Received: 26 September 2016 | Revised: 26 December 2016 | Accepted: 15 March 2017

DOI: 10.1111/1755-0998.12675

RESOURCE ARTICLE

WILEY MOLECULAR ECOLOGY RESOURCES

Unlocking the story in the swab: A new genotyping assay for the amphibian chytrid fungus *Batrachochytrium dendrobatidis*

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Funding information
National Science Foundation, Grant/Award Number: IOS-1354241

Abstract

One of the most devastating emerging pathogens of wildlife is the chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), which affects hundreds of amphibian species around the world. Genomic data from pure Bd cultures have advanced our understanding of Bd phylogenetics, genomic architecture and mechanisms of virulence. However, pure cultures are laborious to obtain and whole-genome sequencing is comparatively expensive, so relatively few isolates have been genetically characterized. Thus, we still know little about the genetic diversity of Bd in natural systems. The most common noninvasive method of sampling Bd from natural populations is to swab amphibian skin. Hundreds of thousands of swabs have been collected from amphibians around the world, but Bd DNA collected via swabs is often low in quality and/or quantity. In this study, we developed a custom Bd genotyping assay using the Fluidigm Access Array platform to amplify 192 carefully selected regions of the Bd genome. We obtained robust sequence data for pure Bd cultures and field-collected skin swabs. This new assay has the power to accurately discriminate among the major Bd clades, recovering the basic tree topology previously revealed using whole-genome data. Additionally, we established a critical value for initial Bd load for swab samples (150 Bd genomic equivalents) above which our assay performs well. By leveraging advances in microfluidic multiplex PCR technology and the globally distributed resource of amphibian swab samples, noninvasive skin swabs can now be used to address critical spatial and temporal questions about Bd and its effects on declining amphibian populations.

KEYWORDS
Batrachochytrium dendrobatidis, genotype, microfluidic multiplex PCR, swab

1 | INTRODUCTION

Emerging infectious diseases (EID) are increasingly recognized as a critical threat to wildlife. EIDs have been responsible for catastrophic declines in many natural systems, for example chytridiomycosis in amphibians, white-nose syndrome in bats, avian malaria in Hawaii and chronic wasting disease in cervids (Atkinson & Samuel, 2010; Foley, Clifford, Castle, Cryan, & Ostfeld, 2011; Saunders, Bartelt-Hunt, & Bartz, 2012; Skerratt et al., 2007). To further understand the origin, spread and transmission dynamics of EIDs of wildlife, biologists have increasingly turned to genetic and genomic tools (Archie, Luikart, & Ezenwa, 2009; Grogan et al., 2014). Advances in DNA extraction, amplification and sequencing technologies can inform wildlife management by allowing effective and timely disease monitoring, identification of disease transmission pathways and reconstruction of evolutionary relationships among pathogens (e.g., Beja-Pereira et al., 2009; Schloegel et al., 2012; Wallace, HoDac, Lathrop, & Fitch, 2007).

Of the many documented wildlife EIDs, few have had such a devastating impact as the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Bd). Bd is a generalist pathogen that infects hundreds of amphibian species around the world. Chytridiomycosis—the disease caused by Bd—has been linked to mass mortality events in many of the most diverse amphibian communities in the world (Berger et al., 1998; Crawford, Lips, & Bermingham, 2010). To date, the most detailed genetic and genomic studies of Bd have relied on pure cultures of the pathogen, which are isolated from infected wild amphibians. Once isolated, pure cultures can be cryoarchived for use in future experiments (Boyle et al., 2003) and can be grown in the laboratory to provide enough genetic material for the application of genome-scale molecular approaches. Molecular studies from pure Bd isolates have been used to produce a Bd phylogeny, reveal complex structural variation in the Bd genome, identify putative virulence genes and catalogue Bd strains that likely result from hybridization (Farrer et al., 2011; Jenkinson et al., 2016; Rosenblum, Poorten, Joneson, & Settles, 2012; Rosenblum et al., 2013). However, pure cultures are laborious to isolate, require access to a sterile laboratory and often necessitate destructive amphibian sampling (Longcore, Pessier, & Nichols, 1999). While some pure Bd cultures have been isolated from toe clips (which is not destructive), toe-clipping can still cause stress to individual amphibians (Mccarthy & Parris, 2004) and still requires access to a sterile laboratory for processing. Therefore, studies using pure isolates generally suffer from limited sample sizes and lack of isolates from remote locations.

