Vol. 123: 213–226, 2017
https://doi.org/10.3354/dao03097

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published March 21

Prevalence and pathogen load estimates for the fungus *Batrachochytrium dendrobatidis* are impacted by ITS DNA copy number variation

Eria A. Rebollar[1,*], Douglas C. Woodhams[2], Brandon LaBumbard[2], Jos Kielgast[3,4], Reid N. Harris[1]

[1]Biology Department, James Madison University, 951 Carrier Dr., MSC 7801, Harrisonburg, Virginia 22807, USA
[2]Department of Biology, University of Massachusetts Boston, 100 Morrissey Blvd., Boston, Massachusetts 02125-3393, USA
[3]University of Copenhagen, Institute of Biology, Section for Freshwater Biology, 2100 Copenhagen, Denmark
[4]University of Copenhagen, Natural History Museum of Denmark, Center for Macroecology, Evolution and Climate, 2100 Copenhagen, Denmark

ABSTRACT: The ribosomal gene complex is a multi-copy region that is widely used for phylogenetic analyses of organisms from all 3 domains of life. In fungi, the copy number of the internal transcribed spacer (ITS) is used to detect abundance of pathogens causing diseases such as chytridiomycosis in amphibians and white nose syndrome in bats. Chytridiomycosis is caused by the fungi *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*), and is responsible for declines and extinctions of amphibians worldwide. Over a decade ago, a qPCR assay was developed to determine *Bd* prevalence and pathogen load. Here, we demonstrate the effect that ITS copy number variation in *Bd* strains can have on the estimation of prevalence and pathogen load. We used data sets from different amphibian species to simulate how ITS copy number affects prevalence and pathogen load. In addition, we tested 2 methods (gBlocks® synthetic standards and digital PCR) to determine ITS copy number in *Bd* strains. Our results show that assumptions about the ITS copy number can lead to under- or overestimation of *Bd* prevalence and pathogen load. The use of synthetic standards replicated previously published estimates of ITS copy number, whereas dPCR resulted in estimates that were consistently lower than previously published estimates. Standardizing methods will assist with comparison across studies and produce reliable estimates of prevalence and pathogen load in the wild, while using the same *Bd* strain for exposure experiments and zoospore standards in qPCR remains the best method for estimating parameters used in epidemiological studies.

KEY WORDS: Chytridiomycosis · Epidemiology · Chytrid fungus · Synthetic standards · Quantitative PCR · Digital PCR · Internal transcribed spacer

INTRODUCTION

The variation in copy number of the ribosomal gene complex, including the internal transcribed spacer (ITS) regions and the ribosomal genes, is a common feature in all 3 domains of life (Acinas et al. 2004, Weider et al. 2005, Nilsson et al. 2008, Pei et al. 2010). In particular, the ITS region has been routinely used as a phylogenetic marker in fungi and has been adopted by the Consortium for the Barcode of Life initiative as the standard marker for DNA-based fungal identification (Smith et al. 2007, Begerow et al. 2010, Schoch et al. 2012). Moreover, the ITS region has been used in the quantification

*Corresponding author: ea.rebollar@gmail.com

of fungal pathogen load both in ecological studies and epidemiological research (Cuenca-Estrella et al. 2009, White et al. 2009, Tellenbach et al. 2010, Horevaj et al. 2011). Although this DNA region has been widely used, in part because its multi-copy nature makes it easy to detect pathogens, its use can cause estimation biases depending on the number of copies present (Bellemain et al. 2010, Lindner & Banik 2011, Schloegel et al. 2012, Longo et al. 2013). One relevant case is the amphibian disease chytridiomycosis, caused by the chytridiomycete fungi *Batrachochytrium dendrobatidis* (*Bd*), and *B. salamandrivorans* (*Bsal*). Chytridiomycosis is an emerging infectious disease that is responsible for population declines and extinctions of amphibians around the world (Skerratt et al. 2007, Fisher et al. 2009, Martel et al. 2014, Yap et al. 2015).

In particular, *Bd* infects the amphibian skin and causes ionic imbalances that lead to cardiac failure (Voyles et al. 2009, 2012). The ability of *Bd* to decimate populations is enhanced by its ability to elude the host acquired immune system by secreting a molecule that inhibits lymphocyte proliferation (Fites et al. 2013). *Bd* can cause precipitous population declines once it arrives in a region with susceptible amphibian populations and optimal conditions for growth and dispersal of the pathogen (Lips et al. 2006, Skerratt et al. 2007, Crawford et al. 2010, Vredenburg et al. 2010).

In the case of chytridiomycosis caused by *Bd*, a few areas in the tropics are still considered to be *Bd*-naive (Dahl et al. 2012, Vredenburg et al. 2012, Bletz et al. 2015a, Rebollar et al. 2016) and therefore, monitoring protocols in these areas are essential to detect the presence of *Bd* when the prevalence and pathogen load may be initially low (Lötters et al. 2011, Lips et al. 2006). In addition, recent studies have identified low *Bd* pathogen load levels in several countries around the world, including South Korea, Vietnam, Cambodia, French Guiana and Panama, where the fungus has probably become endemic (Bataille et al. 2013, Rowley et al. 2013, Courtois et al. 2015, Rodríguez-Brenes et al. 2016).

Boyle et al. (2004) developed a Taqman based qPCR assay with primers that specifically target ITS1 and the beginning of the 5.8S ribosomal gene of *Bd*. This method has been optimized and widely used to detect *Bd* from amphibians in the wild given that it is non-invasive, specific for *Bd*, highly sensitive, and quantitative (Hyatt et al. 2007). At the time of that study, *Bd* zoospores of the isolate used to develop the assay (AAHL 98 1810/3) were thought to have 10 copies of the ITS fragment (Boyle et al. 2004).

Recent studies have determined that the genome of *Bd* is more complex and dynamic than first assumed. *Bd* has several genetic lineages that show multiple genomic rearrangements due to recombination events (Farrer et al. 2011). The genetic structure of these highly polymorphic and recombinant strains suggests a complex evolutionary history of the pathogen, including hybridization events (Rosenblum et al. 2013). Importantly, the number of chromosomes in *Bd* strains can change within a few passages in culture, including isolates that become triploid and tetraploid at some chromosomes (Farrer et al. 2013). Compounding this polyploid phenomenon, the ITS DNA fragment in different *Bd* strains has an extremely variable copy number, thought to range from 10 to 169 copies in a zoospore (Kirshtein et al. 2007, Longo et al. 2013).

Longo et al. (2013) demonstrated that *Bd* reference strains used in this qPCR assay can have different ITS copy numbers, and this may impact pathogen load estimates of *Bd* infection; prior to their work, *Bd* loads had been reported as zoospore equivalents (zeq). Even though Hyatt et al. (2007) considered 0.1 zeq as the minimum detection threshold, most studies consider samples with values below 1 zeq as negative for *Bd* (e.g. Kriger et al. 2007, Kilburn et al. 2010, Woodhams et al. 2012b, 2014). However, the presence of a single copy in a sample can indicate the presence of *Bd* even if the estimate of zeq is <1 (Bletz et al. 2015b). Considering values equivalent to a copy of the ITS region (zeq values <1) can avoid the underestimation of *Bd* prevalence in the wild, and therefore it is essential to consider the copy number of the reference strain used in the qPCR standard curve.

In addition to field studies, infection intensity is also reported from many laboratory experiments, and it is sometimes used to determine infectious zoospore shedding rates and the infection threshold beyond which mortality by chytridiomycosis becomes imminent (DiRenzo et al. 2014). In this context, accurate determination of pathogen loads is critical for epidemiological models and subsequent disease management recommendations (Briggs et al. 2010). Carey et al. (2006) may have been the first to calculate an infection threshold based on dose-response curves for the number of *Bd* zoosporangia that accumulate in boreal toad *Anaxyrus boreas* skin before death by chytridiomycosis (~10^7–10^8 zoosporangia). In a related analysis in mountain yellow-legged frogs *Rana muscosa*, Vredenburg et al. (2010) found that before population declines, frogs reached infection loads of ~10^4 zoospores swab^{-1}.

