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Small Subunit Ribosomal DNA-Based Phylogenetic Analysis of Foliar Nematodes (*Aphelenchoides* spp.) and Their Quantitative Detection in Complex DNA Backgrounds

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ABSTRACT

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Foliar nematodes, plant-parasitic representatives of the genus *Aphelenchoides*, constitute a minority in a group dominated by fungivorous species. Distinction between (mostly harmless) fungal feeding *Aphelenchoides* species and high impact plant parasites such as *A. besseyi*, *A. fragariae*, *A. ritzemabosi*, and *A. subtenuis* is severely hampered by the scarcity of informative morphological characters, some of which are only observable in specific developmental stages. Poor description of a number of non-plant-parasitic *Aphelenchoides* species further complicates identification. Based on (nearly) full-length small subunit ribosomal DNA (SSU rDNA) sequences (≈1,700 bp), a phylogenetic tree was generated, and the four target species appeared as distinct, well-supported groups. Notably, this genus does not constitute a monophyletic group: *A. besseyi* and *A. ritzemabosi* cluster together and they are phylogenetically isolated from *A. fragariae*, *A. subtenuis*, and most other fungivorous species. A phylum-wide SSU rDNA framework was used to identify species-specific DNA motifs. For the molecular detection of four plant-parasitic *Aphelenchoides* species, polymerase chain reaction primers were developed with high, identical annealing temperatures (63°C). Within the molecular framework presented here, these primers can be used for the rapid screening of plant material and soil for the presence of one or multiple foliar nematode species.

Foliar nematodes are the common name of plant-parasitic members of the genus *Aphelenchoides.* Almost all foliar nematodes feed as ecto- or endoparasites on aboveground plant parts, and some species may cause substantial economic losses in both mono- and dicotyledonous crop species. *Aphelenchoides fragariae* is the causal agent of strawberry crimp, and apart from strawberry, this nematode is reported to attack over 100 plant species belonging to various families. "White-tip disease" refers to a characteristic whitening of leaf tips of rice as a result of parasitism by *A. besseyi.* However, rice is not its only host; it also reproduces on strawberry ("summer dwarf") and other plant species such as *Polianthes tuberosa* and *Capsicum annuum* (9). Another economically important member of the genus *Aphelenchoides*, the chrysanthemum foliar nematode *A. ritzemabosi* causes a typical brown discoloration of angular sections between large leaf veins, and chrysanthemum and various other members of the Asteraceae are susceptible hosts. An atypical example of a foliar nematode is the root parasite *A. subtenuis* as it has been shown to feed on roots of narcissus (18). In interaction with other bulbous hosts such as gladiolus, this nematode was found in corms and pseudostems (17). These foliar nematode species all feed on fungi as well (12). Apart from their possible survival on fungi in absence of a plant host, the management of some of the foliar nematodes is hindered by their desiccation tolerance.

Besides foliar nematodes, the genus *Aphelenchoides* harbors over 100 mainly mycetophagous species. Species such as *A. bicaudatus*, *A. composticola*, and *A. saprophilus* are frequently found in habitats that also harbor plant parasites. Only taxonomic experts are able to distinguish between (often) harmless mycetophagous species and true foliar nematodes on the basis of morphological characteristics. It is noted that the systematics of this genus are unstable; Hunt (12) indicated that "many nominal species are inadequately characterized for reliable recognition and the genus is in urgent need of a major revision" (12).

Many taxonomic classifications suggest a common ancestry between the families Aphelenchoididae (*Aphelenchoides* being the most speciose genus of the family) and Aphelenchidae (for most recent overview see De Ley et al. [5]). However, molecular phylogenetics based on full-length small subunit ribosomal DNA (SSU rDNA) sequences suggested a sister relationship between the Aphelenchoididae (*Aphelenchoides*, *Laimaphelenchus*, and *Schistonchus*) and the Parasitaphelenchidae (*Bursaphelenchus* spp.) (10,28), and both families appeared to be only distantly related to the Aphelenchidae (26). The genus *Aphelenchoides* was shown to be polyphyletic as the foliar nematode species *A. besseyi* and *A. ritzemabosi* together with most *Laimaphelenchus* species reside in a subclade robustly separated from the other members of the genus *Aphelenchoides* (26). So far the relatively conserved

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SSU rDNA gene seems to offer sufficient phylogenetic signal for the distinction between *Aphelenchoides* species (26).

Possibilities to explore the ribosomal DNA cistron for *Aphelenchoides* detection purposes have been investigated before. Ibrahim et al. (13) amplified the internal transcribed spacers (ITS) and the entire 5.8S gene from eight *Aphelenchoides* species and looked for restriction fragment length polymorphisms in these ≈1,000-bp fragments. The restriction enzyme *Alu*I was most successful for the differentiation of *Aphelenchoides* species, but failed in the case of *A. besseyi.* More recently, McCuiston et al. (16) concentrated on the ITS1 region (≈170 bp), and on the basis of a framework of 20 ITS1 sequences (including five *Aphelenchoides* species), *A. fragariae*-specific polymerase chain reaction (PCR) primers were developed. ITS regions are relatively variable as they are noncoding. This can be advantageous as these regions show high interspecies variation, but among nematodes the intraspecies variation can be considerable as well (16) (Fig. 1). Hence, for detection purposes thorough insight in ITS sequence variation among populations is a prerequisite. To avoid this and to broaden the applicability of assays, tests for the detection of plant-parasitic nematode species are more and more based on coding regions within the nuclear or mitochondrial genome.

