Journal of Nematology 44(3):284–290. 2012.

Development of PrimeTime-Real-Time PCR for Species Identification of Soybean Cyst Nematode (*Heterodera glycines* Ichinohe, 1952) in North Carolina

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Abstract: Soybean cyst nematode (SCN) is an obligate, sedentary parasite that is a major pathogen of soybean and accounts for an estimated 1 billion dollars in production losses annually in the United States of America. This paper describes the development of a real-time PCR method for rapid, sensitive, species-specific and accurate identification of SCN alone or on mixed populations with other nematodes in North Carolina. The 83-bp DNA fragment of PrimeTime-real-time PCR was designed based on a 477-bp-SCN-SCAR marker previously proved to be SCN-specific. A total of 44 populations including cyst forming nematodes (*Heterodera glycines*, *H. fici*, *H. schachtii*, *H. trifolii*, *Cactodera weissi*, *Globodera tabacum*, *Meloidodera floridensis* and other unidentified cyst nematodes) and non-cyst forming nematodes (*Ditylenchus dipsaci*, *Meloidogyne incognita* and *Xiphinema chambersi*) were tested in this study, all SCN populations are tested positive and non-SCN populations negative. This assay for the detection and identification has been successfully applied for testing a single SCN cyst, a 2nd-stage-SCN juvenile, a single SCN egg, up to ten SCN cysts, a 10-fold dilution of a single 2nd-stage-SCN juvenile and 20-fold dilution of one SCN cyst. The assay is not SCN-race specific. It gave an accurate positive result when SCN is mixed with other cyst species. Also, nematode universal primers/probes for real-time PCR amplification as a nematode endogenous control to detect the presence of 18S ribosomal RNA (rRNA) gene were employed in this assay, so that a SCN-negative sample can be tested to exclude false negative. This method will be very useful for a broad range of research programs as well as the regulatory response and management of SCN in North Carolina and other region of the southeastern U.S.A.

Key words: DNA sequencing, molecular diagnosis, SCAR marker.

Soybean cyst nematode (SCN) was first observed in 1915 in Japan by Hori (1916). This nematode was described as a new species, *Heterodera glycines* (Ichinohe, 1952). SCN has been reported in North America: Canada (Ontario), U.S.A. (Alabama, Arkansas, Delaware, Florida, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Louisiana, Maryland, Minnesota, Michigan, Mississippi, Missouri, Nebraska, New Jersey, North Carolina, North Dakota, Ohio, Oklahoma, Pennsylvania, South Carolina, South Dakota, Tennessee, Texas, Virginia and Wisconsin); South America: Argentina, Brazil, Chile, Colombia, Ecuador; Asia: China (Hebei, Hubei, Heilongjiang, Henan, Jiangsu, Liaoning), Indonesia (Java), Korean peninsula, Japan, Iran, Russia (Amur District in the Far East) and Africa: Egypt (Riggs, 1977; Noel, 1985; Eroshenko et al., 1990; Baldwin and Mundo-Ocampo, 1991; Liu et al., 1997; Evans and Rowe, 1998). In the U.S.A., SCN was first discovered in a bulb-growing area of southeastern North Carolina (Winstead et al., 1955) and subsequently has been reported throughout soybean producing areas in 28 states. SCN is the most damaging pest of soybeans in the U.S. A. Losses from SCN in the U.S.A. have been estimated at $1 billion annually (Wrather et al., 2001). Soybean, *Glycine max*, is the only major economic crop severely affected. Other highly susceptible species are beans (*Phaseolus*), bush clover (*Lespedeza*), vetch (*Vicia*) and ornamental lupines (*Lupinus*). SCN can be spread by anything that moves soil: wind, water, animals (especially birds) and machinery. It is widely considered as a regulatory nematode species and quarantines have been initiated to limit SCN to spread.

