A Rapid Diagnostic for Detection of *Aphelenchoides besseyi* and *A. fujianensis* Based on Real-Time PCR

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Abstract

Aphelenchoides besseyi and *A. fujianensis* have been frequently found in mixed populations associated with forage grass seed in Brazil. The morphological similarity between both species has previously led *A. fujianensis* to be erroneously identified as *A. besseyi*. *A. besseyi* is a quarantine pest in many countries that import Brazilian forage seed; however, there is no current evidence suggesting that *A. fujianensis* is a plant-parasitic species. Two real-time polymerase chain reaction (qPCR) diagnostics were developed to detect each species and an operational envelope was established. A set of primers and hydrolysis probes for each species was designed targeting the large subunit (LSU) region. To assess their specificity, primers and probes

sets were tested with samples of nontarget *Aphelenchoides* and *Paraphelenchus* sp. also frequently associated with forage seed. Experiments using dilutions of purified plasmid standards underpinned the sensitivity of the qPCR assays, which detected as few as 10 copies of target nematode ribosomal DNA. Thus, the developed diagnostics were sufficiently sensitive to detect DNA extracted from a fragment of a single target nematode. There was a positive correlation between copy number of the target species and nematode abundance, suggesting the potential of this method for quantification. Evidence of intra-individual variability among cloned sequences of the LSU region in a single *A. besseyi* population is also reported.

The genus *Aphelenchoides* includes representatives of plant-parasitic, mycophagous, and predatory nematode species distributed across a range of habitats (Hunt 1993). Despite the high number of nominal species in this genus (approximately 200), only a few species have been reported as plant-parasitic (Sánchez-Monge et al. 2015). However, *Aphelenchoides* spp. merited inclusion in the list of economically important nematodes in molecular plant pathology (Jones et al. 2013). Special attention has been paid to *Aphelenchoides besseyi* Christie 1942, *A. fragariae* (Ritzema Bos 1890) Christie 1932, and *A. ritzemabosi* (Schwartz 1911) Steiner and Buhner 1932 due to their economic impact and yield losses in numerous agricultural and horticultural crops (Duncan and Moens 2006; Sánchez-Monge et al. 2015).

A. besseyi, commonly known as rice white tip nematode, is reported to parasitize more than 200 plant species (Duncan and Moens 2006; Cheng et al. 2013), with rice (*Oryza sativa* L.), strawberry (*Fragaria × ananasa* Duch.), and ornamental plants being the most common hosts (Franklin and Siddiqi 1972; Tzeng and Lin 2005). This species is one of the most serious pests affecting global rice production (Nicol et al. 2011), resulting in yield losses up to 71% (Bridge et al. 2005; Cheng et al. 2013; Tülek et al. 2014). In Brazil, *A. besseyi* is widely distributed but economic losses attributed to this species have rarely been reported (Cares et al. 2008; Oliveira and Kubo 2007; Silveira and Curi 1990). Nevertheless, the frequent association of this nematode with forage grass seed represents a phytosanitary barrier for export to many countries where *A. besseyi* is listed as a quarantine pest (Marchi et al. 2007). In addition, this species has been suggested as the causal agent of two new diseases: “black spot disease” on bean (*Phaseolus vulgaris* L.) in Costa Rica (Chaves et al. 2013) and green stem and foliar retention in Brazil (Meyer et al. 2017). This latter, popularly known as “mad soybean”, is responsible for yield losses of up to 100% (Meyer et al. 2017). Thus, it is imperative to ensure

that appropriate phytosanitary measures and management strategies can be implemented against *A. besseyi*, and, therefore, rapid and reliable detection and identification tools are needed.

Difficulties in morphologically identifying *Aphelenchoides* spp. due to the few discriminatory taxonomic characters is conflated by poor descriptions of the numerous nominal species (Hockland 2001; Hunt 1993). This task has been especially challenging in forage grass seed, where a mixed community of morphologically similar but nonpathogenic *Aphelenchoides* spp. are also present, leading to difficulty in attaining a robust diagnosis (Favoreto et al. 2011; Sharma et al. 2001). This was recently underscored by de Jesus et al. (2016), who reported that, in Brazil, *A. fujianensis* Zhou et al. 2010 had occasionally been mistakenly identified as *A. besseyi* and that both species were associated with Brazilian forage seed. This reinforces the urgent need for robust methods of diagnosis, because there is no direct evidence suggesting that *A. fujianensis* is a plant-parasitic species (de Jesus et al. 2016) and forage seed consignments may unnecessarily be rejected for export based on species misidentification.