Previous results (3,26,28) suggested that SSU rDNA contains sufficient phylogenetic signal for the identification of *Aphelenchoides* species. Here an alignment of 48 *Aphelenchoides* SSU rDNA sequences was used in combination with a larger phylum-wide framework comprising ≈2,500 nematode taxa. Apart from a phylogenetic analysis, quantitative PCR-based assays are presented for the detection of four foliar nematode species, namely *A. besseyi*, *A. fragariae*, *A. ritzemabosi*, and *A. subtenuis.* A single PCR program can be used for all assays, and by using phylum-wide unique DNA sequence signatures, these four assays are suitable for detection in both simple (plant material) and complex (soil or substrate) DNA backgrounds. It is noted that by far not all (>100) *Aphelenchoides* species described in literature were included in this paper. In part this is due to the unstable systematic status of this genus and to the very poor description of many species. Hence, specificity of the tests presented here can only be guaranteed within the molecular framework used in this study.

MATERIALS AND METHODS

Taxon sampling and acquisition of SSU rDNA sequences. Nematode collection and identification, followed by lysis, amplification, and sequencing of two overlapping SSU rDNA fragments were performed as described by Holterman et al. (10). Apart from newly generated sequences, all publicly available (nearly) full-length SSU rDNA data from Aphelenchoididae were included. A full list of rDNA sequences and the corresponding GenBank numbers are given in Figure 1.

Phylogenetic analyses. The SSU rDNA trees were constructed using Bayesian inference and the maximum likelihood (ML) method. For the Bayesian analysis, the alignment was divided into a stem and a loop partition according to the secondary structure. For both partitions, GTR + γ + I was used. Four independent runs were made with four Markov chains per run. The program was run for 2 million generations with a sample frequency of 200 generations. The first 60,000 generations were discarded as burn-in. The program Tracer v1.4 (23) was used to check the stabilization of likelihood and parameters.

ML analyses, the RAxML-HPC BlackBox program available at the CIPRES Science Gateway V. 3.1 (http://www.phylo.org/sub_sections/portal/), was used (25). The following parameters were chosen: estimated proportion of invariable sites (GTRGAMMA + I), finding best tree using ML search, bootstrapping halted automatically, and printed branch lengths.

Primer design and specificity testing. For the development of SSU rDNA-based detection assays for *A. besseyi*, *A. fragariae*, *A. ritzemabosi*, and *A. subtenuis*, we essentially followed the procedure as described in Neilson et al. (21). As a starting point, an alignment of approximately 2,500 full-length SSU rDNA sequences was used. The corresponding nematode taxa cover a substantial part of the nematode biodiversity in terrestrial and freshwater habitats in moderate climate zones till genus level. The Linux-based software package ARB (14) was used to identify unique rDNA sequence signatures for each of the four foliar nematode species. PCR primers (Table 1) were developed with a predicted optimal annealing temperature of 63°C. For each of the targeted *Aphelenchoides* species, the closest nontargets (Table 1) were identified by changing the mismatch settings in ARB as described in Neilson et al. (21).

To test the specificity of the primers, bacterial clones harboring a TOPO TA vector with an SSU rDNA fragment of the closest nontarget species were grown at 37°C in 2 ml of Luria-Bertani medium supplemented with ampicillin at 100 μg/ml. Plasmid extraction was performed using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI). DNA concentrations were measured with a NanoDrop spectrophotometer (Thermo Scientific) and adjusted to 10 ng/μl. For the qualitative nematode detection testing, 3 μl of 10^3× diluted sample was used.

For each quantitative PCR reaction, 3 μl of diluted template was mixed with species-specific primers (end concentrations for both primers 200 nM) and 12.5 μl of Absolute SYBR Green fluorescein mix (Thermo Fisher, Wilmington, DE) in a total reaction volume of 25 μl. Thermal cycling was performed on iQ5 (Bio-Rad, Hercules, CA) and the following PCR profile was used: 95°C for 15 min; followed by 60 cycles of 95°C for 30 s, 63°C (or gradient from 61 to 66°C) for 1 min, and 72°C for 30 s. For negative controls, template was replaced by an equal volume of Milli-Q water. Quantitative PCR output is expressed in C_t values (cycle number) at which the reporter dye emission intensity crosses a predetermined threshold. Here, the C_t threshold was set at 80.

Quantitative detection of *Aphelenchoides* species. In order to produce standards to relate C_t to number of target nematodes, quantitative series of microscopically identified nematodes were prepared: 1, 5, 10, 50, and 100 hand-picked individuals of each species were collected in a 0.2-ml tube containing 25 μl of Milli-Q water. For lysis, an equal volume of lysis buffer as described by Holterman et al. (10) was added. Lysis took place in a Thermomixer (Eppendorf, Hamburg, Germany) at 65°C and 750 rpm for 1 h, followed by 5 min incubation at 100°C. Three microliters of 50× diluted sample from each range was used for Q-PCR. As a positive control relevant plasmid DNA was used. See previous section for quantitative PCR conditions. Resulting data were used to define the slope and the *y* intercept of the regression line describing the linear relationship between log (number of nematodes) and the corresponding C_t values.

