MOLECULAR CHARACTERIZATION OF ROOT-LESION NEMATODE SPECIES FROM CORN FIELDS IN NORTH DAKOTA AND EVALUATION OF RESISTANCE IN CORN HYBRIDS

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

The molecular characterization of *Pratylenchus* species determined from D2-D3 of 28S rDNA, ITS of rDNA, and COI of mtDNA regions revealed four *Pratylenchus* species from North Dakota, *P. scribneri*, *P. neglectus*, *Pratylenchus* sp. (ND-2016 isolate HG51), and *Pratylenchus* sp. (ND-2017). They were clustered in four separate clades in the phylogenetic trees indicating the divergence among species. *P. scribneri* and *Pratylenchus* sp. (ND-2016 isolate HG51) were closely associated and *Pratylenchus* sp. (DH-2017) was closely related to *Pratylenchus* sp. (ND-2016 isolate HG51). However, *P. neglectus* was not closely associated with the other three species. Moreover, resistance evaluation of ten corn hybrids to *Pratylenchus scribneri*, *P. neglectus*, and *Pratylenchus* sp. (ND-2017) revealed that 1392 VT2P was moderately resistant to three *Pratylenchus* species. PFS74K89 and 4913 VT2RIB were moderately resistant to two of the three *Pratylenchus* species. X5B-8801, DK 43-46, and DKC 44-13 were susceptible to two of the three *Pratylenchus* species.

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DEDICATION

This work is dedicated to my father Md. Akhtaruzzaman, my mother Moshammad Roksana Begum, my husband Md. Ahasan Habib, my brother Md. Saiful Islam, and my sister Rokeya Zaman Sarah.

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
CA	California
COI	Cytochrome Oxidase Subunit I
CRD	Completely Randomized Design
DNA	Deoxyribonucleic Acid
FAO	Food and Agriculture Organization
GA	Georgia
GPS	Global Positioning System
ITS	Internal Transcribed Spacer
ITS-RFLP	Internal Transcribed Spacer-Restriction Fragment Length Polymorphism
MAD PCR	Multiplex Anti Primer Denaturation Polymerase Chain Reaction
MR	Moderately Resistant Hybrid
MS	Moderately Susceptible Hybrid
mtDNA	Mitochondrial Deoxyribonucleic Acid
ND	North Dakota
NDSU	North Dakota State University
NJ	New Jersey
NY	New York
OR	Oregon
PA	Pennsylvania
PCR	Polymerase Chain Reaction

PPNs.....Plant-parasitic Nematodes

R.....Resistant Hybrid

RAPD.....Random Amplified Polymorphic DNA

RF.....Reproductive Factor

RLN.....Root-lesion Nematode

RNA .....Ribonucleic Acid

rDNA.....Ribosomal Deoxyribonucleic Acid

rRNA.....Ribosomal Ribonucleic Acid

S .....Susceptible Hybrid

US .....United States

USA.....United States of America

USDA.....United States Department of Agriculture-Economic Research Service

UV.....Ultra Violet

CHAPTER 1. INTRODUCTION

Root-lesion nematodes, *Pratylenchus* spp., are one of the most economically important plant-parasitic nematodes of corn as they cause significant yield losses in corn (Castillo and Vovlas 2007). The wide host range, migratory endoparasitic nature, and worldwide distribution made this genus to be the third most important group of plant-parasitic nematodes after root-knot and cyst nematodes (Jones et al. 2013; Sasser and Freckman 1987). They can reproduce sexually and/or asexually depending upon species (Agrios 2005). Corn has fibrous root-system which is favorable for egg-hatch, development, and reproduction of *Pratylenchus* spp. (Norton 1983; De Waele et al. 1988). As a consequence, different species of root-lesion nematodes were reported to cause significant damage to corn such as *Pratylenchus penetrans*, *P. hexincisus*, *P. scribneri*, *P. brachyurus*, and *P. zeae* (Castillo and Vovlas 2007; Windham and Edwards 1999). The first three of these five species are the most important nematode pests in the Midwestern Corn belt causing estimated yield losses of 10 % to 26 % (Castillo and Vovlas 2007; Duncan and Moens 2013, Windham and Edwards 1999). In South Dakota, yield losses of corn due to *P. hexincisus* averaged 9.5 bushels/acre and due to *P. scribneri* averaged 4 bushels/acre (Smolik and Evenson 1987). Another species *P. neglectus* was recorded as a damaging species of wheat in Israel, Mexico, Australia, and Oregon and Washington State in North America (Smiley 2010; Thompson 2008).

Identification and characterization of root-lesion nematode species are important to obtain effective management strategies. Root-lesion nematode species identification based on morphological characters is very time-consuming and needs experienced personnel in nematology because of the morphological similarity in different species and variation in morphology within different population of the same species (Orui 1996; Uehara et al. 1999;

Waeyenberge et al. 2000; Al-Banna et al. 2004; Yan et al. 2008). With the development of Polymerase Chain Reactions (PCR)-based molecular identification techniques, many researchers involved these methods in *Pratylenchus* species diagnosis using variation in rDNA sequences. These techniques became popular because they were simple, rapid, reliable, and did not require extensively trained personnel in nematode taxonomy. D2-D3 of 28S rDNA, 18S rDNA, ITS rDNA, and COI mtDNA regions are commonly being used as molecular markers in specific detection of *Pratylenchus* spp. (Nguyen et al. 2019). These molecular marker-based investigations also provided indispensable information regarding the phylogenetic analysis to ascertain evolution of nematode species and the relationship between closely related species.

In North Dakota, four root-lesion nematode species *Pratylenchus scribneri*, *P. neglectus*, and two new putative *Pratylenchus* spp. have been reported by the nematology group of North Dakota State University from potato, wheat, and two soybean fields, respectively (Yan et al. 2016a; 2016b; 2017a; 2017b). The common ways to manage these root-lesion nematode species are crop rotation, cultural practices, and chemical nematicides. Genetic resistance is the best environmentally friendly approach among sustainable management strategies. The wild corn species *Zea diploperennis* and *Z. mexicana* were reported to have resistance to *P. scribneri* thus they were crossed with corn to obtain fertile hybrids with resistance (Norton et al. 1985; De Waele and Elsen, 2002). Moreover, some corn inberds and hybrids showed some resistance to *P. scribneri* such as B37Ht, B68Ht, SD101, SD102, and SD103 of which SD101 was selected as a source of resistance in breeding (Waudo and Norton 1983; Waudo 1984; Smolik and Wicks III 1987). Wicks et al. (1990) developed and registered *P. hexincisus* and *P. scribneri* resistant yellow corn lines. Some corn inbreds were detected to have susceptibility to *P. scribneri* such as C123Ht, C103, Mo17Ht, C123Ht x Mo17Ht, C123Ht x C103, and A619Ht (Waudo and Norton

1983; Waudo 1984; Smolik and Wicks III 1987). Corn cultivars also showed resistance to another species *P. zeae*. In Nigeria, *P. zeae* resistant corn inbreds were reported including 9450 and 5057, and Western Yellow (Oyekanmi et al. 2007). In Uganda, resistant corn hybrids were reported including CML395/MP709, CML312/5057, CML312/CML206, CML312/CML444, CML395/CML312, and CML312/CML395 (Kagoda 2010). To our best knowledge, resistance of corn hybrids to *P. neglectus* has not been reported. However, the resistance of wheat and barley cultivars to *P. neglectus* were available. Wild and cultivated barley accessions were found to be moderately resistant to *P. neglectus* (Keil et al. 2009). A barley cultivar Harrington showed resistance against *P. neglectus* (May et al. 2016). Moreover, five quantitative trait loci associated with *P. neglectus* resistance in the barley genome were mapped by using a population derived from the cross between winter barley cultivars Igri and Franka (Sharma et al. 2011). Smiley et al. (2014a) revealed that wheat cultivar Alpowa and Louise were susceptible, Perisa 20 was moderately susceptible, and AUS28451 was resistant to *P. neglectus* in field condition. The two new *Pratylenchus* spp. were tested for reproducibility with soybean cultivar Barnes under greenhouse condition and reported to have greatly increased population after 15 weeks of growth (Yan et al. 2017a; 2017b).

The nematode surveys conducted in North Dakota revealed that root-lesion nematodes were found in approximately 20 % of the corn fields surveyed during 2015 and 2016 (Chowdhury et al. 2019). The species identities and characterization of root-lesion nematode species from corn fields of North Dakota have not been reported. In addition, there is no information on the resistance of corn hybrids to root-lesion nematode species from North Dakota. Hence, the objectives of this study were:

1. To identify root-lesion nematode species from corn fields in North Dakota, characterize them by analyzing their DNA sequences and determine their phylogenetic relationship.
2. To evaluate ten corn hybrids used in North Dakota for resistance against *P. scribneri*, *P. neglectus*, and a new putative *Pratylenchus* sp. (ND-2017).

CHAPTER 2. LITERATURE REVIEW

Corn (*Zea mays* L.)

Background of corn

Corn (*Zea mays* L.) is an economically important crop especially in North American continent valued for its multipurpose uses. This is one of the oldest cultivated annual crop belongs to the family Poaceae (Sleper and Poehlman 2006). The species *Z. mays* L. can be divided into 5 subspecies named as *Z. mays* ssp. *mays*, *Z. mays* ssp. *mexicana*, *Z. mays* ssp. *parviglumis*, and two annual teosintes (Iltis and Doebley 1980). *Z. mays* originated from Mexico and Central America (Iltis and Doebley 1980) and domesticated in Balsas River Basin of southwestern Mexico probably 10,000 years back just after the ending of Pleistocene when the environment experienced a drastic change (Hufford et al. 2012; Piperno et al. 2015). Corn is a facultative short-day C4 plant. It has single tall stalk with multiple leaves grows from each node of the stalk. Other major body parts are tassel (male flower) and ears. The ears are harvested after maturation for kernels, silk, husk, and cob. In many regions of the world, corn has been used as staple food particularly in Mexico, Central America, the Andean region of South America, and eastern and southern Africa (Poehlman 1987; Sleper and Poehlman 2006). However, it is also cultivated for livestock feed, ethanol production, and corn starch and corn syrup production (USDA-ERS 2019a). Corn is widely grown in most parts of the world, over a wide range of environmental conditions because of its global and regional importance to millions of people who rely on the crop to achieve food security and livelihoods.

Status of corn production in the world, United States and North Dakota

Corn is one of the major crop grown worldwide after wheat and rice. The global corn production and consumption in 2018 was 43,290 million bushels and 44,511 million bushels

respectively. The U.S. is the leading producer of corn followed by China, Brazil, Argentina, Ukraine, and India. In 2018, The U.S. contributed 14,420 million bushels corn production which was 33.3 % of the world corn production and the average yield was 176.4 bushels/acre (USDA-NASS 2019a). According to 2018 data, in the U.S., 43.6 % of the corn was used for food, seed and fuel ethanol in industries, 41 % was used for animal feed and residual, and 15.4 % was exported to other countries (USDA-ERS 2019a). Therefore, corn production is increasing every year. Corn is the major feed grain crop in the U.S. as it covers more than 95 % of the total feed grain production. The most concentrated corn-producing area in the U.S. is the Heartland region (USDA-ERS 2019b). Top corn-producing states are Iowa and Illinois with 18.09 % and 14.89 % of total U.S. corn production followed by Nebraska, Minnesota, Indiana and South Dakota. North Dakota ranks 11th among all corn-producing states in the U.S. (USDA-NASS 2019a). Corn is the third major crop after soybean and wheat in North Dakota. Generally, two types of corn production occur in North Dakota, corn for grain and corn for silage. In North Dakota, the top three highest corn for grain-producing counties are Sargent, Ransom, and Richland whereas the top three highest corn for silage-producing counties are Barnes, Griggs, and Towner (USDA-NASS 2019b).

