A species-specific qPCR assay for the reptile pathogen Nannizziopsis barbatae and mitochondrial genomes of Nannizziopsis and Paranannizziopsis

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Abstract

Infectious fungal diseases can have devastating effects on wildlife health and our ability to detect emerging fungal pathogens in the wild is considered indispensable for effective management strategies. Several fungi from the genus Nannizziopsis and Paranannizziopsis are emerging pathogens of reptiles and have been observed to cause disease in a wide range of taxa. Nannizziopsis barbatae has become a particularly important pathogen of Australian reptiles with an increasing number of herpetofauna being reported with cases of infection from across the country. Here, we report a species-specific qPCR assay for the rapid detection of N. barbatae and demonstrate its application in a wild urban population of a dragon lizard. In addition, we present the mitochondrial genome sequences and phylogenetic analysis for seven species in this group of fungi to support further research into the evolutionary ecology of these emerging pathogens.

INTRODUCTION

Diseases-causing fungi pose a serious threat to wildlife populations, yet despite the impact they have had on global biodiversity, they are notably understudied (Ghosh, Fisher, & Bates, 2018). New fungal pathogens are emerging that are capable of infecting an increasingly diverse range of taxa and their impacts are being exacerbated by changing climate conditions and globalisation (Fisher et al., 2012; Ghosh et al., 2020). Urbanisation can be a key driver of disease emergence, facilitating transmission due to newly overlapping geographic expansions (Hassell, Begon, Ward, & Fèvre, 2017), increasing the risk of disease spillover from wildlife into humans and other animals (Heesterbeek et al., 2015). Reports of emerging fungal pathogens are rising (Fisher et al., 2020) and over the past few decades, a group of Onygenalean fungi from the genera *Nannizziopsis* ,*Paranannizziopsis* , and *Ophidiomyces* have emerged as a leading cause of severe mycoses in reptiles (Paré & Sigler, 2016).

Fungi from the genus *Nannizziopsis* are capable of causing disease in several species of reptiles and have also been known to infect humans (Nourrisson et al., 2018). Infection in reptiles is contagious and initially presents as cutaneious disease with characteristic lesions forming crusts, ulcers and hyperkeratosis, that often progresses to fatal mycoses (Sigler, Hambleton, & Pare, 2013). Among several species from this genus identified as reptile pathogens, infection with*Nannizziopsis barbatae* has become increasingly observed in free-living populations of Australian reptiles with a wide variety of species being reported with this disease (Peterson et al., 2020). Urban wildlife populations in particular have become a focal point for outbreaks and the need for effective detection and monitoring of pathogen occurrence is considered vital for mitigating the spread and to minimise any potential for transmission to humans (Ghosh et al., 2018). Molecular diagnostic tests are powerful tools for disease surveillance offering a low cost and rapid means to assist in the early

detection in both captive and wild populations (Boyle, Boyle, Olsen, Morgan, & Hyatt, 2004). Such tools also enable long-term tracking of pathogens which facilitate the study of often complex host-pathogen interactions, such as how disease tolerance may effect prevalence and transmission (Seal, Dharmarajan, & Khan, 2021; Tedersoo, Drenkhan, Anslan, Morales-Rodriguez, & Cleary, 2019). Genomic data are valuable resources for the development of diagnostic tools enabling swift identification of target regions for designing highly specific markers, and these data can also serve as a foundation for studies on the molecular basis for pathogen evolution (DeCandia, Dobson, & VonHoldt, 2018; Ghosh et al., 2020, 2021). A rapid diagnostic tool to confirm the presence of*N. barbatae* in clinical samples is currently unavailable. As*Nannizziopsis* fungi are typically first isolated on selective media prior to PCR and sequencing, laboratory diagnosis may involve delays of up to a week due to the slow growth of these species. The aim of this study is to develop a molecular diagnostic for the specific detection of *N. barbatae* infections and to contribute genomic resources for further research into this group of emerging fungal pathogens.