They used the protocols of Boyle et al. (2004) and Hyatt et al. (2007), presumably with the zoospore standards provided by the Australian Animal Health Laboratory, and not from a field isolate, which are often not available because they were not previously isolated. Few studies are available that report infection thresholds at death (e.g. Kinney et al. 2011), but for some species, the infection threshold appears to be high (up to 9.6×10^6 zoospores swab^{-1}; DiRenzo et al. 2014). Using zoospore standards in qPCR from the same isolate used for experimental exposures is one solution that is rarely employed (Ellison et al. 2014).

In this study, we address how variation in copy number of the ITS region from field and reference strains can impact accuracy in estimates of *Bd* prevalence and intensity, especially when the pathogen load is low. Here we consider the *Bd* 'reference strain' to be the one used to obtain and set the standard dilution curve used in the qPCR assay to estimate loads for unknown samples, and the *Bd* 'field strain' as the one present in the unknown samples obtained from the wild. We discuss the consequences of not knowing the copy number of the reference strain used in the qPCR. We also address the limitations of not knowing the copy numbers present in strains from the wild, and the potential issues when different strains of *Bd* coexist within a region (Rodriguez et al. 2014).

In this context, the development of a more accurate and precise method to determine pathogen loads and ITS copy number has become necessary. The use of plasmid or synthetic standards in qPCRs and the implementation of digital PCR (dPCR) are 2 potential strategies to achieve a standardized and replicable method that allows for comparison across studies and allows for reliable estimates of prevalence and pathogen load in the wild. In this study, we determined the copy number of 6 *Bd* strains using the 2 suggested methods: synthetic standards called gBlocks® and dPCR.

The problems and solutions discussed here may have broader implications for applying qPCR results to other ecological and host–pathogen systems. Hence, quantifying the pathogen's ITS copy number for both the reference and field strains is important to accurately detect chytridiomycosis caused by *Bd* and *Bsal* as well as other fungal and bacterial diseases including the white nose syndrome in bats, several plant diseases, and human respiratory diseases including allergies and asthma (Guillemette et al. 2004, Cuenca-Estrella et al. 2009, Muller et al. 2013, Shuey et al. 2014, Smith et al. 2014).

MATERIALS AND METHODS

Effect of ITS copy number variation on pathogen prevalence and pathogen load

Data sets used

To evaluate the effect of ITS copy number on prevalence and pathogen load, we used 10 data sets, 8 of which have been published previously (Woodhams et al. 2008, 2010, 2012a,b, 2014, Rebollar et al. 2014). These study cases comprise 531 individuals across 10 frog species around the world including tropical and temperate species, different developmental stages (tadpoles, metamorphs, and adults), and variable pathogen loads (Table 1). Collections were made on different dates in various regions, and analyses used different reference strains with unknown copy number. It is important to emphasize that we are not testing hypotheses that compare among data sets. We are making comparisons within data sets that were collected under similar conditions. Conclusions based on our analyses should be considered as indicative of the effects of copy number variation and not based on among-species comparisons. These data sets modeled epizootic and enzootic situations. For instance, *Colostethus panamansis* data distribution follows a trend expected in epizootic high-intensity events, whereas the data distribution for *Agalychnis callidryas, Dendropsophus ebraccatus*, and *Craugastor fitzingeri* correspond to enzootic low-intensity events.

DNA extraction and quantitative PCR

We present *Bd* infection data from 10 amphibian host species. DNA extraction methods, qPCR conditions, and reference strains used for *Colostethus panamansis, Lithobates pipiens, Litoria genimaculata, Pelophylax esculentus* complex*, Agalychnis callidryas, Dendropsophus ebraccatus, Craugastor fitzingeri,* and *Alytes obstetricans* have been reported previously (Woodhams et al. 2008, 2010, 2012a,b, 2014, Rebollar et al. 2014).

In addition to the 8 data sets published previously, we included 2 unpublished data sets of *Bombina variegata* and *Lithobates catesbeianus*. In the case of *B. variegata*, microbial swabs were extracted with a Qiagen DNeasy Blood & Tissue Kit according to the manufacturer's protocol including a lysozyme incubation step. In the case of *L. catesbeianus,* DNA extractions were performed using PrepMan®Ultra

Table 1. Data sets used in this study comprise 10 amphibian species across different continents, developmental stages, and infection intensities. Mean pathogen loads (expressed in zoospore equivalents, zeq) were obtained considering only infected individuals

Species	Region	Country	Life stage	Habitat	Sample size/ infected individuals	Mean pathogen load (zeq)	Study
Lithobates catesbeianus	North America	USA	Tadpole	Temperate wetland	24/24	1466.9	D. C. Woodhams et al. (unpubl.)
Lithobates pipiens	North America	USA	Adult	Temperate wetland	28/12	530.58	Woodhams et al. (2012b)
Colostethus panamansis	Central America	Panama	Adult	Tropical rainforest	12/12	190003.2	Woodhams et al. (2008)
Craugastor fitzingeri	Central America	Panama	Adult	Tropical rainforest	37/20	11.56	Rebollar et al. (2014)
Agalychnis callidryas	Central America	Panama	Adult	Tropical rainforest	77/40	0.15	Rebollar et al. (2014)
Dendropsophus ebraccatus	Central America	Panama	Adult	Tropical rainforest	89/39	0.11	Rebollar et al. (2014)
Pelophylax esculentus complex	Europe	Switzerland	Adult	Temperate wetland	100/20	60.19	Woodhams et al. (2012a)
Bombina variegata	Europe	Switzerland	Metamorph	Temperate wetland	50/40	16.55	D. C. Woodhams et al. (unpubl.)
Alytes obstetricans	Europe	Switzerland	Tadpole	Temperate wetland	28/27	4.27	Woodhams et al. (2014)
Litoria genimaculata	Oceania	Australia	Adult	Tropical rainforest	86/34	43.58	Woodhams et al. (2010)

(Applied Biosystems). Samples extracted with Prepman®Ultra were diluted 1/10 for the PCR reaction, and final zoospore estimates took this dilution factor into account. Cq values for both Prepman®Ultra and Qiagen-extracted samples were multiplied considering the final eluted volume (100 µl) in order to obtain the total number of zoospores present in the whole extract. All Cq values were included to estimate zoospore estimates even if the values were very small (values <1).

To determine *Bd* loads in *B. variegata* and *L. catesbeianus*, a qPCR assay was done using *Bd*-specific primers: the ITS1-3 primer (5′-CCT TGA TAT AAT ACA GTG TGC CAT ATG TC-3′) and the 5.8S primer (5′-AGC CAA GAG ATC CGT TGT CAA A-3′) (Boyle et al. 2004). We performed the qPCR reactions according to Boyle et al. (2004). Each assay consisted of a 25 µl reaction volume containing 20 µl of master mix and 5 µl of DNA (both for standards and the sample DNA). Standards included serial dilutions that ranged from 0.1 to 1000 zoospores μl^{-1}. Protocol modifications included running 50 cycles and repeating the analysis when samples run in duplicate yielded standard deviations greater than 0.5. If only 1 well came up positive, we ran the samples in duplicate again. For *B. variegata,* the FastStart Universal Probe Master Mix (ROX) was used with the probe Chytridprobe (6-FAM-CGA GTC GAA CAA AAT-MGB; Roche Applied Science). For *L. catesbeianus,* the TaqMan Universal PCR Master Mix with MGB TaqMan Probe of the same sequence as above (Applied Biosystems) was used. Use of different qPCR assays is part of protocol variation among laboratories that is reflected in published prevalence and pathogen load estimates, but it is a separate issue from copy number variation. All runs were quantified based on zoospore standards acquired from EcoGenics (for *B. variegata*) or Pisces Molecular (for *L. catesbeianus*) and stored at −80°C.

Prevalence analysis

A recent study determined that *Bd* cultured strains isolated from sick frogs from different regions can have a variable number of ITS copies that range from 10 to 144 $zoospore^{-1}$ (Longo et al. 2013). To evaluate changes in prevalence and pathogen load, we investigated the interval reported by those authors, i.e. 10–144.