Identification of cyst nematodes to species based on morphology can be extremely challenging due to the large number of described cyst nematode species, highly variable morphology, limited number of specimens or presence of only juveniles in a given sample and lack of expertise. A number of DNA-based detection technologies have been developed to identify SCN with high sensitivity and specificity including PCR by species-specific primers (Subbotin et al., 2001, Ou et al., 2008), RFLP (Bekal et al., 1997; Szalanski et al., 1997; Subbotin et al., 2000; Zheng et al., 2000) and DNA sequencing (Szalanski et al., 1997; Zheng et al., 2000; Madani et al., 2007). Real-time polymerase chain reaction (PCR) is a laboratory technique that is used to simultaneously amplify and quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of one or more specific sequences in a DNA sample. The procedure follows the general principle of PCR. Its key feature is that the amplified DNA is detected as the reaction progresses in real time, a new approach compared to standard PCR, where the product of the reaction is detected at its end. Real-time PCR applications require the use of fluorescent reagents in order to measure the amount of product amplified. These fluorescent reagents can be either sequence-specific or non-sequence-specific. Common sequence-specific reagents include 5' nuclease probes (including PrimeTime and TaqMan® probes), molecular beacons, hybridization/FRET (Fluorescence Resonance Energy Transfer) probes, and Scorpions® probes. The most common non-sequence-specific reagents are intercalating dyes such as SYBR® Green I or EvaGreenR.

Received for publication January 13, 2011.
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This paper was edited by Kris Lambert.

Real-time PCR assays are simple, fast, sensitive, accurate, allow a high-throughput format, and enable the detection of a series of different agents in the same assay tube.

The Nematode Assay Lab in Agronomic Division of North Carolina Department of Agriculture and Consumer Services (NCDA&CS) is a high-throughput and publicly-operated nematode assay lab in the U.S.A. with about 30,000 soil samples analyzed per year. This lab has provided growers with valuable information on nematode identification, hazard level and management options. Although speciation for cyst nematodes is not normally required, it is needed to support Plant Protection Sector in NCDA&CS Plant Industry Division which is responsible for export certification issues and documentation of distribution of pest. If a nematode cyst is found in soil samples submitted in support of phytosanitary certification, the cyst must be further tested to determine if it is SCN, because Canada and some other countries prohibit the entry of plants with roots or underground pottery of plants grown in soil containing SCN. Additionally, to maintain an accurate known-distribution map for SCN, cysts in routine service samples submitted from counties not previously known to contain SCN, must be positively identified as SCN before the county is added to the national distribution map in the U.S.A. (http://www.plantpath.iastate.edu/dept/labs/tylka/node/21). To be economically feasible, PCR test for these purposes must be able to accurately assay multiple cysts or juveniles per reaction, be sensitive enough to detect one SCN specimen, mixed in with other species and yield a positive result for any races or populations of SCN with sufficient replicates.

In North Carolina, SCN was recorded in over half of the counties in the eastern part of the state (Fig. 1). Other cyst forming nematodes including *Heterodera trifolii*, *Cactodera weissi*, *Globodera tabacum*, *Meloidodera floridensis* and other unidentified cyst nematodes are encountered occasionally in our nematode assays. However, the most common stage of cyst nematode recovered from an assay is the second-stage juvenile, and the identification to species by morphology is impossible. Integrated DNA Technologies (IDT, Coralville, IA, USA) has introduced a new double-quenched probe, PrimeTme real-time PCR assays, which increases the accuracy and reliability of 5' nuclease real-time PCR experiments. While traditional probes have 20-30 bases between the dye and quencher, this novel proprietary probe design positions an internal ZEN quencher nine bases from the 5' fluorophore. This shortened distance, particularly when combined with the standard 3' quencher, significantly decreases background fluorescence and increases sensitivity. The chemical structure of the ZEN quencher stabilizes duplex formation that allows for its use in previously validated sequences. Ou et al. (2008) identified a single-randomly-amplified-polymorphic DNA marker, $OPA06_{477}$, species-specific to SCN. In their study, 37 populations of SCN and populations of *Heterodera avenae*, *H. schachtii*, *Globodera rostochiensis* and *G. pallida* were tested. The objective of the studies reported here was to develop SCN-specific primers and a PrimeTime probe based on the SCN-SCAR-marker genomic sequence (Ou et al., 2008) for a more reliable and sensitive real-time PCR assay regardless of the life stage or abundance of the nematode and even when found in a mixed population with other cyst nematodes.

