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Duplex Real-Time PCR for Rapid Simultaneous Detection of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in Amphibian Samples

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Chytridiomycosis is a lethal fungal disease contributing to declines and extinctions of amphibian species worldwide. The currently used molecular screening tests for chytridiomycosis fail to detect the recently described species *Batrachochytrium salamandrivorans*. In this study, we present a duplex real-time PCR that allows the simultaneous detection of *B. salamandrivorans* and *Batrachochytrium dendrobatidis*. With *B. dendrobatidis*- and *B. salamandrivorans*-specific primers and probes, detection of the two pathogens in amphibian samples is possible, with a detection limit of 0.1 genomic equivalent of zoospores of both pathogens per PCR. The developed real-time PCR shows high degrees of specificity and sensitivity, high linear correlations ($r^2 > 0.995$), and high amplification efficiencies (>94%) for *B. dendrobatidis* and *B. salamandrivorans*. In conclusion, the described duplex real-time PCR can be used to detect DNA of *B. dendrobatidis* and *B. salamandrivorans* with highly reproducible and reliable results.

Chytridiomycosis causes worldwide declines and extinctions of amphibian populations and is one of the most important infectious diseases in amphibians (1–3). *Batrachochytrium dendrobatidis* was the sole *Chytridiomycetes* taxon known to infect vertebrate hosts and to be able to cause this devastating disease (4) until a second chytrid species was isolated from a mortality event that drove the Dutch fire salamander (*Salamandra salamandra*) population nearly to extinction (5, 6). This novel species, *Batrachochytrium salamandrivorans*, cannot be detected with the *B. dendrobatidis*-specific PCR described by Annis et al. (7) or the *B. dendrobatidis*-specific real-time PCR described by Boyle et al. (8). Because both *B. dendrobatidis* and *B. salamandrivorans* are able to cause amphibian chytridiomycosis, the development of a test that would allow fast reliable detection and quantification of these two pathogens is necessary. This test could aid in rapid diagnosis of chytridiomycosis in diseased amphibians but also could be used to map the worldwide distribution of the novel pathogen. Therefore, the aim of this study was to develop a duplex real-time PCR that allows detection of *B. dendrobatidis* and *B. salamandrivorans* in amphibian samples with high sensitivity and specificity.

MATERIALS AND METHODS

Chytrid strains and culture conditions. *B. dendrobatidis* and *B. salamandrivorans* were grown in TGhL broth (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose per liter of distilled water) in 25-cm^3 cell culture flasks and incubated at 20°C (*B. dendrobatidis*) or 15°C (*B. salamandrivorans*). *Homolaphlyctis polyrhiza*, *Gaertneriomyces semiglobifer*, *Geranomyces variabilis*, *Rhizophlyctis rosea*, *Rhizoclosmatium globosum*, *Polychytrium aggregatum*, *Monoblepharis polymorpha*, and *Podochytrium dentatum* (Table 1) were grown in PmTG broth (0.5 g peptonized milk, 0.5 g tryptone, 2.5 g glucose per liter of distilled water) in 25-cm^3 cell culture flasks, with incubation at 23°C. To obtain zoospores of *B. dendrobatidis* and *B. salamandrivorans*, 2 ml of a 5-day-old culture was transferred to TGhL broth with 1% agar plates and incubated for 5 to 7 days at 20°C (for *B. dendrobatidis*) or 15°C (for *B. salamandrivorans*). Zoospores were subsequently collected by flooding the agar plates with 2 ml of filtered (0.2-μm filter) pond water and collecting the fluid. The number of zoospores present in the suspension was determined using a hemocytometer.

Quantitation standards and DNA extracts. Suspensions containing standardized numbers of *B. dendrobatidis* and *B. salamandrivorans* zoospores were prepared as described by Boyle et al. (8). Tenfold serial dilution series ranging from 1,000 to 0.01 genomic equivalents (GEs) of zoospores per real-time PCR mixture were prepared for *B. dendrobatidis* and *B. salamandrivorans*. DNA of the other described *Chytridiomycota* species (Table 1) was prepared from growing cultures with DNA extraction in 100 μl of Prepman Ultra reagent (Applied Biosystems, Foster City, CA), following the DNA extraction method described by Hyatt et al. (9).

