNF)	Feb 2016	Houston	31.4083	-95.1667	21	3[b]	*R. clamitans*
Griffith League Ranch (GLR)	Dec 2015	Bastrop	30.2148	-97.2576	6	0	*R. sphenocephala*
Lakeway (LW)	Mar 2016	Travis	30.3766	-98.0421	6	6	*R. berlandieri*
RRR Ranch (RRR)	Mar 2016	Kaufman	32.5332	-96.4880	47	13	*R. sphenocephala*
Wimberley (WM)	Mar 2016	Hays	29.9955	-98.2163	8	1	*R. berlandieri*
Total					140	41	

[a] includes 1 non-*Bd* isolate
[b] all non-*Bd* isolates

Table 2. Genetic diversity of *Batrachochytrium dendrobatidis* isolates collected in Texas compared to other isolates distributed across the Western Hemisphere. Genetic diversity indices were calculated by averaging across the same six MLST markers.

Region/Population	N [a]	MLG [b]	Genotypic Diversity	Mean Allelic Richness	Clone Corrected Data	
					Observed Heterozygosity	Expected Heterozygosity
Temperate America (*Bd*-GPL)	57	28	0.491	1.81	0.394	0.402
Eastern N.A.	7	7	1.000	1.83	0.429	0.378
Western N.A.	13	13	1.000	1.91	0.385	0.435
Texas	*37*	*15*	*0.405*	*1.72*	*0.387*	*0.348*
Tropical America (*Bd*-GPL)	92	23	0.250	1.68	0.34	0.358
Panama	3	3	1.000	1.59	0.333	0.259
Southeastern Brazil	89	21	0.236	1.67	0.341	0.354
All *Bd*-GPL	**149**	**47**	**0.315**		**0.373**	**0.419**
Hybrids (GPL and *Bd*-Brazil)	3	2	0.667	1.96	0.833	0.438
***Bd*-Brazil**	**25**	**7**	**0.280**	**1.34**	**0.214**	**0.189**
Western Hemisphere	**177**	**56**	**0.316**		**0.370**	**0.506**

[a] Sample size.
[b] Number of multi-locus genotypes

Figure Legends

Fig. 1. Sites where anuran larvae were collected and the proportion of chytrid isolates genetically identified to a known *Bd* strain or other chytrid. The background colors indicate mean maximum temperature in the region (USDA/NRCS - National Geospatial Center of Excellence 2015) with the optimal temperature range for *Bd* growth demarcated in the legend (Piotrowski *et al.* 2004).

Fig. 2. (**A**) STRUCTURE results for $K = 2$ and $K = 3$ averaged from five iterations using CLUMPP. Assignment probabilities to the clusters are represented on the *y*-axis, and each bar represents one of the 68 isolates from a six MLST dataset, including 15 isolates collected for this study and 53 additional strains from Schloegel et al. (2012) and Jenkinson et al. (2016). (**B**) Heat map of pairwise F_{ST} values by origin calculated from the dataset of 68 *Bd* isolates, including 15 isolates from central Texas and 53 additional strains from Schloegel et al. (2012) and Jenkinson et al. (2016).

Mean Max Temperature (C)