An alternative sampling method to collect Bd genetic material is the use of sterile swabs (Hyatt et al., 2007; Retallick, Miera, Richards, & Field, 2006). Bd infects the epidermal cells of amphibians and the keratinized mouthparts of tadpoles (Berger et al., 1998; Fellers, Green, & Longcore, 2001). By swabbing amphibian skin or tadpole mouthparts, Bd genetic material can be collected in an inexpensive and noninvasive manner, allowing for large sample sizes and minimal impacts on host species. Swabs are then typically screened for Bd using a quantitative PCR assay (Boyle, Boyle, Olsen, Morgan, & Hyatt, 2004; Hyatt et al., 2007). To date, Bd swabs have most commonly been used to track Bd prevalence and load in natural populations, infection intensity during laboratory infection experiments and Bd presence in museum specimens (e.g., Cheng, Rovito, Wake, & Vredenburg, 2011; Zhu et al., 2014). Amphibian researchers have collected hundreds of thousands of swabs from myriad host species, geographic localities and time points during Bd outbreaks (Olson et al., 2013). However, the use of swabs for more detailed population genetics studies has been limited by the low quality and quantity of DNA collected using this method. A handful of studies have sequenced genetic markers from swabs, but these have been limited to one or few loci (e.g., Bai, Liu, Fisher, Garner, & Li, 2012; Garland, James, Blair, & Berger, 2011; Goka et al., 2009) and have lacked resolution for addressing key questions about the origin, spread and transmission of Bd.

Here, we present a new method for rapid, cost-effective, multilocus genotyping for Bd swab samples. We selected 192 target regions of the Bd genome based on their ability to capture variation within and between the major clades of the Bd phylogeny. We then developed a custom Bd assay using the Fluidigm Access Array System. This microfluidic multiplex PCR platform amplifies selected regions of the Bd genome and tags individual samples so they can be pooled for Illumina sequencing. The assay is specifically designed to amplify Bd DNA of low quantity and/or quality and is highly scalable for large sample sizes. By leveraging advances in DNA amplification and sequencing technology and the globally distributed resource of amphibian swab samples, we can begin to understand Bd genetic diversity across temporal and spatial scales and finally unlock the story in the swab.

2 | MATERIALS AND METHODS

2.1 | Genomic loci selection and primer design

We used genomic data sets consisting of 29 globally distributed Bd isolates from Rosenblum et al. (2013) and 20 Bd isolates from Farrer et al. (2011) as the basis for developing our genotyping assay. We integrated these two data sets to obtain 76,515 single nucleotide polymorphisms (SNPs) and infer a whole-genome phylogeny (Rosenblum et al., 2013). Using the genomes of these 49 isolates, we selected 192 genomic regions for our new assay (Table S1). We targeted 150- to 200-base pair regions (rather than particular SNPs) to increase the power of our assay and to avoid issues of ascertainment bias. We selected target regions based on three primary criteria:

First, we selected genomic regions that had discriminatory power at different nodes of the Bd phylogeny. Specifically, we chose regions with SNPs that showed high genetic differentiation (F_{ST} values) between clades of interest. We captured variation across five Bd lineages including two main clades within the globally distributed global panzootic lineage (GPL), divergent lineages from South Africa (Bd-Cape) and Europe (Bd-CH) and a deeply divergent group of isolates from Brazil (Bd-Brazil) (Farrer et al., 2011; Rosenblum et al., 2013).

Second, we selected genomic regions in putative mutational hotspots to increase the resolution within the GPL. Using a sliding window analysis, we searched for clusters of informative SNPs that were enriched for rare alleles (defined as a minor allele frequency (MAF) <0.15 in the Rosenblum et al. (2013) genomic data set). In addition, we chose genomic targets that were well dispersed across different chromosomal segments.

Third, we selected several genomic regions to provide a direct comparison to previous Bd studies. We targeted genomic regions previously sequenced in other studies (James et al., 2009; Morehouse et al., 2003; Morgan et al., 2007; Schloegel et al., 2012) and designed additional primers for these regions to fit our target amplicon size and design parameters. In addition, we included a single primer pair to target, *Batrachochytrium salamandrivorans*, a recently described relative of Bd that is particularly deadly to salamanders (Martel et al., 2013). We selected *B. salamandrivorans*-specific

primers for the ITS1, 5.8S rRNA, ITS2 region (GenBank: KC762295) based on Martel et al., 2013. This single *B. salamandrivorans* marker will most likely function as a detection locus rather than as a marker for comparative purposes, but additional *B. salamandrivorans* markers can be added as genomic resources are developed for this species.

2.2 | Primer validation

We designed primers in silico using PRIMER3 version 0.4.0 (Koressaar & Remm, 2007) using Bd isolate JEL423 (Broad Institute, broadinstitute.org) as a reference genome. We optimized for conditions as required for the Fluidigm Access Array System (Fluidigm, South San Francisco, CA, USA) including a melting temperature of 60°C and a 3-bp maximum length for mononucleotide repeats. We selected amplicons of 150–200 bp to increase the potential for informative linked SNPs within regions while keeping amplicon sizes small. We excluded all known polymorphisms from the primer-binding sites to maximize probability of successful primer binding to all Bd samples.