Primer and probe design. The previously described forward primer STerF (5′-TGCTCCATCTCCCCCTCTTCA-3′) and reverse primer STerR (5′-TGAACGCACATTGCACTCTAC-3′) were used to detect the 5.8S rRNA gene of *B. salamandrivorans* (6) (GenBank accession number KC762295). The *B. salamandrivorans*-specific Cy5-labeled probe STerC (5′-ACAAGAAAATACTATTGATTCTCAAACAGGCA-3′), based on the 5.8S rRNA gene of *B. salamandrivorans*, was developed using Kodon (Applied Maths, Kortrijk, Belgium) (Fig. 1). The primer set ITS1-3 Chytr (5′-CCTTGATATAATACAGTGTGCCATATGTC-3′) and 5.8S Chytr (5′-TCGGTTCTCTAGGCAACAGTTT-3′) and the TaqMan probe Chytr MGB2 (5′-CGAGTCGAAC-3′) described by Boyle et al. (8) were used to detect the ITS1 rRNA gene of *B. dendrobatidis*. All primers and probes were checked with BLASTN analysis, to ensure that amplification of genes from other organisms or species was unlikely. For *B. salamandrivorans*, the specificity of the primer set was tested in a SYBR green real-time PCR with DNA extracts of pure *B. salamandrivorans* culture and

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TABLE 1 Overview of the *Chytridiomycota* isolates used to verify the specificity of the real-time duplex PCR for *B. dendrobatidis* and *B. salamandrivorans*

Species	Class	Order	Isolate	Amplification[a]
Batrachochytrium dendrobatidis	*Chytridiomycetes*	*Rhizophydiales*	JEL423	Yes (*B. dendrobatidis*)
Batrachochytrium salamandrivorans	*Chytridiomycetes*	*Rhizophydiales*	AMFP13/1	Yes (*B. salamandrivorans*)
Homolaphlyctis polyrhiza	*Chytridiomycetes*	*Rhizophydiales*	JEL142	No
Rhizophlyctis rosea	*Chytridiomycetes*	*Rhizophlyctidales*	JEL532	No
Gaertneriomyces semiglobifer	*Chytridiomycetes*	*Spizellomycetales*	JEL384	No
Rhizoclosmatium globosum	*Chytridiomycetes*	*Chytridiales*	JEL791	No
Podochytrium dentatum	*Chytridiomycetes*	*Chytridiales*	JEL30	No
Polychytrium aggregatum	*Chytridiomycetes*	*Polychytriales*	JEL109	No
Geranomyces variabilis	*Chytridiomycetes*	*Spizellomycetales*	JEL518	No
Monoblepharis polymorpha	*Monoblepharidomycetes*	*Monoblepharidales*	JEL486	No

[a] The component of the duplex real-time PCR that showed amplification in positive samples is indicated in parentheses.

negative controls, with melting curve analysis and gel electrophoresis of the real-time PCR products (see below).

***B. salamandrivorans* SYBR green real-time PCR.** The *B. salamandrivorans* SYBR green assay was performed on a CFX96 real-time system (Bio-Rad Laboratories, Hercules, CA). A reaction mixture composed of 12.5 μl SYBR green PCR mix (1× SensiMix SYBR No-ROX; Bioline Reagents Ltd., London, United Kingdom), *B. salamandrivorans* forward primer STerF at a concentration of 0.3 μM, *B. salamandrivorans* reverse primer STerR at a concentration of 0.3 μM, 5 μl template, and a volume of RNase- and DNase-free water to a total of 25 μl was used in each reaction. Amplification conditions consisted of 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 62°C for 15 s. A temperature gradient from 60°C to 95°C, with plate reads at every temperature increment of 0.5°C, was used to generate melting curve data.