Biotic constraints of corn production

The naturally occurring pathogens inflict on corn production, which is detrimental to economy and threatening in the regions where it is a staple food commodity. Moreover, fungal infection along with fungal-produced secondary metabolites known as mycotoxins are a dangerous problem in silage producing regions (Pechanova and Pechan 2015). Out of more than 60 diseases of corn, major disease constraints limiting yields in the Northern U.S. are: *Aspergillus* ear rot, *Fusarium* ear rot, *Pythium* damping off, Common smut, Common rust,

Fusarium stalk rot, Gray leaf spot, Northern corn leaf blight, Anthracnose stalk rot, Goss's wilt, Plant-parasitic nematode diseases, *Gibberella* stalk rot, Root rots, and Seedling blights. The fungal disease Southern corn leaf blight epidemic is another major constraint in the U.S. Annual yield loss due to various diseases in the U.S. ranged from 2 to 15 % (Mueller et al. 2016). The prevalent seed and seedling diseases in South Dakota are seed rot, damping-off, and seedling blights resulting in poor plant growth and wilting of young seedlings. Moreover, fungal diseases namely Northern corn leaf blight, Eyespot, Anthracnose; bacterial diseases namely Stewart's disease, Holcus leaf spot, Goss's wilt; viral diseases namely Wheat Streak Mosaic Virus (WSMV) and Maize Dwarf Mosaic Virus (MDMV); and smuts, ear and kernel rots are widespread (Draper et al. 2009). In North Dakota, most prevalent problem is common corn rust followed by northern corn leaf blight and Goss' wilt. Goss' wilt is responsible for maximum yield losses. However, most of the northern corn hybrids have enough resistance to common corn rust and northern corn leaf blight (North Dakota corn council).

Nematode pests of corn

Norton (1983) reported that about 120 species of plant-parasitic nematodes are able to infect corn worldwide of which more than 60 species are constraint of corn production in North America. The most common plant-parasitic nematodes associated with corn in the U.S. are the endoparasitic *Pratylenchus* spp. (root-lesion nematode), *Hoplolaimus* spp. (lance nematode), *Meloidogyne* spp. (root-knot nematode), and ectoparasitic *Belonolaimus* spp. (sting nematode), *Longidorus* spp. (needle nematode), *Paratrichodorus* spp. (stubby-root nematode), *Xiphinema* spp. (dagger nematode), *Paratylenchus* spp. (pin nematode), *Criconemella* spp. (ring nematode), and *Helicotylenchus* spp. (spiral nematode) (Koenning et al. 1999; Tylka et al. 2011). In North Dakota corn fields in 2015 and 2016, the positive fields for plant-parasitic nematodes were 92 %

and 73 %, respectively. Eight genera of plant-parasitic nematodes were detected with variable densities during 2015 and 2016. The nematode distribution and population densities in North Dakota are shown on Fig. 2.1 and Fig. 2.2. (Chowdhury et al. 2017). The most common symptoms due to nematode infestation were stunted plant growth, uneven population, chlorotic plants, and poor ear fill (Draper et al. 2009). Plant-parasitic nematodes were reported to cause more than $8 billion losses in the U.S. (Smiley 2005). Moreover, 5 % to 20 % of yield losses valued $22,992 million were reported from the southern corn-producing states. In 1994, in Florida, *Paratrichodorus* spp. and *Belonolaimus* spp. were responsible for as high as 20 % yield losses. Additionally, in South Carolina, *Helicotylenchus* spp., *Meloidogyne* spp., *Paratrichodorus* spp., and *Pratylenchus* spp. were responsible for 5 to 10 % yield losses (Koenning et al. 1999). In Iowa and Georgia, the annual yield losses in grain corn by plant-parasitic nematodes were 4 % and 7 %, respectively (Society of nematologists 1987). In South Dakota, the estimated yield losses in dry land corn was 9.5 bushels per acre by *Pratylenchus hexincisus* whereas the estimated yield losses in irrigated corn was 4 bushels per acre by *Pratylenchus scribneri* (Smolik and Evenson 1987). Studies during 2012 to 2015 revealed that plant-parasitic nematodes were responsible for the yield loss of approximately 294 million bushels on corn in the U.S. and Ontario, Canada. Therefore, they were considered among top ten disease pathogens of corn in 2012 and 2013 (Muller et al. 2016).

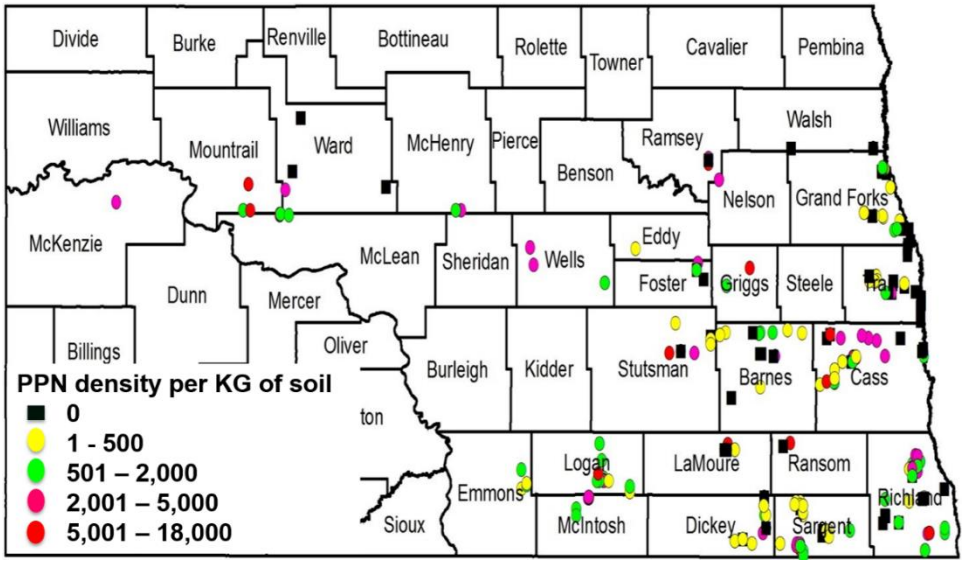

Fig. 2.1. Distribution of plant-parasitic nematodes in Corn fields of North Dakota during 2015 and 2016 (Chowdhury et al. 2017).

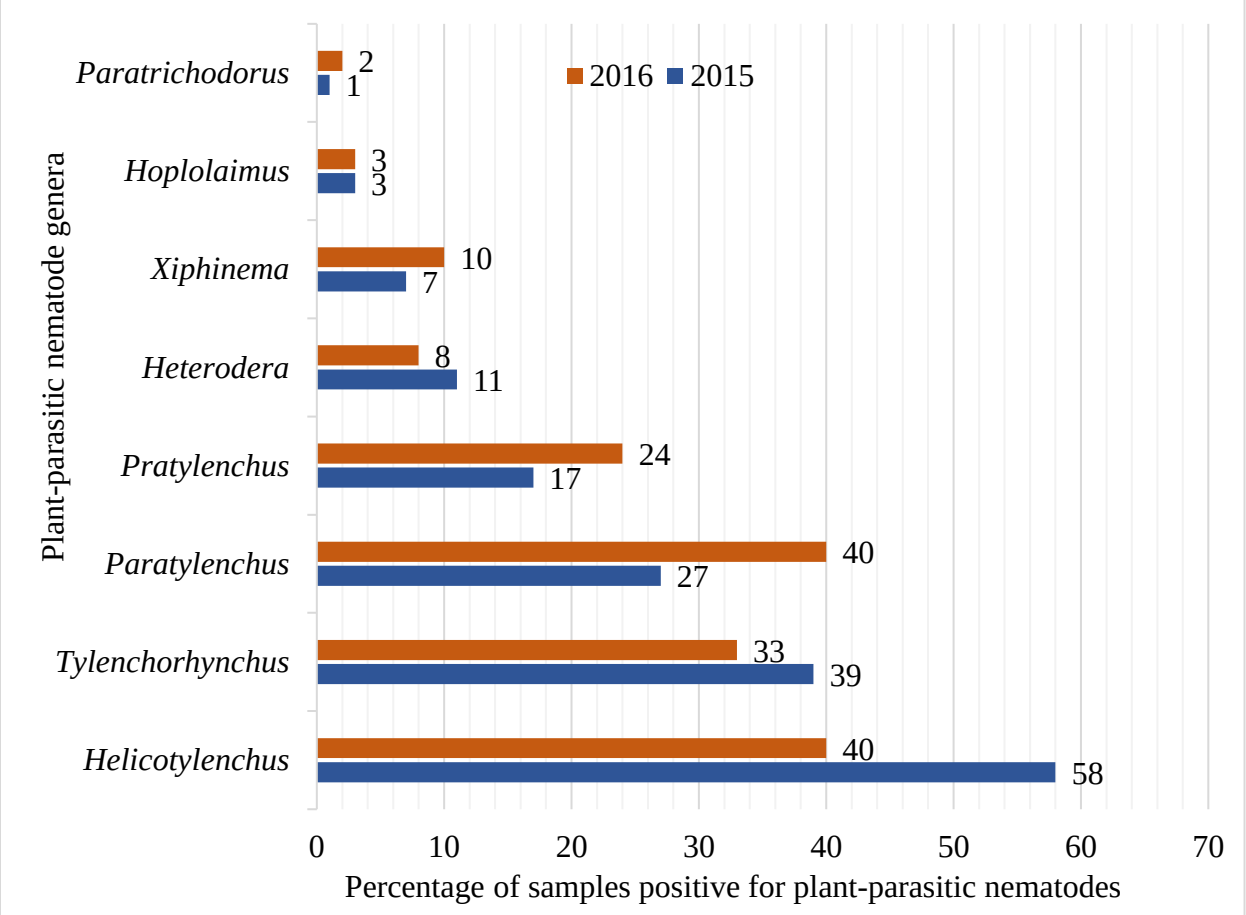

Fig. 2.2. Percentage of plant-parasitic nematodes in Corn fields of North Dakota during 2015 and 2016 (Chowdhury et al. 2017).

Management of plant-parasitic nematodes in corn

The nematodes can be disseminated during movement of soil, plant tissue, farm machinery and equipment, water, animal, and wind from infested to non-infested field. To prevent the spread of nematode from one place to another, the most important management practice is phytosanitary measures including sanitation, use of certified clean plant material, quarantine, and nematode free soil or planting media (Bird 1981; Nicol et al. 2011). Moreover, integrated nematode management approach can help reduce nematode populations below damage threshold levels. Such an approach relies on a combination of control tactics rather than using a single control measure. Integration of cultural practices such as crop rotation, cover crops, planting date, trap crops, roguing or weed management may help reduce the population densities of nematodes (Bird 1981; Brown 1978). Biological control agents (Siddiqui and Mahmood 1999) or resistant cultivars (Kagoda et al. 2011a, 2011b) are other common management strategies. McSorley and Gallaher (1997) described another environmentally friendly approach to management that is yard waste compost. They reported that yard-waste compost (consists of organic matter, Ca, Mg, N, K, P, Cu, Fe, Mn, and Zn) which significantly suppressed the *Paratrichodorus minor* population in soil. Although biological control agents such as bacteria and fungi are environmentally safe and have been effective in controlling nematodes under laboratory conditions. However, their large scale production and field application are laborious and expensive (Siddiqui and Mahmood 1999). In such scenario, despite environmental concerns, nematicides are being used to reduce the economic losses. 1,3-D, terbufos, and carbofuran as well as seed coating with oxamyl and abamectin are some nematicides used effectively against plant-parasitic nematodes (Rich et al. 1985; Todd and Oakley 1995; McGarvey 1982; Cabrera et al. 2009).

Root-lesion nematode (*Pratylenchus* spp.)