Materials and Methods

A total of 44 populations including cyst-forming nematodes (*Heterodera glycines*, *H. fici*, *H. schachtii*, *H. trifolii*, *Cactodera weissi*, *Globodera tabacum*, *Meloidodera floridensis*

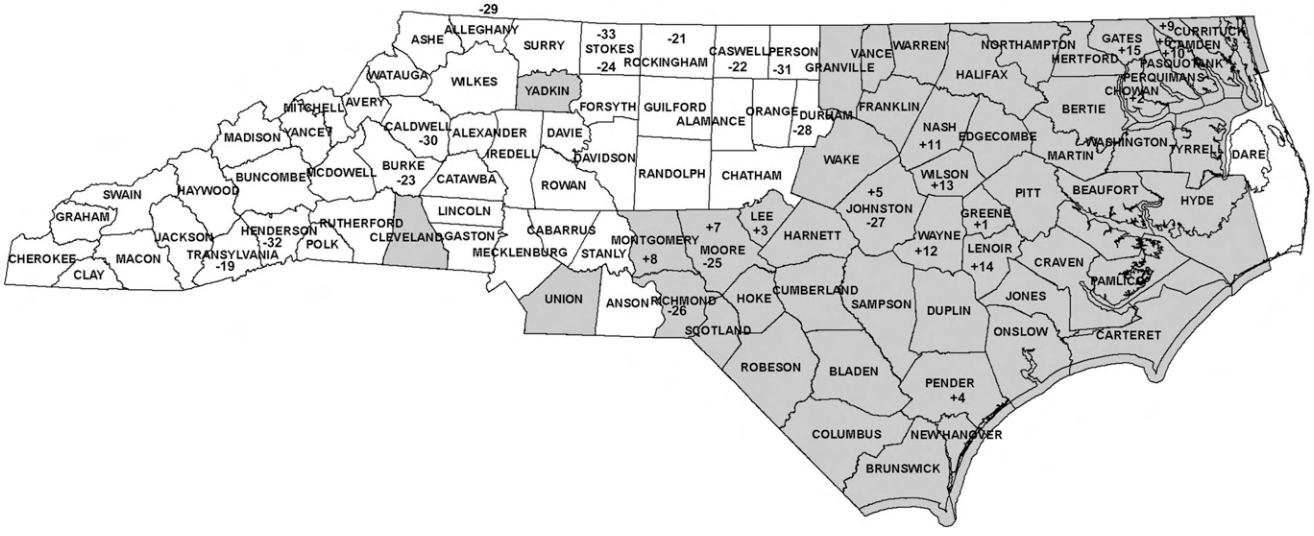

Fig. 1. Known-soybean-cyst nematode distribution in North Carolina (shaded area) and the sampling sites for this study. + = SCN-positive result and – = SCN-negative result in real-time PCR. Numbers corresponding to the population numbers in table 1. Population #3, 7 and 8 were verified by real-time PCR and these counties were added to the distribution map.

and other unidentified cyst nematodes) and non-cyst forming nematodes (*Ditylenchus dipsaci*, *Meloidogyne incognita* and *Xiphinema chambersi*) were tested in this study (Table 1). Among these samples, six populations were greenhouse cultures of SCN from the University of Arkansas designated as race 1, 2, 3, 4, 5 and 14 (Table1, Population #37-42). The sampling sites of cyst forming nematodes from North Carolina in 2007-2010 are mapped in Fig. 1. Some of the samples were from previous export-certification samples or from the North-Carolina-SCN-distribution-verification program. The species identification was based on morphology and confirmed by ribosomal DNA sequencing on 18S, ITS or 28S D2/D3 in a separate project (Table 1). Nematodes were extracted from the soil by a combination of sieving-decanting in an elutriator (Byrd et al., 1976) and the sucrose-centrifugal-flotation method (Jenkins, 1964).

One to ten SCN cysts, 2nd-stage-SCN juveniles or SCN eggs were put on a microscope slide, squashed with a pipette tip under light microscopy and collected into 50µl of AE buffer (10mM Tris-Cl, 0.5mM EDTA; pH9.0) in a 0.5 ml microtube. The samples were stored at -20°C until used. A mixture of ten non-SCN populations (Table 1) each with 1 µl was used as a DNA template in combination with 1 µl of water, 08-00061 or SCN+ to test mixed samples. Three separate samples (Table 1, population #12, 13 and 15) with 10-fold dilution of a 2nd-stage-SCN juvenile and four separate samples (Table 1, population #9, 11, 15 and 16) with 20-fold dilution of a SCN cyst were tested to determine the sensitivity of the assay.