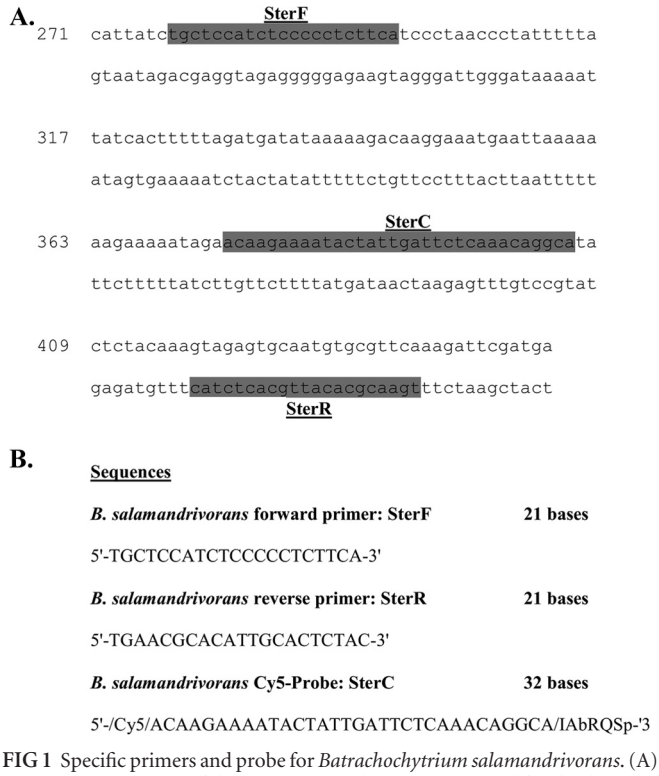

FIG 1 Specific primers and probe for *Batrachochytrium salamandrivorans*. (A) rRNA gene sequences of the ITS1, 5.8S, and ITS2 regions used for the design of the *B. salamandrivorans* primers and probe. (B) *B. salamandrivorans* primer and probe sequences. The sequence used is from GenBank accession number KC762295.

Duplex real-time TaqMan PCR assay optimization. The *B. dendrobatidis* and *B. salamandrivorans* real-time PCR assays were first optimized as simplex assays. Assays were performed on a CFX96 real-time system (Bio-Rad Laboratories, Hercules, CA). Amplification conditions for the simplex and duplex assays consisted of 10 min at 95°C followed by 40 cycles of melting (95°C for 15 s) and annealing/extension (62°C for 1 min). Primer concentrations were optimized in a checkerboard system, with a standard probe concentration of 250 nM. Subsequently, the probe concentrations were optimized with the previously determined optimal primer concentrations. After optimization of the simplex assays, the two PCRs were combined to form the duplex real-time PCR. The precision of the developed duplex real-time PCR assay was evaluated by determining intra- and interassay variability, expressed as the mean coefficient of variation. For the interassay variability, three replicates of the quantitation standard were run in three separate assays; for the intra-assay variability, three replicates were run in one assay. The specificity of the duplex real-time PCR was evaluated by assaying DNA extracts of a wide range of *Chytridiomycota* species (Table 1). Real-time PCR efficiency, slope, and r^2 values were calculated with Bio-Rad CFX Manager v1.6 (Bio-Rad Laboratories, Hercules, CA), with the baseline-subtracted curve-fit setting. Slope and r^2 were calculated with the standard curves determined with the quantitation standards described earlier. Efficiency was calculated as $10^{-1/\text{slope}} - 1$. After optimization of the duplex real-time PCR, a protocol that includes adding bovine serum albumin (BSA) to the PCR mixture was validated as an alternative to diluting samples, in order to alleviate PCR inhibition that could arise due to the nature of amphibian samples (9, 10). BSA (Sigma-Aldrich Inc., Bornem, Belgium) was added to the PCR mixture at a concentration of 400 ng μl^{-1}, as this is the optimal concentration to relieve PCR inhibition (11). Four replicates of the described quantitation standards of *B. dendrobatidis* and *B. salamandrivorans* with added BSA and four replicates without added BSA were run. Mean quantification cycle (C_q) values generated for the two conditions were compared to evaluate any significant effect of the addition of BSA on basic PCR results.