Bd Optimal Temperature

31
30
29
28
27
26
25
24
23
22
21
20
19
18
17
16
15
14
13

Sample size

n = 1

n = 5

n = 10

n = 20

Genotype

Bd-GPL1

Bd-GPL2

Bd-GPL

Other
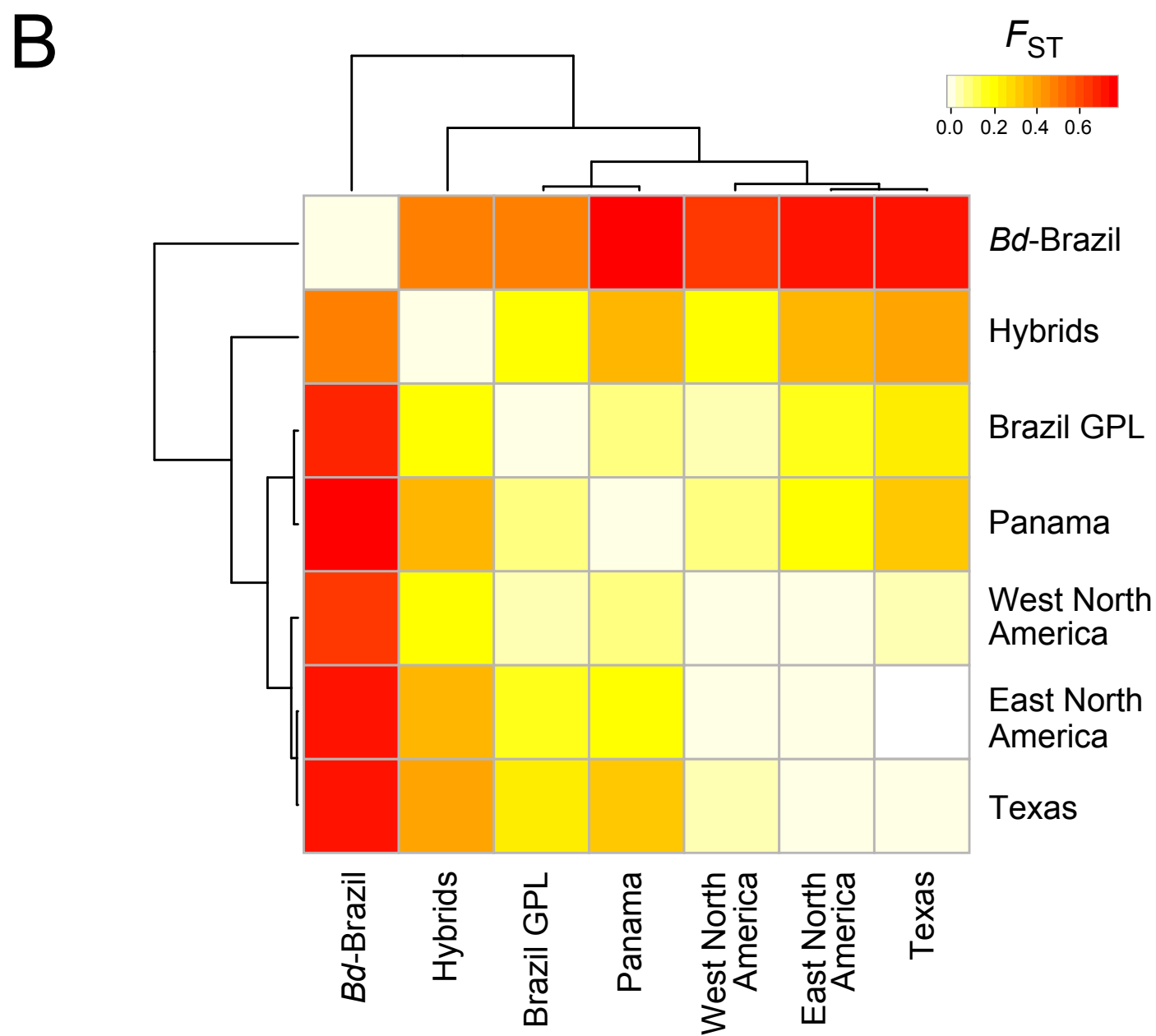