Real time PCR SciTool by IDT (http://www.idtdna.com/Scitools/Applications/RealTimePCR/) was used for primer and probe design. Primers for real time PCR amplification targeting a 83-bp section were forward primer SCNrtF (5' AAATTCCAGGCCGCTATCTC 3': GC content: 50.0%, melting temperature: 54.9°C) and reverse primer SCNrtR (5' CGTGGACTGAACTGGA CAAAG 3': GC content: 52.4%, melting temperature: 55.9°C). Double-quenched probe was SCNrtP (5'/56-FAM/TGGGCTGGG/ZEN/TGCTTCTAGAACTTTT/ 3IABkFQ/3': GC content: 48.0%, melting temperature: 60.5°C). This design is based on 477-bp-SCN-SCAR-marker genomic sequence including forward primer SCNFI and reverse primer SCNRI (Ou et al., 2008, GenBank accession number DQ354374) (Fig. 2). Besides the SCN-specific primer/probe, nematode universal primers for real-time PCR amplification as a nematode endogenous control to detect the presence of 18S ribosomal RNA (rRNA) gene were forward primer Ne18Sf (5' ATTGACGGAAGGGCACCAC 3': GC content: 57.9%, melting temperature: 58.0°C) and reverse primer Ne18Sr (5' GAACGGCCATGCACCAC 3': GC content: 64.7%, melting temperature: 57.4°C). The probe was Ne18Sp (5'/5-TET/ TGCGGCTTAATTTGACTCAACACGGG/ 3IABkFQ/3': GC content: 50.0%, melting temperature: 61.6°C). The sequence design of the universal probe and primers is based on the conserved sites on a multiple alignment from a diverse group of nematodes from genBank including *Anguina tritici* (genBank accession number AY593913), *Ascaris suum* (U94367), *Aphelenchoides besseyi* (AY508035), *A. bicaudatus* (AY284643), *A. blastophtorus* (AY284644), *A. fragariae* (AY284645), *A. fujianensis* (FJ520227), *A. ritzemabosi* (DQ901554), *A. saprophilus* (FJ040408), *A. stammeri* (AB368535), *A. varicaudatus* (HQ283351), *A.* sp. (AY284646, EU287589, EU287591, FJ040411, FJ040412, GU337993-GU337999), *Aphelenchus avenae* (AY284640), *Bursaphelenchus abruptus* (AY508010), *B. doui* (AB299223, FJ501985), *B. sexdentati* (AY508032), *B. xylophilus* (AY508034, FJ520227), *Caenorhabditis elegans* (AY268117), *Cephalobus oryzae* (AF034390), *Cryptaphelenchus* sp. (EU287588), *Ditylenchus dispasi* (AY589297), *Ektaphelenchus obtusus* (AB368532), *Globodera rostochiensis* (EU855120), *Howardula aoronymphium* (AY589304), *H.* sp. (AF519232), *Laimaphelenchus heidelbergi* (EU287587), *L. penardi* (AY593918, EU306346), *L. preissii* (EU287590), *Longidorus macrosoma* (AY580055), *Meloidogyne incognita* (AY284621, U81578), *Myolaimus* sp. (U81585), *Paraphelenchus* sp. (AY284642), *Pristionchus entomophagus* (FJ040441), *Ruehmaphelenchus* sp. (AB368534), *Schistonchus aureus* (DQ912922), *S. centerae* (DQ912923), *S. guangzhouensis* (DQ912924), *S.* sp. ex *Ficus wightiana* (HM151003), *Seinura demani* (FJ969140), *Seinura* sp. (AY284651), *Tylaphelenchus jiaae* (HQ283350) and *Xiphinema bakeri* (AY283173). For *Globodera rostochiensis* (EU855120), these primers target a 142-bp fragment of 18S rRNA of this species. All primers and probes were synthesized by IDT. The probes were labeled with different dyes to allow for duplex real-time PCR.