Amphibian samples. To validate the use of the real-time PCR to detect *B. dendrobatidis* and *B. salamandrivorans* in skin samples from amphibians, we applied the optimized protocol to samples from (i) 10 fire salamanders (*S. salamandra*) experimentally inoculated with *B. salamandrivorans*, (ii) 41 fire salamanders (*S. salamandra*) from the declining population in the Netherlands, (iii) 51 fire salamanders (*S. salamandra*) from a stable Belgian population, and (iv) 27 yellow-bellied toads (*Bombina variegata*) from a healthy Dutch population with known *B. dendrobatidis* infection (see Table 4). The *B. salamandrivorans* infection experiment with fire salamanders was carried out with approval of the ethics committee of the Faculty of Veterinary Medicine, Ghent University (approval no. EC2013/10). Skin swabs were collected by gently rubbing a sterile cotton-tipped swab 10 times across the ventral abdomen, inner thigh, and hind limb digits (9, 12). From dead amphibians, pieces of skin

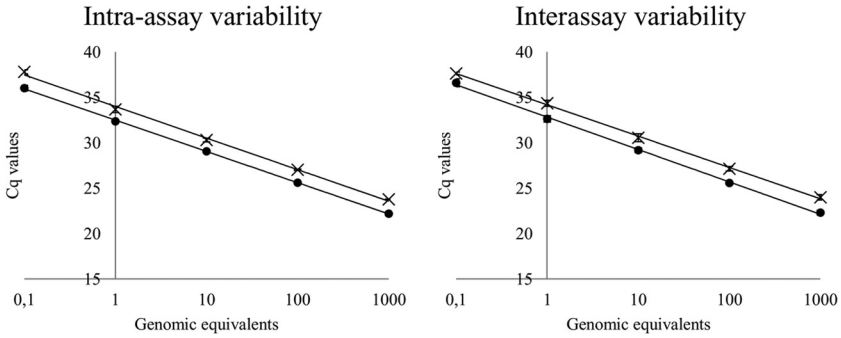

FIG 2 Standard curves for *Batrachochytrium dendrobatidis* and *B. salamandrivorans* generated with the duplex real-time PCR. Standard curves for *B. dendrobatidis* (×) and *B. salamandrivorans* (●) were generated by assaying triplicates of quantitation standards. Error bars represent the standard deviations of the assayed quantitation standard triplicates.

taken from the ventral abdomen (approximate size, 0.25 cm^2) were collected for analysis. DNA was extracted from skin swabs in 100 μl Prepman Ultra (Applied Biosystems, Foster City, CA) (9). DNA was extracted from skin tissue using proteinase K digestion, following the protocol of Bandi et al. (13). After DNA extraction, 1:10 dilutions were prepared, to minimize possible PCR inhibition (9), and were stored at −20°C until further use. All tested samples were run in both simplex and duplex real-time PCR assays, for comparison of the variability in C_q values between the simplex and duplex runs. Samples that did not generate a signal were assigned a C_q value of 40, corresponding to the maximum number of cycles run in this real-time PCR setup. For positive samples, the number of GEs per swab or total skin tissue was calculated with the quantitation standards. A sample was considered positive when the number of GEs per swab/skin tissue exceeded 20, which, because of the dilution of the sample in the process of DNA extraction, corresponded to a detection limit of 0.1 GE per real-time PCR.