Aphelenchoides-free nematode suspensions used as nontarget backgrounds were extracted from freshly harvested narcissus bulbs with adhering soil (Flower Bulbs and Nursery Stock–PPO Lisse, The Netherlands). Suspensions were collected after incubation for 2 days in a mist chamber. Subsamples from suspension were checked microscopically for the absence of target *Aphelenchoides* species. Nontarget backgrounds (≈1,000 individuals for each of the background samples) included plant-parasitic and free-living nematodes.

RESULTS

SSU rDNA-based phylogenetic relationships among Aphelenchoididae. Phylogenetic analysis of 60 full-length SSU rDNA sequences from members of the family Aphelenchoididae (54 sequences) and its close relatives (6 sequences) gave rise to two major subclades (Fig. 1A and B). Notably, foliar nematodes do not constitute a monophyletic group as *A. besseyi* and *A. ritzema-*

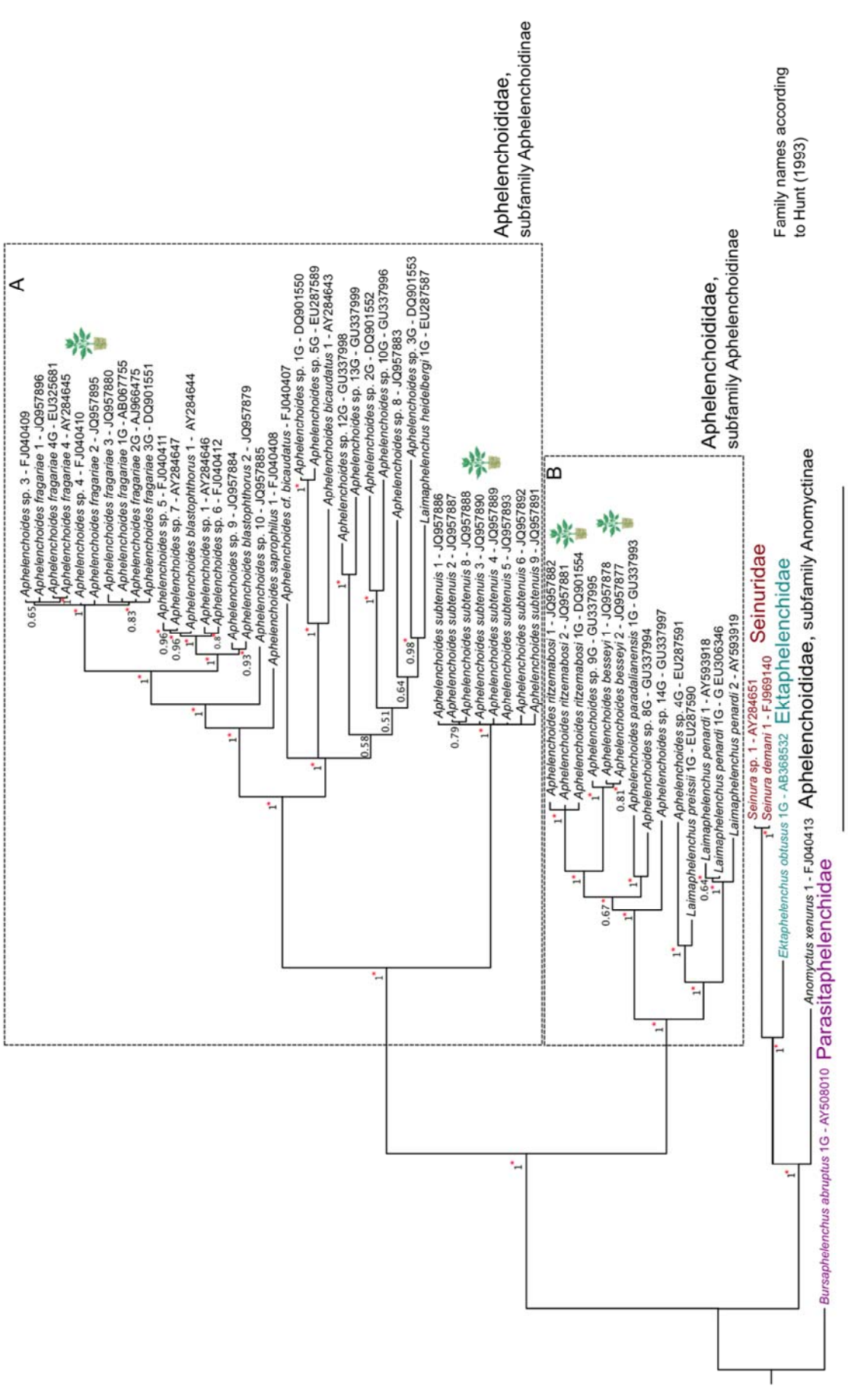

Fig. 1. A small subunit ribosomal DNA-based Bayesian tree of the Aphelenchoididae (Nematoda). Support values (posterior probabilities) are given next to the nodes; nodes marked with an asterisk (*) are highly supported by maximum likelihood (bootstrap > 90%). A 'G' behind a nematode name indicates that the sequence was acquired from GenBank. For the nomenclature of taxonomic groups, we essentially conformed to Hunt (12).