We simulated the proportion of positive samples (prevalence or proportion of infected individuals) based on 3 different thresholds according to different copy numbers per zoospore: (1) zeq ≥ 1 assuming 1 copy zoospore^{-1}, (2) zeq ≥ 0.1 assuming 10 copies zoospore^{-1}, and (3) zeq ≥ 0.0069 assuming 144 copies zoospore^{-1} (Eq. 1). The threshold detection value is

$$ZspT \geq \frac{1sp}{copRS} \quad (1)$$

where $ZspT$ = zoospore threshold value to consider a sample positive, sp = zoospore, and $copRS$ = number of copies zoospore^{-1} in the reference strain. For prevalence, 95% confidence intervals based on a binomial distribution were calculated using the Wilson Interval as described by Kilburn et al. (2010) using R (R Core Team 2014).

Pathogen load analyses

We calculated pathogen load based on 3 scenarios: (A) the field strain having 10 copies and the reference strain 144 copies, (B) the field strain having the same copy number as the reference strain, and (C) the field strain having 144 copies and the reference strain 10 copies. We assumed Scenario B (field strain = reference strain) is the traditional assumption in published studies (Table 1).

The estimated zoospore equivalents are determined by multiplying the number of zoospores from the field strain by the number of copies zoospore^{-1} in the field strain, and dividing this product by the number of copies zoospore^{-1} in the reference strain (Eq. 2).

$$Ezeq = \frac{(zspR \times copFS)}{copRS} \quad (2)$$

where $Ezeq$ = estimated zoospore equivalents, $zspR$ = number of zoospores from the raw data, $copFS$ = number of copies zoospore^{-1} in the field strain, and $copRS$ = number of copies zoospore^{-1} in the reference strain.

Dividing by the number of copies in the reference strain corrects for differences between field and reference strains. For example, in Scenario A, a higher copy number in the reference strain would lead to an underestimate of pathogen load because the Ct values of the standard curve from the reference strains will be shifted toward lower values relative to the field strain Ct value. In Scenario B, the estimation of pathogen load will be accurate. In Scenario C, a lower copy number in the reference strain would lead to an overestimation of pathogen load because the Ct values of the standard curve from the reference strains will be shifted toward higher values relative to the field strain Ct value.

Pathogen load values (quantified as zeq) per species per scenario were obtained by calculating the mean pathogen load from the individuals that were infected (Table 1). These calculations were obtained using SAS (version 9.3).

Statistical analysis

For prevalence, we compared the effect of the 3 thresholds within each species fitting a linear model for categorical data (presence/absence of *Bd* under 3 thresholds) using PROC CATMOD. This procedure fits linear models to functions of response frequencies, and it can be used for linear modeling. For pathogen load, we compared the effect of the 3 scenarios (explained above) within each species using the Wilcoxon non-parametric test since distributions were not normally distributed. We performed the Wilcoxon test for all thresholds per species and also paired comparisons between thresholds within each species (A-B, B-C, A-C). In the case of the paired comparison, we corrected for multiple comparisons using the Bonferroni correction. All statistical tests were conducted using the 0.05 level of significance and were performed using SAS (version 9.3).

qPCR using synthetic standards (gBlocks®) and digital PCR

Bd strain selection

We estimated ITS copy number of 6 *Bd* stains (Table 2) including isolates from 3 lineages: the global panzootic lineage (GPL), Brazilian, and Swiss lineages (for details, see Table S1 in the Supplement at www.int-res.com/articles/suppl/d123p213_supp.pdf). ITS copy number of strains JEL197 and LFT001 had been estimated in previous studies using different methods (Kirshtein et al. 2007, Longo et al. 2013). Isolates were revived from cryopreservation and had similar passage histories before use in each method (gBlocks® or dPCR).

Zoospore quantification and extraction

Bd isolates were grown on 1% tryptone agar plates for 4 to 7 d, until zoospores could be harvested. When

Table 2. Internal transcribed spacer (ITS) copy number in different isolates of *Batrachochytrium dendrobatidis*, estimated using gBlocks® qPCR and digital PCR. nd: not determined; GPL: global panzootic lineage; zsp: zoospore

Isolate	Lineage	Host species and locality	Mean (SD) ITS copies zsp^{-1}		Published ITS copies zsp^{-1}	
			gBlocks® qPCR	Digital PCR	Longo et al. (2013)	Kirshtein et al. (2007)
Campana_H. vireovittatum _13_JLV	GPL	*Hyalinobatrachium vireoittatum*, Campana, Panama	237.2 (94.6)	40.6 (2.1)	nd	nd
VMV 813	GPL	*Lithobates catesbeianus* tadpole, Georgia, USA	199.7 (94.8)	11.3 (1.9)	nd	nd
JEL 197	GPL	Type isolate, *Dendrobates azureus*, National Zoological Park, Washington DC	169.1 (62.3)	7.1 (2.6)	nd	169
JEL 404	GPL	*Lithobates catesbeianus* tadpole, Crocker Pond, Maine, USA	116.8 (61.9)	nd	39.0	nd
LFT 001	Brazilian	São Paulo, Brazil	125.8 (38.0)	nd	125	nd
TG 739	Swiss	*Alytes obstetricans*, Gamlikon, Switzerland	96.2 (69.6)	22.0 (3.1)	nd	nd

ready, plates were flooded with 3 ml 0.5% tryptone broth and allowed to sit for 30 min. The solution was collected and filtered through a cone-shaped filter funnel (8 ml capacity with 10 µm pore size; Chenrus) under vacuum to obtain a pure zoospore stock for each isolate. Zoospores were counted 4 times for accuracy using a hemocytometer (Boyle et al. 2004) and diluted to 10^6 zoospores ml^{-1}. For extractions, 100 µl of the zoospore dilution was added to a 1.5 ml Eppendorf tube to make a 10^5 zoospore extraction. To this, we added 100 µl Prepman® Ultra reagent (Applied Biosystems), following the DNA extraction protocol described by Hyatt et al. (2007). Each extraction was performed in triplicate for each *Bd* isolate, and we assumed 100% extraction efficiency for each sample. Serial dilutions were performed for each extraction and ranged from 10^4 to 10^2.

qPCR using synthetic standards (gBlocks®)

To analyze samples, qPCR Taqman assays were conducted using an Agilent Mx3005P system and followed the protocol set by Boyle et al. (2004). The primers used to detect the *Bd* ITS1 rRNA gene are listed above. The Taqman probe, Chytr MGB2, used was 5′-CGA GTC GAA C-3′. The gBlocks® gene fragments (Integrated DNA Technologies) specific to the *Bd* ITS1 rRNA gene sequence by Boyle et al. (2004) were included in each qPCR plate as synthetic standards in $\log_{10}$ increments (10^2 to 10^6 copies; in triplicate) for quantification of ITS copies zoospore^{-1} for each *Bd* isolate. To estimate ITS copies, we took the copies calculated from the qPCR, genetic equivalents (GE), and multiplied them by the dilution factor of 20 to estimate the number of copies in 100 µl. We then divided the GE number by the number of zoospores from the serial dilutions. We averaged all the estimated ITS copy numbers (n = 9 samples) together to get an average copy number per *Bd* isolate.

Digital PCR

Four isolates were studied by digital PCR (dPCR) on the QX100 Droplet Digital PCR System (Bio-Rad). Zoospores from 4 isolates were cultured and quantified as above. Exactly 100 000 zoospores were extracted using 110 µl Prepman®Ultra reagent. Samples were diluted 1000-fold, and 4 µl (400 zoospores total) were used in each 20 µl reaction. Two technical replicates of the 4 biological replicates from each *Bd* isolate (8 samples per isolate total) were tested. Samples were processed according to the manufacturer's protocol (QX 100 Droplet Generator, Instruction Manual Catalogue no. 186-3002, Bio-Rad Laboratories).

Determining infection threshold associated with mortality

We re-calculated the infection threshold (zoospores swab^{-1}) reported in 3 studies on golden frogs of the genus *Atelopus* (Becker et al. 2011, DiRenzo et al. 2014, Ellison et al. 2014) based on our gBlocks® standard estimates of ITS copy number (Table S1 in the Supplement) for the isolates used in the reported exposure experiments. Infection thresholds at mortality were compared across host species and *Bd* isolates.