Reliable and fast DNA-based detection and identification tools such as polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP), conventional PCR, or real-time PCR (qPCR) have proved valuable for the diagnosis of various species of plant-parasitic nematodes (Braun-Kiewnick et al. 2016; Hübschen et al. 2004; Oliveira et al. 2005; Rybarczyk-Mydlowska et al. 2012). qPCR assays have been successfully used for detection and quantification of economically important plant-parasitic nematodes, including *Pratylenchus* spp. (Koyama et al. 2016; Oliveira et al. 2017; Yan et al. 2012, 2013), *Globodera pallida* and *G. rostochiensis* (Nakhla et al. 2010), *Meloidogyne* spp. (Sapkota et al. 2016; Zhao et al. 2010), *Rotylenchulus reniformis* (Sayler et al. 2012), *Xiphinema index* (Van Ghelder et al. 2015), and *Bursaphelenchus xylophilus* (Ye and Giblin-Davis 2013). However, few molecular assays for detection yet alone quantification of *Aphelenchoides* spp. have been reported (Devran et al. 2017; Ibrahim et al. 1994; McCuiston et al. 2007; Rybarczyk-Mydlowska et al. 2012). Ibrahim et al. (1994) employed internal transcribed spacer (ITS)-PCR-RFLP to discriminate various species of *Aphelenchoides*, wherein the restriction enzyme *AluI* was the most successful for species differentiation, although not for *A. besseyi*. Rybarczyk-Mydlowska et al. (2012) developed qPCR assays for four *Aphelenchoides* spp. based on the small subunit ribosomal DNA (SSU rDNA), including *A. besseyi*. Recently, Devran et al. (2017) developed specific-primers for *A. besseyi*, also

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based on the SSU rDNA, using conventional PCR. However, these diagnostics were not challenged against *A. fujianensis*, which is closely related to *A. besseyi* (Zhou et al. 2010). This is critical, because the SSU rDNA region of the two sibling species is highly conserved, thus creating a risk of a false positive for *A. besseyi*, especially in analysis of forage grass seed in Brazil, where both *Aphelenchoides* spp. are often mixed. Thus, the objective of this study was to develop and validate a fast and robust qPCR method for accurate detection of both *A. besseyi* and *A. fujianensis* for phytosanitary use applicable to nematode abundances seen in the Brazilian commercial forage grass industry.

Materials and Methods

Nematode and DNA extraction. Nematodes were extracted from forage grass and rice seed from disparate geographic areas (Table 1) according to Coolen and D'Herde (1972), or cultivated in vitro (de Jesus et al. 2016). DNA was extracted from single nematodes. Individual nematodes were transferred to separate microtubes containing 18 µl of 1× PCR buffer (Promega) and approximately five 1-mm glass beads (Thistle Scientific) (de Jesus et al. 2016). The nematode cuticle was disrupted in a Retsch M300 tissue disruptor for 30 s at 30 Hz; thereafter, 2 µl of proteinase K (100 µg ml⁻¹) was added to each microtube. Microtubes were incubated as follows: 60°C for 60 min, 95°C for 15 min, and 15°C for 2 min. Extracted DNA was stored at -20°C and thereafter used for both conventional PCR and qPCR assays.

Probes and primers design. To facilitate designing specific primers and probes for detection of *A. besseyi* and *A. fujianensis*, an alignment of 81 large subunit (LSU) rDNA sequences of superfamily Aphelenchoidea sourced from GenBank was used, including recently published sequences of both target species (de Jesus et al. 2016). The hydrolysis probes were labeled with 6-carboxyfluorescein at the 5' end and a black hole quencher at the 3' end in accordance with the guidelines proposed by Bustin et al. (2009). The primers and probes were designed and synthesized by PrimerDesign (Table 2). The specificity of each primer-probe set was analyzed in silico by comparison with several nematode DNA sequences from GenBank using BLAST. This allowed a comparison to be made of putatively named and unnamed *Aphelenchoides* spp. that were not available for qPCR testing in this study.