The 25-µl PCR contained 10-µl Cepheid OmniMix™ HS master mix (Takara Bio Inc., Otsu, Shiga, Japan) (One bead reconstituted with 30 µl water), 2.5 µl each of 2 µM forward primer, reverse primer and probe, 6.5 µl water and 1 µl of DNA template. The 2-step thermal cycling program was as follows: denaturation at 95°C for 2 min, followed by 50 cycles of denaturation at 95°C for 10 s, annealing and extension at 62°C for 45 s in Cepheid SmartCycler II system (Sunnyvale, CA, USA). Water was used as negative control in all experiments. Duplex real-time PCR was performed with two sets of primers and probes in one reaction tube.

Results

Results of all real-time PCR assays are shown in Table 1. All assays were 100% specific and accurate for detection of SCN with FAM threshold cycle (Ct value) from 25-42 (Table 1 and one example test in Fig. 3). Some soybean cyst nematode samples and all other non-SCN samples were tested positive for the presence of nematode 18S rRNA with TET Ct from 23-33 (Table 1). All six SCN samples designated as race 1, 2, 3, 4, 5 and 14 reared in

TABLE 1. Nematode species and populations used in this study, genes sequenced by DNA sequencing and real-time PCR result shown on Ct value (Threshold cycle: The intersection between an amplification curve and a threshold line).

Population #	Lab No. or code	Nematode material	Host	Locality	18S	ITS	28S	FAM Ct	TET Ct [d]
Heterodera glycines									
1	07-27835[a]	1 cyst	Strawberry	Pikeville, Greene, NC				24.99	
2	08-00061	1 cyst, mixed with 10 non-SCN samples	Soybean	Edenton, Chowan, NC				24.75, 27.33	23.60
3	08-00240[b]	1 cyst	Soybean	Sanford, Lee, NC				33.07	33.15
4	08-00705[a]	1 cyst	Strawberry	Burgaw, Pender, NC				37.45	
5	08-18975	2 cyst	Cotton	Princeton, Johnston, NC				26.62	
6	08-22550	1 cyst	Soybean	Elizabeth City, Pasquotank, NC				36.98	
7	09-05730[b]	1 cyst	Soybean	Moore, NC				33.46	
8	09-24067[b]	3 cysts	Soybean	Troy, Montgomery, NC				33.24	27.53
9	10-00363	1 cyst, 1/20 of 1 cyst	Soybean	South Mills, Camden, NC				29.24, 35.11	28.34
10	10-00447	1 cyst	Soybean	Elizabeth City, Pasquotank, NC				27.05	
11	10-18187	1 cyst, 1/20 of 1 cyst	Soybean	Nashville, Nash, NC		+		34.24, 31.48	30.25
12	10-18445	10 cysts, 1 j, 1 egg, 1/10 of 1 j	Cotton	Princeton, Wayne, NC				27.78, 34.68, 37.12, 41.64	
13	10-25196	8 cysts, 1 j, 1 egg, 1/10 of 1 j	Soybean	Spring Hope, Wilson, NC				27.04, 34.21, 34.54, 37.50	
14	10-25578	10 cysts, 1 j, 1 egg	Soybean	Kinston, Lenoir, NC				28.28, 39.25, 38.71	
15	10-27277	1 cyst, 1 j, 1 egg, 1/20 of 1 cyst, 1/10 of 1 j	Soybean	Gates, Gates, NC				34.17, 34.74, 37.51, 36.50, 35.31	
16	SCN+	1 cyst, 1/20 of 1 cyst, mixed with 10 non-SCN samples	Soybean	NC		+	+	24.77, 26.44, 26.79	26.45
37	SCN Race 1	1 cyst	Soybean	Greenhouse culture, Fayetteville, AR				31.84	27.09
38	SCN Race 2	1 cyst	Soybean	Greenhouse culture, Fayetteville, AR				30.19	23.10
39	SCN Race 3	1 cyst	Soybean	Greenhouse culture, Fayetteville, AR				28.10	
40	SCN Race 4	1 cyst	Soybean	Greenhouse culture, Fayetteville, AR				31.12	
41	SCN Race 5	1 cyst	Soybean	Greenhouse culture, Fayetteville, AR				27.21	
42	SCN Race 14	1 cyst	Soybean	Greenhouse culture, Fayetteville, AR				28.59	
43	SCN PT	1 cyst	Soybean	Pine Tree Research Station, Colt, AR				32.20	24.89
44	SCN Mariana	1 cyst	Soybean	Lon Mann Cotton Research Station, Mariana, AR				29.32	
Heterodera fici									
17	Hf[b]	1 cyst	*Ficus elastica*	Newport News, VA		+	+	0	31.10
Heterodera schachtii									
18	Hs[c]	1 cyst	Cabbage	Greenhouse culture, Raleigh, NC		+	+	0	24.50
Heterodera trifolii									
19	08-26199[c]	6 cysts	Daylily	Brevard, Transylvania, NC	+	+		0	30.79
20	Ht[c]	1 cyst	*Trifolium pretense*	Ellicott City, MD		+	+	0	24.15

(*Continued*)

TABLE 1. Continued.