We subsequently tested all designed primers using in silico PCR in GENEIOUS version 7.1.2 (Kearse et al., 2012) to assess specificity and confirm that targets were single-copy loci. We ensured no overlap between forward and reverse primers of nearby target regions, we verified inclusion of SNPs in the target sequences, and we confirmed that all primer-binding sites were located in conserved regions of the Bd genome. Finally, we assessed phylogenetic resolution of our assay by aligning target sequences from Bd strains in all major clades.

2.3 | Primer pooling

Pooling primers allows more high-throughput workflows, but multiplex PCR methods can be prone to amplification failure if primer pools are not constructed carefully (e.g., dimer interactions among primers, amplification bias towards more robust amplicons, differences in GC content among amplicons (Suzuki & Giovannoni, 1996; Baskaran et al., 1996; Markoulatos, Siafakas, & Moncany, 2002). We therefore carefully assigned our primers to pools that minimized adverse interactions. We constructed primer pools in 48 wells (four primer pairs per pool). As an initial screen for primer pooling, we sorted primer pairs based on target regions being on different chromosomal segments. We then screened for potential interprimer interactions using Thermo Fisher Scientific multiple primer analyzer tool (https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html). Additionally, we kept amplicon sizes within 20% of the average length within each primer pool to avoid amplification biases resulting from combining PCR products of different sizes. Furthermore, we calculated GC content of all amplicons using the Biostrings package in R (Pagès, Aboyoun, Gentleman, & DebRoy, 2016) to ensure combined primer pairs would target regions with GC content within 20% of the average GC content among amplicons in each well.

2.4 | Sample selection and DNA isolation

Following genomic target selection and primer design, we selected samples for assay validation with two primary goals. Our first goal was to assess how accurately our 192 genomic targets could recover the basic topology of the Bd phylogeny presented in Rosenblum et al. (2013). Therefore, we selected 28 Bd isolates that were globally dispersed and from a variety of amphibian host species (Table S2). Our sampling focused on the GPL and the divergent Bd-Brazil clade, but was not exhaustive and did not include *B. salamandrivorans*. We also included isolates from three non-Bd chytrids: *Homolaphlyctis polyrhiza*, *Entophlyctis helioformis* and *Rhizophydium brooksianum* to test the specificity of the primers. We extracted isolate DNA using a modified version of Zolan and Pukkila (1986) phenol–chloroform protocol (Joneson, Stajich, Shiu, & Rosenblum, 2011).

Our second goal was to test assay sensitivity to DNA quantity. We included three replicates each of serial diluted Bd DNA extractions at 0.1, 1, 10 and 100 genome equivalents (GE). We also included 48 field-collected swab samples ranging in Bd copy number from 1.6 to 116,706. We extracted DNA from the swab samples using the manufacturer's recommendations for Qiagen DNeasy Kits (Qiagen, Valencia, CA, USA). Copy number for the swabs was calculated using a plasmid-based qPCR standard. Average copy number is a commonly reported measure of Bd infection intensity; therefore, we chose swab samples with different Bd loads to assess assay performance.

2.5 | Microfluidic PCR and Illumina sequencing

The Fluidigm Access Array platform allows users to perform multiple (48 primer pairs) PCRs across multiple (48) DNA samples in parallel through a two-staged multiplex amplification (2,304 simultaneous PCRs). During PCR, all amplicons are tagged with (i) dual barcodes to distinguish each sample and (ii) sequencing adapters suitable for downstream Illumina sequencing. After amplification, all samples are pooled for sequencing and can later be demultiplexed by their respective barcodes.

To improve microfluidic PCR success, we used a pre-amplification step that first enriches DNA template for the targeted genomic regions. Pre-amplification is especially beneficial when working with low quantities of template DNA (e.g., swab samples). We performed pre-amplification reactions according to the manufacturer's protocol (Fluidigm, South San Francisco, CA, USA). We cleaned pre-amplified products using ExoSAP-IT, followed by a 1:5 dilution in water.

We used both our pre-amplified diluted products and primer pair pools in Access Array amplification and Illumina sequencing. Briefly, samples and primer pools were loaded into Fluidigm's integrated fluidic circuits (IFC) and multiplex PCRs occurred in the Fluidigm FC1 Cycler at the University of Idaho IBEST Genomics Resources Core. Sequencing was performed on the Illumina MiSeq using the 300-bp paired-end kits at the University of Idaho IBEST Genomics Resources Core. We processed our samples on a quarter of a sequencing plate (resulting in ~4 million reads) to generate ~200× coverage for each unique amplicon (i.e., each combination of sample

and target). We chose a relatively high level of sequencing effort to increase number of reads per samples and minimize missing data given our input of samples of variable DNA quantity and quality.