Conventional PCR and standard curve generation. Genomic DNA was extracted from single nematodes representative of each

species from known populations of *A. besseyi* (DJ11) and *A. fujianensis* (DJ01) (Table 1), as described previously. Amplification of the LSU region of both targets was performed using the universal primers D2A (5' CAAGTACCGTGAGGGAAAGTTG 3') and D3B (5' TCGGAAGGAACCACTACTA 3'), according to Ye et al. (2007). Briefly, PCR were prepared in a 15-µl final reaction volume containing reagents with the following: 1× PCR High-Fidelity buffer (Invitrogen), 2 mM MgSO₄ (Invitrogen), 0.2 mM each dNTP (Promega), 0.4 mM each primer, 1 U of High-Fidelity Platinum Taq polymerase (Invitrogen), 1 µl of DNA, and sterile water to complete the volume of each reaction. PCR conditions consisted of denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 2 min. A final extension was performed at 72°C for 10 min (Ye et al. 2007).

PCR products were purified using a Wizard SV Gel and PCR Clean-Up System kit (Promega), inserted into the pGEM-T Easy vector (Promega), and transformed into DH5α *Escherichia coli* electro-competent cells (Invitrogen) according to the protocols provided by the manufacturer. Thereafter, cloned plasmids were extracted and purified using a GeneJET plasmid miniprep kit (Thermo Scientific), sequenced to confirm the identity of each insert, and subsequently used to generation of standard curves. In total, 10 clones of *A. fujianensis* and 9 of *A. besseyi* were sequenced. The cloned LSU sequences were deposited in GenBank under accession numbers KT692690, KT692694, and KY123694 to KY123700 (*A. besseyi*) and KT692683, KT692691, KT692695, KT692699, and KY123688 to KY123693 (*A. fujianensis*).

Cloned plasmid DNA concentrations were measured by Nanodrop spectrophotometer. The number of copies of each LSU insert was determined using the online tool DNA Copy Number and Dilution Calculator of Thermo Scientific (<https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html#legacy=www.thermoscientificbio.com>). After linearization with *NotI* enzyme (Promega), initial concentrations were adjusted in nuclease-free water to 1.0 × 10⁹ copies µl⁻¹ (Daniell et al. 2012). A 10-fold serial dilution (from 10⁸ to 10 copies µl⁻¹) was prepared in duplicate to establish standard curves for qPCR assays.

qPCR assay. qPCR amplifications were performed using a StepOne Real-Time PCR System (Applied Biosystems) in a 20-µl

Table 1. List of populations of superfamily Aphelenchoidea used in the study

Species, origins	Host	Code	Number of specimens tested ^a
<i>Aphelenchoides besseyi</i> ^b			
Japan	<i>Oryza sativa</i>	DJ06S, DJ07S	48
Costa Rica	<i>Phaseolus vulgaris</i>	DJ11 ^c	...
Brazil	<i>O. sativa</i>	DJ12S, DJ15S	...
Spain	<i>O. sativa</i>	DJ18S	...
<i>A. fujianensis</i> ^b			
Brazil	<i>Brachiaria brizantha</i>	DJ01, DJ08, DJ10, DJ14, DJ16	159
	<i>B. decumbens</i>	DJ02, DJ03, DJ17	...
	<i>Panicum maximum</i>	DJ04	...
	Unidentified forage grass seed	DJ13	...
	<i>O. sativa</i>	DJ05F, DJ12F, DJ15F	...
Japan	<i>O. sativa</i> 'Hinohikari'	DJ06F, DJ07F	...
<i>A. fragariae</i>			
United Kingdom	<i>Woodwardia fimbriata</i>	Frag1, Frag3 ^d	4
United States	Unknown	FragNC, Frag99 ^e	...
<i>A. ritzemabosi</i> ^f			
United Kingdom	Unknown	Ritz1, Ritz2	2
<i>Aphelenchoides</i> sp.			
Brazil	<i>P. maximum</i>	S10.2	1
<i>Paraphelenchus</i> sp.			
Brazil	<i>P. maximum</i>	S4.1, S5.1	4
	<i>B. ruzizensis</i>	S11.1, S11.3	...

^a Number of specimens tested in real-time polymerase chain reaction (qPCR) assay.

^b Those populations were molecular and morphologically characterized by de Jesus et al. (2016).

^c Sample used only as *A. besseyi* standard DNA to generate standard curves in qPCR assays.

^d Nematodes provided by Idowu Rotifa (Scottish Agricultural College, UK).

^e DNA samples provided by Dr. Weimin Ye (North Carolina Department of Agriculture & Consumer Services).