Population #	Lab No. or code	Nematode material	Host	Locality	18S	ITS	28S	FAM Ct	TET Ct [d]
Cactodera weissi									
21	07-27182[c]	1 cyst	Tobacco	Wentworth, Rockingham, NC	+	+		0	24.78
22	08-11074[c]	1 cyst	Tobacco	Yanceyville, Caswell, NC		+		0	24.86
23	09-01783[a]	1 cyst	Shaded tree	Morganton, Burke, NC		+		0	29.34
Globodera tabacum									
24	08-04211[c]	5 cyst	Tobacco	Westfield, Stokes, NC	+			0	26.10
Meloidodera floridensis									
25	08-10238[c]	4 j	Azalea	Pinehurst, Moore, NC	+	+		0	27.76
26	09-00151[c]	4 j	Grass	Rockingham, Richmond, NC	+	+		0	29.93
27	09-20530[c]	1 j	Azalea	Smithfield, Johnston, NC				0	30.15
Unidentified cyst nematodes									
28	07-27462[a]	1 cyst	Daylily	Durham, Durham, NC				0	32.71
29	08-24870[a]	1 j	Boxwood	Sparta, Alleghany, NC				0	28.32
30	09-24040[a]	1 cyst	Ornamental	Lenoir, Caldwell, NC				0	28.70
31	10-02895	1 cyst	Tobacco	Timberlake, Person, NC				0	26.35
32	10-24518	1 cyst	Tomato	Hendersonville, Henderson, NC				0	24.93
33	10-27083	1 cyst	Tobacco	Danbury, Stokes, NC				0	30.63
Ditylenchus dipsaci									
34	Dd	1 j	Garlic	Ottawa, Canada		+		0	30.84
Meloidogyne incognita									
35	Mi	1 female	Tomato	Raleigh, Wake, NC				0	32.09
Xiphinema chambersi									
36	10-23191[c]	1 female	Daylily	Lumberton, Robeson, NC				0	28.50

[a] Recovered previously from export-certification samples.
[b] SCN-distribution-verification samples.
[c] DNA sample used for a mixed-non-SCN DNA sample.
[d] Blank result means no real-time PCR performed on a SCN-positive sample.

greenhouse from the University of Arkansas were tested SCN-positive. No fluorescent signals were obtained for other cyst-forming nematodes or *Ditylenchus dipsaci*, *Meloidogyne incognita* and *Xiphinema chambersi*. A mixture of ten non-SCN samples were tested as negative, but positive when a SCN was spiked in the sample (Table 1, population #2 and 16). This assay works the same with sample comprised of a single cyst or up to ten cysts (Table 1). Similar results were obtained for a single 2nd-stage juvenile or a single egg, but the Ct value is higher than that of a cyst sample. Both 10-fold dilution of a single-2nd-stage-SCN juvenile and 20-fold dilution of a single-SCN cyst yielded reliable results similar to non-diluted samples with little variation on the Ct value. Duplex real-time PCR was not successful for all samples. Only TET dye can be detected for the presence of nematode 18S ribosomal RNA (rRNA) gene, but not the FAM dye for the presence of SCAR DNA fragment in SCN. However, these assays were all successful when the reactions were performed separately.