2.6 | SNP genotyping and analysis

Starting with raw sequences, we used DBCAMPLICONS (https://github.com/msettles/dbcAmplicons) to process reads and identify consensus sequences and putative alleles for each sample and primer pair. Briefly, each read was demultiplexed for each sample and primer, using the sample-specific dual barcode combinations (edit distance ≤1bp) and target-specific primers (Levenshtein distance ≤4 with final four bases as perfect matches, resulting in firm ends). Primer sequences were then removed. Representative sequences for each sample and amplicon were then identified using the reduce_amplicons R script within the dbcAmplicons repository (https://github.com/msettles/dbcAmplicons/blob/master/scripts/R/reduce_amplicons.R). Paired reads were overlapped and joined into a single continuous sequence using FLASH2 (https://github.com/dstreett/FLASH2). Lastly, reads for each sample and primer pair were collapsed to their most often occurring amplicon length variant (all other reads were not included in the consensus or ambiguity sequences). Ambiguity sequences contain IUPAC ambiguity codes at positions with possible polymorphisms. Consensus sequences (with and without ambiguities) were generated when each variant was present in at least five reads and at least 5% of the total number of reads.

Whole-genome data from 25 isolates were cleaned and analysed as done in Rosenblum et al. (2013). This data set contained 22 isolates previously published in Rosenblum et al., 2013 and three isolates from Panama that have not been published (Campana, JEL410, Rio Maria). Briefly, variants were called using the best practices for the GATK v.3.4 pipeline (McKenna et al., 2010). The GATK tools RealignerTargetCreator and IndelRealigner were used to realign reads around insertions/deletions, and the HAPLOTYPECALLER and GENOTYPEGVCFS tools were used to call variants. Custom R scripts were used to filter genotypes based on quality and depth. SNPs were encoded to represent homozygous for ancestral allele (0), heterozygous (1) and homozygous for derived allele (2). A data set consisting of 20,535 SNPs was used to create a maximum parsimony phylogenetic tree with 100 bootstraps using the PHANGORN package in R (Schliep, 2011). For the Bd Fluidigm Access Array data, the consensus sequences for all shared amplicons were concatenated to form a single consensus sequence. These sequences were then aligned using MUSCLE v3.8 (Edgar, 2004) and alignments were visually inspected to check for errors. A maximum parsimony phylogenetic tree with 100 bootstrap replicates was created using the PHANGORN package (Schliep, 2011) in R (version 3.2.2) with UM142 as the designated outgroup. Each phylogenetic tree was plotted in R using the phytools package (Revell, 2012) to create a cophylogeny that is rotated to minimize the distance between each sample pair. Three of the 28 isolates included in the Bd Fluidigm Access Array study were omitted from this analysis because whole-genome sequences were not available. Finally, to assess swab performance, we conducted a local regression comparing the log Bd copy number to the number of unique amplicons returned. We used the loess function in R and then a recursive decision tree in the R package rpart (Therneau, Atkinson, & Ripley, 2010) to classify swabs into higher-performing and lower-performing groups.

3 | RESULTS

3.1 | General assay performance

Of the 192 target loci, 190 (99%) successfully amplified (produced a consensus sequence) for at least one of the 96 samples (Figure 1). The vast majority of target loci amplified consistently across the pure Bd culture samples. For the pure Bd isolates, we obtained an average of 182.5 (95%) loci per sample, resulting in concatenated consensus sequences of more than 20,000 base pairs in length. All pure Bd isolates, including the most divergent UM142 and LFT001, had similar amplification profiles and low locus dropout rates. We also used standard dilutions of pure Bd culture to test assay sensitivity. Number of loci successfully sequenced increased predictably with Bd genomic equivalents (GE). An average of 2.3 loci were returned for 0.1 GE, 29.3 for 1 GE, 41 for 10 GE and 93.3 for 100 GE. Also as expected, the non-Bd isolates amplified only a small number of target regions. We obtained an average of eight loci for *Homolaphlyctis polyrhiza*, 23 loci for *Entophlyctis helioformis* and 24.5 loci for *Rhizophydium brooksianum*.

3.2 | Swab performance

The number of loci successfully sequenced was positively associated with swab Bd load (LOESS span $\alpha = 0.75$, degree of polynomial fit $\lambda = 2$, Figure 2). The break between the higher-performing and lower-performing groups of swabs fell at a Bd load of 2.203 log10 Bd copy numbers (or 159.6 Bd DNA copies). All of the samples in the higher-performing group returned at least 100 loci and an average of 169.6 loci (n = 17, standard deviation = 23.8). The lower-performing group returned an average of 41.2 loci (n = 30, standard deviation = 39.7). However, there were some outliers in each group. One swab sample that would be included in the high-performing group (average copy number of 91,698) only returned a single locus. This outlier was excluded from the curve fit and decision tree calculation is displayed in Figure 2, as well as the average loci data presented above. Additionally, two samples grouped with the lower-performing swabs (average copy number of 35.6 and 59.0) returned a surprisingly large number of loci (149 and 163, respectively).