^f Nematodes provided by Dr. Thomas Prior (The Food Environment Research Agency, UK).

reaction volume containing 1× Precision Fast 2× Real-Time PCR Mastermix (PrimerDesign), 1 µl of primer–probe master mix (primer at 6 pg µl⁻¹ and probe at 3 pg µl⁻¹), and 2 µl of DNA (cloned plasmid DNA or DNA extracted from single nematodes). The standard amplification protocol was 95°C for 20 s followed by 40 cycles at 95°C for 1 s with 62°C for 20 s. All reactions were performed in duplicate and included positive (standard DNA) and negative (sterilized pure water) controls. Data were analyzed using StepOne software (version 2.1; Applied Biosystems).

Average cycle of quantification (C_q) values from each standard DNA dilution were plotted against the logarithm (log₁₀) of their

initial template copy numbers and standard linear regressions were generated by a correlation coefficient (R^2) of the plotted points. From the slope of each standard curve, PCR amplification efficiency (E) was calculated using the equation $E = (10^{-\text{slope}} - 1) \times 100$. Each amplification curve was characterized by its C_q and visualized and analyzed by plotting fluorescence (normalization of the reporter dye fluorescence [ΔR_n]) against C_q.

Specificity and sensitivity of *A. besseyi* and *A. fujianensis* qPCR assays. To determine the limit of detection of each diagnostic, plasmid DNA of *A. besseyi* and *A. fujianensis* were diluted serially from 10⁸ to 10 copies µl⁻¹ and amplified according to qPCR conditions

Table 2. Real-time polymerase chain reaction diagnostic primer–probe sets designed for the detection of *Aphelenchoides besseyi* and *A. fujianensis*

Target (GenBank access code)	Primer–probe ^a	Sequence (5'–3')	Position	T _m ^b	GC (%) ^c	Product length (bp)
<i>A. besseyi</i> (KT692690)	AbF	GTAATGAAAGTGAGACATTGGATG	516	55.1	37.5	70
	AbR	GATGTTGCGCGCCGGA	585	57.7	68.8	...
	AbP	CGAAACGCCCCAACGCTCACATCGTC	...	...	60.0	...
<i>A. fujianensis</i> (KT692691)	AfF	GTAATGAAAGTGAGACTCTTGGAG	525	56.0	41.7	73
	AfR	GGCGATGTTGCACGAAGAA	597	56.9	52.6	...
	AfP	TCGAAACGCGCCCCGTTTCACATC	...	...	61.0	...

^a Primer–probe sets were designed and synthesized by PrimerDesign Ltd. (Southampton, UK). F = forward primer, R = reverse primer, and P = hydrolysis probe.

^b Melting temperature.

^c GC = Percentage of guanine and cytosine.

Fig. 1. Amplification curves obtained for specificity tests of real-time polymerase chain reaction diagnostics. **A**, Specificity test of *Aphelenchoides besseyi* diagnostic challenged against plasmid DNA of *A. fujianensis* (AphF) from 10⁸ to 10⁶ copies µl⁻¹. **B**, Specificity test of *A. fujianensis* diagnostic challenged against plasmid DNA of *A. besseyi* (AphB) from 10⁸ to 10⁶ copies µl⁻¹. Specificity test of **C** and **E**, *A. besseyi* diagnostic and **D** and **F**, *A. fujianensis* diagnostic challenged against *A. fragariae* (Frag1, FragNC, and Frag99), *A. ritzemabosi* (Ritz1 and Ritz2), *Aphelenchoides* sp. (S10.2), and *Paraphelenchus* sp. (S4.1, S5.1, S11.1, and S11.3). NTC = nontarget control.

described previously. Additionally, serially diluted plasmid DNA from *A. fujianensis* (from 10^8 to 10^6 copies μL^{-1}) was used to test the specificity of the *A. besseyi* diagnostic in the presence of high concentrations of nontarget DNA. The same was done for the *A. fujianensis* diagnostic using serially diluted plasmid DNA from *A. besseyi*. The range of concentrations used was based on previous tests, because $<10^5$ copies μL^{-1} of nontarget DNA generated no amplification.