DISCUSSION

Ou et al. (2008) identified a single randomly amplified polymorphic DNA marker, $OPA06_{477}$, species-specific to SCN and designed a pair of species-specific primers to identify SCN by PCR. These primers would not amplify *Heterodera avenae*, *H. schachtii*, *Globodera rostochiensis* and *G. pallida* in their tests. Based on this result, we designed new primers for real time PCR amplification targeting a 83-bp section of 477-bp $OPA06_{477}$. Blastn search of this DNA fragment against nucleotide collection database yielded no other match, except for itself (DQ354374). This fragment had exact match with

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GGACCCTGACCAAAAAGTTTCCGCTGGTAGGATTTTAAGTTGAAGTGTTGGCTAAAAGTTAAAAAAAATT
SCNFI forward primer                                              SCNrtF
CCAGGCCGCTATCTCTAACCCCC***TGGGCTGGGTGCTTCTAGAACTTTT***CAGTACCAACTTTGTCCAGTTC
                        Probe                                SCNrtR
AGTCCACGTTGAATAACTCATTAGGCTTTGACCTGAGCGCAGGGTGGCCAGCAGACTTATTGTAGAGCTT
TAAAAGCCCGATTTTTCCAAATTTTAAAATTTCGAAAATTTTCAAAATTTTTCGAGCTAGGCCTAAAAAA
CTGTTTTCGGGTTTTTGAATTTTAGGATTTTTTAACGAATTTTCCCAGAGAAACGGAGACCACTTCCAAA
ACATAGTTTTTCGAGCTCTATCGATTGGTAATTTTTTCGGAAAATTTTCGATTTTTTTTCGGAAATAGAA
AAATTTTTTGGCCTCAGCGGGACATTTGAGTCCCGGGGCCCATAACTCGTCAGGGTCC
                                  SCNRI reverse primer
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FIG. 2. Forward primer (SCNrtF), probe and reverse primer (SCNrtR) design based on 477-bp-SCN-SCAR-marker genomic sequence including forward primer SCNFI and reverse primer SCNRI (Ou et al., 2008, GenBank accession number DQ354374).

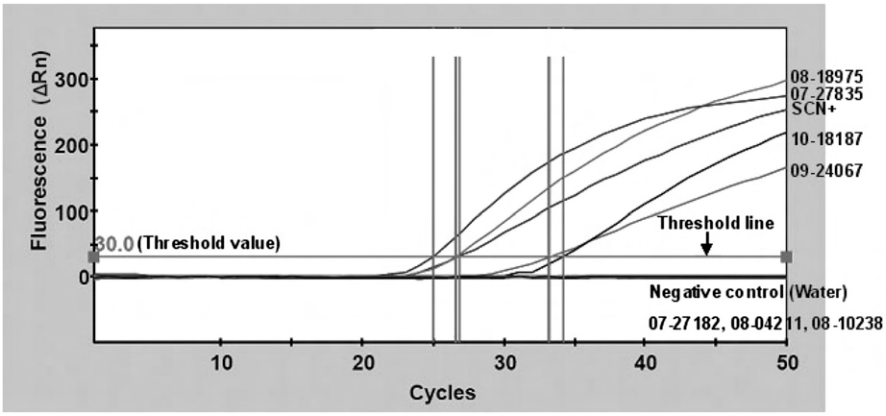

FIG. 3. One example assay of real-time PCR results from Cepheid SmartCycler II system. Curve designations are sample lab No. or code in Table 1. Samples are SCN-positive above threshold line and SCN-negative below threshold line.

three sequences of *Heterodera glycines* strain OP25 (ABLA01010051, ABLA01013933, ABLA01035857) from whole genome shotgun sequence database and 91-99% maximum identity with 16 sequences of *Heterodera glycines* strain OP25. Besides, there is no significant match with our designed primers and probe in genBank which ensures the specificity of this assay. As shown in this study, all assays were highly specific for detection of SCN regardless of the races, but not to any other cyst-forming nematodes including *Heterodera fici*, *H. schachtii*, *H. trifolii*, *Cactodera weissi*, *Globodera tabacum*, *Meloidodera floridensis*, some unidentified cyst nematodes or non-cyst-forming nematodes including *Ditylenchus dipsaci*, *Meloidogyne incognita* and *Xiphinema chambersi*. This technique is rapid (<3 h) and therefore of great help in processing samples required for certain phytosanitory certificates or SCN-distribution verification in North Carolina.