RESULTS AND DISCUSSION

Assay optimization. The primer and probe concentrations used in the duplex real-time PCR were the lowest concentrations that yielded the highest ΔR_n (defined as the R_n value [the fluorescence emission of the reporter dye, normalized to the background fluorescence] of a reaction mixture containing all the reaction components [including the template] minus the R_n value of an unreacted negative control) and lowest quantification cycle (C_q) values, respectively, in a checkerboard system. This resulted in a PCR mixture of 25 μl per reaction composed of 12.5 μl TaqMan PCR mix (1× iQ Supermix; Bio-Rad Laboratories, Hercules, CA), *B. salamandrivorans* forward primer STerF at a concentration of 0.3 μM, *B. salamandrivorans* reverse primer STerR at a concentration of 0.3 μM, *B. salamandrivorans* Cy5-labeled probe STerC at a concentration of 0.1 μM, *B. dendrobatidis* forward primer ITS1-3 Chytr at a concentration of 0.9 μM, *B. dendrobatidis* reverse primer 5.8S Chytr at a concentration of 0.9 μM, *B. dendrobatidis* FAM-labeled probe Chytr MGB2 at a concentration of 0.15 μM, and 5 μl template. The amplification conditions were identical to the conditions used in the *B. dendrobatidis*-specific real-time PCR (8) with the exception of an increase in the annealing/extension temperature from 60°C to 62°C. No differences between PCR results were found when a serial 10-fold dilution series of *B. dendrobatidis* DNA was assayed in triplicate with standard and elevated annealing/extension temperatures. All *B. salamandrivorans*-positive samples, as determined with the *B. salamandrivorans* PCR (6) or by immunohistochemistry (9), and positive controls generated a single peak in the SYBR green real-time PCR melting curve analysis, with a constant melting temperature (T_m) of 75.5°C. Gel electrophoresis of the PCR product of *B. salamandrivorans*-positive samples and positive controls always generated a single DNA band at the expected size of approximately 160 bp. Negative samples and negative controls did not generate a peak in the melting curve analysis or generate a visible band in gel electrophoresis, indicating that no nonspecific binding of the primers occurred.

Sensitivity and specificity. The sensitivity of the duplex real-time TaqMan PCR assay was tested with the described quantitation standards of *B. dendrobatidis* and *B. salamandrivorans*. Triplicates of serial dilution series ranging from 0.01 GE to 1,000 GEs of zoospores of the two pathogens were assayed with the duplex real-time PCR (Fig. 2). Although some of the 0.01-GE samples did generate C_q values, these were not consistent. The remaining concentrations of the 10-fold dilution series of both pathogens were detected with the duplex real-time PCR assay in all replicates. This demonstrates that the limit of detection of the *B. salamandrivorans* component of this duplex real-time PCR is 0.1 GE per PCR, which is similar to that for *B. dendrobatidis* in the *B. dendrobatidis*-specific real-time PCR (8). A detection limit lower than 1 GE of *B. salamandrivorans* suggests the presence of a high copy number of the ITS1 region, as already demonstrated for certain *B. dendrobatidis* strains (14, 15). This ability of the duplex real-time PCR to detect the two pathogens at low levels makes it ideal for early pathogen detection in environmental screening and in the diagnosis of chytridiomycosis. To ensure that no interference occurred when DNA of *B. dendrobatidis* and *B. salamandrivorans* was present in a sample, quantitation standards with DNA of both pathogens were assayed. This resulted in C_q values very similar to

TABLE 2 Assay precision, efficiencies, and linear correlations of the *B. dendrobatidis* and *B. salamandrivorans* real-time duplex PCRs

Species and variability experiment	Efficiency (%)	Linear correlation (r^2)	Coefficient of variation (% [mean ± SD])
B. dendrobatidis			
Intra-assay	94.1	0.997	0.56 ± 0.34
Interassay	99.4	0.996	0.99 ± 0.39
B. salamandrivorans			
Intra-assay	95.7	0.999	0.39 ± 0.48
Interassay	96.0	0.997	0.98 ± 0.27

TABLE 3 Effect of adding BSA (400 ng μl^{-1}) to the duplex real-time PCR mixture on C_q values generated with *B. dendrobatidis* and *B. salamandrivorans* quantitation standards[a]

Genomic equivalent(s)	C_q (mean ± SD) for:			
	B. dendrobatidis		*B. salamandrivorans*	
	With BSA	Without BSA	With BSA	Without BSA
1,000	24.95 ± 0.02	23.96 ± 0.04	23.00 ± 0.04	22.90 ± 0.10
100	27.22 ± 0.03	27.21 ± 0.07	26.32 ± 0.06	26.34 ± 0.17
10	30.48 ± 0.10	30.55 ± 0.11	29.69 ± 0.15	29.67 ± 0.17
1	33.53 ± 0.10	33.67 ± 0.32	33.32 ± 0.62	32.99 ± 0.19
0.1	37.33 ± 0.17	37.43 ± 0.23	36.18 ± 0.23	36.18 ± 0.16

