**Molecular Characterization and Identification of the New Root-lesion Nematode Species on Soybean in North Dakota**

TECHNICAL REPORT

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Plant-parasitic nematodes are a group of pests that are becoming increasingly important as a crop production limiting factor for growers world-wide. Among these pests, soybean cyst nematode (SCN), root-lesion nematode, lance nematode and root knot nematode are considered highly aggressive to soybeans and can be devastating to soybean production if left unchecked. Root-lesion nematodes are vermiform (motile), endo-parasitic organisms that are widely distributed throughout the world and have a wide host range. In North Dakota, during 2015 and 2016, a soil survey of soybean fields was conducted to determine the prevalence and distribution of plant-parasitic nematode in the fields. During this survey six soil samples were collected from a soybean field in Richland County. After extracting nematodes from these samples, it was evident that root-lesion nematodes were present in all of the samples with population densities ranging from 125 to 2,000 nematodes per kg of soil. Morphological measurement of adult males and females as well as DNA sequencing of two genomic regions revealed that this nematode differs from other known species of root-lesion nematodes in both morphology and DNA sequences, allowing us to conclude that this is a new species of root-lesion nematode. This new North Dakota species has never been reported in any literature prior to 2017. Thus, an efficient and sensitive detection system is necessary to identify this nematode species; however, distinction between root-lesion nematode species is difficult and time consuming based on morphology using traditional microscopic methods. Molecular technology provides a powerful platform for rapid and sensitive detection of root-lesion nematodes at species level. Therefore, the objective of this research was to develop a new real-time PCR assay for specific detection and identification of the new root-lesion nematode species found in North Dakota, characterize this species and determine its evolutionary relationship with other species of root-lesion nematodes.

To achieve these objectives soil samples were collected from the field where the new root-lesion nematode species was first detected. Nematodes were then extracted from these soil samples using sieving, decanting and sucrose centrifugation methods. Root-lesion nematodes were collected from the soil extracts based on their morphological features. DNA was extracted from these root-lesion nematodes using the Proteinase K method, which involves chopping the nematodes in a 10 µl nematode suspension of double distilled water (ddH2O) then adding it to a centrifuge tube containing 2 µl of 10x PCR buffer, 2 µl Proteinase K (600 µg/ml), and 6 µl of ddH2O. The tubes were then stored at -20°C for 30 minutes and then incubated at 65°C for 1 hour followed by 95°C for 10 minutes.

The DNA samples from the new root-lesion nematode were then prepared for sequencing by amplifying the ITS (internal transcribed spacer) region of the ribosomal DNA using the universal primer set rDNA-2v /rDNA1 via conventional PCR. The samples were then purified and sent for DNA sequencing by GenScript (GenScript, Piscataway, NJ). The DNA sequence data were edited with EditSeq module of DNASTAR software package to remove vector sequences and messy regions. Clustal X software was then used to determine consensus sequence by aligning edited nucleotide sequences from three individuals of the population.

The ITS sequences of 20 other root-lesion nematodes were also retrieved from GenBank to compare and identify the unique regions in the ITS sequence of the new root-lesion nematode species. The DNA sequences were then aligned and a primer set, ITS1F (forward, 5’-TGTGTGCGAATGTTCCTG-3’) and ITS1R (reverse, 5’- CGTATGTTTTATATGGGGACTC-3’), specific to the region of ITS rDNA of the target root-lesion nematode was then designed. The specificity of the primer set was examined in-silico using the Blast-search function of the NCBI GenBank. Results of the in-silico analysis revealed no perfect match with other plant-parasitic nematode sequences in GenBank. The primers were then synthesized by Eurofin MWG operon LLC (Huntzville, AL).

The PCR amplification performance of the primer set at different annealing temperatures (55, 56, 57, 58, 59 and 60°C) was evaluated. Although the ITS1F/ITS1R primer set was specifically able to amplify the target root-lesion nematode DNA at different annealing temperatures tested, the best amplification occurred at 58°C. The optimal amplification condition for conventional PCR was then established as initial denaturation for 3 min at 94°C followed by 35 cycles of denaturing at 94°C for 45s, annealing for 60s at 58°C, extension for 60s at 72°C and a final extension for 10 min at 72°C. The optimal amplification condition for real-time PCR was established as incubation for 4 min at 95°C and 35 cycles of 95°C for 10s and 58°C for 30s.

The specificity of this primer set was then tested using conventional PCR and real-time quantitative PCR. DNA from multiple isolates of the target species as well as multiple isolates of five other confirmed species of root-lesion nematodes (*Pratylenchus scribneri*, *P. neglectus*, *P. thornei*, *P. penetrans*, and *Pratylenchus* sp. ND-2016) were used to evaluate the primer specificity. Seven other genera of plant-parasitic nematodes (*Paratylenchus* sp., *Paratrichodorus* sp., *Tylenchorhynchus* sp., *Helicotylenchus* sp., *Heterodera glycines*, *Heterodera schachtii,* *Hoplolaimus* sp., and *Xiphinema* sp.) and two genera of non-plant parasitic nematodes were also used in the specificity test (Table 1). The specificity tests confirmed that this primer set was successfully and consistently able to amplify the target root-lesion nematode DNA and it did not amplify the DNA of non-target species.