RESULTS

Copy number variation in the reference strain affects prevalence estimates

We determined prevalence based on 3 different scenarios varying the defined threshold to call a sample positive or negative for *Bd* (Eq. 1): (1) using 1 zeq as the cut-off, which means the cut-off is the same as the typically unknown copy number of the reference strain, (2) assuming 10 copies zoospore^{-1} (0.1 zeq as the cut-off), and (3) assuming 144 copies (0.0069 zeq as the cut-off). There are no changes in prevalence using a different cutoff if the intensity of the infection is high (Table 1, Fig. 1). This is the case for *Lithobates catesbeianus*, *L. pipiens*, *Colostethus panamansis*, and *Litoria genimaculata*, which are the species with highest loads (Table 1, Fig. 1). However, if within a data set some of the zoospore values are <1, then prevalence changes depending on the threshold used. This is the case for *Craugastor fitzingeri*, *Agalychnis callidryas*, *Dendropsophus ebraccatus*, *Pelophylax esculentus* complex, *Bombina variegata* and *Alytes obstetricans* (Fig. 1). Within each of these species, with the exception of *P. esculentus* complex, the differences among thresholds are statistically significant: *C. fitzingeri* (χ^2 = 11.70, df = 2, p = 0.003), *A. callidryas* (χ^2 = 58.11, df = 2, p < 0.0001), *D. ebraccatus* (χ^2 = 44.40, df = 2, p < 0.0001), *B. variegata* (χ^2 = 8.82, df = 2, p = 0.0121) and *A. obstetricans* (χ^2 = 11.70, df = 2, p = 0.0029). For instance, the estimate of prevalence for *A. obstetricans* increased 40% when comparing the 1 zeq threshold with the 0.0069 zeq threshold. In the case of *D. ebraccatus*, the prevalence would change from 0 to 30%, depending on the threshold used (Figs. 1 & 2).

Copy number variation in the reference and field strains affects the estimate of pathogen load

We determined pathogen load simulating 3 different scenarios (A, B, C) according to the known span of ITS copy number that could be present in the field and in the reference strain (Longo et al. 2013) using Eq. 2 (Fig. 2).

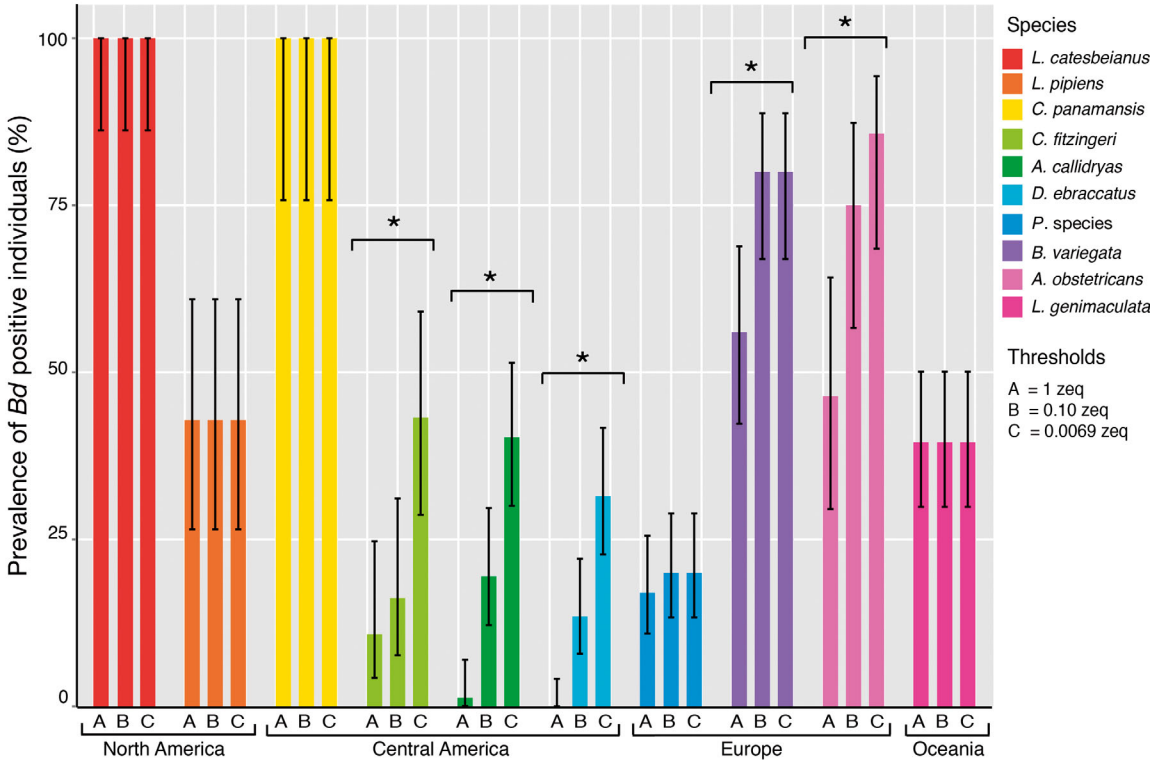

Fig. 1. *Batrachochytrium dendrobatidis* (*Bd*) prevalence of 10 frog species depending on 3 different thresholds to determine which samples are considered positive: A corresponds to values ≥1 zoospore equivalent (zeq) (1 copy zoospore^{-1}), B corresponds to values ≥0.1 zeq (10 copies zoospore^{-1}), and C corresponds to values ≥0.0069 zeq (144 copies zoospore^{-1}). Error bars represent 95% confidence Intervals. Full genus names are indicated in Table 1. *: overall significant differences across threshold within each species (p = 0.05). The '*P.* species' label corresponds to the *Pelophylax esculentus* complex

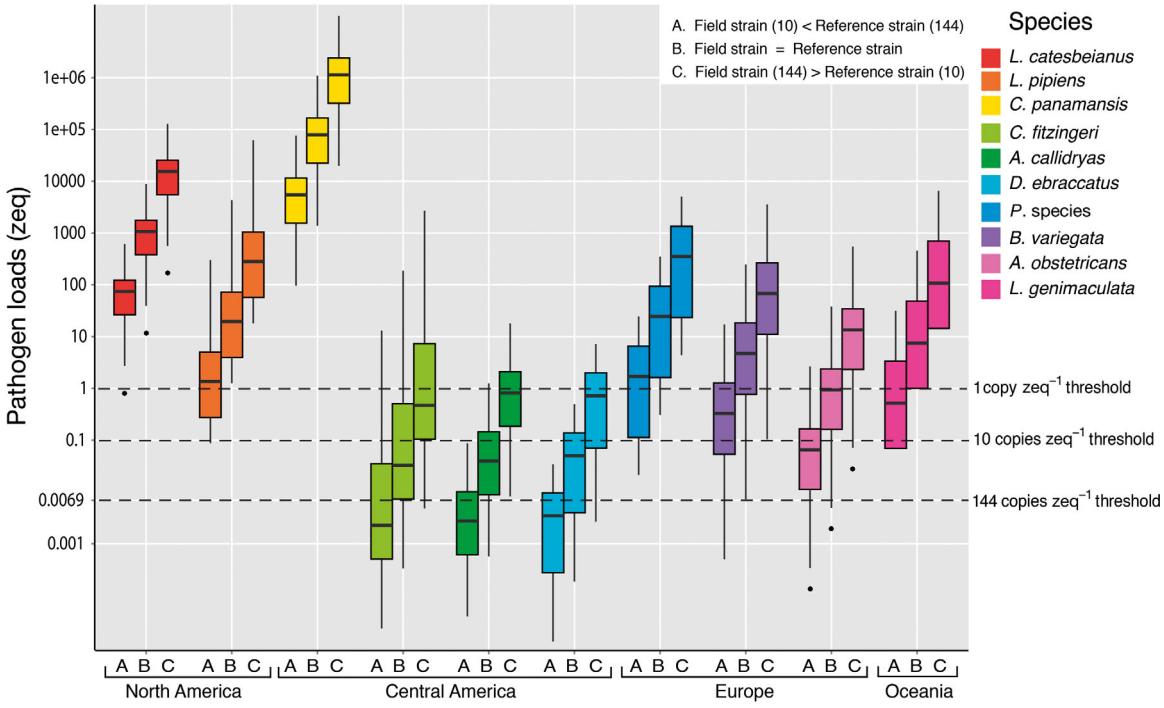

Fig. 2. Pathogen load (zoospore equivalents, zeq) of 10 frog species based on 3 scenarios depending on the internal transcribed spacer (ITS) copy number of the field and the reference strain. Scenario A: field strain = 10 copies and reference strain = 144 copies. Scenario B: field strain = reference strain. Scenario C: field strain = 144 copies and reference strain = 10 copies. Full genus names are indicated in Table 1. All overall and paired comparisons within each species data set obtained with the Wilcoxon test were significant (all $p < 0.01$) with the exception of A-B and B-C comparison in *L. pipiens* ($\chi^2 = 5.6033$, df = 1, $p = 0.0537$). The '*P.* species' label corresponds to the *Pelophylax esculentus* complex. Dashed horizontal lines indicate the 3 zeq thresholds used to determine *Batrachochytrium dendrobatidis* (*Bd*) prevalence