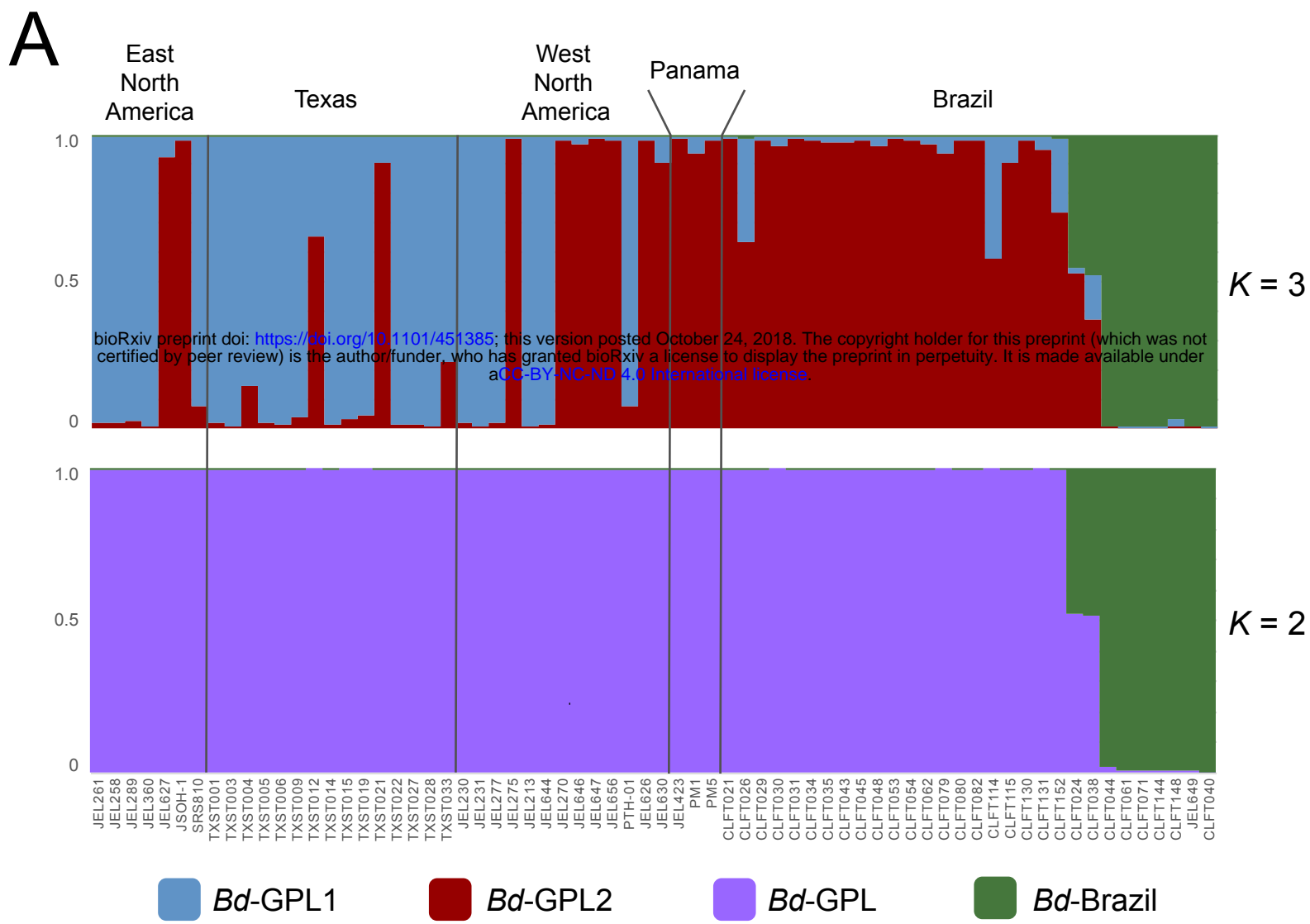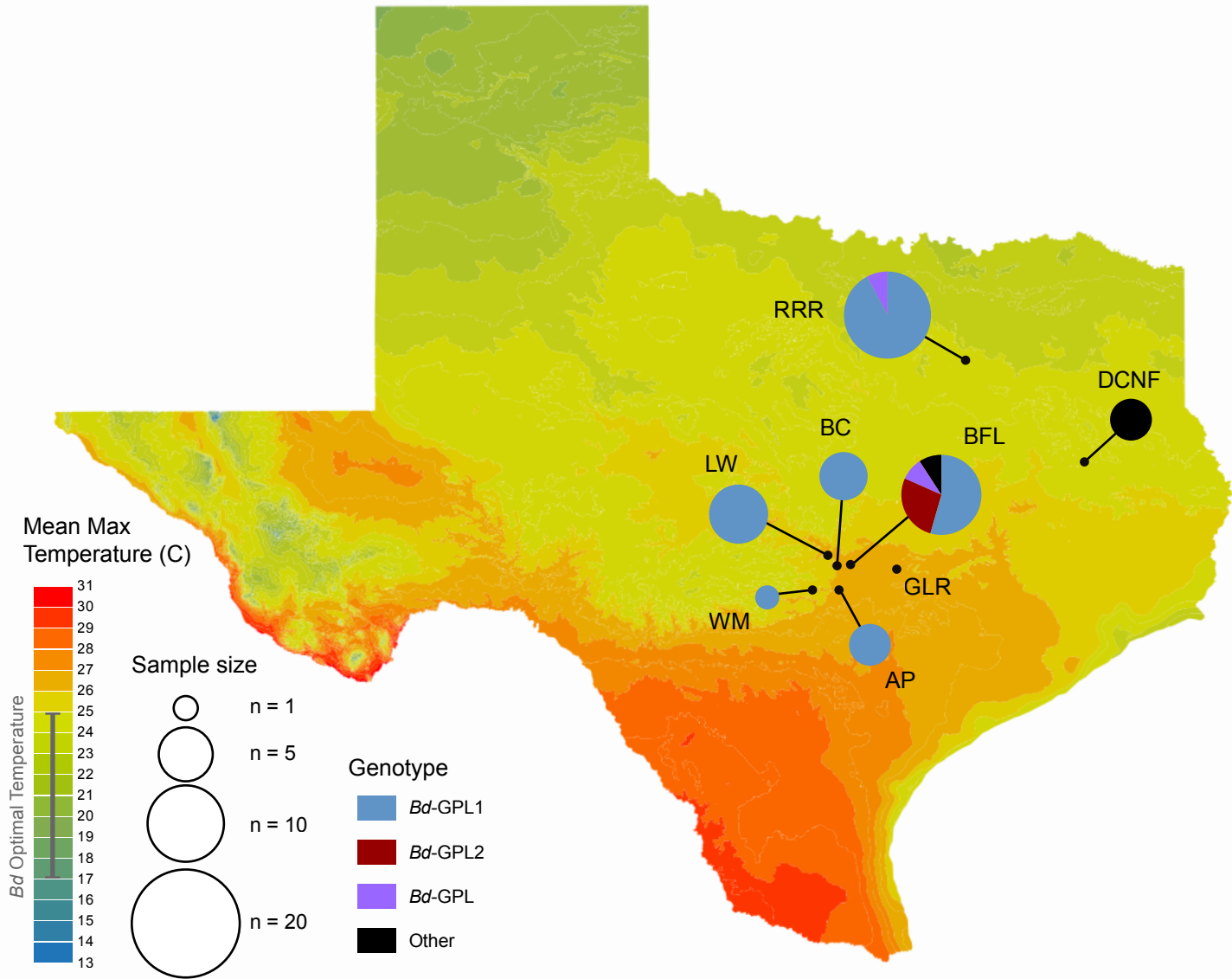
APPENDICES