Root-lesion nematode (*Pratylenchus* spp.) in corn

Root-lesion nematodes (*Pratylenchus* spp.) are one of the major nematode pests in corn, distributed worldwide and cause significant yield losses in corn (Norton 1983, 1984; Castillo and Vovlas 2007). They are migratory endo-parasitic in nature as they penetrate the root system, feed and multiply inside the root tissue thus injure the root system resulting in brown to black lesion on the root tissue. Life cycles of these nematodes have five stages, beginning with an egg followed by four juvenile stages, then to an adult (Decraemer and Hunt 2006). They have vermiform body shape in all stages of their life cycle except egg. Their sexual identities are recognized at adulthood. They can reproduce sexually and/or by parthenogenesis, depending upon species (Agrios 2005). There are more than 68 species of *Pratylenchus* have been known worldwide, 27 of them present in North America, and at least 5 of those cause significant damage to corn: *P. penetrans*, *P. hexincisus*, *P. scribneri*, *P. brachyurus*, and *P. zeae* (Castillo and Vovlas 2007; Windham and Edwards 1999). The first three of these five are the most damaging pests in the Midwestern Corn belt and created estimated yield losses of 10 % to 26 % (Castillo and Vovlas 2007; Duncan and Moens 2013; Windham and Edwards 1999). In South Dakota, yield losses of corn due to *P. hexincisus* averaged 9.5 bushels/acre and *P. scribneri* averaged 4 bushels/acre (Smolik and Evenson 1987). The root-lesion nematodes are mostly associated with corn as the fibrous root-system of corn favors the growth and reproduction of *Pratylenchus* spp. (Norton 1983). De Waele et al. (1988) showed that corn roots influence *Pratylenchus* spp. egg hatch as well. Norton (1983) reported that per gram dry root of an infected plant contained as high as 10,000 to 40,000 *Pratylenchus* spp. In Michigan, the estimated damage threshold for *Pratylenchus* spp. was 250 per gram of dry root on corn whereas in

Midwest, the average *Pratylenchus* spp. population has been estimated to be around 1,000 per gram of dry root (Batista da Silva 2013). Population densities of *Pratylenchus* spp. have a positive correlation with yield losses of corn (McSorley and Dickson 1989; Tarte 1971). Other hosts of *Pratylenchus* spp. include soybean, sorghum, potato, rye, as well as a variety of grasses and weed species (Barker and Olthof 1976; Bélair et. al. 2007; Castillo and Vovlas 2007; Jordaan and De Waele 1988). Pathogenicity varies by species and can be a determining factor in the effective use of rotation control methods.

Pratylenchus scribneri

Pratylenchus scribneri is one of the most economically important species in Midwestern Corn Belt. This species does not produce adult male thus it reproduces by parthenogenesis. It has a wide host range including corn, barley, soybean, potato, sugar beet, broccoli, strawberry, onion, tomato, and peach (Castillo and Vovlas 2007). It is known that corn yield has a positive correlation with root density in the upper 15 cm (Kuchenbuch and Barber 1987). Interestingly, MacGuidwin and Stanger found approximately 50 % of *P. scribneri* populations were also found in the upper 15 cm of corn root systems (MacGuidwin and Stanger 1991). This finding also demonstrates that *P. scribneri* is associated with yield losses. *Pratylenchus scribneri* has interaction with other pathogens. Palmer et al. (1967), showed a synergetic interaction between *P. scribneri* and *Fusarium moniliforme* affecting the fresh weight of corn more than when either organism was present alone. Although the common ways to manage *P. scribneri* are crop rotation and cultural practices, several nematicides are used by the growers. For example, application of terbufos and carbofuran at planting and carbofuran at post-planting significantly reduced *P. scribneri* population in soil and roots (Todd and Oakley 1995). Use of resistant hybrids is the best nematode control means. There is very less studies on resistance of corn

hybrids to *P. scrbneri*. The wild corn species *Zea diploperennis* and *Z. mexicana* were reported to have resistance to *P. scribneri* (Norton et al. 1985). Later on, many breeders crossed *Z. diploperennis* with corn to obtain fertile hybrids (De Waele and Elsen 2002). Corn inbreds B37Ht and B68Ht were reported to have some resistance against *P. scribneri* and corn inbreds C123Ht, C103, Mo17Ht, C123Ht x Mo17Ht, and C123Ht x C103 showed susceptibility to *P. scribneri* (Waudo and Norton 1983; Waudo 1984). Smolik and Wicks III (1987) reported that corn inbreds SD101, SD102, and SD103 were found resistant to *P. scribneri* among which, SD101 was selected as a source of resistance. Moreover, inbred A619Ht was found susceptible to both *P. hexincisus* and *P. scribneri* (Smolik and Wicks III 1987). Wicks et al. (1990) developed and registered *P. hexincisus* and *P. scribneri* resistant yellow corn line. In North Dakota, *P. scribneri* was first reported from a potato field in 2015 (Yan et al. 2016a). However, their effect on corn yield and resistance to corn hybrids have not yet been reported.

Pratylenchus neglectus

Pratylenchus neglectus is another economically important species distributed mostly in Australia, North America, South Africa, Sudan, Spain, Italy, France, and Canada. Their wide host range includes corn, wheat, chickpea, vetch, barley, oat, alfalfa, sorghum, potato, peach, pear, apple, canola, annual medic, and wheat grass (Taylor 2000). *Pratylenchus neglectus* has been found to cause yield losses from 10 % to 30 % (Vanstone et al. 1998, 2002, 2008; Taylor 2000). In Israel, Mexico, and Oregon and Washington State in North America, *P. neglectus* is responsible for significant yield losses in wheat and other host crops (Smiley 2010). *P. neglectus* does not produce adult male thus it reproduces by parthenogenesis. One interesting character of *P. neglectus* is that they can survive in dry soil for more than 15 months by entering anhydrobiosis state (Taylor 2000). Therefore, it is menacing in dryland cropping regions.

Moreover, *P. neglectus* has interaction with other pathogens. Taheri et al. (1994) reported that the presence of *Microdochium bolleyi* and *Fusarium* spp. significantly increased *P. neglectus* population and wheat root infection. The economic damage thresholds for *P. neglectus* are reported at 2,000 nematodes/kg dry soil in Oregon and Washington states (Smiley et al. 2005). Taylor et al. (2000), used the reproduction ability of *P. neglectus* for host suitability evaluation where eighty-one cultivars from 12 field crop species were tested for resistance. They were grouped as good host, moderate host and poor host based on multiplication rate. Vanstone et al. (1998) demonstrated that resistance and tolerance to *P. neglectus* often coincide in wheat. The *P. neglectus* resistance in almonds was identified and used in breeding through the use of resistant root stock (Marull et al. 1990) and in oats (Townshend 1989). Wild and cultivated barley accessions were found to be moderately resistant to *P. neglectus* (Keil et al. 2009). Sharma et al. (2011) mapped five quantitative trait loci associated with *P. neglectus* resistance in barley genome. May et al. (2016) revealed that barley cultivar Harrington showed resistance against *P. neglectus.* Smiley et al. (2014) revealed that wheat cultivar Alpowa and Louise were susceptible, Perisa 20 was moderately susceptible, and AUS28451 was resistant to *P. neglectus* in field condition. In North Dakota, *P. neglectus* was first reported from a wheat field in 2015 (Yan et al. 2016b). However, their effect on corn yield and resistance to corn hybrids has yet to be reported.

New *Pratylenchus* species reported in North Dakota

There are more than 70 species of *Pratylenchus* have been known worldwide. With the advancement of detection, many new *Pratylenchus* spp. are being found in various regions. Two new un-named *Pratylenchus* spp. have been reported from North Dakota crop fields. The first one, *Pratylenchus* sp. ND-2016 isolate HG51 was first reported from a soybean field in Hankinson, Richland County, North Dakota with a density of 150 to 875 per kg soil during 2015

and 2016. After morphometric measurement and molecular screening, this species was found distinct from other known *Pratylenchus* spp. (Yan et al. 2017a). Another new *Pratylenchus* spp., *Pratylenchus* sp. ND-2017 was first reported from a soybean field in Walcott, Richland County, North Dakota with a density of 125 to 2,000 per kg soil during 2015 and 2016 (Yan et. al. 2017b). After morphometric measurement and molecular screening, this species was found distinct from the *Pratylenchus* sp. ND-2016 isolate HG51 and other known *Pratylenchus* spp. (Yan et. al. 2017b). The two new putative *Pratylenchus* spp. were screened for reproducibility with soybean cultivar Barnes under greenhouse condition and reported to have greatly increased population after 15 weeks of growth (Yan et al. 2017a, 2017b). However, their effect on corn yield and resistance to corn hybrids has yet to be reported.

Molecular identification and characterization of *Pratylenchus* spp.

Molecular identification up to species is based on protein profiling were widely used previously to identify *Pratylenchus* spp. Payan and Dickson (1990) analyzed protein polymorphism and genetic diversity of *P. scribneri* and *P. brachyurus* using Iso-Electric Focusing (IEF). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was used to analyze patterns of extracted proteins of *Pratylenchus* to identify different species which included *P. vulnus*, *P. goodeyi*, *P. coffeae*, *P. scribneri* and *P. thornei* (Jaumot et al. 1997). Species-specific enzyme markers have been effectively used to determine intra-specific variation in populations from various geographical locations along with species identification (Andrés et al. 2000).

The development of DNA sequencing and PCR technique replaced these protein- or isoenzyme-based diagnostic techniques. Random Amplification of Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Internal Transcribed Spacer-Restriction

Fragment Length Polymorphism (ITS-RFLP), Sequence Characterized Amplified Region-Polymerase Chain Reaction (SCAR-PCR), multiplex PCR, and satellite DNA sequences were DNA and PCR-based diagnostic methods used in *Pratylenchus* spp. identification. These techniques have revolutionized molecular characterization of *Pratylenchus* spp. as well as provided indispensable information regarding phylogenetic analysis (Yan et al. 2012; Carrasco-Ballesteros et al. 2007; Al-Banna et al. 2004; Troccoli et al. 2008; Subbotin et al. 2008). In particular, RAPD was used to classify seven *Pratylenchus* spp. in Brazil and combination of RAPD and SCAR-PCR techniques was used to detect *P. thoreni* (Siciliano-Wilcken et al. 2002; Carrasco-Ballesteros et al. 2007). Tan (2012) used nucleic acid based diagnostics (e.g. ITS based PCR) for *Pratylenchus* spp. identification. Besides, multiplex anti primer denaturation PCR (MAD PCR) was developed by Tan (2012) which was a combination of a qPCR technology and auto sticky PCR. This technique was used to detect several species at the same time (multiplexing). Recently, D2-D3 of 28S rDNA, 18S rDNA, ITS rDNA, and COI mtDNA regions are largely being used as molecular markers in specific detection of *Pratylenchus* spp. (Nguyen et al. 2019). In addition, the real-time PCR assay was used to identify *P. scribneri* and *P. thoreni* (Huang and Yan 2017; Yan et al. 2012). *P. penetrans* was successfully detected directly from soil samples using a SYBR Green I-based qPCR assay (Baidoo et al. 2017).

Phylogenetic relationships of *Pratylenchus* species

The phylogenetic analysis helps to understand the process of organismal evolution and relationships between organisms from DNA or protein variation patterns. This can be the only way to detect the evolutionary information especially when there is lack of fossil data or when morphological features are limited. The phylogenetic analysis provides information regarding the ancestor of a taxon, sister taxa, evolutionary distance between species, and the comparison

between closely related species more precisely and rapidly. This study also acts as the basis for comparative genomics. The phylogenetic tree graphically represents the evolutionary relationship of a target species. Due to the overlapping morphological features, the presence of morphotypes, invertebrate nature, together with the presence of intraspecific and interspecific genetic variation in root-lesion nematodes, identification and separation of these species have become challenging. The lack of informative morphological characters as well as high economic impact on plants prompted researchers to study the phylogenetic relationship of plant parasitic nematodes.

Formerly, the phylogeny and evolution of *Pratylenchus* spp. were determined based on morphological data (Ryss 2002). The morphological tree was constructed by cladistics methods (Handoo et al. 2002). However, the molecular phylogenetic trees inferred from 28S rDNA sequences were not fully similar to the morphological tree due to study of limited and different set of species in both cases (Handoo 2008). The first molecular study of phylogenetic tree on *Pratylenchus* spp. was constructed using D3 segment of rDNA (Al-Banna et al. 1997). Subsequently, the second study on the *P. coffeae* species complex was developed using both D2 and D3 rDNA regions (Duncan et al. 1999). Carta et al. (2001) has restored *Pratylenchus* monophyly by incorporating more *Pratylenchus* species and different outgroups. The study of De Luca et al. (2004) revealed the high variability among individuals of *P. neglectus*. In recent studies, direct sequencing based on ITS-1 rDNA followed by phylogenetic analysis including determination of heterogeneity between species gave more intricate insight regarding the relationship between the species (Machado et al. 2015). Nguyen et al. (2017) used D2-D3 of 28S rDNA and ITS rDNA genes to study the phylogenetic relationship of a new *Pratylenchus*

species, *Pratylenchus haiduongensis*, associated with carrot in Vietnam. They revealed that *P. haiduongensis* had close relation with *P. parazeae, P. zeae,* and *P. bhattii*.