3.3 | Phylogenetic discriminatory power

Our sequence data recapitulated the known phylogenetic relationships of Bd isolates based on whole-genome data. For example, all isolates previously described as GPL (Rosenblum et al., 2013) were correctly assigned to the GPL clade using our amplicon data (Figure 3). Additionally, the two isolates previously described as

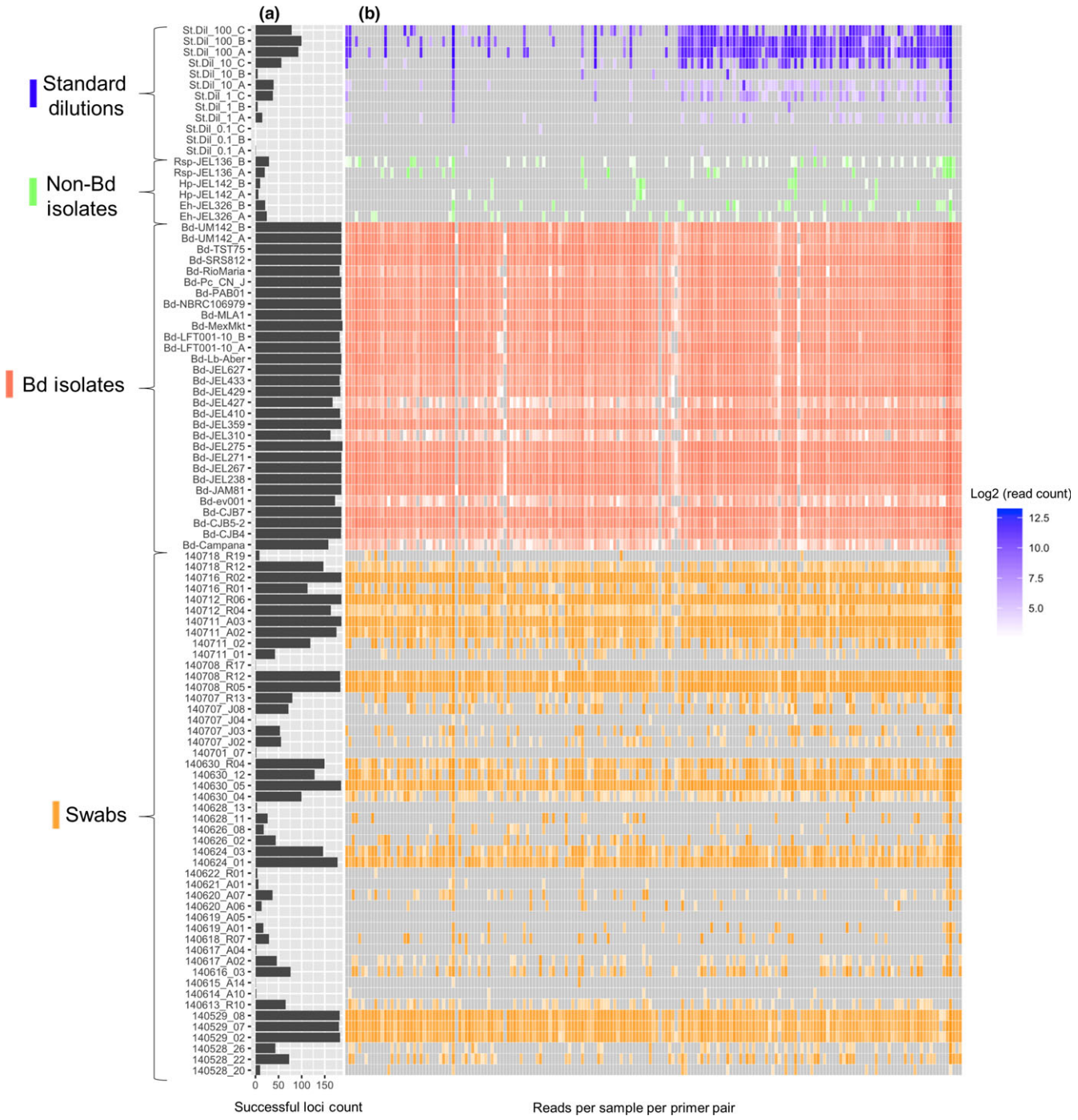

FIGURE 1 Sample performance. Sample names are given as row labels. (a) Barplot showing the number of successful loci recovered per sample (192 possible). (b) Heatmap showing the log2 count of reads per sample and loci (192 possible loci from left to right on the *x*-axis). Samples that are in the standard dilution series are in blue, non-Bd pure culture samples in green, Bd pure culture in red and swab samples in orange. The intensity of the colour represents the number of reads per sample/locus with more intense colour representing more reads associated with that sample/locus pair (unsuccessful amplification shown in grey). [Colour figure can be viewed at wileyonlinelibrary.com]

belonging to the Bd-Brazil clade were correctly placed outside of the GPL. Overall, almost all of the nearest-neighbour relationships were conserved in the phylogeny produced from the amplicon data set and the phylogeny produced from the whole-genome data set. However, placement of isolates into GPL subclades was not always consistent across each data set (Figure 3).