MATERIALS AND METHODS

Sample culturing and processing

Swab samples were obtained from population of free-living easter water dragons (*Intellagama lesueurii*) with a history of infection with*N. barbatae* in Brisbane city's Roma Street Parkland (-27deg27'46'S, 153deg1'11'E), Queensland, Australia. Skin lesions from animals displaying characteristic signs of infection were sampled with sterile rayon dry transfer swabs (Copan Diagnostics Inc.) and placed on ice before transportation to the laboratory to be stored at -20oC until processed to extract DNA using the Wizard Genomic DNA Purification Kit (Promega). Type cultures were purchased from the UAMH Centre for Global Microfungal Biodiversity in Toronto, Canada. *N. barbatae* strain USC001 was isolated and sequenced previously (Powell et al., 2021). Fungal cultures were grown on potato dextrose agar at 25°C for up to three weeks to obtain sufficient growth. Plate cultures were scraped, and DNA was purified using the DNeasy Plant Mini kit (Qiagen) with 5 mm Stainless Steel Beads (Qiagen). Purified DNA was then quantified and stored at -20°C. Short-read sequencing was undertaken using the Nextera DNA Flex library kit and run on the Illumina NextSeq platform at Centre for the Analysis of Genome Evolution & Function at the University of Toronto.

Assembly of mitochondrial genomes

Sequencing reads were trimmed and filtered for quality using Trimmomatic v0.36 (Bolger, Lohse, & Usadel, 2014) before use with the GetOrganelle v1.7.3.5 tool (Jin et al., 2020) with the parameters -R 10 -k 21,45,65,85,105 -F fungus_mt for *de novo* assembly incorporating the SPAdes v3.13.0 assembler (Prjibelski, Antipov, Meleshko, Lapidus, & Korobeynikov, 2020) and Bowtie2 v2.4.2 (Langmead & Salzberg, 2012). Protein-coding sequences were predicted using Mitos2 webserver (Donath et al., 2019) using the RefSeq 89 Fungi database. Coding sequences were manually inspected using Geneious Prime software v2022.2.1 (Biomatters Ltd., New Zealand). Mitochondrial circular map was drawn using OGDRAW v1.3.1 (Greiner, Lehwark, & Bock, 2019). Newly assembled mitochondrial genomes were submitted to the NCBI Genbank under accessions listed in TABLE 1.

Comparative analysis

A total of 13 protein coding genes (PCGs) could be consistently annotated across all genomes generated in this study. The PCGs*cox1* , *cox2* , *cox3* , *atp6* , *atp8* ,*atp9* , *nad1* , *nad2* , *nad3* , *nad4* ,*nad4L* , *nad5* and *nad6* from 13 fungal strains were concatenated and aligned using MAFFT v7.221 (Katoh & Standley, 2013) and a small number of regions containing gaps were eliminated. PhyML v3.3.20180214 (Guindon et al., 2010) was used to construct the maximum-likelihood tree using the LG method with 250 bootstrap replicates. The tree was visualised in Geneious Prime. Whole mitochondrial genome alignments were performed using the Mauve v1.1.3 (Darling, Mau, & Perna, 2010) plugin in Geneious Prime.

qPCR assay design

Extracted DNA from fungal cultures was checked with PCR using ITS gene fragment primers forward 5'-GCATCGATGAAGAACGCAGCGA-3' and reverse 5'-GGYCAGCKCCGGCCGGGTC-3' used in a pre-

vious study (Peterson et al., 2020) to confirm that purified DNA from the reference strains could be successfully amplified. Primers and probe were selected by manually examining nucleotide alignments of each coding sequence for regions of dissimilarity. The intronic region of the *N. barbatae* NADH dehydrogenase subunit I (*nad1*) gene was selected due to a larger number of polymorphisms compared with other genes (FIGURE 1). A custom primer and probe set was developed using the PrimerQuest Tool (Integrated DNA Technologies, Inc) and produced with the 6-FAM five-prime reporter dye and the ZEN/Iowa Black FQ double-quenched probe system (Integrated DNA Technologies, Inc). The qPCR reactions consisted of 10 µl SensiFAST Probe No-ROX Kit (Meridian Bioscience), 0.4 µM each of forward primer 5'-TGATCATGTTTAGTCTCTGAAGGT-3' and reverse primer 5'-AGGCTAAGCTGATTTGGTCTC-3', 0.1 µM of the probe 5'-6-FAM/TCCCTGCTG/ZEN/ATTGCCATATATTAGGT/FQ/-3', and 2 µl DNA template made up to a volume of 20 µl using molecular grade water. The cycling parameters included an initial denaturation step of 95°C for 5 mins followed by 35-40 cycles of 95°C for 20 seconds and 63°C for 30 seconds using a Rotor-Gene Q HRM 5plex Platform (Qiagen). The resulting 114 bp amplicon was confirmed as the correct region using Sanger sequencing. A positive result was called for any sample crossing the cycle threshold (C_t) as determined by the Rotor-Gene Q software.