bosi, two major impact plant parasites, reside in a subclade (Fig. 1B), distinct from most other foliar nematode species (Fig. 1A). This division is supported by two independent phylogenetic methods, namely Bayesian inference and ML. Subclade B comprises a split between *A. besseyi*, *A. ritzemabosi*, and the recently described *A. paradalianensis* (4) on the one hand, and *Laimaphelenchus preissii* and *L. penardi* on the other. Subclade A is characterized by a sister relationship between *A. subtenuis* and representatives of other *Aphelenchoides* species (and *L. heidelbergi*). Other members of the superfamily Aphelenchoidoidea, namely Parasitaphelenchidae, Ektaphelenchidae, and Seinuridae, were used as outgroup. Noteworthy is the predatory nature of the *Seinura* spp., an unusual feeding type among the Aphelenchoidoidea; with rapid stylet thrusts it punctures the cuticle of its prey, after injection of pharyngeal gland secretions, the body content is ingested.

A remarkable characteristic of this *Aphelenchoides* phylogenetic tree is the inclusion of a relatively large number of sequences (number = 21) for which the corresponding nematode could not be identified to species level. The group consists of 11 accessions from GenBank ("G" behind the name), and 10 accessions generated in this research (for example "*Aphelenchoides* sp. 2"). This illustrates the paucity of informative characters for many species within this genus. In a few cases "*Aphelenchoides* sp." sequences reside within a well-supported monophyletic group with limited intraspecies variation. *Aphelenchoides* sp. 2, 3, and 4 are positioned within a cluster of *A. fragariae* sequences, and we assume that *Aphelenchoides* sp. 2, 3, and 4 in fact are representatives of this species. The same holds for *Aphelenchoides* sp. 9G, which is positioned in a small cluster of *A. besseyi* sequences. We assume that *Aphelenchoides* sp. 9G belongs to this species. A number of other "*Aphelenchoides* sp." sequences such as *Aphelenchoides* sp. 1G, 5G, and 12G (etc.) could not be assigned to any *Aphelenchoides* species.

Although it is realized that a part of the *Aphelenchoides* biodiversity is not represented in this tree—and even this statement cannot be substantiated further due to poor description of many nominal species (12)—some foliar nematode species appeared as well-supported distinct groups with limited intraspecies variation in this SSU rDNA-based phylogenetic analysis. This prompted us

TABLE 1. Primer combinations and close nontarget sequences used in detection assays for four *Aphelenchoides* species: *A. besseyi*, *A. fragariae*, *A. ritzemabosi*, and *A. subtenuis*[a]

Target species	Primer combinations, positions, and product lengths	Closest nontargets
A. subtenuis	1454: 5′-gtagttggattgtctcgcc	*Anomyctus xenurus* 1 (FJ040413)
	1458: 5′-atgacttgtcttgagcacg	*Aphelenchoides ritzemabosi* 1
		Aphelenchoides ritzemabosi 2
	Primer 1454 based on SNP at position 589	*Aphelenchoides* sp. 10
	Primer 1458 based on SNP at position 697	*Alaimus parvus* 1 (AY284738)
	Product length: 144 bp	*Diplopeltula* sp.1 (EF591329)
		Granonchulus sp. 1 (AY593953)
		Heterodera goettingiana 1 (EU669915)
		Plectonchus sp. 1 (AY593920)
		Pratylenchus neglectus 1 (EU669923)
		Tylolaimophorus typicus 2 (JQ957901)
A. besseyi	1770: 5′-gcgggattcgtggttc*t	*Acrobeloides cf thornei* 1 (JQ957903)
	1772: 5′-cgacatgccgaaacatgag	*Ascolaimus cf elongates* 2 (EF591330)
		Aphelenchoides ritzemabosi 2
	Primer 1454 based on SNP at position 1317	*Clavicaudoides trophurus* 1 (AY284772)
	Primer 1458 based on SNP at position 1608	*Deladenus durus* 1 (JQ957898)
	Product length: 325 bp	*Domorganus macronephritices* 2 (FJ969122)
		Ethmolaimus pratensis 1 (AY593942)
		Panagrobelus stammeri 1 (AF202153)
		Paracyatholaimus intermedius 3 (JQ957906)
		Rotylenchus uniformis (AY593882)
		Tripyla cf filicaudata 1 (AY284730)
A. fragariae	1469: 5′-cttatcgcacgactttacg	*Anomyctus xenurus* 1 (FJ040413)
	1472: 5′-tcaaagtaatccgcatccaat	*Anoplostoma* sp. 2 (FJ040492)
		Aphelenchoides sp. 8
	Without LNA	*Aphelenchoides cf bicaudatus* 1
	1844: 5′-ttatcgcacgactttacg	*Aphelenchoides saprophilus* 1
	1847: 5′-caaagtaatccgcatccaat	*Aphelenchoides* sp. 1
		Aphelenchoides sp. 5
	Primer 1454 based on SNP at position 223	*Pratylenchus penetrans* 1 (EU669925)
	Primer 1458 based on SNP at position 655	*Neopsilenchus magnidens* 1 (AY284585)
	Product length: 470 bp	*Symplocostoma* sp. 1 (FJ040502)
A. ritzemabosi	1496: 5′-cgctggtgggtttcga	*Anomyctus xenurus* 1 (FJ040413)
	1499: 5′-cccgctaagaaatgatcac*c	*Aphelenchoides composticola* 1
		Aphelenchoides sp. 8
	Primer 1454 based on SNP at position 986	*Aphelenchoides* sp. 10
	Primer 1458 based on SNP at position 1299	*Geomonhystera villosa* 1 (EF591334)
	Product length: 347 bp	*Globodera* sp. 4 (JQ957897)
		Laimaphelenchus penardi 1
		Ogma menzeli 1 (EU669919)
		Oncholaimidae sp. 1 (FJ040493)
		Paratylenchus straeleni 2 (AY284630)
		Rotylenchus sp. 1 (AY284608)
		Synonchiella sp. 1 (FJ040468)
		Steinernema glaseri 2 (FJ040422)