Appendix 1. Amphibian larvae and adults sampled for this study with collector number (TM = Thomas Marshall, MF = Michael Forstner Tissue Catalog), host species (*Rs = Rana sphenocephala, Rb = R. berlandieri, Rc = R. clamitans, Ps = Pseudacris streckeri*), host COI GenBank accession number, isolate number, chytrid taxon (*Bd = Batrachochytrium dendrobatidis, Uh = Uebelmesseromyces harderi, Ch = Chytriomyces* sp*., Hc = Hyaloraphidium curvatum*), chytrid 18S GenBank accession number, and locality information.

Host ID	Host Species	COI	Isolate ID	Species/Strain	18S	County	Site
TM001	Rs	MG969227				Bastrop	GLR
TM002	Rs	MG969228				Bastrop	GLR
TM003	Rs	MG969229				Bastrop	GLR
TM004	Rs	MG969230				Bastrop	GLR
TM005	Rs	MG969231				Bastrop	GLR
TM006	Rs	MG969232				Bastrop	GLR
TM007	Rb	MG969233				Hays	Abby's Pond
TM008	Rb	MG969234				Hays	Abby's Pond
TM009	Rb	MG969235				Hays	Abby's Pond
TM010	Rc	MG969236				Houston	DCNF
TM011	Rc[a]					Houston	DCNF
TM012	Rc	MG969237				Houston	DCNF
TM013	Rc	MG969238				Houston	DCNF
TM014	Rc	MG969239				Houston	DCNF
TM015	Rb	MG969240	TXST001	*Bd*-GPL1	MG979804	Hays	Abby's Pond
TM016	Rb[a]		TXST002	*Bd*-GPL1	MG979805	Hays	Abby's Pond
TM017	Rb	MG969241	TXST003	*Bd*-GPL1	MG979806	Hays	Abby's Pond
TM018	Rs	MG969242				Kaufman	RRR Ranch
TM019	Rs	MG969243				Kaufman	RRR Ranch
TM020	Rs	MG969244	TXST004	*Bd*-GPL1	MG979807	Kaufman	RRR Ranch
TM021	Rs	MG969245				Kaufman	RRR Ranch
TM022	Rs	MG969246				Kaufman	RRR Ranch
TM023	Rs	MG969247	TXST005	*Bd*-GPL1	MG979808	Kaufman	RRR Ranch
TM024	Rs	MG969248				Kaufman	RRR Ranch
TM025	Rs	MG969249				Kaufman	RRR Ranch
TM026	Rs	MG969250	TXST006	*Bd*-GPL1	MG979809	Kaufman	RRR Ranch
TM027	Rs	MG969251				Kaufman	RRR Ranch
TM028	Rs	MG969252	TXST007	*Bd*-GPL1	MG979810	Kaufman	RRR Ranch
TM029	Rs	MG969253	TXST008	*Bd*-GPL1	MG979811	Kaufman	RRR Ranch
TM030	Rs	MG969254	TXST009	*Bd*-GPL1	MG979812	Kaufman	RRR Ranch
TM031	Rs	MG969255				Kaufman	RRR Ranch
TM032	Rs	MG969256	TXST010	*Bd*-GPL1	MG979813	Kaufman	RRR Ranch
TM033	Rs	MG969257	TXST011	*Bd*-GPL1	MG979814	Kaufman	RRR Ranch
TM034	Rs	MG969258	TXST012	*Bd*-GPL	MG979815	Kaufman	RRR Ranch
TM035	Rs	MG969259				Kaufman	RRR Ranch
TM036	Rs	MG969260	TXST013	*Bd*-GPL1	MG979816	Kaufman	RRR Ranch
TM037	Ps	MG969261				Travis	Bee Cave
TM038	Rb[a]		TXST014	*Bd*-GPL1	MG979817	Travis	Bee Cave
TM039	Rb[a]		TXST015	*Bd*-GPL1	MG979818	Travis	Bee Cave
TM040	Ps	MG969262				Travis	Bee Cave
TM041	Rb	MG969263	TXST016	*Bd*-GPL1	MG979819	Hays	Wimberley
TM042	Rb	MG969264				Hays	Wimberley
TM043	Rb	MG969265				Hays	Wimberley
TM044	Rb	MG969266				Hays	Wimberley
TM045	Rs	MG969267	TXST017	*Bd*-GPL1	-	Kaufman	RRR Ranch
TM046	Rs[a]					Kaufman	RRR Ranch
TM047	Rs	MG969268				Kaufman	RRR Ranch
TM048	Rs[a]		TXST018	*Bd*-GPL1	MG979820	Kaufman	RRR Ranch