RESULTS

Mitochondrial genome assembly and phylogenetics

Short-read shotgun sequencing produced a minimum 150-fold read coverage per sample that was used to reconstruct the mitochondrial genomes from six *Nannizziopsis* and one *Paranannizziopsis* reference strains. Variability in mitochondrial DNA length between species ranged from 24.5 to 30.8 Kb (TABLE 1). The overall GC content was fairly consistent among species ranging between 23.4 to 24.5%, consistent with the AT-rich composition of fungal mitochondrial genomes. The *N. barbatae* mtDNA is gene-dense and contains the same gene order reported for other Onygenaceae (FIGURE 2) (de Melo Teixeira, Lang, Matute, Stajich, & Barker, 2021).

A total of 3,780 positions were aligned across the 13 PCGs annotated for each of the genomes used in this study (FIGURE 3). Topology of the*Nannizziopsis* branches in the phylogenetic tree resemble results from previous studies (Peterson et al., 2020; Sigler et al., 2013) showing congruence between the approach used in this study and the conserved nucleotide targets of the ITS and 28S ribosomal regions. Only two out of the 3,757 aligned positions observed between the assemblies of *N. barbatae* were different suggesting a high level of conservation in PCGs between these strains. Likewise, only two out of the 3,760 aligned amino acids between *N. vriesii* and*N. dermatiditis* were found to be different. Given the high degree of sequence conservation between these two pairwise comparisons, whole mtDNA alignments were performed to determine the degree of similarity at the nucleotide level. Whole mtDNA comparisons between *N. barbatae*strains USC001 and UAHM 11185 resulted in 1,675 differences being observed among 27,757 aligned nucleotide positions (93.97% identical). However, the alignment between *N. vriesii* and *N. dermatiditis* resulted in only 154 differences out of 24,572 aligned nucleotide positions (99.37% identical). The*N. barbatae* strain USC001 assembly is slightly larger than the UAHM 11185 strain owing to the inclusion of two introns, one in the *cox1* gene and another in the large ribosomal subunit RNA gene (*rnl*) gene of approximately 1 Kb each in size. The *cox1* intron was found to contain an LAGLI-DADG endonuclease motif and the *rnl* intron an GIY-YIG endonuclease motif. The smaller assembly size for *N. vriesii* when compared with *N. dermatiditis* is due to the former missing an approximately 1 Kb intron in the *nad1* gene, explaining the absence of PCR amplification with the primers from this assay. This intron also contains an GIY-YIG endonuclease motif.

Species-specific qPCR performance

Using our qPCR assay, *N. barbatae* DNA from two different isolates was successfully detected in the absence of all other*Nannizziopsis* species in this study, including the closely related *N. crocodili* . These results suggest that mtDNA targets can be used to distinguish between species of *Nannizziopsis*fungi. A small degree of non-specific fluorescence was observed from*N. dermatitidis* DNA at the optimal probe annealing T_m of 60°C owing to a lower amount of variation in this species at the probe target location (FIGURE

1). This was resolved without sacrificing reaction efficiency by raising the annealing temperature to 63°C. The probe sequence confers the specificity in the assay as the PCR primer sequences were conserved across *Nannizziopsis* species and could produce an amplicon from all but *N. vriesii* (Supplementary Figure 1).

Standard curve DNA was diluted across genome equivalent (GE) values 400,000 GE, 40,000 GE, 4,000 GE, 400 GE, 40 GE, 4 GE and 0.4 GE with each standard prepared in triplicate reactions in two separate qPCR runs to evaluate for reproducibility (FIGURE 4). The reaction efficiency ranged from 0.89 to 1.01 and the R^2 values were greater than 0.99 for both runs (ranging from 0.996 – 0.999). The C_t values of the quantitation standards were also consistent between runs with each dilution crossing the cycle threshold at approximately the same cycle number.

To test the performance of this assay to detect the presence of *N. barbatae* from field collected samples, a total of 96 skin swab samples were obtained from 85 individual animals that were either healthy or displaying various levels of disease severity (see example in Supplementary Figure 2) over the course of 2021 and tested using this protocol. A total of 67 swab samples returned a positive result for infection with *N. barbatae* (TABLE 2).