[a] All primer combinations were tested in the annealing temperature of 63°C. For the use of locked nucleic acids (LNAs), we essentially adhered to Nakitandwe et al. (20).

to investigate whether this gene could be used for the molecular identification of four plant-parasitic *Aphelenchoides* species.

The use of SSU rDNA sequence motifs for the (quantitative) detection of four plant-parasitic *Aphelenchoides* species. A phylum-wide database comprising approximately 2,500 (nearly) full-length SSU rDNA sequences was used as a starting point to develop detection assays for four plant-parasitic *Aphelenchoides* species, namely *A. besseyi*, *A. fragariae*, *A. ritzemabosi*, and *A. subtenuis*. The software package ARB (14) was used to identify species-characteristic sequence motifs and close nontarget taxa for these particular fragments within the SSU rDNA. It is worth mentioning that these close nontargets are not necessarily systematically related to the target species. For the detection of *A. besseyi*, the most optimal primer combination (1770/1772, Table 1) was designed on the basis of two typical SSU rDNA motifs. As shown in Table 1, a series of nematode species was identified within the SSU rDNA database with similar motifs. Apart from *A. ritzemabosi*, this list includes taxa that are taxonomically unrelated to *A. ritzemabosi* such an *Acrobeloides* (Cephalobidae), an *Ethmolaimus* (Ethmolaimidae), a *Panagrobelus* (Panagrolaimidae), and a *Rotylenchus* (Hoplolaimidae) species (three bacterivores and one plant parasite, respectively).

Similarly, PCR primer combinations were developed for the other three selected *Aphelenchoides* species (Table 1). To optimize specificity, PCR primers were designed to work optimally at a high annealing temperature (63°C). Moreover, all PCR primers have the same annealing temperature. As a consequence of this uniformity, these *Aphelenchoides* tests can be run in parallel in any combination.

In Figure 2A, the procedure for the testing of candidate *Aphelenchoides* species-specific primer combinations is illustrated. For detection of *A. subtenuis*, the annealing temperature characteristics were tested for primer combination 1454/1458 (Table 1) on plasmid DNA harboring the relevant SSU rDNA fragments. As can be seen in Figure 2A, the optimal temperature (the temperature giving the lowest C_t value) is indeed 63°C. Subsequently, the specificity of primer combination 1454/1458 was tested with all relevant target species (cloned SSU rDNA fragments from three *A. subtenuis* populations) and 11 close nontarget species. As shown in Figure 2B1, none of the close nontarget species gave a significant PCR signal after 60 cycles. In Figure 2B2, the negative first derivative of the melting curve (temperature-dependent dissociation of the amplicon) is shown. It is noted that this particular amplicon gives rise to an asymmetric melting pattern.

To translate C_t values into numbers of nematodes, a calibration curve was generated. To this end, a series of 1, 5, 10, 50, and 100 hand-picked *A. subtenuis* individuals was analyzed twice (technical replication). The results of the analyses with primer combination 1454/1458 is shown in Figure 2C. A robust linear relationship (R^2 = 0.99) was found between C_t values the log [number of target nematodes]. The relationship is characterized by a slope of –5,437 (slope is negative as more target DNA results in lower C_t values), and an intercept (C_t value corresponding with a single target nematode) of 30.65 cycles. Values on the *x* axis are negative as only a small fraction of the nematode lysate was used for analysis.

In essence, the same procedure was followed for *A. besseyi*, *A. fragariae*, and *A. ritzemabosi*. The selected primer combinations 1770/1772, 1469/1472 (1844/1847), and 1496/1499 (for primers see Table 1) were tested against 11, 10, and 13 close nontargets. The results are shown in Figure 3A1, B1, and C1. It was not always possible to design primers that give no detectable signal at all for all close nontargets. In case of the specificity test for *A. fragariae*, the gap between the latest target and the earliest nontarget signal is 29.3 cycles, which will be sufficient for most practical applications. In addition, the negative first derivatives of the melting curves are shown in Figure 3A2, B2, and C2.