To determine the amplification efficiency of the primer set in real-time PCR assay, a standard curve was generated (Figure 1) based on the linear regression between the quantification cycle (Cq) values and the log values of numbers of the target nematode. DNA from 4 individuals of the target root-lesion nematodes were subjected to 2-fold sequential serial dilution and 9 observations (3 biological replicates with 3 technical replicates) from each dilution level were used to generate the standard curve. The equation for the standard curve was determined as y = -3.474x + 29.002, with an R² value of 0.9467 and an amplification efficiency (E) of 94.02%. Thus, the standard curve generated shows a strong negative correlation between the Cq values from real-time PCR and the log values of numbers of the target nematode with high amplification efficiency. To check the amplification specificity, melting curve analysis was also performed at the end of real-time PCR amplification, which showed a single peak, indicating the target species-specific amplicons were formed in the assay (Figure 2).

The sensitivity of the primer set was also determined for the real time PCR assay. The assay was able to amplify the DNA of 4 individuals diluted 8 times (4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125), however, DNA from the 9th dilution (0.015625) was not amplified in the assay, suggesting this primer set could detect an equivalent to 1/32 of the DNA of a single nematode.

A total of 27 fields were selected for soil sample collection based on our previous work to test the real-time PCR assay. Nematodes were extracted from these soil samples as described above and root-lesion nematodes along with other plant-parasitic nematodes were identified and quantified based on their morphological features under a light microscope. Twenty of these samples were found to be positive with root-lesion nematodes. The population densities in these fields ranged from 15 to 360 root-lesion nematodes per 100 cc of soil. DNA was extracted from the root-lesion nematodes obtained from each of the samples. These DNA samples were used to test whether the real-time PCR assay can accurately detect and differentiate this new species from other root-lesion nematode species. The root-lesion nematode species identities in these samples were confirmed by species-specific PCR or DNA sequencing (Table 2). Out of the 20 samples, 11 were determined to be *P. neglectus* whereas 8 were *P. scribneri* through species specific conventional PCR.The ITS1F/ITS1R primer set designed in this study could not amplify any of those 19 samples, however, it was able to amplify the DNA of root-lesion nematodes obtained from a field sample that was collected from a field that is neighboring the field where the new root-lesion nematode was first detected. Thus, the results indicate that the primer set designed is able to accurately detect and differentiate the new species from other root-lesion nematode species in North Dakota.

To molecularly characterize the new root-lesion nematode species, DNA was extracted from single target nematodes. Three genomic regions (D2-D3 of 28S rDNA, ITS of rDNA, [cytochrome oxidase subunit I](https://en.wikipedia.org/wiki/Cytochrome_c_oxidase_subunit_I)gene) were amplified and sequenced. Nucleotide sequences of these three genomic regions from other *Pratylenchus* species were retrieved from GenBank. Phylogenetic analysis was conducted using MEGA7 software. Three phylogeny trees based on the three genomic regions were constructed using maximum likelihood method. The new root-lesion nematode species was found to be present in a separate clade, indicating the divergence among species (Figure 3).

The new molecular assay developed in this study provides a strong, robust platform that can be used to specifically and sensitively detect the new root-lesion nematode species detected in North Dakota soybean fields. This method of detection allows us to avoid the time-consuming and labor-intensive steps of root-lesion nematode species identification based on morphology under a microscope. Establishing an efficient and quick detection method for the new species of root-lesion nematode can allow us to move faster to nematode risk assessment and management steps for infested fields.

**Table 1.** Specificity of ITS1F and ITS1R primer set designed to identify the new root-lesion nematode species detected in a soybean field of North Dakota.

|  |  |  |  |
| --- | --- | --- | --- |
| Sample IDa | Species | # of Nemab  | PCR Assayc |
| Conventional | Real-time PCR (Cq) |
| HG 50-1 | New *Pratylenchus* sp. | 1 | + | 29.87±0.5 |
| HG 50-2 | New *Pratylenchus* sp. | 2 | + | 27.6±0.2 |
| HG 50-3 | New *Pratylenchus* sp. | 4 | + | 26.43±0.05 |
| HG 50-4 | New *Pratylenchus* sp. | 2 | + | 28.91±0.4 |
| HG 50-5 | New *Pratylenchus* sp. | 2 | + | 27.7 ± 0.1 |
| HG 50-6 | New *Pratylenchus* sp. | 2 | + | 28.02±0.59 |
| HG 51 | *Pratylenchus* sp. ND 2016 | 2 | - | N/A |
| Ps1 | *P. scribneri* | 2 | - | N/A |
| Ps2 | *P. scribneri* | 2 | - | N/A |
| Pn1 | *P. neglectus* | 2 | - | N/A |
| Pn2 | *P. neglectus* | 2 | - | N/A |
| Pt | *P. thornei* | 2 | - | N/A |
| Pp | *P. penetrans* | 2 | - | N/A |
| Tyl | *Tylenchorhynchus* sp. | 2 | - | N/A |
| Spi | *Helicotylenchus* sp. | 2 | - | N/A |
| Xph | *Xiphinema* sp. | 2 | - | N/A |
| Prt | *Paratylenchus* sp. | 2 | - | N/A |
| Ptr | *Paratrichodorus* sp. | 2 | - | N/A |
| Hop | *Hoplolaimus* sp, | 2 | - | N/A |
| SCN | *Heterodera glycines* | 2 | - | N/A |
| SBCN | *H. schachtii* | 2 | - | N/A |
| NPN1 | Non-plant parasitic nematode | 2 | - | N/A |
| NPN2 | Non-plant parasitic nematode | 2 | - | N/A |