4 | DISCUSSION

Here, we present a new genotyping assay for the pathogenic fungus Bd that uses a microfluidic PCR approach optimized for low-quality/quantity DNA. We targeted a carefully curated set of 192 highly polymorphic regions of the Bd genome (each 150–200 bp long). Our

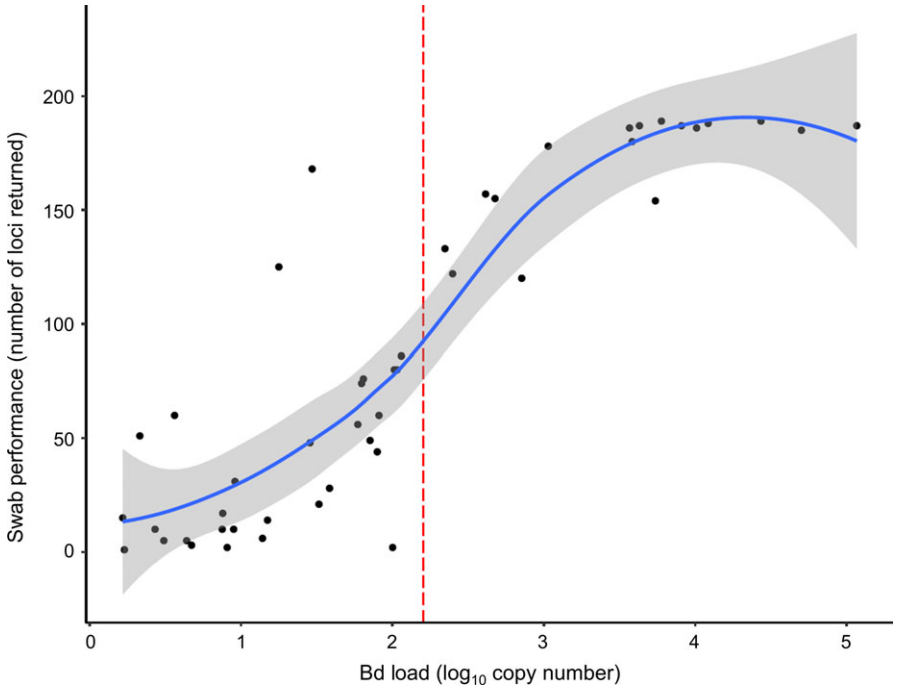

FIGURE 2 Scatterplot of swab performance (number of loci returned) compared to swab quality (log10 Bd copy number). The local regression (blue curve) and 95% confidence interval for best fit of points (grey curve) are shown. The red dotted line indicates the separation between the higher-performing and lower-performing samples at 2.203 log10 Bd copy number (159.6 Bd DNA copies). [Colour figure can be viewed at wileyonlinelibrary.com]

amplicon approach reduces ascertainment bias that can be problematic in other SNP-based genotyping approaches (e.g., Albrechtsen, Nielsen, & Nielsen, 2010). The new assay performed exceptionally well not only for pure Bd cultures but also for samples collected noninvasively from swabbing host skin (Figures 1 and 2). Swabs with a reasonable starting amount of Bd (more than 150 Bd genomic equivalents) returned robust sequence data. Our assay also has the power to accurately discriminate among major Bd clades, and 192 markers were sufficient to reconstruct the basic Bd tree topology generated previously from whole-genome sequence data (Figure 3). Our approach is highly scalable (samples are individually barcoded and pooled before sequencing), and can support much larger sample sizes than alternative sequencing approaches. Additionally, the per-sample cost for our assay (~$25) is more than an order of magnitude less than for whole-genome sequencing (Table S3). Other studies have successfully implemented a similar microfluidic workflow to genotype low-quality DNA from noninvasively collected samples, including historical fish scales (Smith et al., 2011) and bird feathers (Ruegg et al., 2014).

Given its low cost, high scalability and reliable data generation for low DNA quantity samples, our new assay will help address many outstanding questions in the Bd disease system. In particular, swab samples will become a revitalized resource. Swabs have been the preferred method for large-scale collection of Bd DNA because they are minimally invasive, do not require a sterile laboratory for collection and can be easily stored and transported for long periods of time. Hundreds of thousands of amphibian skin swab samples have already been collected (e.g., see Olson et al., 2013) and can now be genotyped to reveal spatial and temporal dynamics of Bd in natural systems. For example, one exciting spatial application of this genotyping assay is the potential to create a global Bd genotype map. Bd swabs from around the world can be affordably genotyped, and genotypes could be integrated into Bd mapping efforts (e.g., http://www.bd-maps.net/; Olson et al., 2013). The result would be a public-access database that includes not only Bd presence/absence data but also genotype information. Each successive sample would add more resolution to the global Bd map, allowing Bd researchers to make more effective inferences and predictions about the distribution and spread of different Bd clades, as well as provide a tool for rapid disease surveillance. Understanding the spatial distribution of Bd clades is especially important given that certain strains of Bd are known to be more pathogenic than others (e.g., Berger, Marantelli, Skerratt, & Speare, 2005) and could pose threats if introduced to novel areas.