TM049	Rs[a]		TXST019	*Bd*-GPL1	MG979821	Kaufman	RRR Ranch
TM050	Rs	MG969269				Kaufman	RRR Ranch
TM051	Rs	MG969270				Kaufman	RRR Ranch
TM052	Rs	MG969271				Kaufman	RRR Ranch
TM053	Rs	MG969272				Kaufman	RRR Ranch
TM054	Rs	MG969273				Kaufman	RRR Ranch
TM055	Rs[a]					Kaufman	RRR Ranch
TM056	Rs[a]					Kaufman	RRR Ranch
TM057	Rs[a]	MG969274				Kaufman	RRR Ranch
TM058	Rs	MG969275				Kaufman	RRR Ranch
TM059	Rs	MG969276				Kaufman	RRR Ranch
TM060	Rs	MG969277				Kaufman	RRR Ranch
TM061	Rs	MG969278				Kaufman	RRR Ranch
TM062	Rs	MG969279				Kaufman	RRR Ranch
TM064	Rs[a]					Kaufman	RRR Ranch
TM065	Rs[a]					Kaufman	RRR Ranch
TM066	Rs	MG969280				Kaufman	RRR Ranch
TM067	Rs	MG969281				Kaufman	RRR Ranch
TM068	Rs	MG969282				Kaufman	RRR Ranch
TM069	Rs[a]					Kaufman	RRR Ranch
TM070	Rs[a]					Kaufman	RRR Ranch
TM071	Rs[a]					Kaufman	RRR Ranch
TM072	Rs[a]					Kaufman	RRR Ranch
TM074	Rb	MG969283	TXST020	*Bd*-GPL1	MG979822	Travis	BFL
TM075	Rb	MG969284	TXST021	*Bd*-GPL2	MG979823	Travis	BFL
TM076	Rb	MG969285	TXST022	*Bd*-GPL1	MG979824	Travis	BFL
TM077	Rb	MG969286				Travis	BFL
TM078	Rb	MG969287				Travis	BFL
TM079	Rb	MG969288				Travis	BFL
TM080	Rb	MG969289				Travis	BFL
TM081	Rb	MG969290	TXST023	*Bd*-GPL1	MG979825	Travis	BFL
TM082	Rb	MG969291				Travis	BFL
TM083	Rb	MG969292				Travis	BFL
TM084	Rb	MG969293				Travis	BFL
TM085	Rb	MG969294				Travis	BFL
TM086	Rb	MG969295	TXST024	*Bd*-GPL1	MG979826	Travis	BFL
TM087	Rb	MG969296				Travis	BFL
TM088	Rb	MG969297	TXST025	*Bd*-GPL1	MG979827	Travis	BFL
TM089	Rb	MG969298	TXST038	*Hc*	MG979828	Travis	BFL
TM090	Rb	MG969299	TXST026	*Bd*-GPL2	MG979829	Travis	BFL
TM091	Rb	MG969300	TXST027	*Bd*-GPL1	MG979830	Travis	Lakeway
TM092	Rb	MG969301	TXST028	*Bd*-GPL1	MG979831	Travis	Lakeway
TM093	Rb	MG969302	TXST029	*Bd*-GPL1	MG979832	Travis	Lakeway
TM094	Rb	MG969303	TXST030	*Bd*-GPL1	MG979833	Travis	Lakeway
TM095	Rb	MG969304	TXST031	*Bd*-GPL1	MG979834	Travis	Lakeway
TM096	Rb	MG969305	TXST032	*Bd*-GPL1	MG979835	Travis	Lakeway
TM097	Rb	MG969306				Travis	BFL
TM098	Rc	MG969307				Houston	DCNF
TM099	Rc	MG969308				Houston	DCNF
TM100	Rc	MG969309				Houston	DCNF
TM101	Rc[a]					Houston	DCNF
TM102	Rc	MG969310				Houston	DCNF
TM103	Rc	MG969311				Houston	DCNF
TM104	Rc	MG969312				Houston	DCNF
TM105	Rc	MG969313				Houston	DCNF
TM106	Rc	MG969314				Houston	DCNF
TM107	Rc	MG969315				Houston	DCNF
TM108	Rc	MG969316	TXST039	*Uh*	MG979836	Houston	DCNF
TM109a	Rc	MG969317	TXST040	*Uh*	MG979837	Houston	DCNF
TM109b	Rc	MG969318	TXST041	*Ch*	MG979838	Houston	DCNF
TM110	Rc	MG969319				Houston	DCNF
TM111	Rc	MG969320				Houston	DCNF