DISCUSSION

This study offers a rapid method for the detection of *N. barbatae*DNA from cultured and field collected samples. Diagnostic confirmation had previously relied on cultivation and subsequent metabarcoding analysis to confirm presence of *Nannizziopsis* infection (Peterson et al., 2020) leading to lengthy delays in obtaining results due to the slow growth characteristics of these species (Paré & Sigler, 2016).

Our qPCR assay was able to correctly distinguish DNA samples of *N. barbatae* from five other species of *Nannizziopsis* . The assay was sufficiently sensitive to detect the presence of less than one genomic equivalent per reaction, owing to the presence of multiple copies of the mitogenome for every nuclear genome. We expect there to be an average of between six to nine mitochondrial genome copies per nuclear genome for the *N. barbatae* isolates sequenced in this study based on differences observed in the amount of sequencing read coverage between the nuclear and mitochondrial genome. However, we caution that this may be highly variable and with only a few isolates sequenced to date, the use of higher limit of detection should be considered until sufficient data on copy number can be attained.

We also demonstrate the utility of this assay to screen a free-living population of infected reptiles. In addition to the 67 samples that tested positive for infection with *N. barbatae* , there were seven instances of what appear to be false negative results out of 74 visibly diseased animals from this screening set. As these occurred only in the mildly diseased animals (rating 1 or 2, Supplementary Table 1), we posit that this is likely the result of the state of the infected site being small and difficult to recover fungal DNA, and/or variation in the swab sampling techniques from different handlers that yielded lower sample material. There were no false positive results observed in this sample set suggesting no contaminating sources of non-target DNA were encountered.

We report that the high similarity between the species *N. vriesii*and *N. dermatitidis* extends across the entire mitochondrial genome with the notable exception of an intron present in the*nad1* gene in *N. dermatitidis* . This high level of similarity suggests some taxonomic revision may be appropriate to either group these two species together or split the two strains of*N. barbatae* . The varying occurrence of introns was also observed between the two strains of *N. barbatae* in this study isolated from infected reptiles approximately 10 years apart. Each of these introns was found to contain either an LAGLI-DADG or GIY-YIG endonuclease domain motif. These domains encode homing nucleases, suggesting these introns possess a capacity for self-splicing (Megarioti & Kouvelis, 2020). Intra-specific variations in the presence of mitochondrial introns has been reported for other species of fungi (Deng et al., 2020; Freel, Friedrich, Hou, & Schacherer, 2014) and the non-uniform inclusion of introns in the mitochondrial genes found in these closely related species may indicate the activity of mobile elements during their evolutionary history. An intron is present in the*nad1* gene of all *Nannizziopsis* and*Paranannizziopsis* isolates included in this study with the exception of *N. vriesii* (UAMH 3527) which was originally isolated in 1972, two decades earlier than any of the other fungal species.

This strain of *N. vriesii* was also found in Europe, quite distant from each of the other strains that were found in either Australia or North America. For each *N. barbatae* isolate, this intron is 1,047 bp in length and was dissimilar in only two positions, which are outside of the assay target region, despite UAMH 1185 being isolated from its reptile host over 10 years prior and from a geographic location separated by more than 700 km. Even though the *nad1* gene intron contains an GIY-YIG motif, it is conserved in all but one species of*Nannizziopsis* included in this study and could be amplified using the same primer pair in each case. We believe this DNA target is stable enough for further applications using this assay for the detection of*N. barbatae* .

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AUTHOR CONTRIBUTIONS

The study was conceived by DP and CHF. Sequence data was generated by DP and BS. Data analysis and assay validation was performed by DP. CHF provided funding. DP wrote the manuscript with input from all authors.

DATA AVAILABILITY STATEMENT

The mitochondrial genome assemblies produced in this study are available from the NCBI GenBank under the accession numbers listed in TABLE 1.

ETHICAL NOTE

Our study was part of ongoing research conducted with approval from the University of the Sunshine Coast (animal ethics approval numbers ANS1858 and ANA20161).

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TABLE AND FIGURES

TABLE 1 Information and statistics for the mitochondrial genome assemblies for each fungal species.