DNA extracted from 159 individual of *A. fujianensis* obtained from 15 populations (DJ01, DJ02 DJ03, DJ04, DJ05F, DJ06F, DJ07F, DJ08, DJ10, DJ12F, DJ13, DJ14, DJ15F, DJ16, and DJ17) and 48 *A. besseyi* from 5 populations (DJ06S, DJ07S, DJ12S, DJ15S, and DJ18S) acquired from either cultured populations or rice seed (Table 1) were used in qPCR assays to estimate the copy number of target DNA present in individual nematodes. Those same putative *A. fujianensis* samples were used to test the specificity of the *A. besseyi* qPCR diagnostic and those putative *A. besseyi* samples were used to test the specificity of the *A. fujianensis* diagnostic. Moreover, the specificity of both qPCR diagnostics was also determined using DNA from closely related nontarget species; namely, *A. fragariae*, *A. ritzemabosi*, *Aphelenchoides* sp., and *Paraphelenchus* sp. (Table 1).

Quantitative detection of nematodes. To correlate the copy number of target species to nematode abundance, duplicate series of microscopically identified *A. besseyi* and *A. fujianensis* were prepared as follows: 1, 3, 6, and 10 individuals of each species were hand picked, DNA extracted, and qPCR run as previously described.

Statistical analysis. qPCR data derived from individual nematodes from distinct populations of *A. besseyi* and *A. fujianensis* were used for calculation of mean DNA copy number. Linear discriminant analysis (LDA) was performed to generate a discriminant function for the qPCR diagnostics. Using InfoStat (v. 2014), scores generated by the discriminant function were subject to a classification tree analysis to determine the cut-off value for each target that facilitated separation of samples positive for *A. besseyi* from those positive for *A. fujianensis* (Di Rienzo et al. 2014).

Results

Specificity of qPCR assays. At nematode abundances typically found associated with forage seed, the qPCR assays reliably discriminated the

two target species. Initially, the specificity of the *A. besseyi* primers and probe was tested using serial plasmid dilutions of both target species at concentrations of 10^8 to 10^6 copies μL^{-1} (equivalent to 14,164 to 142 *A. besseyi* and 27,548 to 275 *A. fujianensis* individuals). Specificity was found to be high, with at least a fivefold specificity between these closely related targets (Fig. 1A and B). Cross-reactions were only noted when the *A. besseyi* primers and probe were tested against the highest concentrations (equivalent to 27,548 *A. fujianensis*), generating a signal equivalent to 0.02 individual *A. besseyi* nematode. The primers and probe for *A. fujianensis* were even more specific: when challenged with a serial dilution of plasmid DNA of nontarget *A. besseyi*, a lower signal was observed. Using the *A. fujianensis* primers and probe, 10^8 copies of *A. besseyi* (equivalent to 14,164 individuals) generated a signal equivalent to 0.002 individual *A. fujianensis* nematode (Fig. 1B). To date, such high abundances (14,000 to 27,500) have not been found associated with forage seed and represent 283 to 184 times greater than what is reasonably likely to be encountered.

The specificity of *A. besseyi* and *A. fujianensis* primers and probe was further challenged with genomic DNA from single nematodes obtained from known populations of both species that generated, on the whole, no amplification signal or very few low-level random signals (data not shown). No cross-reactions were observed among closely related tested species, *A. fragariae*, *A. ritzemabosi*, *Aphelenchoides* sp., and *Paraphelenchus* sp. (Fig. 1C to F) for either qPCR primer–probe set.

Using DNA extracts from single nematodes, cross-reactions with the *A. fujianensis* primer–probe set were observed from five putative *A. besseyi* nematodes identified from a single population (DJ18S). To understand this unexpected result, DNA from the five individual nematodes was amplified by standard PCR and the LSU region sequenced. One of the seven sequences expected to be *A. besseyi* had 99% affinity to *A. fujianensis*, contrary to unequivocal identification based on morphology and morphometric (Table 3). One possible explanation of the unexpected cross-reaction was the presence of intra-individual LSU variation. To test this hypothesis, five individuals from each of two populations (DJ07S and DJ18S) of *A. besseyi* were randomly selected and the DNA extracted from each individual nematode. PCR products of the LSU region from each individual nematode were cloned and sequenced. Cloned sequences from 8 of the 10

Table 3. Number of clones derived from single nematodes from populations DJ07S and DJ18S and their respective identity based on GenBank database