Depending on the ITS copy number ratio of the field strains to the reference strains, the pathogen load values can vary 2 orders of magnitude (Fig. 2). Compared with the situation where the copy number of the field and the reference strains is the same (i.e. Scenario B), pathogen load can be 1 order of magnitude higher or lower depending on which strain has higher copy numbers (i.e. A and C). The same trends are observed in all species independently of the intensity and distribution of the data in each species. All species showed overall significant differences among the 3 different scenarios (Fig. 2). All paired comparisons within each species were significantly different, with the exception of the A-B and B-C comparison in *L. pipiens*, which after correcting for multiple comparisons were not significant ($\chi^2 = 5.6033$, df = 1, $p = 0.0537$).

ITS copy number estimation using synthetic standards (gBlocks®) and digital PCR

We quantified the ITS1/5.8S copy number from 6 *Bd* isolates using qPCR with gBlocks® standards, and 4 *Bd* isolates using dPCR (Table 2). The values for qPCR with gBlocks® standards ranged from 12.7 to 237.2 copies zoospore^{-1}, while dPCR ranged from 7.1 to 40.6 copies zoospore^{-1}. We determined that the ITS copy number of 2 strains from 2 lineages estimated using gBlocks® coincides with the copy number determined in previous studies (Kirshtein et al. 2007, Longo et al. 2013), whereas dPCR estimates were consistently lower. While the copy numbers estimated using gBlocks® are similar to previous studies, standard deviations are larger than values obtained with dPCR. However, this is likely due to averaging values from 3 extractions of different zoospore dilutions for gBlocks® compared to only 1 extraction per *Bd* isolate for dPCR (see 'Materials and methods'). We provide copy number estimates for additional *Bd* strains in Table S1.

Determining infection threshold associated with mortality

Infection thresholds were recalculated from experimental exposure studies on harlequin toads in

the genus *Atelopus*. Mean pathogen load at death ranged from 3.2×10^6 to 6.8×10^6 zoospores swab^{-1}, and they were only slightly reduced from published estimates for each of the isolates when corrected for ITS copy number (Fig. S1 in the Supplement). Isolates JEL310 and JEL423 showed similar ranges of infection, and pathogen load was similar for *A. zeteki* and *A. glyphus*. The highest pathogen load from the studies listed in Table 2 was from a population of Panamanian rocket frogs *C. panamansis,* on the verge of collapse from an epizootic (Woodhams et al. 2008), reported at 1.9×10^5, and recalculated in Fig. 2 to show a potential infection threshold up to 2.7×10^6. Thus, depending on the ITS copy number in the field strain, *C. panamansis* may function similarly to *Atelopus* in the field as a zoospore 'super-shedder' (DiRenzo et al. 2014).

DISCUSSION

Prevalence and pathogen load can be misestimated due to variation in the ITS copy number

Our analysis demonstrates that by incorrectly or arbitrarily assigning a detection threshold, prevalence can be underestimated by up to 40% (Fig. 1) and would be further underestimated if the reference strain has a higher copy number than 144 (Fig. 3A, Table 2). In sum, the more copies the reference strain has, the lower the threshold of detection is when estimating prevalence (Eq. 1). It is quite possible, then, that *Bd* prevalence and pathogen loads from around the world have been incorrectly estimated.

Pathogen load can be under- or overestimated by at least 2 orders of magnitude depending on the copy number ratio of the field strain and the reference strain (Fig. 2). If the copy number of the samples collected in the field is somewhat similar to the copy number present in the reference strain, then pathogen load estimates will be accurate. However, if the field strain has a lower copy number than the reference strain, the intensity of the infection will be underestimated. When the field strain has a higher copy number than the reference strain, intensity values will be overestimated (Fig. 3B).

Prevalence can be incorrectly estimated when pathogen loads are low (Fig. 1). There are at least 3 situations where pathogen load is expected to be low and accurate estimates are needed. First, *Bd* has been documented to move in waves and invade naive populations (Lips et al. 2006, Briggs et al. 2010, Lips 2016). If there is a chance to invoke disease mitigation measures (Woodhams et al. 2011, Bletz et al. 2013, Scheele et al. 2014), it is imperative to have an early warning system for the emergence of *Bd*. Thus, it is critical to avoid underestimating prevalence. Second, some species seem to be resistant to *Bd*, and low pathogen loads have been recorded (Table 1; Swei et al. 2011, Bataille et al. 2013, Rowley et al. 2013). Similarly, amphibian species in tropical lowlands tend to have low intensity (e.g. Kielgast et al. 2010, Rebollar et al. 2014, Courtois et al. 2015, Rodríguez-Brenes et al. 2016), perhaps due to *Bd* being out of its optimal temperature range (Rödder et al. 2009). In this context, to accurately track the infection dynamics, it is again necessary to have accurate estimates of prevalence and pathogen loads. Third, in the case of describing the effects of experimental

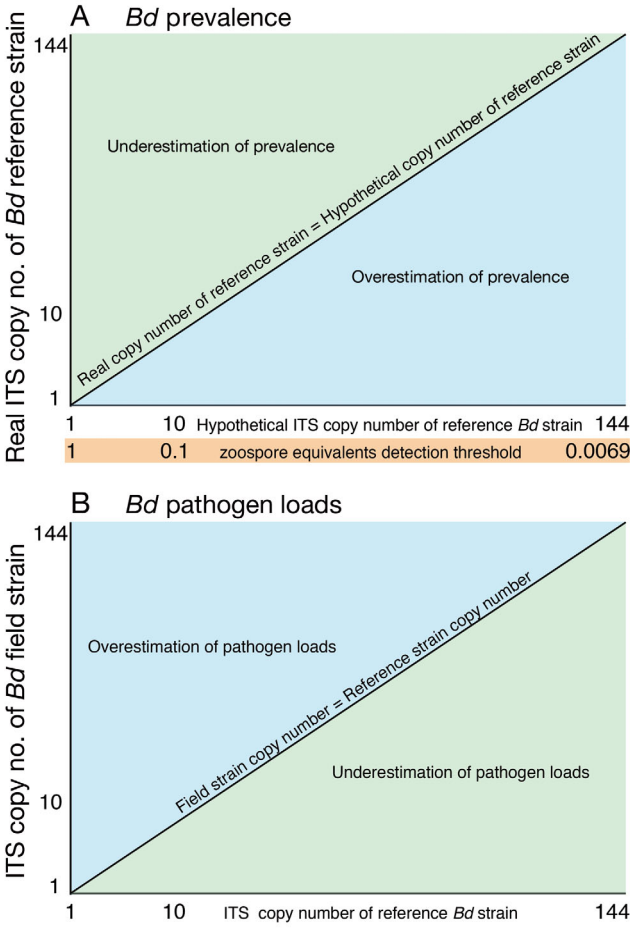

Fig. 3. Effect of internal transcribed spacer (ITS) copy number on *Batrachochytrium dendrobatidis* (*Bd*) prevalence and pathogen load. (A) In the case of prevalence, discrepancies occur when the assumed or hypothetical copy number of the reference strain deviates from the real reference strain copy number. (B) In the case of pathogen load, discrepancies occur when the copy number of the reference strain deviates from the copy number of the field strain

treatments of chytridiomycosis, an accurate estimate of prevalence is essential, otherwise a treatment may be deemed effective when in reality low infection loads are present that could with time build up and cause disease re-emergence. One example of this case is the study conducted by Woodhams et al. (2012b), in which *Alytes obstetricans* tadpoles were treated with 3 different antifungal agents and *Bd* zoopore loads were measured. The threshold used to detect *Bd* loads dramatically affects the interpretation of treatment results. When no threshold was employed, almost all frogs, regardless of treatment, were considered infected. At higher thresholds, treatment appeared to eliminate infections (Woodhams et al. 2012b).