Management of root-lesion nematodes (*Pratylenchus* spp.) in corn

The common management practices to control the root-lesion nematodes in corn fields are cultural practices including sanitation, crop rotation, cover crops, and chemical nematicides. Despite of environmental concerns, nematicides are being used to reduce the economic losses in corn production. In Iowa, a combination of 1,3-D and carbofuran was used which resulted in good control of *Pratylenchus hexincisus* (Norton and Hinz 1976). Moreover, pre-plant treatments with 1,2-D-1,3-D or carbofuran were found effective against *P. brachyurus* and *P. zeae* (Rich et al. 1985). Additionally, the application of terbufos and carbofuran at planting time and carbofuran at post-planting time was found effective against *P. scribneri* (Todd and Oakley 1995). However, seed treatment with nematicides is a more efficient and relatively environmentally friendly method for nematode management. Seed treatment could be the least expensive and easiest method of nematicide application. Indeed, it is the least contaminating for the environment compared to other forms of nematicide application. According to McGarvey (1982), seed coating with oxamyl could protect the plants from *P. penetrans* and *Meloidogyne hapla Chitwood* and enhanced plant growth. Furthermore, Cabrera et al. (2009) reported that abamectin was very effective in reducing *P. zeae* penetration into corn roots up to 80 %.

Undoubtedly, the use of resistant cultivars is one of the most environmentally friendly approaches to management. Few corn inbreds with plant parasitic nematode resistance genes were known (Windham and Edwards 1999). Of 129 known corn germplasms, four have successful nematode resistance genes (Young 1998). Unfortunately, root-lesion nematode-resistant commercial corn hybrids are yet to be reported. During 1980's several experiments

were done to check the resistance of corn hybrids. Already existed corn lines, inbreeds and hybrids were being screened for resistance to *Pratylenchus* spp. Among them, corn inbreds Mo17Ht, B73Ht, and B73HtxMo17Ht were found to support *P. scribneri* reproduction (Waudo and Norton 1986). Smolik and Wicks (1987) found that corn inbred line SD45 was found to support the reproduction of *P. scribneri* and *P. hexicinsus.* However, corn inbred lines Mo17 and B73 were potential for root-lesion nematode resistance hence commonly used in corn breeding programs (Georgi et al. 1983). During 2010's, Kagoda et al. (2011a) reported six corn hybrids namely CML395/MP709, CML312/5057, CML312/CML206, CML312/CML444, CML395/CML312, and CML312/CML395 that performed best under *P. zeae* infested conditions. Interestingly, corn inbreds MP709 and CML206 had the highest general combining ability for *P. zeae* resistance and most of the dominant *P. zeae* resistant genes derived from parents MP709, CML206, 5057, and CML444 in all their crosses (Kagoda et al. 2011b). To our best knowledge, resistance of corn hybrids to *P. neglectus* has not been reported. The migratory nature of root-lesion nematodes made the development of resistant corn hybrid more challenging and expensive. Identification and development of resistant inbred or hybrid against species of root-lesion nematodes will help improve grain yield under nematode infested conditions.

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CHAPTER 3. MOLECULAR CHARACTERIZATION AND IDENTIFICATION OF ROOT-LESION NEMATODE SPECIES FROM CORN FIELDS IN NORTH DAKOTA

Abstract

Root-lesion nematodes (RLN), *Pratylenchus* spp., are one of the major plant-parasitic nematodes in agriculture and known as one of the most detrimental nematode pests in corn in the U.S. Identification and characterization of RLN species are important to obtain effective management strategies. Therefore, it was imperative to identify species of RLN in corn fields of North Dakota (ND), characterize them by analyzing their DNA sequences, and determine the phylogenetic relationship among the species. To achieve the objectives, soil samples were collected arbitrarily from 50 corn fields of ND in 2017 and 2018. Out of these samples, 17 samples (34 %) were found positive for RLN after nematode extraction by sugar centrifugal flotation method. The RLN densities varied from 200 to 2,100 nematodes/kg of soil. The positive samples of RLN were identified to species level by species-specific PCR and DNA sequencing of three genomic regions including D2-D3 of 28S rDNA, internal transcribed spacer (ITS) of rDNA, and cytochrome oxidase subunit one (COI) of mtDNA. Out of the 17 samples, six were identified as *P. scribneri*, four were new putative *Pratylenchus* sp. ND-2016 isolate HG51, three were new putative *Pratylenchus* sp. ND-2017, one was *P. neglectus*, and three of the fields were found to contain a mixed population of *P. neglectus* and *P. scribneri*. The neighbor-joining trees for the three regions were constructed independently to better understand the phylogenetic relationship of these species. In the three phylogenetic trees, four species were clustered in four separate clades indicating the divergence among species. Among the four species, *P. scribneri* and new putative *Pratylenchus* sp. ND-2016 isolate HG51 were more closely associated with

each other. The new putative *Pratylenchus* sp. ND-2017 were also closely related to the *Pratylenchus* sp. ND-2016 isolate HG51. However, *P. neglectus* was not closely associated with other three species. The lowest intra-species variation (0.52 to 3.16 %) was found in the *Pratylenchus* sp. ND-2017 populations whereas the highest intra-species variation (11.93 to 24.21 %) was found in the *P. neglectus* populations. Molecular characterization of these four species will be helpful for understanding the evolutionary relationships of RLN and for facilitating species identification and management of RLN in infested corn fields.

Keywords: Species-specific PCR, DNA sequencing, Phylogenetic tree, *P. scribneri*, *P. neglectus*, *Pratylenchus* sp. ND-2016 isolate HG51, *Pratylenchus* sp. ND-2017, D2-D3 of 28S rDNA, ITS of rDNA, COI of mtDNA.

Introduction

Root-lesion nematodes, *Pratylenchus* spp., are one of the most economically important plant-parasitic nematodes. They are widespread in all agricultural regions of the world and cause significant yield losses (Castillo and Vovlas 2007). The wide host range, migratory endoparasitic nature, and worldwide distribution made this genus to be the third most important group of plant-parasitic nematodes after root-knot and cyst nematodes (Jones et al. 2013; Sasser and Freckman 1987). They have four motile life stages and in all motile life stages, they move freely from the soil to the roots to feed and reproduce. Thus they create numerous local tissue lesions, which serves as an entrance for the secondary pathogens such as bacteria or fungi (Rybarczyk-Mydlowska 2013). Moreover, the damaged tissues fail to uptake water and nutrient by the roots resulting in poor plant growth and yield (Orion et al. 1984; Farsi et al. 1993). They do not produce permanent feeding site in the roots like root-knot and cyst nematodes thus their presence is less obvious and quantification is more difficult. Therefore, detailed research on molecular

characterization and biology of root-lesion nematodes had been comparatively neglected until recently.

Out of more than 80 well-known species of *Pratylenchus* worldwide, 27 have been present in North America. *P. penetrans*, *P. hexincisus*, *P. scribneri*, *P. neglectus*, and *P. crenatus* are common pathogen of corn to north-central US and *P. penetrans*, *P. hexincisus*, *P. scribneri*, *P. brachyurus*, and *P. zeae* have reported to cause significant damage to corn (MacGuidwin and Bender 2016; Castillo and Vovlas 2007; Windham and Edwards 1999). Among them, *P. penetrans*, *P. hexincisus*, and *P. scribneri* have been the most damaging pests in the Midwestern Corn Belt by creating estimated yield losses of 10 % to 26 % (Castillo and Vovlas 2007; Duncan and Moens 2013, Windham and Edwards 1999). In South Dakota, yield losses of corn due to *P. hexincisus* averaged 9.5 bushels/acre and *P. scribneri* averaged 4 bushels/acre (Smolik and Evenson 1987). Moreover, *P. penetrans* was devastating for potato, pea, carrot, bean, and ornamentals in Europe (Talavera et al. 2001; Pudasaini et al. 2007), and wheat and barley in Australia and Canada (Nicol and Rivoal 2007). Another species *P. neglectus* was recorded as a damaging species of wheat in Israel, Australia, Mexico, and Oregon and Washington State in North America (Smiley 2010; Thompson 2008) and reported to causes yield losses of 8-36 % (Smiley et al. 2005). This species can cause 10-30 % yield losses depending on infected crop species (Vanstone et al. 1998, 2002, 2008; Taylor 2000). Youssef (2013) demonstrated that the densities of *P. zeae* in corn roots were negatively correlated with grain yield. *P. zeae* mostly infected sugarcane in Queensland, Australia (Blair et al. 1999a, 1999b).

Root-lesion nematode species identification based on morphological characters is very time-consuming, and needs experienced personnel because of the morphological similarity in different species and morphological variation within a species (Orui 1996; Uehara et al. 1999;

Waeyenberge et al. 2000; Al-Banna et al. 2004; Yan et al. 2008). With the development of Polymerase Chain Reactions (PCR)-based molecular identification techniques, many researchers involved these methods in *Pratylenchus* species diagnosis using variation in rDNA sequences. These techniques became popular because they were simple, rapid, reliable, and did not require extensively trained personnel in nematode taxonomy. Previously, Random Amplified Polymorphic DNA (RAPD) polymerase chain reactions were used to differentiate *P. vulnus* isolates (Pinochet et al. 1994). Afterward, molecular tools such as Internal Transcribed Spacer-Restriction Fragment Length Polymorphism (ITS-RFLP), Sequence Characterized Amplified Region-Polymerase Chain Reaction (SCAR-PCR), multiplex PCR, and satellite DNA sequences have been commonly used in species identification and evaluation of genetic diversity of root-lesion nematodes (Al-Banna et al. 1997; Troccoli et al. 2008; Waeyenberge et al. 2009). Tan (2012) used nucleic-acid based diagnostics (e.g. ITS based PCR) for four *Pratylenchus* spp. (*P. thornei*, *P. penetrans*, *P. neglectus*, and *P. zeae*) identification and phylogenetic analysis. In addition, Multiplex Anti Primer Denaturation PCR (MAD PCR) was developed by Tan (2012) which was a combination of a qPCR technology and auto sticky PCR. This technique was used to detect several species at the same time (multiplexing). D2-D3 of 28S rDNA, 18S rDNA, ITS rDNA, and COI mtDNA regions are commonly being used as molecular markers in specific detection of *Pratylenchus* spp. (Nguyen et al. 2019). Moreover, the real-time PCR assay was used to identify *P. scribneri* and *P. thornei* (Huang and Yan 2017; Yan et al. 2012). *P. penetrans* was successfully detected directly from soil samples using an SYBR Green I-based qPCR assay (Baidoo et al. 2017). *P. thornei*-specific PCR primers were designed from 28S rDNA, ITS rDNA, COI mtDNA, and ß-1,4-endoglucanase gene and other specific genes (Castillo and Vovlas 2007). *P. neglectus*-specific primer set PNEG/D3B was designed by Al-Banna et al.

(2004) which could separate *P. neglectus* from *P. vulnus*, *P. scribneri*, *P. thoreni*, *P. penetrans*, and *P. brachyurus*. The modified primer set PNEG-F1/D3B5 was able to separate *P. neglectus* in DNA extracts from soil (Yan et al. 2008). Moreover, *P. scribneri*-specific primer sets PSCR/D3B (Al-Banna et al. 2004) and PSC3 (Gray et al. 2011) were reported to detect *P. scribneri* by conventional PCR.