Nematode code	Clones with identity to <i>A. besseyi</i> /total number (%)	Percent coverage/identity to <i>A. besseyi</i> (DQ328684)	Clones with identity to <i>A. fujianensis</i> /total number (%)	Percent coverage/identity to <i>A. fujianensis</i> (KT692691)
DJ18S ^a	6/7 (86)	98/99	1/7 (14)	100/98
DJ18SA	18/18 (100)	97/99	0/18 (0)	...
DJ18SC	16/20 (80)	97/100	4/20 (20)	98/97–98
DJ18SG	6/15 (40)	97/100	9/15 (60)	98–99/97
DJ18SL	20/20 (100)	98/100	0/20 (0)	...
DJ18SM	19/19 (100)	98/100	0/19 (0)	...
DJ07SB	19/19 (100)	97/100	0/19 (0)	...
DJ07SG	17/17 (100)	97/99	0/17 (0)	...
DJ07SL	18/18 (100)	97/99	0/18 (0)	...
DJ07SH	15/15 (100)	98/100	0/15 (0)	...
DJ07SI	18/18 (100)	98/99	0/18 (0)	...

^a First specimen in which intra-individual variability was previously detected in the large subunit region.

Table 4. Performance characteristics of real-time polymerase chain reaction (qPCR) diagnostic assay using plasmid DNA for *Aphelenchoides besseyi* and *A. fujianensis*

Species	Number of experiments	Limit of detection		Linear regression		
		Lowest concentration (copy number of target)	Average Cq ^a	Slope ^b	R ^{2c}	E (%) ^d
<i>A. besseyi</i>	2	10	33.1; 34.3	−3.32; −3.31	0.998; 0.999	100; 100
<i>A. fujianensis</i>	2	10	33.8; 34.1	−3.56; −3.47	0.996; 0.999	90.8; 95.7

^a Cq = cycle of quantification.

^b Slope of the linear regression line.

^c Square regression coefficient.

^d Efficiency of amplification.

putative individual *A. besseyi* had high affinity to *A. besseyi*. However, cloned sequences from two individuals had affinity to both *A. besseyi* and *A. fujianensis* (Table 3) (i.e., 13 of 35 cloned LSU sequences of two individuals previously identified taxonomically as *A. besseyi* had affinity to *A. fujianensis*).

Sensitivity of qPCR assays. The sensitivity of the qPCR assays was measured with a 10-fold serial dilution of plasmid DNA and reliably detected 10 copies μL^{-1} of template DNA per reaction. There was a negative correlation between Cq values and $\log_{10}$ of the initial known DNA quantities ($R^2 \geq 0.996$) over a range of 10^8 to 10 copies μL^{-1} , suggesting a strong linear relationship between target DNA concentrations and Cq values (Table 4).

DNA extracted from individual nematodes *A. besseyi* ($n = 48$) and *A. fujianensis* ($n = 159$) was tested against primer–probe sets developed for both target species. Each tested qPCR diagnostic detected single nematodes from the respective species but not the nontarget species. Quantification of DNA from individual nematodes was performed by comparison of the fluorescence signals with that obtained from standard plasmid DNA dilutions. Based on genomic DNA extracted from single nematodes, the estimated number of LSU rDNA copies per nematode was $3.63 \times 10^4 \pm 2.11 \times 10^4$ copies for *A. fujianensis* and $7.06 \times 10^4 \pm 5.7 \times 10^4$ copies for *A. besseyi*,

corresponding to average Cq values of 24 ± 1.7 and 25 ± 1.1 , respectively (Table 5).

Detection and quantification of *A. besseyi* and *A. fujianensis* using qPCR assays. Target copy number data estimated from individual nematodes were subjected to LDA and the scores generated by the discriminant function underwent tree classification analysis for determining a cut-off value that separated each target species, reducing the potential of false positives. The discriminant function $y = 0.41 + 0.00031B - 0.00031F$ (where B and F represent the estimated copy numbers derived by the *A. besseyi* and *A. fujianensis* diagnostics, respectively, after challenged by DNA from the same sample) generated a cut-off value of 0.506 that clearly separated *A. besseyi* and *A. fujianensis* (Fig. 2). For quantification, there was a significant positive correlation for *A. besseyi* ($r = 0.97$, $P = 0.014$) and for *A. fujianensis* ($r = 0.99$, $P = 0.004$) between copy number of the target species and abundance of nematodes tested (Fig. 3).