In the case of samples with low pathogen loads, e.g. where fractions of zeq are obtained, qPCR replicates and controls are especially important to make sure these values are not false positives. On the one hand, negative controls are essential to rule out the possibility of background amplification. Since negative controls do not show any amplification, replicated amplification of unknown samples indicates pathogen presence, even if the zoospore counts are very low. This would be the case for zeq values <1 which indicate that *Bd* is present in the sample. A recent study comparing different extraction methods has shown that known zoospore quantities of 1 and 10 can yield consistent values <1 zeq, demonstrating that these values need to be taken into consideration in future studies (Bletz et al. 2015b). On the other hand, the use of positive controls is important since they will produce a stronger standard curve and therefore zeq estimates will be more accurate.

As demonstrated above, not knowing the ITS copy number of the qPCR reference strain can lead to errors in estimating prevalence and pathogen loads of *Bd*. In addition, not knowing the copy number of the field samples is problematic for estimating *Bd* pathogen load (Fig. 3). A further complication is that more than 1 strain of *Bd* can co-occur at the same location and on the same individuals, and therefore the pathogen load estimated will be affected by the number of copies present in each of the field strains (Schloegel et al. 2012, Rodriguez et al. 2014). In addition to field studies, infection studies will also need to calculate the ITS copy number of the reference strains and the strain used for the infections, since several studies have attempted to determine mortality thresholds based on zoospore estimates (Carey et al. 2006, Vredenburg et al. 2010, Kinney et al. 2011, DiRenzo et al. 2014). However, when the strain used for the infection study is the same as the strain used as a standard, it is perfectly acceptable to use genomic zoospore standards (quantified by microscope counts) as the standards. In this case (e.g. Ellison et al. 2014; Fig. S1), ITS copy number does not matter, and the results will be accurate.

Synthetic standards and dPCR are potential solutions to the variation in copy number of *Bd* strains

We consider that there are 2 potential solutions to the problem of copy number variation in fungi and specifically in *Bd* strains: (1) Synthetic ITS fragments such as gBlocks® can be used to either correlate a known concentration of the DNA fragment to the number of copies present on the reference strain or to prepare a standardized dilution series that can be used instead of the reference strain standards. The use of ITS synthetic templates would allow for consistency across studies since it does not depend on the changing copy nature of *Bd* strains. Current efforts include the use of synthetic templates containing specific ITS sequences that allow for the detection of *Bd* and *Bsal* simultaneously (J. Kerby pers. comm.). However, this method still lacks a way to correlate amplicon abundance with zoospore counts from samples obtained in the wild. (2) Recent developments in dPCR platforms offer a convenient way to circumvent the problems associated with using reference strain standard curves in qPCR. The dPCR method does not use a standard curve for quantification and has been particularly successful in elucidating issues pertaining to variation in the number of gene copies within a genome (Weaver et al. 2010, Pinheiro et al. 2012, Whale et al. 2012). In effect, dPCR removes the problem of mismatching a reference strain to an unknown strain. The method is fundamentally different in conceptual approach to DNA quantification. DNA copies in a sample are quantified by fractionating it into thousands of independent end-point PCR reactions with known volume. Based on the ratio of individual positive and negative amplification outcomes, the number of copies and thereby target concentration can be calculated based on a Poisson distribution (Vogelstein & Kinzler 1999, Baker 2012, Huggett & Whale 2013). The standard diagnostic protocol for *Bd* (Boyle et al. 2004) can be readily implemented on a dPCR platform to quantify the number of ITS target copies in a field or isolate sample independent of a standard. The dPCR method has been demonstrated to be more sensitive and robust for samples of low DNA

concentration, such as residual HIV and leukemia-related genes (Ross & Branford 2011, Whale et al. 2012, Hindson et al. 2013, Strain & Richman 2013). Furthermore, dPCR appears to be notably more resistant to PCR inhibitors (Hoshino & Inagaki 2012, Dingle et al. 2013).

Both of these methods were used in this study to determine the copy number of 6 *Bd* strains, and our results support previous studies showing that copy number variation exists within and between strains (Kirshtein et al. 2007, Farrer et al. 2013, Longo et al. 2013). We obtained different copy number values using these different methods: values obtained with gBlocks® were similar to previous studies, suggesting that this method may be accurate. Differences in gBlocks® and dPCR results may be caused by differences in the accuracy and precision of the quantification methods or by rapid changes in ITS copy number of *Bd* strains with minor differences in passage through culture. While it is beyond the scope of this study, we feel that in order to determine which of the methods is more accurate in determining pathogen loads, it will be necessary to run gBlocks® standards using dPCR. Moreover, it will be necessary to increase replications, as well as evaluate both methods using the same *Bd* strains with the same culture passages, using the same extraction methods.

CONCLUSIONS

We tested how changes in copy number of the ITS region could greatly affect prevalence and pathogen load estimates of the most widely applied diagnostic assay for *Bd*. We performed simulations of variations in ITS copy number using 10 authentic data sets from 4 different continents spanning multiple species and levels of infection. We demonstrate that a copy number discrepancy between reference and field strains can create a large bias that hampers epidemiological interpretation (Fig. 3). Both pathogen load and prevalence may be poorly estimated, and therefore, not only studies directly pertaining to pathogen loads, but also results of surveys mapping presence/absence may have been affected.

We presented evidence that current standards in *Bd* diagnostics have important limitations that likely obscure our understanding of this host–pathogen system. We propose ways to move forward by improving current diagnostics which include the use of matching strains for zoospore standards and experimental exposure studies, synthetic standards (such as gBlocks® or from plasmids), or implementing new assays like dPCR that do not require the use of reference strains. Our preliminary comparison of gBlocks® and dPCR estimates of pathogen load suggest that dPCR estimates are consistently lower and very precise; however, the strains used on each method had slightly different passage histories. Both methods represent improvements of the original qPCR method. Ultimately, convergence onto 1 standard method across laboratories will facilitate comparison of results. For now, the use of gBlocks® seems to solve some of the limitations of the traditional qPCR approach, although this method may lack precision due to the way gBlocks® are prepared (diluted, stored, and measured), thus making it a less standardized method. In contrast, dPCR is a more precise and sensitive method, but further testing is needed to determine the accuracy and precision of dPCR in comparison to gBlocks®. Given that *Bd* strains can change ITS copy number with time in culture (Farrer et al. 2011), using genomic standards from the same *Bd* strain and prepared at the same time as an exposure experiment will provide the best estimates for epidemiological parameters based on quantification of infectious zoospores rather than ITS copies. If synthetic or plasmid standards or dPCR are used, determination of the ITS copy number in the *Bd* isolate at the time of experimental use is critical for accurate zoospore quantification.

Assessing *Bd* prevalence and pathogen load is essential to understanding the dynamics of chytridiomycosis and its consequences in amphibian communities all over the world. Therefore, the genomic complexity of fungi and the multi-copy nature of the ribosomal gene complex needs to be considered to accurately estimate and characterize the dynamics of fungal diseases in natural populations. In addition, the development of alternative molecular methods like dPCR and the design of new molecular primers for single copy markers have a great potential for the study of several fungal diseases that are impacting other organisms, such as bats, plants, fishes, and corals (Fisher et al. 2012, Muller et al. 2013, Gozlan et al. 2014).