a DNA from six different isolates of the target root-lesion species, five other confirmed species of root-lesion nematodes, eight other genera of plant-parasitic nematodes, and two genera of non-plant parasitic nematodes.

b For the samples having the target root-lesion nematode, DNA was extracted from 1, 2 or 4 nematodes whereas for each of the samples with non-target species, DNA was extracted from 2 nematodes.

c For conventional PCR, + = positive amplification and - = no amplification. For real-time PCR, quantification cycle (Cq) value is presented as the mean ± standard deviation of three replicates and N/A = not amplified.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Field ID | County  | RLN/100 cc of soila | Conventionalb | Real-time PCR  | Species Identity |
| PNEG-F1/ D3B5  | PsF7/PsR7 | ITS1F/ ITS1R | ITS1F/ITS1R (Cq)c |
| SCN 366 | Grand Forks | 60 | + | - | - | N/A | *P. neglectus* |
| SCN 311 | Nelson | 30 | + | - | - | N/A | *P. neglectus* |
| SCN 388 | Grand Forks | 25 | + | - | - | N/A | *P. neglectus* |
| SCN 207 | Cass | 242 | + | - | - | N/A | *P. neglectus* |
| Russ Field | Wells | 203 | + | - | - | N/A | *P. neglectus* |
| SCN 188 W | Cass | 45 | + | - | - | N/A | *P. neglectus* |
| SCN 188 E | Cass | 45 | + | - | - | N/A | *P. neglectus* |
| SCN 310 | Nelson | 31 | + | - | - | N/A | *P. neglectus* |
| SCN 215 | Grand Forks | 120 | + | - | - | N/A | *P. neglectus* |
| SCN 222 | Cass | 100 | + | - | - | N/A | *P. neglectus* |
| SCN 7 | Cass | 60 | + | - | - | N/A | *P. neglectus* |
| SCN 55 | Richland | 75 | - | + | - | N/A | *P. scribneri* |
| 50 RL 1 | Richland | 21 | - | + | - | N/A | *P. scribneri* |
| 50 RL 2 | Richland | 28 | - | + | - | N/A | *P. scribneri* |
| 50 RL 4 | Richland | 360 | - | + | - | N/A | *P. scribneri* |
| C3L | Sargent | 75 | - | + | - | N/A | *P. scribneri* |
| C14L | Dickey | 140 | - | + | - | N/A | *P. scribneri* |
| SCN 48 | Richland | 15 | - | + | - | N/A | *P. scribneri* |
| 50 RL 5 | Richland | 28 | - | + | - | N/A | *P. scribneri* |
| 50 RL 3 | Richland | 342 | - | - | + | 27.4 ± 0.1 | *New Pratylenchus* sp. |
| HG 50 Field Soil | Richland | 360 | - | - | + | 27.7 ± 0.1 | *New Pratylenchus* sp. |
|  |  |  |  |  |  |  |  |

**Table 2.** Root-lesion nematodes collected from fields with soybean or a history of soybean in different locations in North Dakota used to test the real-time PCR assay.

a Nematodes were extracted from soil samples using centrifugal sugar floatation method and the population density of root-lesion nematode was determined under a microscope based on their morphology.

b Species-specific primers used for conventional PCR specific to *Pratylenchus neglectus* (PNEG-F1/ D3B5), *P. scribneri* (PsF7/PsR7), and the target new species of root-lesion nematode (ITS1F/ITS1R).

c Quantification cycle (Cq) value for the real-time PCR assay is presented as the mean ± standard deviation of three replicates, and N/A = not amplified.

**Figure 1.** Standard curve of the real-time PCR assay with high amplification efficiency (E) and strong correlation (R²) between quantification cycle values from real-time PCR (y-axis) and log values of the nematode numbers (x-axis).



**Figure 2.** Melting curves of the new root-lesion nematode species-specific amplicons with melting temperature at 81.5 °C, showing the specificity of the new primers designed.



**Figure 3.** Phylogenetic relationship of the new *Pratylenchus* sp*.* (Hg50) detected in a soybean field in North Dakota (red arrow) with other *Pratylenchus* species based on ITS-rDNA.