[a] Four replicates with and four replicates without added BSA were assayed in order to evaluate the effect on standard dilutions of *B. dendrobatidis* and *B. salamandrivorans* DNA. No significant difference was found between the two conditions for any of the dilutions for either pathogen (*t* test, $P > 0.05$).

the values obtained with single-strain standards, indicating that accurate quantification can be performed with samples containing DNA of the two pathogens. To verify the specificity of the duplex real-time PCR, a total of 10 different isolates belonging to the class *Chytridiomycota*, including *B. dendrobatidis* and the *B. salamandrivorans* type strain, were assayed (Table 1). The real-time PCR amplified only *B. dendrobatidis* and *B. salamandrivorans* of all included isolates, indicating a high degree of specificity.

Assay performance and precision. Assay performance and precision were evaluated with the described quantitation standards of *B. dendrobatidis* and *B. salamandrivorans*. High linear correlation ($r^2 > 0.995$) and amplification efficiency (>94%) values for *B. dendrobatidis* and *B. salamandrivorans* in intra-assay and interassay variability experiments, together with low (<1%) intra- and interassay variabilities, demonstrate that the developed duplex real-time PCR has a good performance over the tested quantitation range, with highly reproducible results (Table 2). These traits make the duplex real-time PCR highly suitable for use in screening surveys and disease diagnosis.

PCR protocol with bovine serum albumin. Adding BSA to the duplex real-time PCR mixture at a final concentration of 400 ng μl^{-1} did not significantly affect the generated C_q values (*t* test, $P > 0.05$) (Table 3). This allows BSA to be used as an alternative to sample dilution in order to alleviate the influence of PCR inhibitors present in amphibian samples.

Amphibian samples. To validate the developed duplex real-time PCR, amphibian skin swabs and skin tissue were assayed (Table 4). The samples included known negative and positive *B. dendrobatidis* and *B. salamandrivorans* samples. All tested samples were run in the simplex and duplex real-time PCR assays to compare the variability in C_q values between the simplex and duplex runs (Fig. 3). Very little variation was found between the results of the duplex real-time PCR and both simplex real-time PCRs, as indicated by a high degree of correlation ($r^2 > 0.995$) for the C_q values from the simplex and duplex runs for *B. dendrobatidis* and *B. salamandrivorans*. In the setup used in this study, the lowest detectable number of GEs per swab was 20, which corresponds to 0.1 GE per PCR. Dilution occurring in the process of DNA extraction and in the prevention of PCR inhibition accounts for this difference in detection limits between swabs and reactions. All samples that tested negative in the *B. dendrobatidis* and *B. salamandrivorans* simplex PCR assays also tested negative in the duplex PCR assay. The samples that tested positive in the *B. dendrobatidis* or *B. salamandrivorans* simplex PCR assay also tested positive for the corresponding pathogen in the duplex PCR assay. The noninvasive sampling technique (skin swabbing) resulted in overall lower GE numbers than the invasive technique (skin tissue collection) (Table 4). In the *S. salamandra* samples taken from the declining Dutch population, skin swabs were collected from live and apparently healthy animals, while skin tissues were collected from animals found dead on site. A possible explanation for the higher number of GEs found in the skin tissue samples could therefore be a more advanced disease state of the animals, accompanied by increased infection intensity. The smaller difference between GE numbers in the skin swab and skin tissue samples from the *B. salamandrivorans*-positive fire salamanders (*S. salamandra*) in the *B. salamandrivorans* infection experiment could be explained by the short period of time between swabbing and the animals dying due to infection with *B. salamandrivorans*. For *B. dendrobatidis*, a threshold in infection intensity (mean of >10,000

TABLE 4 Overview of the amphibian samples used to validate the real-time duplex PCRs for *B. dendrobatidis* and *B. salamandrivorans*