Some of the above mentioned molecular techniques also provided indispensable information regarding the phylogenetic analysis. The nematodes play important role in geographic evolution and plant-parasitic nematodes have great economic impact on plants as mentioned before. Therefore, it is necessary to understand the evolution of plant-parasitic nematodes which will help to understand the host-nematode interaction mechanism and management strategies. Previously, evolutionary information of nematodes relied on morphological and fossil information. The phylogenetic analysis can be the only way to detect the evolutionary information from DNA or protein variation especially when there is a lack of fossil data or when morphological features are limited. The phylogenetic analysis provides information regarding the ancestor of a taxon, sister taxa, evolutionary distance between species, and the comparison between closely related species more precisely and rapidly. This study also acts as the basis for comparative genomics. The phylogenetic tree graphically represents the evolutionary relationship of a target species. Due to the overlapping morphological features, the presence of morphotypes, invertebrate nature, together with the presence of intraspecific and interspecific genetic variation in root-lesion nematodes, identification and separation of these species have become challenging. The lack of informative morphological characters, as well as the high economic impact of root-lesion nematodes on plants, prompted researchers to study the phylogenetic relationship of root-lesion nematodes. Some molecular markers are used for

phylogenetic analysis of root-lesion nematodes. Al-Banna et al. (1997) at first determined the phylogenetic relationship of the genus *Pratylenchus* using 26S rDNA. Subbotin et al. (2008) used D2-D3 of 28S rRNA and 18S rRNA regions to analyze the phylogenetic framework for the genus *Pratylenchus.* Palomares-Rius (2014) used D2-D3 of 28S, partial 18S, and ITS rRNA regions as markers to determine the relationship of *P. oleae* n. sp. with *P. dunensis*, *P. penetrans*, and *P. pinguicaudatus*. The phylogenetic analysis based on ITS-1 rDNA including determination of heterogeneity between species gave more intricate insight regarding the relationship between the species (Machado et al. 2015). Nguyen et al. (2017) used D2-D3 of 28S rDNA and ITS rDNA genes to study the phylogenetic relationship of a new *Pratylenchus* species, *Pratylenchus haiduongensis*, associated with carrot in Vietnam. They revealed that *P. haiduongensis* had close relation with *P. parazeae*, *P. zeae*, and *P. bhattii*.

In North Dakota, during 2015 and 2016, four species of root-lesion nematodes (*P. neglectus*, *P. scribneri*, and two new putative *Pratylenchus* spp.) were reported for the first time based on morphological measurements and sequence information of 28S rDNA and/or ITS rDNA (Yan et al. 2016a, 2016b; Yan et al. 2017a, 2017b) from different crop fields. Molecular characterization and phylogenetic analysis of these species are important to obtain effective management strategies. The nematode surveys conducted in North Dakota revealed that root-lesion nematodes were found in approximately 20 % of the corn fields surveyed in 2015 and 2016 (Chowdhury et al. 2019). The specific identities and characterization of root-lesion nematode species from corn fields of North Dakota have not been reported. Therefore, the aims of this study were to 1) identify root-lesion nematode species from corn fields in North Dakota, 2) characterize them by analyzing their DNA sequences from three genomic regions including D2-D3 of 28S rDNA, Internal Transcribed Spacer (ITS) of rDNA, and Cytochrome Oxidase

Subunit One (COI) of mtDNA, and 3) determine the phylogenetic relationship among the species.

Materials and methods

Soil sample collection

Soil samples were arbitrarily collected during the summer time of 2017 and 2018. Samples were collected across North-East, East, South-East, and East-Central counties of North Dakota, where corn are grown in rotation with soybean, dry bean, wheat and potato (Fig. 3.1). In 2017, six samples were collected from four counties and in 2018, 44 samples were collected from 10 counties of North Dakota. A total of 50 soil samples were collected from 50 fields in 10 counties (Table 3.1). Approximately one kg of soil was collected from each sampling field. Global Positioning System (GPS) co-ordinates were recorded for each sampling field using GPS navigator system (Garmin Drive 51 USA LM GPS Navigator System, OR, USA). The soil was collected from each field using 2.5 cm diameter soil probes (Gempler's model L Sampler, Madison, WI). Each soil core was taken following a zig-zag pattern by removing the top dry surface soil and collecting soil from up to 30 cm depth around the rhizosphere region. The soil cores were subsequently placed in properly labeled plastic bags, stored in a cooler box during sampling and transportation and subsequently kept in a cold room at 4 °C until nematode extractions were performed.

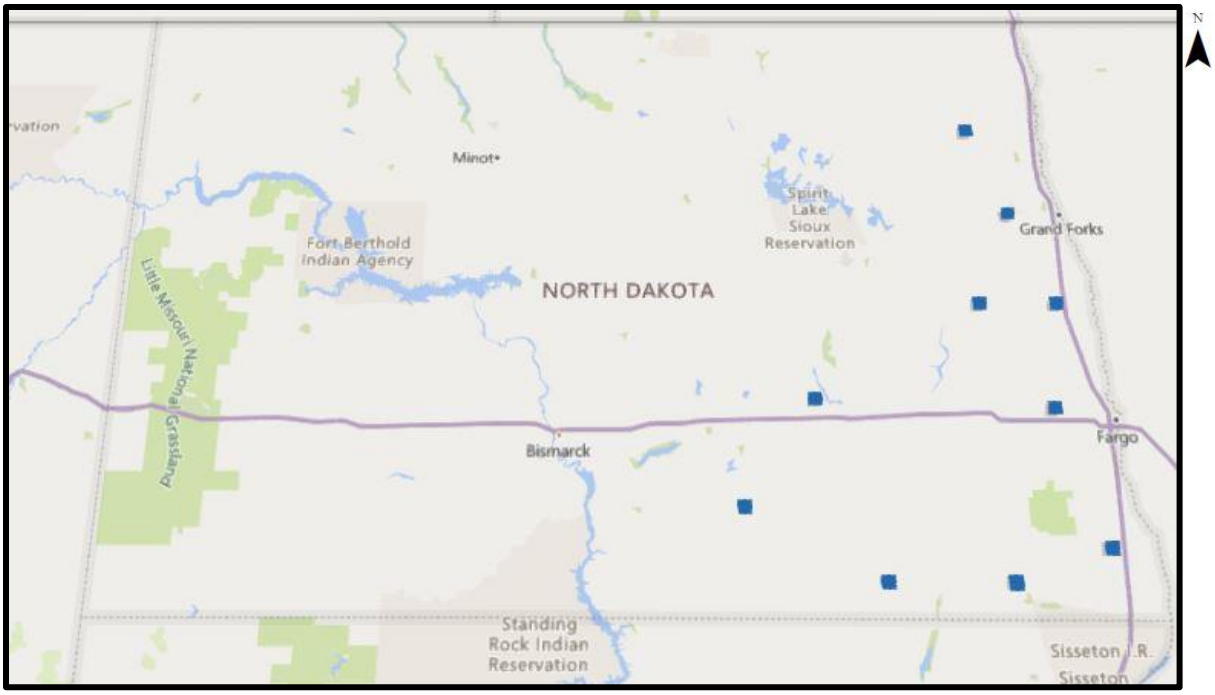


Fig. 3.1. Map of North Dakota where each blue square index represents one county selected for sampling.

Table 3.1. The number of soil samples collected and counties surveyed in 2017 and 2018 from corn fields in North Dakota.

Year[a]	Number of Samples	Counties	Total Counties[b]
2017	6	Richland, Grand Forks, Sargent, Stutsman	4
2018	44	Richland, Walsh, Grand Forks, Cass, Traill, Dickey, Sargent, Stutsman, Logan, Steele	10

[a]Years in which samples were collected.
[b]Total counties covered during 2017 and 2018.

Nematode extraction, identification, and quantification

Nematode extractions were attempted to be done within two weeks of sampling to prevent changes in nematode populations. All the samples were extracted and quantified in the Nematology Laboratory at North Dakota State University. The entire soil of a sample was mixed thoroughly in a mixing tray (36 cm x 27 cm) to maintain the uniformity of nematode densities in

the entire sample. Subsequently, 200 g of sub-sample was taken from each composite sample and nematodes were extracted using sieving and decanting and sugar centrifugal-floatation technique (Jenkins 1964). The extracted nematodes were collected using a 20 μm (#635) sieve and then transferred to a 50 ml nematode suspension vial. The nematodes suspensions were stored in 4 °C refrigerator for further processing.

One ml of nematode suspension was taken into a counting slide (Chalex Co., Portland, OR, USA) and placed under an inverted transmitted light microscope at 100X magnification (Zeiss Axiovert 25, Carl Zeiss Microscopy, NY). Plant-parasitic nematodes were categorized by genera based on some key morphological features such as body shape and size, stylet type, stylet length, mouth type, lip region, esophageal overlap, vulva position, and tail type (Fortuner 1988; Mai et al. 1996) and subsequently counted following the method described by Plaisance and Yan (2015). The densities of plant-parasitic nematodes were determined as the total number of individual nematodes of a genus in 200 g of soil.

DNA extraction from root-lesion nematodes

Molecular examinations were performed to identify root-lesion nematodes to species level. All molecular examinations were performed from the positive samples of root-lesion nematodes obtained by nematode extraction. Six adults *Pratylenchus* spp. from each nematode suspension were handpicked based on their morphological features (Fortuner 1988; Mai et al. 1996) under an inverted transmitted light microscope at 100X magnification (Zeiss Axiovert 25, Carl Zeiss Microscopy, NY) inside laminar flow hood and placed on a concave glass slide (Labscientific Inc., Livingston, NJ, USA). DNA was extracted from a single individual adult nematode using Proteinase K method (Huang and Yan 2017) for direct DNA sequencing. A single nematode was handpicked from the previously picked nematodes, and placed on another

concave glass slide containing 10 µl double-distilled water and chopped in that concave glass slide. Then, nematode suspension containing nematode pieces (10 µl) was pipetted into a 0.5-ml sterile Eppendorf tube containing 2 µl of 10x PCR buffer, 2 µl of Proteinase K (600 µg/ml), and 6 µl of double-distilled water. Tubes containing chopped nematodes and lysis solution were incubated at -20 ºC for 20 mins followed by 65 ºC for 1 hour to digest the cell proteins and then tubes were incubated at 95 ºC for 10 minutes to eliminate the activity of Proteinase K (Huang and Yan 2017). Finally, when the DNA extracts were cool down to room temperature, they were centrifuged at 10,000 rpm for 2 min to spin down all the DNA extracts to the bottom of the tubes. The resulting 20 µl DNA extracts were stored at -20 °C for further experiments. Thus, 6 DNA extracts were obtained from each field sample (n= 6 per field) for further sequencing. In case of species-specific PCR, DNA was extracted from three adult nematodes using the same DNA extraction protocol.

Species-specific PCR and DNA sequencing

Molecular identification of root-lesion nematodes to species level was performed by species-specific PCR and direct DNA sequencing methods. A total of seven pairs of primers were used to conduct these examinations. The primer pair D3A/D3B was used as universal primer to check the quality of DNA before species-specific PCR and the primer pairs PsF7/PsR7, Pn-ITS-F2/Pn-ITS-R2, and PNEG-F1/D3B5 were used as species-specific primers. Three genomic regions including D2-D3 of 28S rDNA, Internal Transcribed Spacer (ITS) of rDNA, and Cytochrome Oxidase Subunit One (COI) of mtDNA were amplified and sequenced for direct DNA sequencing. The primer pairs D2A/D3B, rDNA2-V/rDNA1, and JB3/JB4.5 were used for direct DNA sequencing. The elaborate information about the primers used for species-specific PCR and direct DNA sequencing are given in Table 3.2. All the PCR reactions were performed

using the Bio-Rad T100 Thermal Cycler (Hercules, CA, USA). The details about PCR amplification conditions with reaction volume for seven primer pairs were compiled in Table 3.3.

Table 3.2. List of primers used in species-specific PCR and DNA sequencing.

Target	Primer name	Sequence (5' -> 3')	Target size	References
D2-D3 of 28S rDNA	D3A	GACCCGTCTTGAAACACGGA	360 bp	Al-Banna L et al. 1997
	D3B	TAGGAAGGAACCAGCTACTA		
P. scribneri	PsF7	AGTGTTGCTATAATTCATGTAAAGTTGC	136 bp	Huang and Yan 2017
	PsR7	TGGCCAGATGCGATTCGAGAGGTGT		
P. neglectus	Pn-ITS-F2	GGCACTGTGCGAAGTGTCCG3	234 bp	Yan et al. 2013
	Pn-ITS-R2	TTAACACCTCAGGCGTCATGTAC		
P. neglectus	PNEG-F1	CGCAATGAAAGTGAACAATGTC	144 bp	Yan et al. 2008
	D3B5	AGTTCACCATCTTTCGGGTC		
D2-D3 of 28S rDNA	D2A	ACAAGTACCGTGAGGGAAAGTTG	~750 bp	Subbotin et al. 2006
	D3B	TCGGAAGGAACCAGCTACTA		
ITS of rDNA	rDNA2-V	TTGATTACGTCCCTGCCCTTT	varied	Cherry et al. 1997
	rDNA1	ACGAGCCGAGTGATCCACCG		
COI of mtDNA	JB3	TTTTTTGGGCATCCTGAGGTTTAT	~450 bp	Derycke et al. 2010
	JB4.5	TAAAGAAAGAACATAATGAAAATG		

Table 3.3. PCR amplification conditions with reaction volume and primers used for molecular identification of root-lesion nematodes.