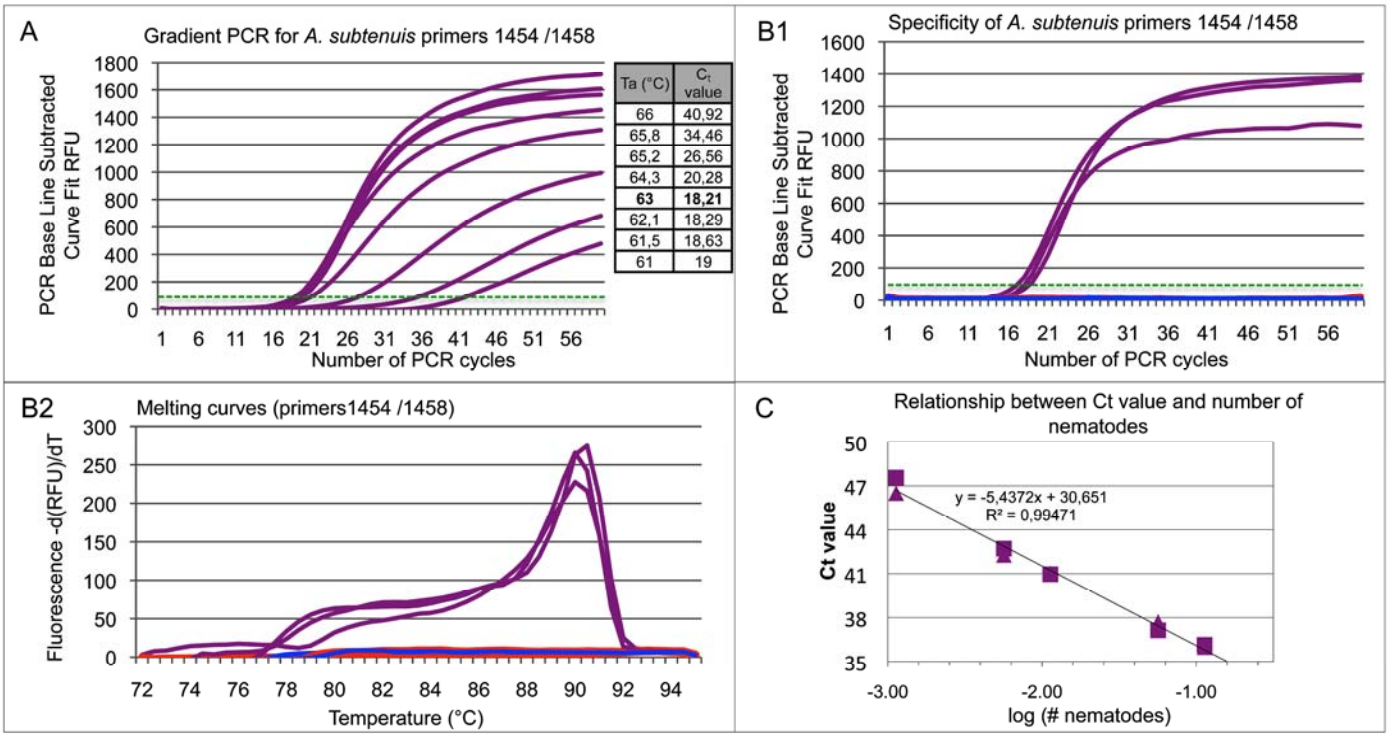

Fig. 2. Development of a foliar nematode-specific primer combination. *Aphelenchoides subtenuis* is used as an example to illustrate the procedure followed for primer development. **A,** All primers were designed to have optimal annealing temperature (T_a) of 63°C, with C_t values rapidly increasing above target temperature plus 1°C. **B1,** Specificity test of an *A. subtenuis* primer combination with plasmid DNAs from three target species, small subunit ribosomal DNA fragments from 11 potential false positives (Table 1) and a negative water control. **B2,** Graph showing the negative first derivatives of the melting curve of the amplicons shown in B1. **C,** The relationship between C_t values and numbers of nematodes for quantification of densities. Series of hand-picked individuals of *A. subtenuis* were lysed and amplified with primer combination 1454/1458. Two times five C_t values were used to define the slope and the *y* intercept of the regression line describing the linear relationship between log (number of nematodes) and the corresponding C_t values.

In several cases, locked nucleic acids (LNAs) were included to optimize primer specificity. For those who would prefer not to use LNAs, we tested whether conventional primers would give similar specificity. In the case of the *A. fragariae* primers 1469/1472, removal of a single nucleotide at the 5′ end resulted in primers (1844/1847) with the same optimal annealing temperature (63°C) and almost the same specificity (data not shown).