Amphibian species	Origin (coordinates and/or reference) and yr	Health status	Skin sample type (*n*)	No. PCR positive (species)	Mean GEs/swab for positive samples (range)[a]
Fire salamander (*Salamandra salamandra*)	Bunderbos, Netherlands (N50°55′, E5°45′), 2010	Declining	Swab (33)	13 (*B. salamandrivorans*)	219.8 (60–1,750)
			Tissue (8)	4 (*B. salamandrivorans*)	2,398.3 (242–10,180)
	Merelbeke, Belgium (N50°57′, E3°43′), 2012	Healthy	Swab (51)	0	NA
	B. salamandrivorans infection experiment (6), 2013	Diseased	Swab (5)	5 (*B. salamandrivorans*)	6,920 (1,572–10,740)
			Tissue (5)	5 (*B. salamandrivorans*)	10,915 (3,420–19,380)
	B. salamandrivorans infection experiment (6), 2013	Healthy	Swab (5)	0	NA
			Tissue (5)	0	NA
Yellow-bellied toad (*Bombina variegata*)	't Rooth, Netherlands (N50°50′, E5°47′) (A. Spitzen-van der Sluijs, A. Martel, C. A. Hallmann, W. Bosman, T. W. J. Garner, P. van Rooij, R. Jooris, F. Haesebrouck, and F. Pasmans, submitted for publication), 2013	Healthy	Swab (27)	14 (*B. dendrobatidis*)	175.4 (20–1,488)

[a] Mean values of genomic equivalents (GEs) of zoospores per swab of *B. dendrobatidis* and *B. salamandrivorans* for the positive samples were calculated with the included quantitation standards for *B. dendrobatidis* and *B. salamandrivorans*. NA, not applicable.

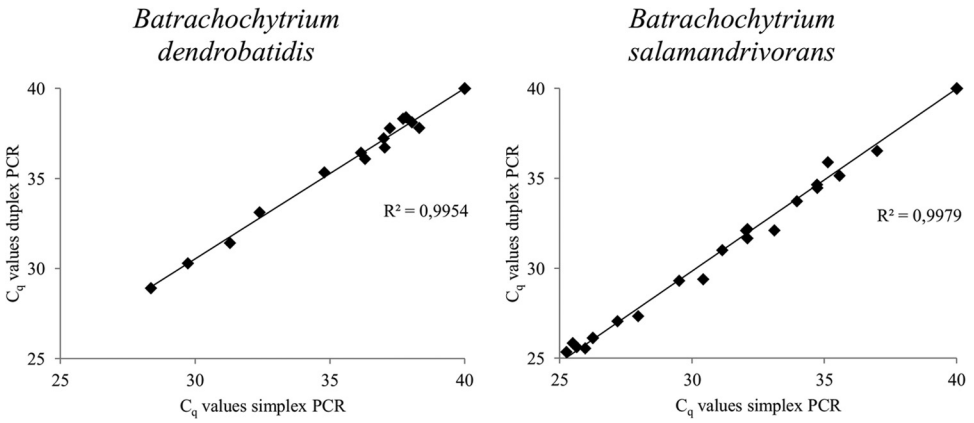

FIG 3 C_q values generated by assaying amphibian samples with the *Batrachochytrium dendrobatidis* and *B. salamandrivorans* duplex real-time PCR assay and both simplex real-time PCR assays. An overview of all assayed amphibian samples is presented in Table 1. Samples that did not generate a signal were assigned a C_q value of 40, corresponding to the maximum number of cycles run in this real-time PCR setup.

GEs per swab) predicts whether an amphibian population will decline due to *B. dendrobatidis* infection (16). In the *B. salamandrivorans* infection experiment, the mean value for *B. salamandrivorans* GEs per swab was comparable to this threshold (mean of 6,920 GEs per swab), indicating that this could also be the case for *B. salamandrivorans*.

Conclusion. The described *B. dendrobatidis* and *B. salamandrivorans* duplex real-time PCR can be used to accurately and reliably detect these two pathogens in amphibian samples. The real-time PCR can be used to aid in chytridiomycosis disease diagnosis and in mapping of the worldwide distribution of *B. dendrobatidis* and *B. salamandrivorans*.

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