In addition to using swab genotypes to understand the spatial distribution of Bd clades around the world, this assay can be used to answer key questions about temporal dynamics in the Bd system. For example, one promising application of this assay is to better infer the origin and introduction history of Bd in different parts of the world. To understand how long Bd has been present in an area and when it arrived, researchers have turned to swabbing the skin of preserved museum specimens (e.g., Cheng et al., 2011; Zhu et al., 2014). Additionally, new DNA extraction techniques have opened the possibility of obtaining reliable Bd DNA from formalin-fixed specimens (Hykin, Bi, & McGuire, 2015; Richards-Hrdlicka, 2012). By integrating new extraction techniques with the genotyping assay described here, museum specimens can now be used to reveal the historical occurrence of Bd, document how and when Bd introductions occurred and understand long-term temporal dynamics of the major Bd clades.

We expect this assay to contribute substantially to understanding Bd-related amphibian declines, but it is important to note

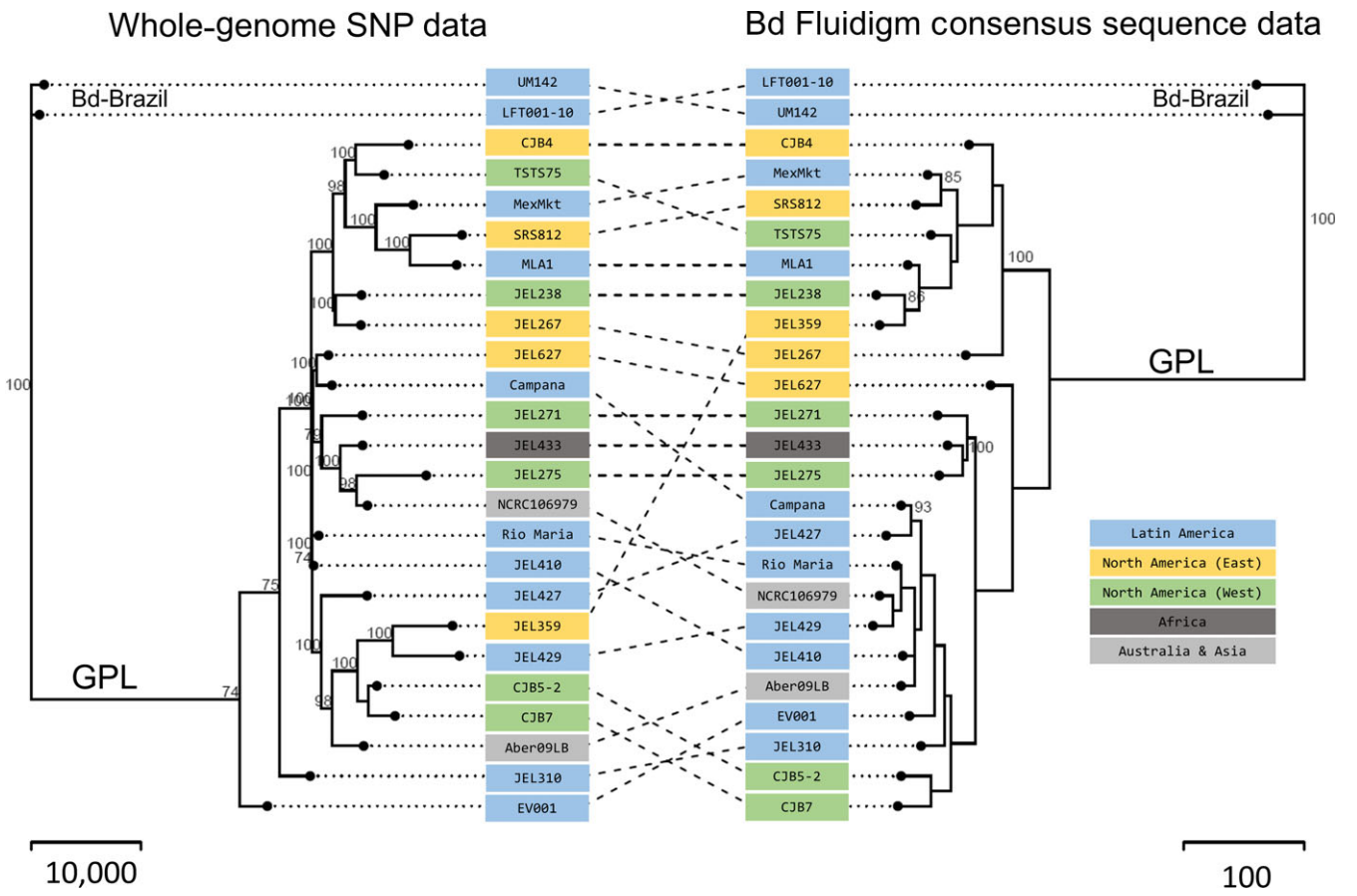

FIGURE 3 Comparison of maximum parsimony phylogenies with 100 bootstrap replicates for 25 Bd isolates created using whole-genome SNP data (left) and consensus sequences obtained using the Bd Fluidigm Access Array assay (right). The geographic origin of each isolate is indicated by colour. Only isolates for which whole-genome data were available were included in this analysis. [Colour figure can be viewed at wileyonlinelibrary.com]

limitations of the approach. The assay works best with at least a moderate amount of input Bd DNA. The threshold amount of Bd DNA for our higher-performing swabs (~150 genome equivalents/1 μl) is higher than many positive Bd skin swabs, especially in areas where Bd appears to be endemic such as Asia (Bataille et al., 2013) and Brazil (Rodriguez, Becker, Pupin, Haddad, & Zamudio, 2014). This may introduce a bias towards recovering more virulent and/or faster-growing strains if the assay is only applied to samples with high loads. Additionally, swab samples sometimes fail to detect Bd infection altogether and often underreport Bd infection loads (Shin, Bataille, Kosch, & Waldman, 2014). However, a subset of swabs in our study with very low Bd loads produced a robust set of amplicons, so some samples with lower Bd loads could be successfully analysed using this platform. Further, one high-load swab failed for unknown reasons. The occasional sample failure with a PCR-based method is not surprising especially given that swab samples are often extracted using relatively inexpensive extraction methods that can contain PCR inhibitors. The probability of sample success can be increased through precipitating and concentrating sample extracts. In addition, we cannot predict how well the current panel of primers will work for novel strains yet to be discovered. We designed primers to amplify across diverse Bd isolates, and we obtained high-quality data from diverse isolates both within and outside the