Discussion

This study reports the first species-specific qPCR primers and hydrolysis probes for quantitative detection of the sibling species *A. besseyi* and *A. fujianensis*. The use of hydrolysis probes ensures higher specificity compared with SYBR Green, reducing the probability of nonspecific

Table 5. Performance characteristics of real-time polymerase chain reaction (qPCR) diagnostic assay using DNA from single nematodes for *Aphelenchoides besseyi* and *A. fujianensis*^a

Species	Number of nematodes ^b	Limit of detection		Linear regression		
		Mean number of copies of target in positive samples $\pm$ SD	Average Cq in positive samples $\pm$ SD ^c	Slope $\pm$ SD ^d	$R^2 \pm$ SD ^e	E (%) $\pm$ SD ^f
<i>A. besseyi</i>	43	$7.06 \times 10^4 \pm 5.7 \times 10^4$	25 ± 1.1	-3.467 ± 0.127	0.996 ± 0.004	95 ± 4.5
<i>A. fujianensis</i>	159	$3.63 \times 10^4 \pm 2.11 \times 10^4$	24 ± 1.7	-3.333 ± 0.038	0.999 ± 0.0003	100 ± 1.4

^a Data are means $\pm$ standard deviation (SD) from six experiments.

^b DNA samples investigated for intra-individual variation in the 28S region were not considered here.

^c Cq = cycle of quantification.

^d Slope of the linear regression line.

^e Square regression coefficient.

^f Efficiency of amplification.

Fig. 2. Classification tree analysis and determination of the threshold that discriminates positive samples of *Aphelenchoides besseyi* from *A. fujianensis* based on the score generated by the discriminant function ($y = 0.41 + 0.00031B - 0.00031F$, where B represents the copy number of target estimated for a sample using the *A. besseyi* diagnostic and F represents the copy number of target estimated for a sample using the *A. fujianensis* diagnostic). The threshold (0.506) is indicated by a continuous line, showing the separation between the positive samples for *A. besseyi* (triangle) and those positive for *A. fujianensis* (cross).

fluorescence signal because, in theory, specific hybridization between probe and target DNA is required to generate a fluorescent signal (Scheda et al. 2013). In general, although hydrolysis probes have greater specificity, they are susceptible to template mutations. Thus, mutations in the probe hybridization site may reduce efficiency and sensitivity of the qPCR assay or may block the probe binding completely, leading to false-negative results (Nagy et al. 2016; Yao et al. 2006). Analysis of intraspecific variability in *A. fujianensis* detected the presence of mutation at the probe site of a single cloned individual (Supplementary Table S2) but this did not affect the efficiency of amplification of the target genomic DNA from single nematodes (Table 5).

It is acknowledged that qPCR for detection and quantification of plant-parasitic nematodes is faster, more sensitive, and more accurate than traditional techniques (e.g., morphological identification, PCR-RFLP, and conventional PCR) (Castagnone-Sereno et al. 2011). The diagnostics developed in this study were sufficiently sensitive to detect the DNA extracted from single nematodes, and discriminatory at abundances associated with forage grass seed when challenged against closely related species (*A. fragariae*, *A. ritzenbosi*, *Aphelenchoides* sp., and *Paraphelenchus* sp.) that are known to coexist associated with forage grass seed. It should be noted that *A. bicaudatus*, *A. sexlineatus* (Favoreto et al. 2011), and *A. subtenius* (Sharma et al. 2001) may also occur in association with forage seed but, due to unavailability of material and absence of their LSU sequences in public databases, they were not included in this study. However, future tests should be conducted to verify the specificity of the newly developed primers and probes with these species.

In this study, we sought to identify the working envelope of the developed diagnostics. When challenged with nematode abundances that represented a multiplication factor of 184 and 283 more than what is encountered with forage grass seed (27,548 and 14,164

individuals of *A. fujianensis* and *A. besseyi*, respectively), a nonspecific amplification of plasmid DNA equivalent to 0.02 and 0.002 *A. besseyi* and *A. fujianensis*, respectively, was generated. Typical populations of both species in forage grass seed in Brazil are in the region of a few hundred 10 g^{-1} of seed. Thus, an extreme level of nematode abundance would be required to trigger a cross-reaction resulting in a false positive equivalent to that generated by a single nematode. In pragmatic terms, this is sufficient resolution for rapid phytosanitary screening of forage grass seed, and a working envelope has been established that will form part of the operational risk register for the phytosanitary laboratory.