Acknowledgements. We thank Lisa K. Belden, Myra C. Hughey, and Daniel Medina for developing the field design and collecting samples in Panama. Thanks to Leyla Davis, Gabriel Erni, Holly Archer, Franklin Román-Rodríguez, and Valerie McKenzie for help sampling amphibians and qPCR analyses. We thank Ollie Hyman and Molly C. Bletz for reviewing this paper and contributing to ideas. This project was funded by the National Science Foundation Dimensions of Biodiversity program under grant DEB-1136602 to R.N.H.

LITERATURE CITED

Acinas SG, Marcelino LA, Klepac-Ceraj V, Polz MF (2004) Divergence and redundancy of 16S rRNA sequences in genomes with multiple rrn operons. J Bacteriol 186: 2629–2635

Baker M (2012) Digital PCR hits its stride. Nat Methods 9: 541–544

Bataille A, Fong JJ, Cha M, Wogan GOU and others (2013) Genetic evidence for a high diversity and wide distribution of endemic strains of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* in wild Asian amphibians. Mol Ecol 22:4196–4209

Becker MH, Harris RN, Minbiole KP, Schwantes CR and others (2011) Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. EcoHealth 8:501–506

Begerow D, Nilsson H, Unterseher M, Maier W (2010) Current state and perspectives of fungal DNA barcoding and rapid identification procedures. Appl Microbiol Biotechnol 87:99–108

Bellemain E, Carlsen T, Brochmann C, Coissac E, Taberlet P, Kauserud H (2010) ITS as an environmental DNA barcode for fungi: an *in silico* approach reveals potential PCR biases. BMC Microbiol 10:189

Bletz MC, Loudon AH, Becker MH, Bell SC, Woodhams DC, Minbiole KPC, Harris RN (2013) Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. Ecol Lett 16:807–820

Bletz MC, Rosa GM, Andreone F, Courtois EA and others (2015a) Widespread presence of the pathogenic fungus *Batrachochytrium dendrobatidis* in wild amphibian communities in Madagascar. Sci Rep 5:8633

Bletz MC, Rebollar EA, Harris RN (2015b) Differential efficiency among DNA extraction methods influences detection of the amphibian pathogen *Batrachochytrium dendrobatidis.* Dis Aquat Org 113:1–8

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Org 60:141–148

Briggs CJ, Knapp RA, Vredenburg VT (2010) Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. Proc Natl Acad Sci USA 107:9695–9700

Carey C, Bruzgul J, Livo L, Walling M and others (2006) Experimental exposures of boreal toads (*Bufo boreas*) to a pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*). EcoHealth 3:5–21

Courtois EA, Gaucher P, Chave J, Schmeller DS (2015) Widespread occurrence of *Bd* in French Guiana, South America. PLOS ONE 10:e0125128

Crawford AJ, Lips KR, Bermingham E (2010) Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. Proc Natl Acad Sci USA 107:13777–13782

Cuenca-Estrella M, Meije Y, Diaz-Pedroche C, Gomez-Lopez A and others (2009) Value of serial quantification of fungal DNA by a real-time PCR-based technique for early diagnosis of invasive aspergillosis in patients with febrile neutropenia. J Clin Microbiol 47:379–384

Dahl C, Kiatik I, Baisen I, Bronikowski E and others (2012) *Batrachochytrium dendrobatidis* not found in rainforest frogs along an altitudinal gradient of Papua New Guinea. Herpetol J 22:183–186

Dingle TC, Sedlak RH, Cook L, Jerome KR (2013) Tolerance of droplet-digital PCR vs real-time quantitative PCR to inhibitory substances. Clin Chem 59:1670–1672

DiRenzo GV, Langhammer PF, Zamudio KR, Lips KR (2014) Fungal infection intensity and zoospore output of *Atelopus zeteki*, a potential acute chytrid supershedder. PLOS ONE 9:e93356

Ellison AR, Tunstall T, DiRenzo GV, Hughey MC and others (2014) More than skin deep: functional genomic basis for resistance to amphibian chytridiomycosis. Genome Biol Evol 7:286–298

Farrer RA, Weinert L, Bielby J, Garner TWJ and others (2011) Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. Proc Natl Acad Sci USA 108:18732–18736

Farrer RA, Henk DA, Garner TWJ, Balloux F, Woodhams DC, Fisher MC (2013) Chromosomal copy number variation, selection and uneven rates of recombination reveal cryptic genome diversity linked to pathogenicity. PLOS Genet 9:e1003703

Fisher MC, Garner TWJ, Walker SF (2009) Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. Annu Rev Microbiol 63:291–310

Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, McCraw SL, Gurr SJ (2012) Emerging fungal threats to animal, plant and ecosystem health. Nature 484:186–194

Fites JS, Ramsey JP, Holden WM, Collier SP and others (2013) The invasive chytrid fungus of amphibians paralyses lymphocyte responses. Science 342:366–369

Gozlan RE, Marshall WL, Lilje O, Jessop CN, Gleason FH, Andreou D (2014) Current ecological understanding of fungal-like pathogens of fish: What lies beneath? Front Microbiol 5:62

Guillemette T, Iacomi-Vasilescu B, Simoneau P (2004) Conventional and real-time PCR-based assay for detecting pathogenic *Alternaria brassicae* in cruciferous seed. Plant Dis 88:490–496

Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN and others (2013) Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat Methods 10:1003–1005

Horevaj P, Milus EA, Bluhm BH (2011) A real-time qPCR assay to quantify *Fusarium graminearum* biomass in wheat kernels. J Appl Microbiol 111:396–406

Hoshino T, Inagaki F (2012) Molecular quantification of environmental DNA using microfluidics and digital PCR. Syst Appl Microbiol 35:390–395

Huggett JF, Whale A (2013) Digital PCR as a novel technology and its potential implications for molecular diagnostics. Clin Chem 59:1691–1693

Hyatt AD, Boyle DG, Olsen V, Boyle DB and others (2007) Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis.* Dis Aquat Org 73:175–192

Kielgast J, Röder D, Veith M, Löters S (2010) Widespread occurrence of the amphibian chytrid fungus in Kenya. Anim Conserv 13:36–43

Kilburn VL, Ibáñez R, Sanjur O, Bermingham E, Suraci JP, Green DM (2010) Ubiquity of the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis*, in anuran communities in Panamá. EcoHealth 7:537–548

Kinney VC, Heemeyer JL, Pessier AP, Lannoo MJ (2011) Seasonal pattern of *Batrachochytrium dendrobatidis* infection and mortality in *Lithobates areolatus*: affirma-

tion of Vredenburg's '10,000 Zoospore Rule'. PLOS ONE 6:e16708

Kirshtein JD, Anderson CW, Wood JS, Longcore JE, Voytek MA (2007) Quantitative PCR detection of *Batrachochytrium dendrobatidis* DNA from sediments and water. Dis Aquat Org 77:11–15

Kriger KM, Pereoglou F, Hero JM (2007) Latitudinal variation in the prevalence and intensity of chytrid (*Batrachochytrium dendrobatidis*) infection in eastern Australia. Conserv Biol 21:1280–1290

Lindner DL, Banik MT (2011) Intragenomic variation in the ITS rDNA region obscures phylogenetic relationships and inflates estimates of operational taxonomic units in genus *Laetiporus*. Mycologia 103:731–740

Lips KR (2016) Overview of chytrid emergence and impacts on amphibians. Philos Trans R Soc Lond B Biol Sci 371: 20150465

Lips KR, Brem F, Brenes R, Reeve JD and others (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proc Natl Acad Sci USA 103:3165–3170

Longo AV, Rodriguez D, da Silva LD, Toledo LF, Mendoza Almeralla C, Burrowes PA, Zamudio KR (2013) ITS1 copy number varies among *Batrachochytrium dendrobatidis* strains: implications for qPCR estimates of pathogen load from field-collected amphibian skin swabs. PLOS ONE 8: e59499

Lötters S, Kielgast J, Bielby J, Schmidtlein S and others (2009) The link between rapid enigmatic amphibian decline and the globally emerging chytrid fungus. EcoHealth 6:358–372