TM112	Rc	MG969310				Houston	DCNF
TM113	Rc[a]					Houston	DCNF
TM114	Rb	MG969321				Travis	BFL
TM115	Rb	MG969322				Travis	BFL
TM116	Rb	MG969323				Travis	BFL
TM117	Rb[a]					Travis	BFL
TM118	Rb	MG969324				Travis	BFL
TM119	Rb	MG969325				Travis	BFL
TM120	Rb	MG969326				Travis	BFL
TM121	Rb	MG969327	TXST033	*Bd*-GPL	MG979839	Travis	BFL
TM122	Rb	MG969328	TXST034	*Bd*-GPL1	MG979840	Travis	BFL
TM123	Rb	MG969329				Travis	BFL
TM124	Rb	MG969330				Travis	BFL
TM125	Rb	MG969331	TXST035	*Bd*-GPL2	MG979841	Travis	BFL
TM126	Rb	MG969332				Travis	BFL
TM127	Rb	MG969333	TXST036	*Bd*-GPL1	-	Travis	Bee Cave
TM128	Rb	MG969334	TXST037	*Bd*-GPL1	MG979842	Travis	Bee Cave
TM129	Rb	MG969335				Hays	Wimberley
TM130	Rb	MG969336				Hays	Wimberley
TM131	Rb	MG969337				Hays	Wimberley
TM132	Rb	MG969338				Hays	Wimberley
TM133	Rb	MG969339				Travis	BFL
TM134	Rb	MG969340				Travis	BFL
TM135	Rb	MG969341				Travis	BFL
TM136	Rb	MG969342				Travis	BFL
TM137	Rb	MG969343				Travis	BFL
TM138	Rb	MG969344				Travis	BFL
TM139	Rb	MG969345				Travis	BFL
TM140	Rb	MG969346				Travis	BFL
TM141	Rb	MG969347				Travis	BFL
MF8122[b]	Rb	MG969220				Bastrop	30.201034, -97.221376
MF8124[b]	Rb	MG969221				Bastrop	30.201034, -97.221378
MF17750[b]	Rb	MG969222				Brewster	29.1796932, -102.9955709
MF17751[b]	Rb	MG969223				Brewster	29.1796932, -102.9955709
MF17752[b]	Rb	MG969224				Brewster	29.1796932, -102.9955709
MF17753[b]	Rb	MG969225				Brewster	29.1796932, -102.9955709
MF21852[b]	Rb	MG969226				Jim Hogg	27.1633606, -98.54593658

[a] Host species assignment based on morphology and collection locality.

[b] Adult *Rana berlanderi* used as genetic references.

3

Appendix 2. *Bd* Isolates Analyzed for this Study with Lineage, Location Collected, and Original Publication. This table includes the 68 isolates, including 15 Texas isolates, included in the STRUCTURE and pairwise F_{ST} analyses using six MLST markers.