Cloning and subsequent sequencing of PCR products of the LSU region of 35 clones derived from two individual nematodes (DJ18SC and DJ18SG) from a single population (population DJ18S) of taxonomically identified *A. besseyi* showed the presence of two LSU variants, one that had affinity to *A. besseyi* ($n = 22$ clones) and the other affinity to *A. fujianensis* ($n = 13$ clones) (Table 3) (i.e., intra-individual variation within individual putative *A. besseyi*). This fully accounts for the cross-reaction observed when individuals from this population were used in qPCR assays. Thus, the cross-reactions did not occur due to the lack of specificity of the qPCR diagnostic but, instead, were due to intra-individual variability present in a few tested individuals of a single atypical population of *A. besseyi*.

Intra-individual variability of ribosomal cistrons is rare compared with the ITS but has been observed in some metazoans (Carranza et al. 1996; McTaggart and Crease 2009), including nematodes (Bik et al. 2013; Nyaku et al. 2013). In the flatworm *Dugesia mediterranea*, two variants of the SSU region were found coexisting in the same individual. The analysis of the secondary structure of these variants indicated that both were functional, although only one type had been transcribed into RNA (Carranza et al. 1996). In the plant-parasitic nematode *R. reniformis*, two distinct sequence variants in

Fig. 3. Real-time polymerase chain reaction quantification of **A**, *Aphelenchoides besseyi* ($P = 0.014$) and **B**, *A. fujianensis* ($P = 0.004$). R^2 = coefficient of determination.

the SSU region were identified (Nyaku et al. 2013), and these authors considered this as a result of a recombination event during unequal crossing over between sister chromatids or rDNA between the chromosomes X and Y, carrying coevolution of these sequences in these chromosomes. Thus, the presence of variant sequences with high affinity to *A. fujianensis* coexisting in specimens of *A. besseyi* could be evidence of a recent hybridization event, or even that these sibling species recently diverged from a common parental lineage. However, further research is needed to elucidate how often this occurs in ribosomal cistrons of plant-parasitic nematodes, especially in *A. besseyi*. This is especially relevant because these genomic regions are the target of many recently developed molecular diagnostics (Devran et al. 2017; Rybarczyk-Mydłowska et al. 2012).

We noted a wide variation in the copy number of DNA estimated from single nematodes of both species and among specimens of the same species. Potential explanations for this variation include (i) limited DNA homogenization due to the presence of glass beads in the microtubes; (ii) variation in the efficiency of DNA extraction; and (iii) random selection of nematodes at different life stages, pregnant females, or even spermatheca filled with sperm, the latter relevant for *A. besseyi*. However, the addition of a DNA internal control to monitor the efficiency of DNA extraction (Daniell et al. 2012) and removal of glass beads before using the DNA in qPCR assays should reduce variation in the estimate of copy number in a single nematode. Notwithstanding the large variation in the estimate of DNA copy number, there was high correlation between copy number and nematode abundance (Fig. 3).

The influence of nematode developmental stage on the fluorescence signal of hydrolysis probes has been reported by Van Ghelder et al. (2015), wherein the Cq values were slightly lower in samples with equal numbers of juveniles and adult *X. index*. In contrast, Yan et al. (2013), using SYBR Green, reported no significant difference between the Cq values among different stages of *Pratylenchus neglectus*, including eggs, suggesting that the amount of DNA is similar in different stages of development of this nematode. These contradictory results may be due to the use of different qPCR methodologies (hydrolytic probes versus SYBR Green).

Interpretation of Cq ≥ 35 is often a challenge in qPCR assays because they may indicate the detection of low amounts of target DNA or be caused by poor amplification efficiency, as much as can be derived from nonspecific probe hydrolysis or even probe degradation (Nagy et al. 2016). To underpin the diagnostic and provide phytosanitary laboratories with additional provenance to support their findings, we propose the use of LDA and tree classification analyzes to generate a discriminant equation and threshold value, respectively, to robustly separate *A. besseyi* and *A. fujianensis*, especially when Cq ≥ 35 are observed for an unknown sample of DNA. This provides an underpinning framework to minimize the risk of generating false positives, and tree classification analysis has been used in medical and health care applications for >30 years (Krasteva et al. 2015; Podgorelec et al. 2002; Silvera et al. 2014).

Future research should be conducted to fully explore results presented in this study, including screening of additional populations of *A. besseyi* to estimate the frequency of the sequence variant noted in population DJ18S from Spain, and whether it occurs in Brazilian *A. besseyi* populations. Intra-individual variability of the ribosomal cistrons in *A. besseyi* clearly needs to be further investigated. This is especially pertinent because the target genomic region has been used for the development of many molecular diagnostics to discriminate numerous nematode species without consideration of such intra-individual cistron variability.

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