Name of primer	DNA template	Final reaction volume	Amplification conditions	
D3A/D3B and rDNA2-V/rDNA1	1.5 µl	16 µl	94°C 3 minutes	
			94°C 45 seconds	40 cycles
			55°C 1 minute	
			72°C 1 minute	
			72°C 10 minutes	
PsF7/PsR7, Pn-ITS-F2/Pn-ITS-R2, and PNEG-F1/D3B5	1.5 µl	16 µl	94°C 3 minutes	
			94°C 40 seconds	35 cycles
			60°C 50 seconds	
			72°C 1 minute	
			72°C 10 minutes	
D2A/D3B	3 µl	25 µl	94°C 3 minutes	
			94°C 30 seconds	40 cycles
			50°C 1 minute	
			72°C 1 minute	
			72°C 10 minutes	
JB3/JB4.5	1.5 µl	25 µl	94°C 3 minutes	
			94°C 30 seconds	40 cycles
			50°C 30 seconds	
			72°C 1 minute	
			72°C 10 minutes	

After completion of PCR cycles, agarose gel electrophoresis was performed using Owl™ EasyCast™ Wide-Format Horizontal Electrophoresis Systems (Model D3-14 System, Thermo-Fisher Scientific, Pittsburgh, PA, USA) at 100 V for 25 minutes where 3 µl of PCR product was mixed with 3 µl of 2x loading dye and a total of 5 µl of the mixture was loaded in 2 % agarose gel. The gel was visualized under UV light and AlphaImager® Gel Documentation System (Proteinsimple Inc., Santa Clara, CA, USA) was used for documenting banding patterns of PCR products. In the case for direct sequencing, amplified DNA was purified from the remaining PCR product using E.Z.N.A. Cycle Pure Kit (Omega Biotek Inc, Doraville, GA, USA) following the

protocol recommended by the manufacturer and sent for DNA sequencing to GenScript (GenScript, Piscataway, NJ).

Sequence analysis and phylogenetic analysis

The DNA sequences obtained from three genomic regions (D2-D3 of 28S rDNA, ITS of rDNA, and COI of mtDNA) were aligned using the sequence alignment tool, Clustal X under default parameter settings (Thompson et al. 1997). The BLAST tool in National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) was used to compare and identify similarity with the known nematode species sequences previously deposited in the GenBank database. The top species with highest sequence identity were selected as candidate species as proposed by Altschul et al. (1990). The species identity was confirmed after analyzing sequences from three genomic regions. The nucleotide length, percentage of variation (insertions/deletions, transitions and transversions) of intra-species were analyzed for sequence divergence analysis. The phylogenetic trees for the three genomic regions (D2-D3 of 28S rDNA, ITS of rDNA, and COI of mtDNA) were constructed independently using MEGA X version 10.0.5 software (Kumar et al. 2018) to better understand the phylogenetic relationship of these species. The corresponding sequences and outgroup taxa used in phylogenetic tree construction were retrieved from the NCBI nucleotide database. The sequences obtained from our study and additional sequences obtained from NCBI nucleotide database were aligned using CLUSTAL W (Thompson et al. 1994), using default parameters. Finally, the trees were constructed using the Neighbor-Joining method. Bootstrap support for trees was generated with 1000 replicates searches.

Results

Occurrence of plant-parasitic nematodes

During 2017 and 2018 survey period, eighty-eight percent of the samples tested were found positive for plant-parasitic nematodes. There were eight genera of plant-parasitic nematodes detected from corn fields in North Dakota after nematode extraction by sugar centrifugal floatation method. The occurrence frequency of eight genera of plant-parasitic nematodes were shown in Fig. 3.2. Among the eight genera, most prevalent genus was *Helicotylenchus* (spiral nematode), was detected in 50 % of the tested samples with the highest density of 4,125 nematodes/kg of soil. The *Pratylenchus* (root-lesion nematode) was the second most prevalent genus. Of 50 samples collected, 17 samples (34 %) were found positive for root-lesion nematodes. The densities of this nematode varied from 200 to 2,100 nematodes/kg of soil (Table 3.4). The highest density of this nematode detected in Richland County of North Dakota. Other six genera include *Tylenchorhunchus* (stunt nematode; incidence: 22 %; highest density: 1,500 nematodes/kg of soil), *Heterodera* (cyst nematode; 20 %; 2,200), *Paratylenchus* (pin nematode; 12 %; 900), *Aphelenchoides* (foliar nematode; 4 %; 625), *Xiphinema* (dagger nematode; 2 %; 400), and *Paratrichodorus* (stubby root nematode; 2 %; 150).

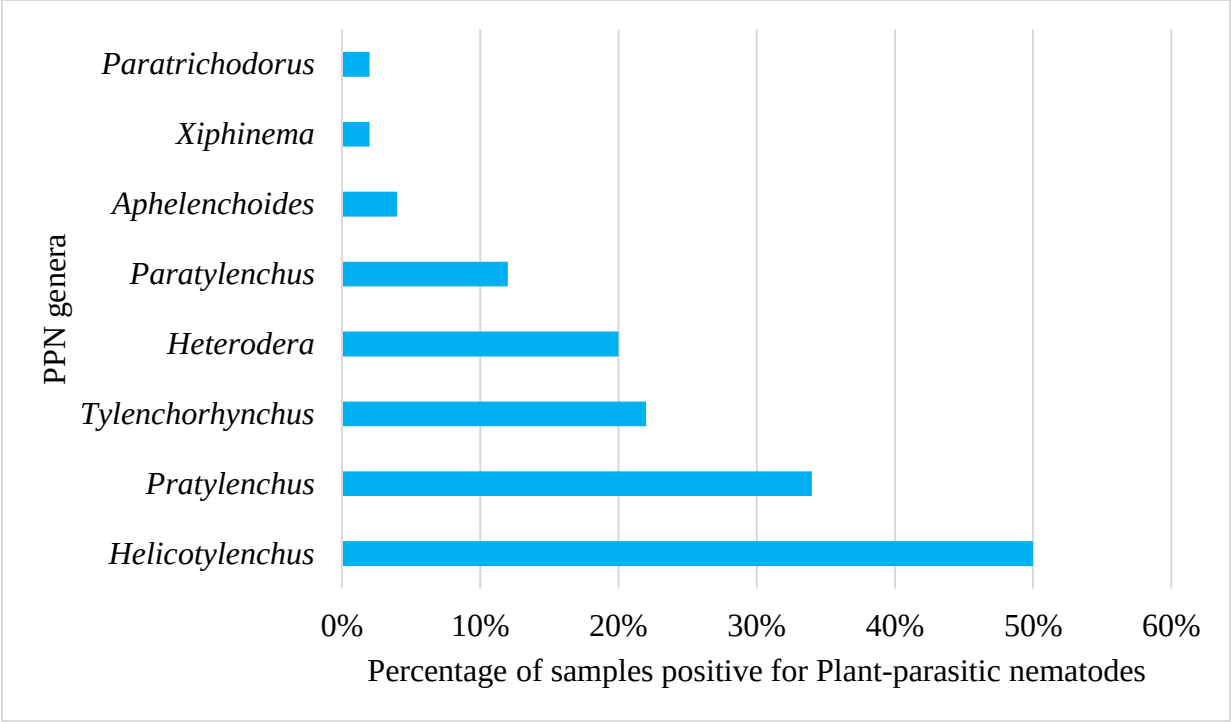
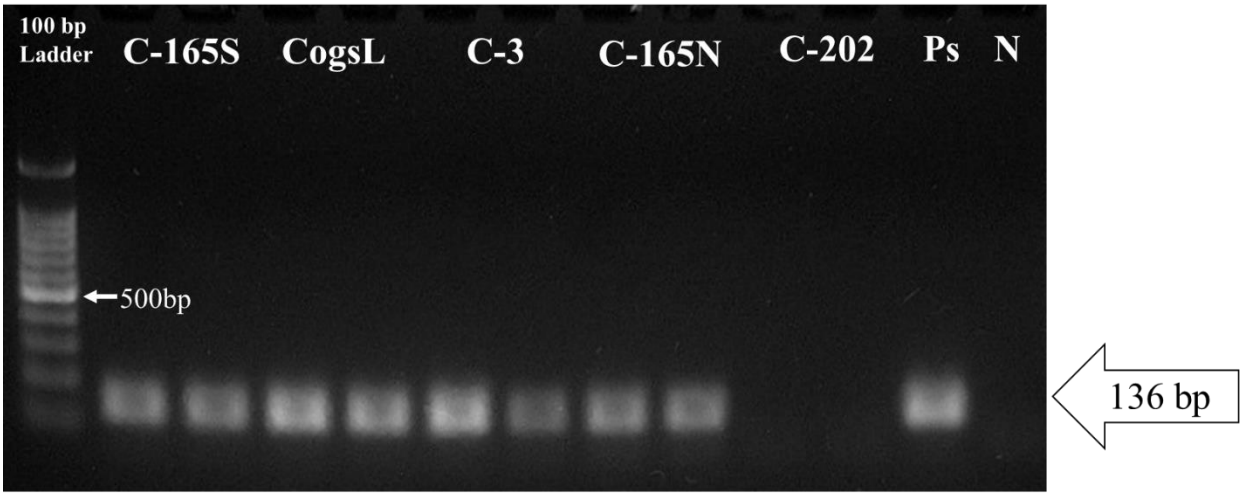


Fig. 3.2. Occurrence frequency of plant-parasitic nematodes (PPN) in 50 soil samples from North Dakota corn fields in 2017 and 2018.

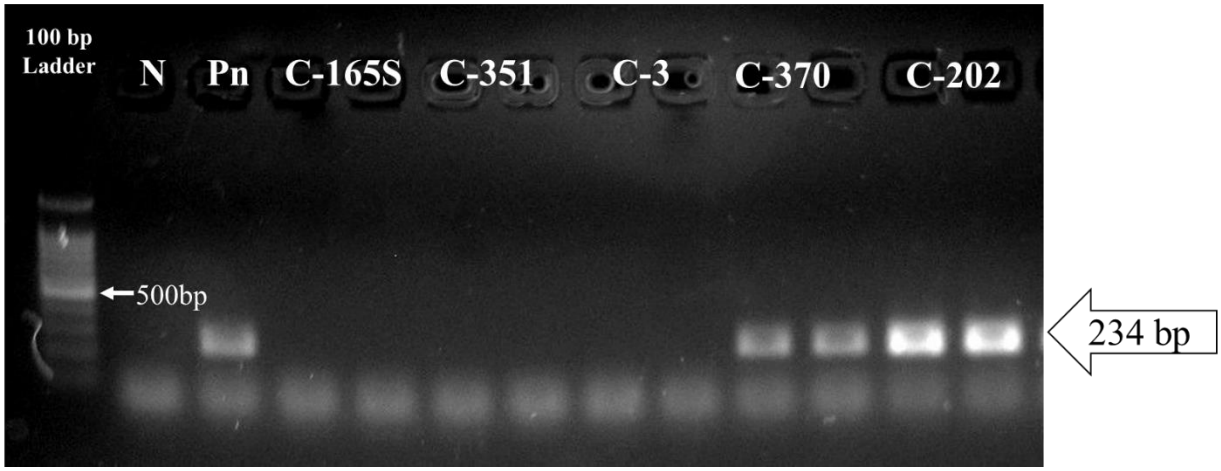
Species identification of root-lesion nematodes by species-specific PCR

Three species-specific primer pairs (PsF7/PsR7, PNEG-F1/D3B5, and Pn-ITS-F2/Pn-ITS-R2) were used for species-specific PCR examination (Table 3.2 and 3.3). Species-specific PCR revealed that six of the samples (37.5 %) were amplified with only *P. scribneri*-specific primers PsF7/PsR7 and did not amplified with *P. neglectus*-specific primers PNEG-F1/D3B5, and Pn-ITS-F2/Pn-ITS-R2. The samples produced approximately 136 bp DNA fragment with *P. scribneri*-specific primers PsF7/PsR7 and identified as *P. scribneri*. Therefore, *P. scribneri* was considered as dominant species in North Dakota corn fields. One sample was amplified with *P. neglectus*-specific primers Pn-ITS-F2/Pn-ITS-R2 and did not amplified with *P. scribneri*-specific primers PsF7/PsR7. The sample produced approximately 234 bp DNA fragment with *P. neglectus*-specific primers Pn-ITS-F2/Pn-ITS-R2 therefore detected as *P. neglectus*. Three samples that had higher densities of root-lesion nematode were amplified with both *P. scribneri*-

specific and *P. neglectus*-specific primers. Thus they were considered as mixed population of *P. neglectus* and *P. scribneri*. Rest of the seven samples did not amplified with *P. scribneri*-specific and *P. neglectus*-specific primers. Some examples of species-specific PCR examination are shown in Fig. 3.3, 3.4, and 3.5.