Real-time PCR yields precise and accurate results with the least amount of starting material, theoretically down to a single copy of a transcript. In this study, we used 1 μl nematode template out of 50 μl in AE buffer to run a 25-μl PCR reaction and tested 1-10 cysts, single 2nd-stage juvenile, single egg, 10-fold dilution of a single 2nd-stage juvenile and 20-fold dilution of a single cyst. All of the results revealed significant fluorescent signals (Ct value from 25 to 42). This sensitivity and accuracy made real-time PCR an ideal method for species diagnosis. In a real world application, a single cyst or 2nd-stage juvenile would be available in the assay which has sufficient material to replicate and thus there is no need to further dilute the sample.

In year 2007, 2008 and 2009, three cyst nematode samples (Table 1, population #3, 7 and 8 and Fig. 1) were morphologically identified as SCN. These identification results were confirmed by real-time PCR in this study. These samples were collected from soybean fields adjacent to the known-SCN-positive counties in North Carolina indicating the spread of SCN within this state. These findings have been officially added to SCN-distribution map in the U.S.A.

Soil samples for export-phytosanitary certificates in North Carolina are usually from ornamental plants growing in containers with compost mix, but some are from plants in field soil. Occasionally, some cyst nematodes were detected from these samples in the stage of either cyst females or second-stage juveniles. In this study, two strawberry samples (Table 1, population #1 and 4) were tested positive and four ornamental samples (Table 1, population #23 and 28-30) were tested negative. Although SCN has a very narrow host range, other plants can still host this species and have the risk to carry this nematode to a new location. This real-time PCR assay is ideal for this purpose because conclusive results can be obtained in a few hours and short turn-around time is guaranteed.

In the state of North Carolina, several other cyst nematodes are present besides SCN, including *Heterodera trifolii*, *Globodera tabacum*, *Cactodera weissii* and *Meloidodera floridensis*. If a sample of any of these tests is negative for SCN, we have already developed a PCR and DNA sequencing protocol on ribosomal DNA 18S, 28S D2/D3 and ITS. These sequences are available for comparison for some of the samples (Table 1). Therefore, PCR and DNA sequencing can be employed when needed for any SCN-negative samples to further identify the nematode in the sample. Besides, nematode universal primers/probes for real-time PCR amplification as a nematode endogenous control to detect the presence of 18S ribosomal RNA (rRNA) gene were employed in this assay, so that a SCN-negative sample can be tested to exclude false negative. Duplex real-time PCR was performed in this study but not successfully, only universal 18S rRNA fragment can be detected. rRNA is a house-keeping gene with numerous copies in one nematode, which could dominant in PCR amplification over SCAR DNA in a SCN sample.

The morphology of cyst can be very variable in size, color and shape within one species or one population, and it is possible that a field has multiple species which makes species identification more challenging. In this

study, a sample consisting of a mixture of ten non-SCN DNAs tested SCN-negative, but it was tested SCN-positive when spiked with a SCN sample (Table 1, population #2 and 16). Thus, multiple unknown cysts can be combined in one tube as a single assay unit to detect the presence of SCN. This can significantly reduce the number of assays required to test all cysts found in a sample and therefore reduce the cost and time.

The approach presented in this paper is the first to use PrimeTime-real-time PCR to identify SCN. It is simple, fast, sensitive, reliable and accurate regardless of the life stage, abundance of the nematode, SCN alone or mixed with other species. The sample can be diluted for numerous replicates and stored in freezer for future confirmation and reference. The major shortfall is the high cost of the instruments, supplies and reagents used in real-time PCR with the minimum cost of supplies and reagents about $3.00 per assay.

Acknowledgments. We thank NCDA&CS Plant Industry Division for the use of its real-time PCR equipments, supplies and partial financial support; Dr. Robin M. Giblin-Davis at the Fort Lauderdale Research and Education Center, University of Florida for critical review of the manuscript; Dr. Robert T. Robbins from Dept. Plant Pathology, University of Arkansas, Dr. Zafar Handoo from USDA-ARS Nematology Laboratory, Dr. Qing Yu from Invertebrate Biodiversity, Agriculture and Agri-Food Canada, and Dr. Rick Davis from the Dept. Plant Pathology, North Carolina State University, Mrs. Kathy Imbriani from NCDA&CS Plant Industry Division for providing nematode materials; and Dr. Ken Ahlstrom from NCDA&CS Plant Industry Division for making nematode sampling and SCN distribution map of NC. Mention of a trade name or commercial product in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the North Carolina Department of Agriculture & Consummer Services.

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