Species	Strain	GenBank Accession	Mitogenome size (bp)	GC %	Country	Y
Nannizziopsis barbatae	USC001	CM026550.1	30,107	23.7	Australia	2
Nannizziopsis barbatae	UAMH 11185	ON968427	27,824	23.5	Australia	2
Nannizziopsis crocodili	UAMH 9666	ON968429	30,841	23.7	Australia	1
Nannizziopsis vriesii	UAMH 3527	ON968426	24,569	24.5	Netherlands	1
Nannizziopsis dermatitidis	UAMH 7582	ON968430	25,568	24.2	Canada	1
Nannizziopsis hominis	UAMH 7860	ON968431	30,161	23.8	USA	1
Nannizziopsis guarroi	UAMH 10352	ON968428	30,493	23.6	USA	2
Paranannizziopsis australasiensis	UAMH 10439	ON968432	31,311	23.4	Australia	2

TABLE 2. Summary of qPCR assay screening of skin swabs from visibly diseased or otherwise healthy dragons accompanied with assigned disease severity rating.

Disease rating	Disease rating	qPCR Positive	qPCR Positive	qPCR Negative	qPCR Negative	qPC
1. Mild	1. Mild	33	33	6	6	6
2. Mild-moderate	2. Mild-moderate	13	13	1	1	1
3. Moderate	3. Moderate	11	11	0	0	0
4. Moderate-Severe	4. Moderate-Severe	9	9	0	0	0
5. Severe	5. Severe	1	1	0	0	0
0. No obvious lesions	0. No obvious lesions	0	0	22	22	22
Totals			67	67	29	

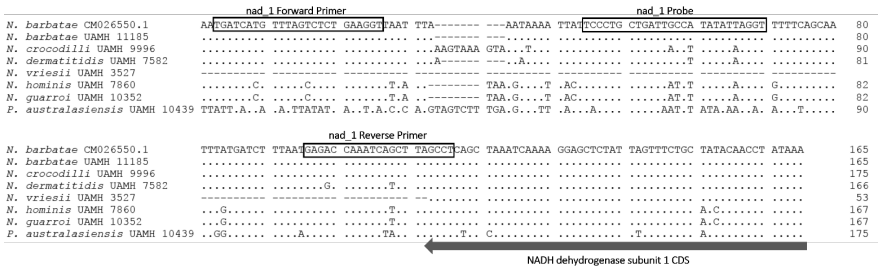

FIGURE 1 Fungal mtDNA sequence alignment of the *nad1* gene showing positions of the *N. barbatae* - specific primers and probe used in this study. Dots indicate bases identical to the *N. barbatae* CM026550.1 reference sequence. The assay produces an amplicon 114 bp in length.

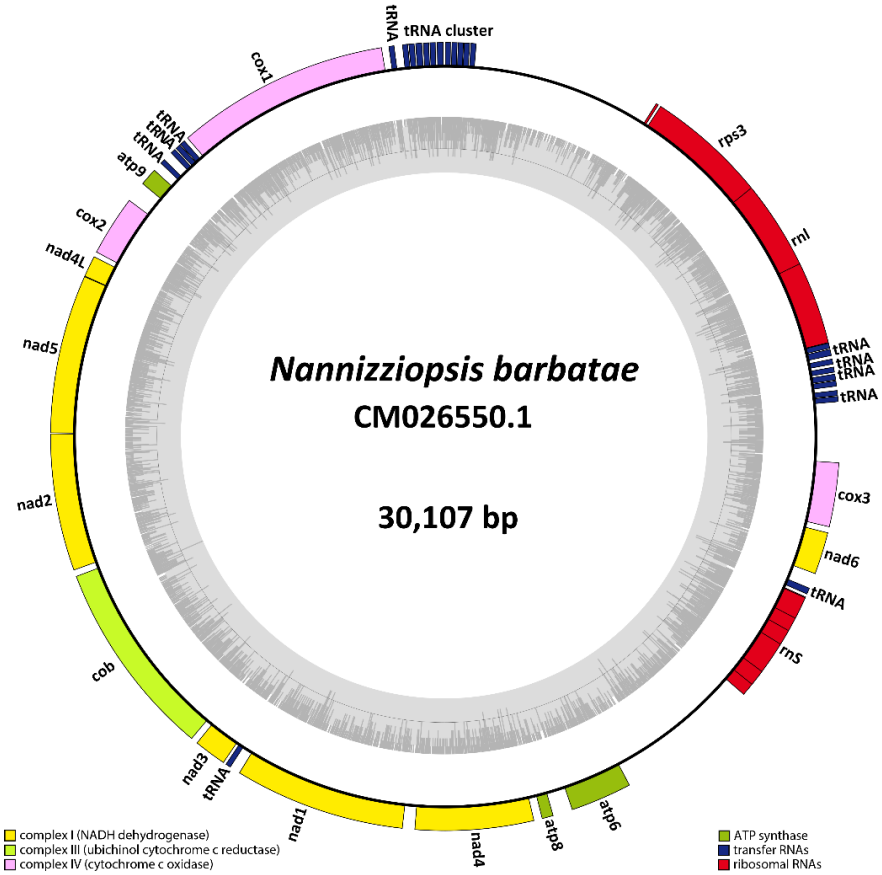

FIGURE 2 Circular representation of the mitochondrial genome of *N. barbatae* strain USC001 isolated from an infected Eastern water dragon. Protein coding genes are represented by coloured bars. GC content is represented in the internal circle.