Isolate	Lineage	Location	Source
TXST001	GPL1	Texas	Collected for this study
TXST003	GPL1	Texas	Collected for this study
TXST004	GPL1	Texas	Collected for this study
TXST005	GPL1	Texas	Collected for this study
TXST006	GPL1	Texas	Collected for this study
TXST009	GPL1	Texas	Collected for this study
TXST012	GPL	Texas	Collected for this study
TXST014	GPL1	Texas	Collected for this study
TXST015	GPL1	Texas	Collected for this study
TXST019	GPL1	Texas	Collected for this study
TXST021	GPL2	Texas	Collected for this study
TXST022	GPL1	Texas	Collected for this study
TXST027	GPL1	Texas	Collected for this study
TXST028	GPL1	Texas	Collected for this study
TXST033	GPL	Texas	Collected for this study
JEL213	GPL1	W. North America	Schloegel et al. 2012
JEL230	GPL1	W. North America	Schloegel et al. 2012
JEL231	GPL1	W. North America	Schloegel et al. 2012
JEL258	GPL1	E. North America	Schloegel et al. 2012
JEL261	GPL1	E. North America	Schloegel et al. 2012
JEL270	GPL2	W. North America	Schloegel et al. 2012
JEL275	GPL2	W. North America	Schloegel et al. 2012
JEL277	GPL1	W. North America	Schloegel et al. 2012
JEL289	GPL1	E. North America	Schloegel et al. 2012
JEL360	GPL1	E. North America	Schloegel et al. 2012
JEL423	GPL2	Panama	Schloegel et al. 2012
JEL626	GPL2	W. North America	Schloegel et al. 2012
JEL627	GPL2	E. North America	Schloegel et al. 2012
JEL630	GPL2	W. North America	Schloegel et al. 2012
JEL644	GPL1	W. North America	Schloegel et al. 2012
JEL646	GPL2	W. North America	Schloegel et al. 2012
JEL647	GPL2	W. North America	Schloegel et al. 2012
JEL656	GPL2	W. North America	Schloegel et al. 2012
JSOH-1	GPL2	E. North America	Schloegel et al. 2012
PM1	GPL2	Panama	Schloegel et al. 2012
PM5	GPL2	Panama	Schloegel et al. 2012
PTH-001	GPL1	W. North America	Schloegel et al. 2012
SRS810	GPL1	E. North America	Schloegel et al. 2012
CLFT026	GPL	Brazil	Jenkinson et al. 2016
CLFT029	GPL2	Brazil	Jenkinson et al. 2016

CLFT030	GPL2	Brazil	Jenkinson et al. 2016
CLFT031	GPL2	Brazil	Jenkinson et al. 2016
CLFT034	GPL2	Brazil	Jenkinson et al. 2016
CLFT035	GPL2	Brazil	Jenkinson et al. 2016
CLFT038	Hybrid	Brazil	Jenkinson et al. 2016
CLFT040	*Bd*-Brazil	Brazil	Jenkinson et al. 2016
CLFT043	GPL2	Brazil	Jenkinson et al. 2016
CLFT044	*Bd*-Brazil	Brazil	Jenkinson et al. 2016
CLFT045	GPL2	Brazil	Jenkinson et al. 2016
CLFT048	GPL2	Brazil	Jenkinson et al. 2016
CLFT053	GPL2	Brazil	Jenkinson et al. 2016
CLFT054	GPL2	Brazil	Jenkinson et al. 2016
CLFT061	*Bd*-Brazil	Brazil	Jenkinson et al. 2016
CLFT062	GPL2	Brazil	Jenkinson et al. 2016
CLFT071	*Bd*-Brazil	Brazil	Jenkinson et al. 2016
CLFT079	GPL2	Brazil	Jenkinson et al. 2016
CLFT080	GPL2	Brazil	Jenkinson et al. 2016
CLFT082	GPL2	Brazil	Jenkinson et al. 2016
CLFT114	GPL	Brazil	Jenkinson et al. 2016
CLFT115	GPL2	Brazil	Jenkinson et al. 2016
CLFT130	GPL2	Brazil	Jenkinson et al. 2016
CLFT131	GPL2	Brazil	Jenkinson et al. 2016
CLFT144	*Bd*-Brazil	Brazil	Jenkinson et al. 2016
CLFT148	*Bd*-Brazil	Brazil	Jenkinson et al. 2016
CLFT152	GPL	Brazil	Jenkinson et al. 2016