Lötters S, Rödder D, Kielgast J, Glaw F (2011) Hotspots, conservation, and diseases: Madagascar's megadiverse amphibians and the potential impact of chytridiomycosis. In: Zachos FE, Habel JC (eds) Biodiversity hotspots. Springer, Berlin, p 255–274

Martel A, Blooi M, Adriaensen C, van Rooij P and others (2014) Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science 346:630–631

Muller LK, Lorch JM, Lindner DL, O'Connor M, Gargas A, Blehert DS (2013) Bat white-nose syndrome: a real-time TaqMan polymerase chain reaction test targeting the intergenic spacer region of *Geomyces destructans*. Mycologia 105:253–259

Nilsson RH, Kristiansson E, Ryberg M, Hallenberg N, Larsson KH (2008) Intraspecific ITS variability in the kingdom fungi as expressed in the international sequence databases and its implications for molecular species identification. Evol Bioinform Online 4:193–201

Pei AY, Oberdorf WE, Nossa CW, Agarwal A and others (2010) Diversity of 16S rRNA genes within individual prokaryotic genomes. Appl Environ Microbiol 76: 3886–3897

Pinheiro LB, Coleman VA, Hindson CM, Herrmann J, Hindson BJ, Bhat S, Emslie KR (2012) Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. Anal Chem 84:1003–1011

R Core Team (2014) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. www.r-project.org

Rebollar EA, Hughey MC, Harris RN, Domangue RJ, Medina D, Ibáñez R, Belden LK (2014) The lethal fungus *Batrachochytrium dendrobatidis* is present in lowland tropical forests of Far Eastern Panamá. PLOS ONE 9: e95484

Rebollar EA, Hughey MC, Medina D, Harris RN, Ibáñez R, Belden LK (2016) Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. ISME J 10: 1682–1695

Rödder D, Kielgast J, Bielby J, Schmidtlein S and others (2009) Global amphibian extinction risk assessment for the panzootic chytrid fungus. Diversity 1:52–66

Rodriguez D, Becker CG, Pupin NC, Haddad CFB, Zamudio KR (2014) Long-term endemism of two highly divergent lineages of the amphibian-killing fungus in the Atlantic Forest of Brazil. Mol Ecol 23:774–787

Rodríguez-Brenes S, Rodriguez D, Ibáñez R, Ryan MJ (2016) Spread of amphibian chytrid fungus across lowland populations of Túngara frogs in Panamá. PLOS ONE 11: e0155745

Rosenblum EB, James TY, Zamudio KR, Poorten TJ and others (2013) Complex history of the amphibian-killing chytrid fungus revealed with genome resequencing data. Proc Natl Acad Sci USA 110:9385–9390

Ross DM, Branford S (2011) Minimal residual disease: the advantages of digital over analog polymerase chain reaction. Leuk Lymphoma 52:1161–1163

Rowley JJL, Hoang HD, Le DTT, Dau VQ, Neang T, Cao TT (2013) Low prevalence or apparent absence of *Batrachochytrium dendrobatidis* infection in amphibians from sites in Vietnam and Cambodia. Herpetol Rev 44: 466–469

Scheele BC, Hunter DA, Grogan LF, Berger L and others (2014) Interventions for reducing extinction risk in chytridiomycosis-threatened amphibians. Conserv Biol 28:1195–1205

Schloegel LM, Toledo LF, Longcore JE, Greenspan SE and others (2012) Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. Mol Ecol 21:5162–5177

Schoch CL, Seifert KA, Huhndorf S, Robert V and others (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Natl Acad Sci USA 109:6241–6246

Shuey MM, Drees KP, Lindner DL, Keim P, Foster JT (2014) Highly sensitive quantitative PCR for the detection and differentiation of *Pseudogymnoascus destructans* and other *Pseudogymnoascus* species. Appl Environ Microbiol 80:1726–1731

Skerratt LF, Berger L, Speare R, Cashins SD and others (2007) Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth 4: 125–134

Smith ME, Douhan GW, Rizzo DM (2007) Intra-specific and intra-sporocarp ITS variation of ectomycorrhizal fungi as assessed by rDNA sequencing of sporocarps and pooled ectomycorrhizal roots from a *Quercus* woodland. Mycorrhiza 18:15–22

Smith DS, Rocheleau H, Chapados JT, Abbott C and others (2014) Phylogeny of the genus *Synchytrium* and the development of TaqMan PCR assay for sensitive detection of *Synchytrium endobioticum* in soil. Phytopathology 104:422–432

Strain MC, Richman DD (2013) New assays for monitoring residual HIV burden in effectively treated individuals. Curr Opin HIV AIDS 8:106–110

Swei A, Rowley JJL, Rödder D, Diesmos MLL and others (2011) Is chytridiomycosis an emerging infectious disease in Asia? PLOS ONE 6:e23179

Tellenbach C, Grünig CR, Sieber TN (2010) Suitability of quantitative real-time PCR to estimate the biomass of fungal root endophytes. Appl Environ Microbiol 76:5764–5772

Vogelstein B, Kinzler KW (1999) Digital PCR. Proc Natl Acad Sci USA 96:9236–9241

Voyles J, Young S, Berger L, Campbell C and others (2009) Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. Science 326:582–585

Voyles J, Vredenburg VT, Tunstall TS, Parker JM, Briggs CJ, Rosenblum EB (2012) Pathophysiology in mountain yellow-legged frogs (*Rana muscosa*) during a chytridiomycosis outbreak. PLOS ONE 7:e35374

Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ (2010) Dynamics of an emerging disease drive large-scale amphibian population extinctions. Proc Natl Acad Sci USA 107:9689–9694

Vredenburg VT, Preez L, Raharivololoniaina L, Vieites DR, Vences M, Weldon C (2012) A molecular survey across Madagascar does not yield positive records of the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. Herpetol Notes 5:507–517

Weaver S, Dube S, Mir A, Qin J and others (2010) Taking qPCR to a higher level: Analysis of CNV reveals the power of high throughput qPCR to enhance quantitative resolution. Methods 50:271–276

Weider LJ, Elser JJ, Crease TJ, Mateos M, Cotner JB, Markow TA (2005) The functional significance of ribosomal (r)DNA variation: impacts on the evolutionary ecology of organisms. Annu Rev Ecol Evol Syst 36:219–242

Whale AS, Huggett JF, Cowen S, Speirs V and others (2012) Comparison of microfluidic digital PCR and conventional quantitative PCR for measuring copy number variation. Nucleic Acids Res 40:e82

White PL, Perry MD, Barnes RA (2009) An update on the molecular diagnosis of invasive fungal disease. FEMS Microbiol Lett 296:1–10

Woodhams DC, Kilburn VL, Reinert LK, Voyles J and others (2008) Chytridiomycosis and amphibian population declines continue to spread eastward in Panama. EcoHealth 5:268–274

Woodhams DC, Kenyon N, Bell SC, Alford RA and others (2010) Adaptations of skin peptide defences and possible response to the amphibian chytrid fungus in populations of Australian green-eyed treefrogs, *Litoria genimaculata.* Divers Distrib 16:703–712

Woodhams DC, Bosch J, Briggs CJ, Cashins SD and others (2011) Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. Front Zool 8:8

Woodhams DC, Bigler L, Marschang R (2012a) Tolerance of fungal infection in European water frogs exposed to *Batrachochytrium dendrobatidis* after experimental reduction of innate immune defenses. BMC Vet Res 8: 197

Woodhams DC, Geiger CC, Reinert LK, Rollins-Smith LA and others (2012b) Treatment of amphibians infected with chytrid fungus: learning from failed trials with itraconazole, antimicrobial peptides, bacteria, and heat therapy. Dis Aquat Org 98:11–25

Woodhams DC, Brandt H, Baumgartner S, Kielgast J and others (2014) Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. PLOS ONE 9:e96375

Yap TA, Koo MS, Ambrose RF, Wake DB, Vredenburg VT (2015) Averting a North American biodiversity crisis. Science 349:481–482

Editorial responsibility: Louise Rollins-Smith, Nashville, Tennessee, USA

Submitted: November 17, 2015; Accepted: December 15, 2016
Proofs received from author(s): March 4, 2017