To further verify the specificity of the *Aphelenchoides* primers, target nematodes were added to *Aphelenchoides*-free nematode suspensions from soil. Twenty independent nematode suspensions that had been checked microscopically for the absence of *Aphelenchoides* spp. were used as backgrounds. For each experiment, five hand-picked target nematodes were added to the nematode suspension (≈1,000 individuals extracted narcissus bulbs with adhering soil), and after DNA extraction the newly developed SSU rDNA-based primers were used to check whether *A. fragariae* and *A. ritzemabosi* could be detected. In the *A. fragariae*-spiked samples, the target could be detected whereas *A. ritzemabosi* primers gave no signal at all (Table 2). In the *A. ritzemabosi*-spiked samples, the targets were always detected, but for three samples an *A. fragariae* signal was found as well (backgrounds 11, 13, and 14). The most plausible explanation for this result would be the presence of a small number of (probably one) *A. fragariae* individuals in the supposedly *Aphelenchoides*-free nematode suspension (with the detection assay presented here, a single *A. fragariae* gives a C_t value around 37 cycles [data not shown]).

DISCUSSION

Foliar nematodes, plant-parasitic representatives of the genus *Aphelenchoides*, are problematic in more than one aspect. Although the genus receives most attention because of a few notorious plant parasites, by far most species are—from a plant production point of view—harmless fungivores. The distinction between foliar nematodes and fungal feeding *Aphelenchoides* species on the basis of morphological characteristics is often problematic. This is not only due to the absence of a reliable identification key for this genus, but also to the (very) poor description of numerous nominal species (12). To assess whether the SSU rDNA gene could be used for the molecular recognition of foliar nematode species, a phylogenetic analysis was made. The resulting tree showed a major, well-supported split among the foliar nematodes; whereas *A. fragariae* and *A. subtenuis* reside in