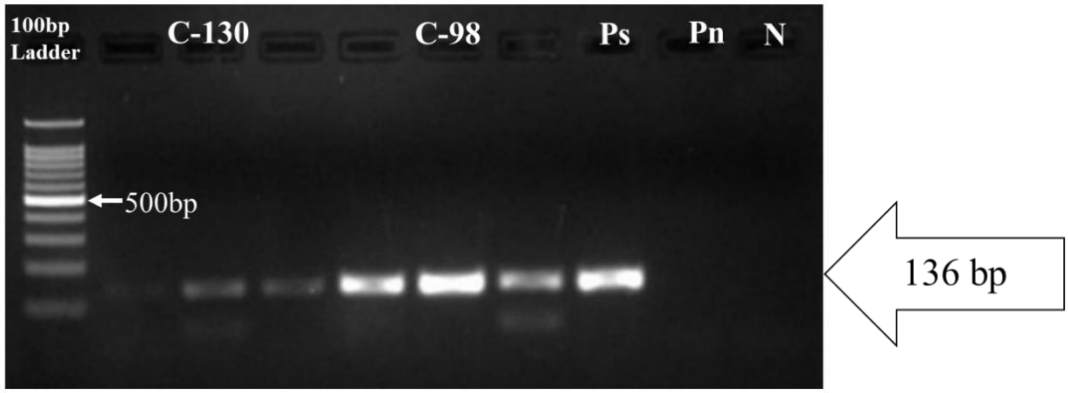


Amplified with *P. scribneri* specific primers

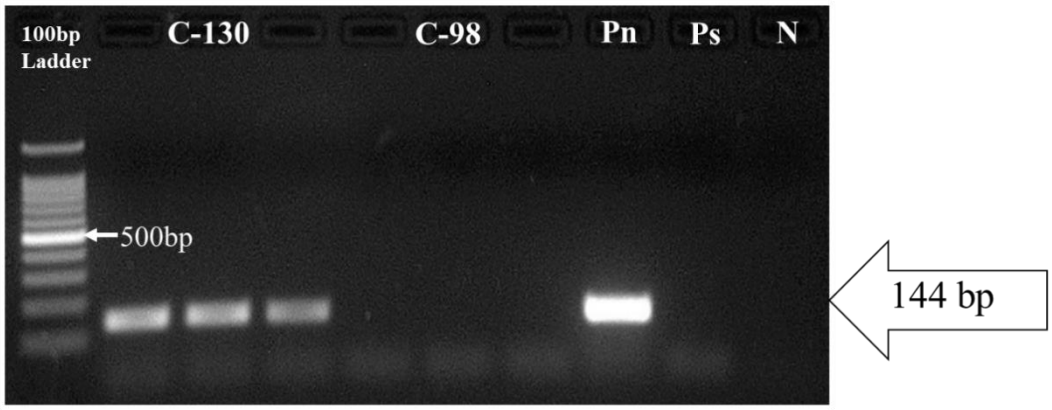


Amplified with *P. neglectus* specific primers

Fig. 3.3. Identification of *Pratylenchus* spp. by species-specific PCR. DNAs were amplified with the *P. scribneri*-specific primers PsF7/PsR7 (Huang and Yan 2017) and *P. neglectus*-specific primers Pn-ITS-F2/Pn-ITS-R2 (Yan et al. 2013). C-165S, CogsL, C-3, C-165N, C-202, C-351, and C-370 indicate field ID (each with 2 replicates), Ps - *P. scribneri* (positive control), Pn - *P. neglectus* (positive control), and N - water (negative control).

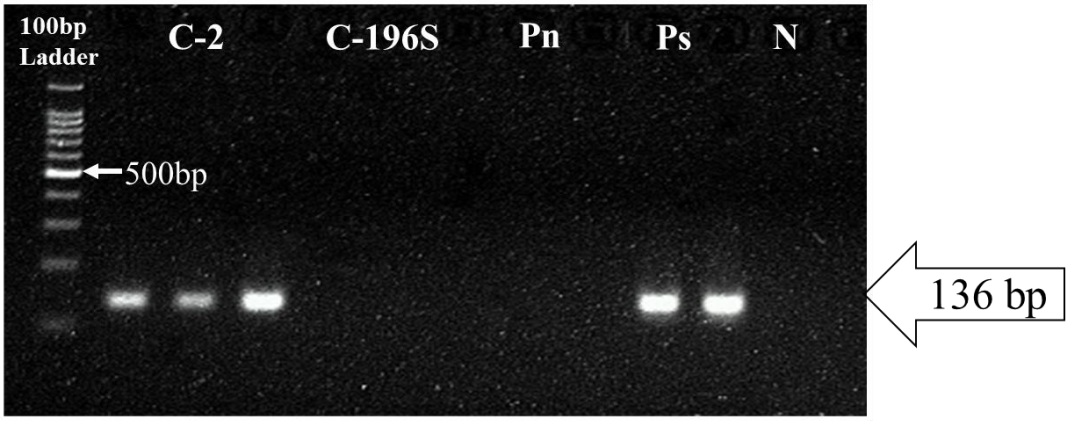


Amplified with *P. scribneri* specific primers

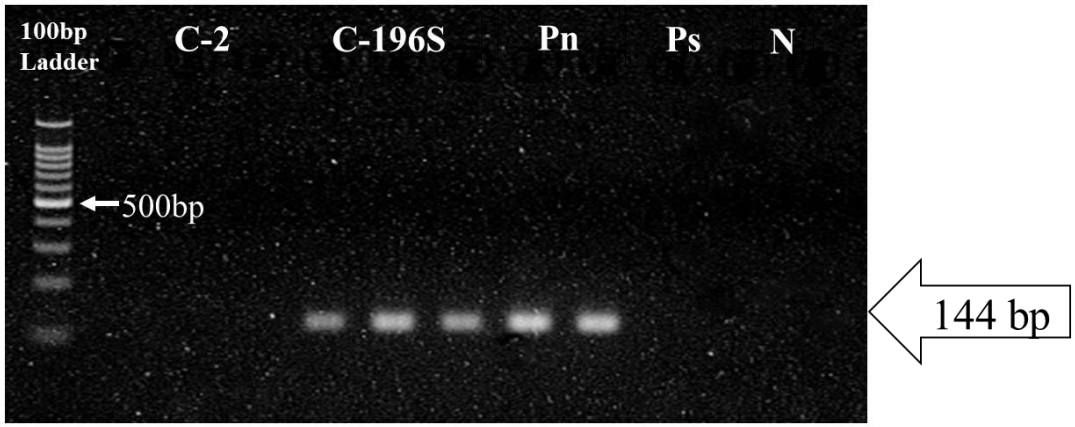


Amplified with *P. neglectus* specific primers

Fig. 3.4. Identification of *Pratylenchus* spp. by species-specific PCR. DNAs were amplified with the *P. scribneri*-specific primers PsF7/PsR7 (Huang and Yan 2017) and *P. neglectus*-specific primers PNEG-F1/D3B5 (Yan et al. 2008). C-130 and C-98 indicate field ID (each with 3 replicates), Ps - *P. scribneri* (positive control), Pn - *P. neglectus* (positive control), and N - water (negative control).



Amplified with *P. scribneri* specific primers



Amplified with *P. neglectus* specific primers

Fig. 3.5. Identification of *Pratylenchus* spp. by species-specific PCR. DNAs were amplified with the *P. scribneri*-specific primers PsF7/PsR7 (Huang and Yan 2017) and *P. neglectus*-specific primers PNEG-F1/D3B5 (Yan et al. 2008). C-2 and C-196S indicate field ID (each with 3 replicates), Pn - *P. neglectus* (positive control, each with 2 replicates), Ps - *P. scribneri* (positive control, each with 2 replicates), and N - water (negative control).

Species identification of root-lesion nematodes by DNA sequencing

The direct DNA sequencing of D2-D3 of 28S rDNA, ITS of rDNA, and COI of mtDNA genomic regions were performed to confirm the species identity obtained by species-specific PCR. The DNA sequencing of D2-D3 expansion segments of 28S rDNA region revealed that eight of the samples showed 98 % to 100 % sequence similarity to different *P. scribneri* populations (KX842628, KX842627, KX842625, MK209593, KY424300, and JX047001), two

of the samples showed 98 % to 99 % sequence similarity to different *P. neglectus* populations (JX261951 and KY468854), four of the samples showed 98 % sequence similarity to new putative *Pratylenchus* sp. ND-2016 isolate HG51 population (KY200665), and three of the samples showed 100 % sequence similarity to new putative *Pratylenchus* sp. ND-2017 population (KX889989) upon sequence BLAST in NCBI.

Moreover, The direct DNA sequencing of ITS of rDNA region revealed that eight of the samples showed 95 % to 100 % sequence similarity to different *P. scribneri* populations (KX842626, KT873860, KX842625, MH729053, and KY424229), two of the samples showed 89 % and 98 % sequence similarity to different *P. neglectus* populations (FR692286 and KY424242), four of the samples showed 98 % to 100 % sequence similarity to new putative *Pratylenchus* sp. ND-2016 isolate HG51 population (KY200666), and two of the samples showed 92 % and 98 % sequence similarity to new putative *Pratylenchus* sp. ND-2017 population (KX889990) upon sequence BLAST in NCBI.

The direct DNA sequencing of third genomic region COI of mtDNA revealed that nine of the samples showed 96 % to 100 % sequence similarity to different *P. scribneri* populations (MH016378, KY424093, KY424091, and KY424092), one of the samples showed 99.73 % sequence similarity to *P. neglectus* population (KY424103), four of the samples showed 93 % to 94 % sequence similarity to new putative *Pratylenchus alleni* population (MK045330), and three of the samples showed 84.6 % sequence similarity to new putative *Pratylenchus* sp. CD1302 population (KU198944) upon sequence BLAST in NCBI.

After considering the results of species-specific PCR and direct DNA sequencing of three genomic regions it was summarized that six of the tested samples were *P. scribneri*, four were

new putative *Pratylenchus* sp. ND-2016 isolate HG51, three were new putative *Pratylenchus* sp. ND-2017, one was *P. neglectus* and three were found to contain a mixed population of *P. neglectus* and *P. scribneri* (Table 3.4).

Table 3.4. Sampling, population densities, and species identity of root-lesion nematodes.

Sl. No.	Sample ID	Sampling Date	Density (/kg of soil)	Species Identity[a]
1	C-130	7/31/2017	1,680	*P. neglectus*+*P. scribneri*
2	Cogswell (CogsL)	9/14/2017	655	*P. scribneri*
3	C-2	8/9/2017	1,100	*P. scribneri*
4	C-98	9/21/2017	700	*P. scribneri*
5	HG-50	9/29/2017	1,033	*P.* sp. ND-2017
6	HG-51	9/8/2017	800	*P.* sp. ND-2016 isolate HG51
7	C-351	5/29/2018	1,500	*P.* sp. ND-2016 isolate HG51
8	C-159	7/12/2018	200	*P.* sp. ND-2017
9	C-366	9/28/2018	2,000	*P.* sp. ND-2017
10	C-370	7/9/2018	2,100	*P. neglectus*+*P. scribneri*
11	C-3	7/12/2018	1,125	*P. scribneri*
12	C-165N	7/12/2018	450	*P. scribneri*
13	C-165S	9/28/2018	300	*P. scribneri*
14	C-202	7/12/2018	300	*P. neglectus*
15	C-196S	7/9/2018	1,500	*P. neglectus*+*P. scribneri*
16	C-151	7/12/2018	450	*P.* sp. ND-2016 isolate HG51
17	C-152	7/12/2018	300	*P.* sp. ND-2016 isolate HG51

[a]Both species-specific PCR and DNA sequencing were used as species identification method for all the samples. The species were identified by *P. scribneri* and *P. neglectus* species-specific PCR and sequencing of D2-D3 of 28S rDNA and/or ITS rDNA and/or COI of mtDNA.