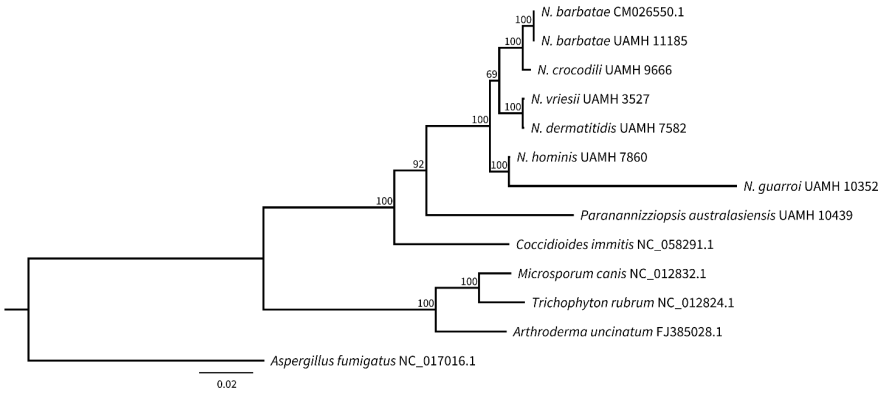

FIGURE 3 Maximum-likelihood phylogenetic tree showing the evolutionary relationship between the seven mitochondrial genomes annotated in this study and with other species of onygenales fungi. *Aspergillus fumigatus*, a member of the Eurotiales, was used as the outgroup. The alignment contained 13 concatenated mitochondrial protein-coding genes. Bootstrap support values obtained with 250 replicates are included at the nodes.

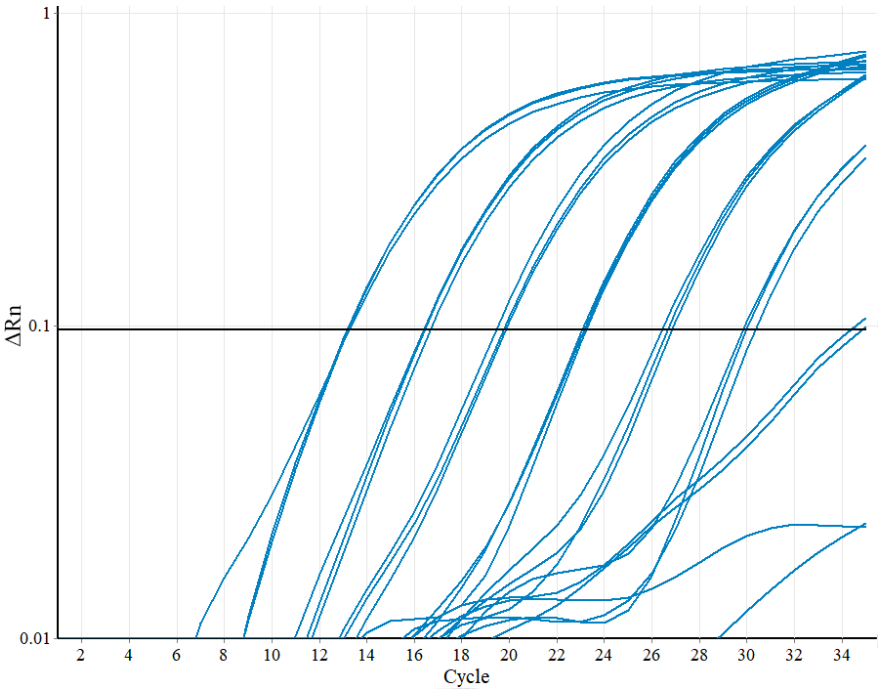

FIGURE 4 Amplification plot of the *N. barbatae* -specific qPCR assay fluorescence values showing performance of DNA standards over 7 orders of magnitude.