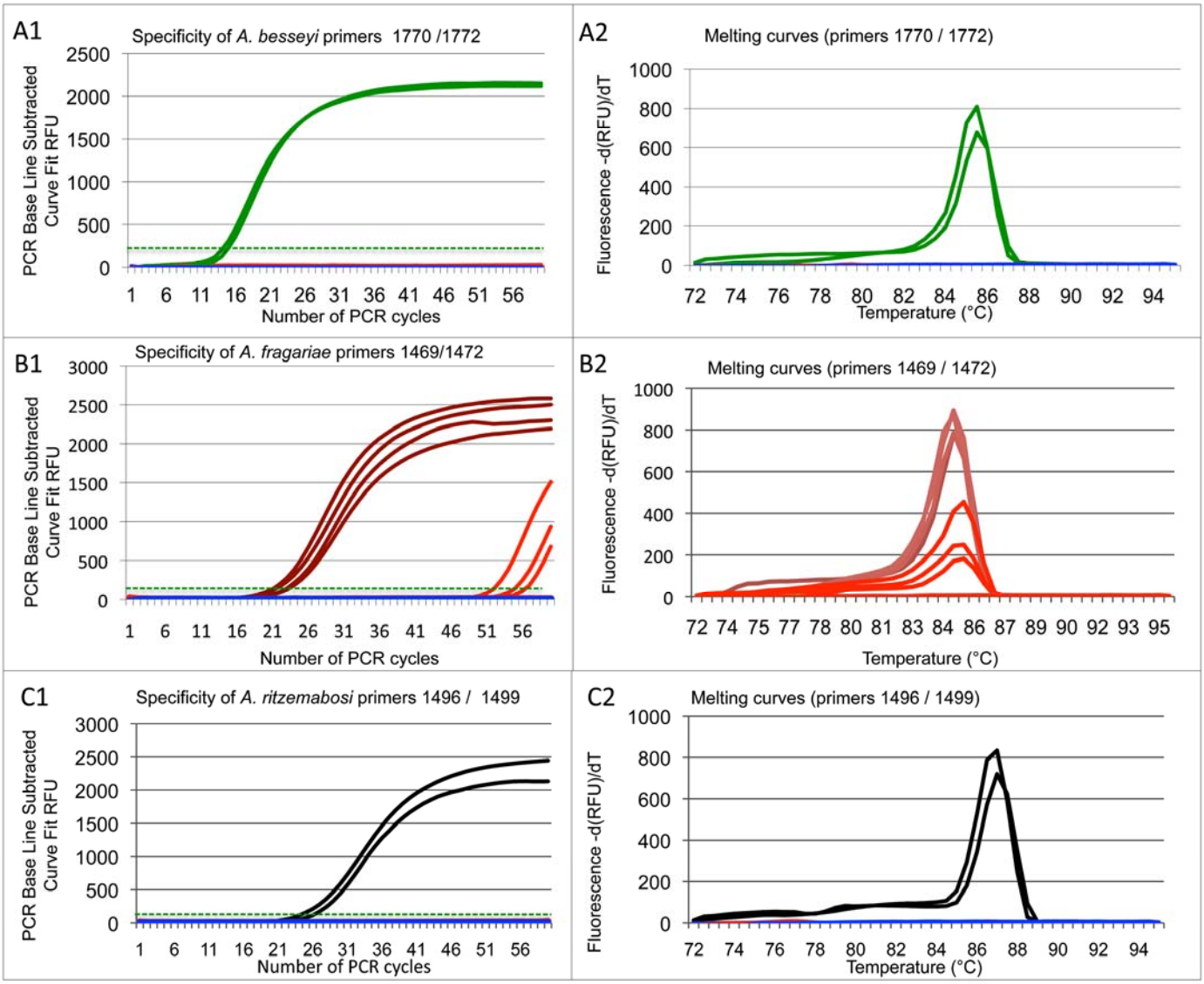

Fig. 3. Testing of polymerase chain reaction assays for the detection of three foliar nematode species. **A1,** Specificity test of an *Aphelenchoides besseyi* primer combination with plasmid DNAs from target and close nontarget species as given in Table 1, and **A2,** graph showing the negative first derivatives of the melting curve of the amplicons **B1 and B2,** *idem* for *A. fragariae* and **C1 and C2,** *idem* for *A. ritzemabosi.*

subclade A, two other foliar nematode species, *A. besseyi* and *A. ritzemabosi*, are positioned in subclade B (Fig. 1). In two previous analyses with fewer representatives from this particular group a similar split has been observed. Based on 13 and 18 *Aphelenchoides* sequences, respectively, Chizhov et al. (3) and Van Megen et al. (26) mentioned two separate clades uniting the plant-parasitic aphelenchids. *A. subtenuis* residing in a sister position vis-à-vis all other subclade A members, is known to infect roots, an atypical characteristic among the plant-parasitic *Aphelenchoides* species. The tail tip morphology seems to support the positioning of *A. subtenuis.* Except for *A. bicaudatus*, the *Aphelenchoides* species represented in subclade A all bear a single, poorly to well-developed mucro ("a sharp point at the end of, in this case, nematode's tail") devoid of any processes.

A remarkable feature of this phylogenetic tree is the positioning of two members of the predatory genus *Seinura* among fungivorous and plant-parasitic relatives. Upon puncturing the cuticle, the prey is immobilized by the injection of pharyngeal gland secretions, and the predigested body content is taken up. Possibly, *Seinura* is not the only predaceous nematode among the Aphelenchoidoidea as there is one report about *L. penardi* feeding on other nematodes (15).

Application of real-time PCR for the quantitative detection of plant-parasitic nematode species has rapidly gained popularity. Quantitative molecular assays have been developed for several high impact species such as *Pratylenchus penetrans* (24), *P. thornei* (27), the soybean cyst nematode *Heterodera glycines* (7), and the potato cyst nematodes *Globodera rostochiensis* (22) and *G. pallida* (19). All aforementioned assays are based on species-specific motifs in one of the two (noncoding) internal transcribed spacers (ITS1 or ITS2) located within the ribosomal DNA cistron. In a few occasions, authors selected coding regions; for the development of a quantitative assay for the pinewood nematode *Bursaphelenchus xylophilus*, Huang et al. (11) preferred the DNA topoisomerase I gene, whereas the SSU rDNA was shown to be suitable for the detection of the potato cyst nematode *G. rostochiensis* (10).

Ideally, molecular detection of plant-parasitic nematode species would be based on a DNA region showing minimal intraspecific and maximal interspecific variation. Hence, DNA regions that are used to study relationships among populations are less suitable for species detection. ITS regions were shown to be informative to study the relatedness between *G. pallida* populations (2,8), isolates of the burrowing nematode *Radopholus similis* (6), and populations of the Columbia lance nematode *Hoplolaimus* (*Basirolaimus*) *columbus* (1). Hence, the use of the ITS regions for species detection purposes requires a thorough inventory of the intraspecific variation. This information would allow for the distinction between population and species-specific polymorphisms, and robust detection assays could be based on the latter. For foliar nematode species detection, we preferred to use an SSU rDNA, a relatively conserved coding region within the ribosomal DNA cistron with for these four *Aphelenchoides* species low intraspecific variation. We showed that this gene harbors sufficient informative nucleotide positions for real-time PCR-based detection, and the assays presented here are technically straightforward and easily implementable (i.e., requires standard laboratory equipment) in agricultural research and service and inspection laboratories.

TABLE 2. To verify the specificity of the small subunit ribosomal DNA (SSU rDNA)-based primers, target nematodes were added to 20 independent *Aphelenchoides*-free nematode backgrounds (each ≈1,000 nematodes)[a]

Aphelenchoides species	Background sample ID	Number of target nematodes	SSU rDNA-based detection	
			C_t (*A. fragariae*)	C_t (*A. ritzemabosi*)
A. fragariae	6	5	34.00	N/A
	7		33.25	N/A
	8		33.09	N/A
	9		33.25	N/A
	10		32.57	N/A
	35		32.60	N/A
	36		32.72	N/A
	37		32.34	N/A
	38A		32.31	N/A
	38B		32.91	N/A
A. ritzemabosi	11	5	37.67	34.33
	12		N/A	36.53
	13		36.92	36.26
	14		37.22	35.78
	15		N/A	35.40
	16		N/A	34.44
	17		N/A	35.39
	18		N/A	34.75
	19		N/A	34.24
	20		N/A	34.62

[a] The background suspensions were extracted from narcissus bulbs with adhering soil. Individual nematode suspensions were checked microscopically for the absence of *Aphelenchoides* spp.

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