GPL. However, relatively few loci amplified for distantly related non-Bd chytrids, so it remains to be seen how much deeper phylogenetic diversity can be captured by the current primer pool. Additionally, this study is limited in its power to assess the efficacy of our primer pool to distinguish among all major Bd clades because we focused on samples from the GPL and Bd-Brazil clades (Table S2). Future applications will include samples from other Bd clades and from *B. salamandrivorans*. Finally, researchers have not yet thoroughly characterized genetic diversity in Bd populations over fine spatial or temporal scales. Although our target amplicons are effective for distinguishing among major Bd clades, they may not provide sufficient resolution for finer scale questions. However, there is room to expand the number of target loci as other Fluidigm Access Array designs have included up to 10 primer pairs per pool (for a total of 480 target loci) (e.g., Halbritter et al., 2012). By increasing the number of target regions and/or base pairs per target, this assay could be extended to address questions about Bd dynamics across deeper or finer temporal and spatial scales.

In summary, the genotyping assay described here uses microfluidic, multiplex PCR technology to create a highly scalable workflow with the specific aim of garnering genotype data from Bd skin swabs. Our assay focuses on Bd genotyping, but skin swabs can also be used for population genetics studies of amphibians (e.g., Prunier et al., 2012). Thus, extensions of our assay could be used to understand patterns of genetic variation in both host and pathogen simultaneously. Ultimately, the approach described here expands the utility of noninvasive sampling methods for understanding disease-related amphibian declines, an important advance for studying imperiled amphibian species. The application of this tool to swab samples collected across species, life stages, continents and decades will be a critical step forward in the study of Bd and for amphibian conservation planning.

ACKNOWLEDGEMENTS

This work was supported by an NSF GRFP to AQB and an NSF IOS grant to EBR (IOS-1354241). We thank Cori Richards-Zawacki for sharing Bd swab extracts. We thank Katy Richards-Hrdlicka and Andrew O'Guin at Fluidigm and Dan New and Alida Gerritsen at the University of Idaho IBEST Genomics Resources Core for assistance with troubleshooting.

AUTHOR CONTRIBUTIONS

A.Q.B., T.J.P., M.L.S. and E.B.R. designed the research; A.Q.B., A.P.R., T.J.P. and J.E. performed the research; A.Q.B., A.P.R., R.J.P. and M.L.S. analysed the data; A.Q.B., A.P.R., T.J.P., J.E., M.L.S. and E.B.R. wrote the manuscript.

DATA ACCESSIBILITY

Supporting data have been accessioned to Dryad (https://doi.org/10.5061/dryad.33st2).

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How to cite this article: Byrne AQ, Rothstein AP, Poorten TJ, Erens J, Settles ML, Rosenblum EB. Unlocking the story in the swab: A new genotyping assay for the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *Mol Ecol Resour*. 2017;17:1283–1292. https://doi.org/10.1111/1755-0998.12675