Intra-species genetic diversity of root-lesion nematodes

To determine the intra-species genetic diversity, the sequences of seven populations of *P. scribneri*, four populations of new putative *Pratylenchus* sp. ND-2016 isolate HG51, three populations of new putative *Pratylenchus* sp. ND-2017, and two populations of *P. neglectus* were compared independently by making alignment using the multiple sequence alignment tool, ClustalW under default parameter settings (Thompson et al. 1997) (Fig. 3.6).

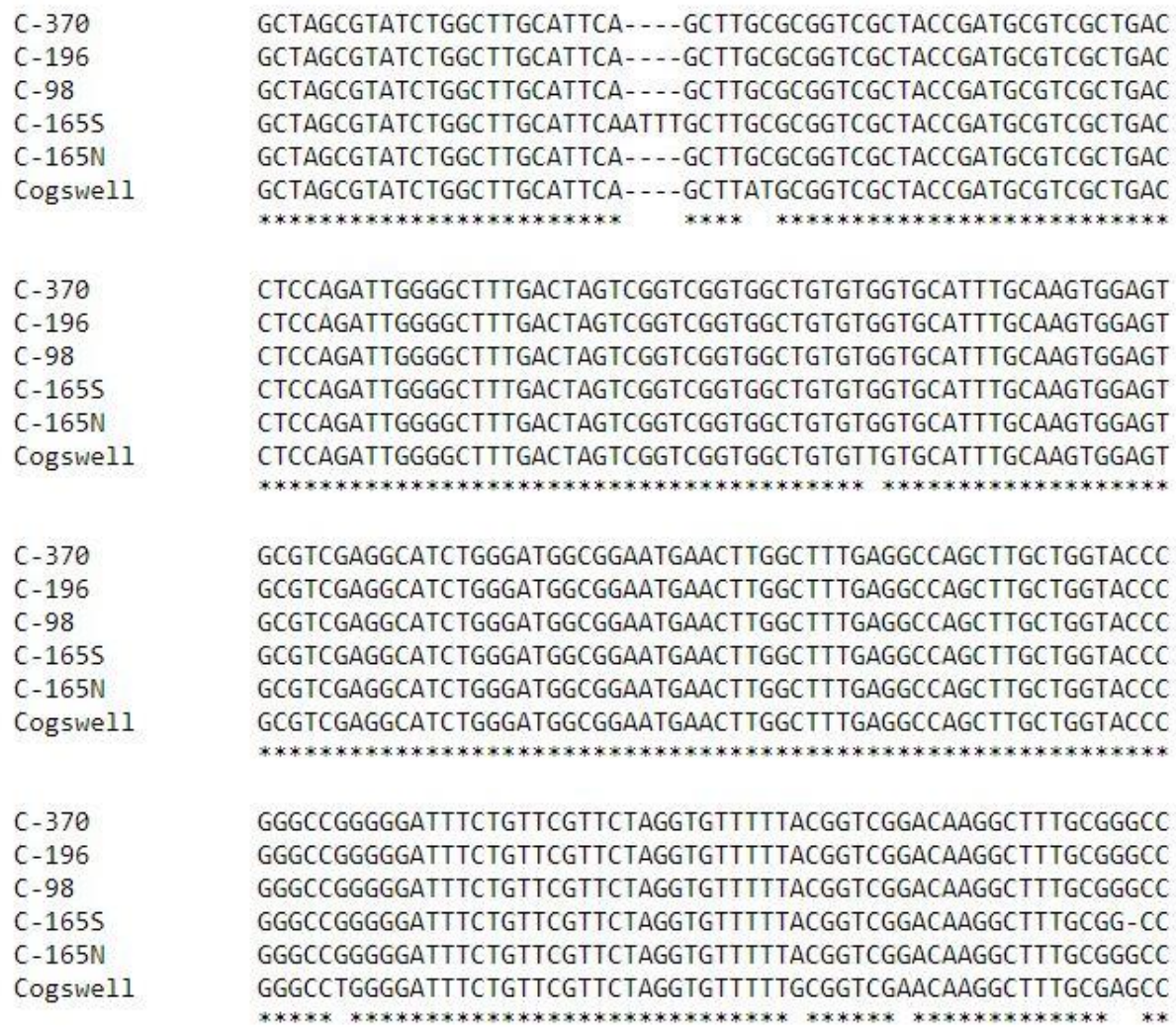


Fig. 3.6. Partial alignment of six sequences of D2-D3 of 28S rDNA of *P. scribneri* amplified by primer set D2A/D3B showing insertions/deletions.

The sequence length of seven populations of *P. scribneri* obtained from D2-D3 of 28S rDNA region ranged from 601 to 689 bp. The variation within species in this region was very low (1.92 %). The sequence length of four populations of new putative *Pratylenchus* sp. ND-2016 isolate HG51 obtained from same region ranged from 659 to 761 bp with a very high intra-species variation (19.77 %). Moreover, the length of nucleotides of two populations of *P. neglectus* obtained from this region ranged from 547 to 665 bp with the highest intra-species variation (24.21 %). The sequence length of three populations of new putative *Pratylenchus* sp.

ND-2017 ranged from 617 to 641 bp where the variation within this species was lower (3.16 %) (Table 3.5).

In case of ITS of rDNA region, the sequence length of seven populations of *P. scribneri* ranged from 529 to 605 bp with high intra-species nucleotide variation (13.59 %). The sequence length of four populations of new putative *Pratylenchus* sp. ND-2016 isolate HG51 ranged from 479 to 570 bp with a lower intra-species nucleotide variation (3.14 %) in this region. The length of nucleotides of two populations of *P. neglectus* obtained from this region ranged from 413 to 417 bp with high intra-species nucleotide variation (11.93 %). The length of nucleotides of two populations of new putative *Pratylenchus* sp. ND-2017 ranged from 578 to 651 bp with a lower intra-species nucleotide variation (2.76 %) (Table 3.5).

In case of COI of mtDNA region, the sequence length of eight populations of *P. scribneri* ranged from 373 to 429 bp with high intra-species nucleotide variation (11.19 %). The sequence length of four populations of *P. alleni* (new putative *Pratylenchus* sp. ND-2016 isolate HG51) ranged from 414 to 444 bp. with very high intra-species nucleotide variation (14.69 %). The sequence length of only one population of *P. neglectus* was 374 bp. The length of nucleotides of three populations of *Pratylenchus* sp. CD1302 (new putative *Pratylenchus* sp. ND-2017) ranged from 377 to 384 bp with the lowest intra-species nucleotide variation (0.52 %) (Table 3.5).

Table 3.5. Percentage of sequence (base-pair) variation in three genomic regions including D2-D3 of 28S rDNA, ITS of rDNA, and COI mtDNA for four species of *Pratylenchus* found in corn fields of North Dakota.

Species ID	Region[a]	Indel[b] (%)	Transition[c] (%)	Transversion[d] (%)	Total variation[e] (%)	Total length[f]
P. scribneri	D2-D3 of 28S rDNA	0.74	1.03	0.14	1.92	677
P. sp. ND-2016 isolate HG51	D2-D3 of 28S rDNA	7.06	10.01	2.7	19.77	779
P. neglectus	D2-D3 of 28S rDNA	22.15	1.79	0.28	24.21	727
P. sp. ND-2017	D2-D3 of 28S rDNA	3.16	0	0	3.16	727
P. scribneri	ITS of rDNA	3.07	8.25	2.27	13.59	618
P. sp. ND-2016 isolate HG51	ITS of rDNA	0.87	1.75	0.52	3.14	572
P. neglectus	ITS of rDNA	2.85	5.99	3.09	11.93	421
P. sp. ND-2017	ITS of rDNA	0.61	1.84	0.31	2.76	652
P. scribneri	COI of mtDNA	2.59	6.51	2.09	11.19	430
P. sp. ND-2016 isolate HG51	COI of mtDNA	11.28	2.98	0.43	14.69	470
P. sp. ND-2017	COI of mtDNA	0	0.52	0	0.52	384

[a]Genomic regions including D2-D3 of 28S rDNA, ITS of rDNA, and COI of mtDNA.
[b]Insertion/deletion mutation (Insertions are mutations in which extra base pairs are inserted into a new place in the DNA and deletions are mutations in which a section of DNA is lost, or deleted).
[c]Transition mutation (A point mutation in DNA involving substitution of one base pair for another by replacement of one purine by another purine and of one pyrimidine by another pyrimidine but without change in the purine-pyrimidine orientation).
[d]Transversion mutation (a point mutation in DNA, where a single purine is changed for a pyrimidine, or vice versa).
[e]Sum of variation of insertions/deletions, transition, and transversion in one genomic region of one species.
[f]Total sequence length (base-pair).

Phylogenetic relationship of root-lesion nematodes

The neighbor-joining tree was constructed independently for D2-D3 of 28S rDNA, ITS of rDNA, and COI of mtDNA genomic regions. The neighbor-joining tree obtained from D2-D3 of 28S rDNA region revealed that, seven populations of *P. scribneri* clustered together except

one. The four *Pratylenchus* sp. ND-2016 isolate HG51 populations were divided into two sub-groups. The three *Pratylenchus* sp. ND-2017 populations clustered together and the two *P. neglectus* populations were divided into two sub-groups (Fig. 3.7).

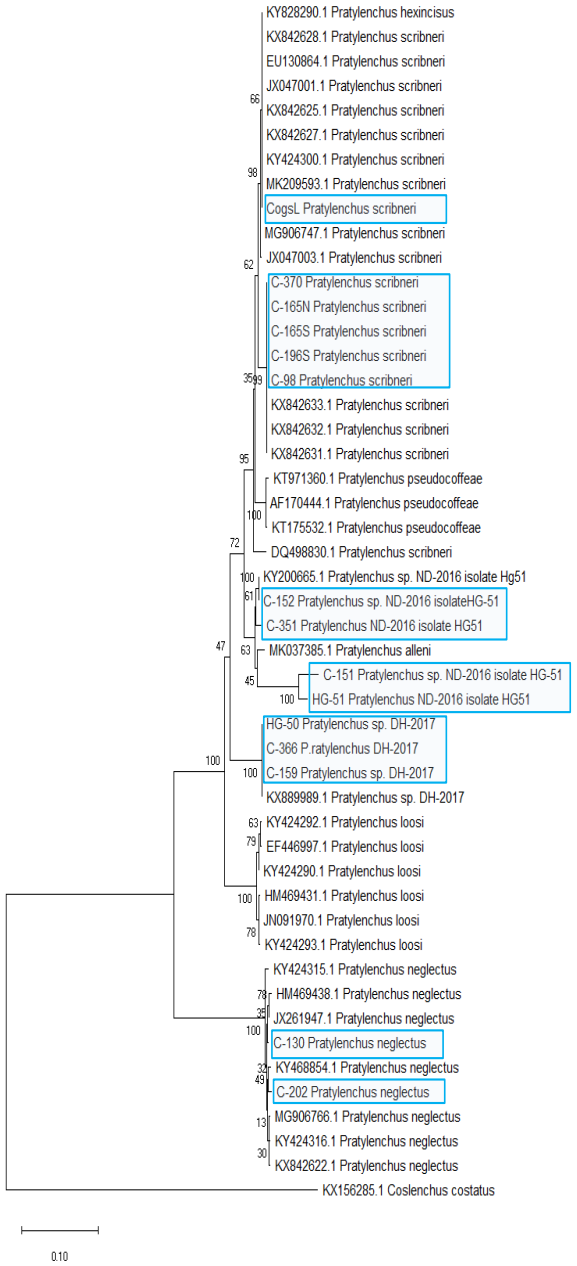


Fig. 3.7. Phylogenetic relationships of different *Pratylenchus* spp. found in North Dakota corn fields from D2-D3 of 28S rDNA region with other *Pratylenchus* species based on Neighbor-joining tree analysis using MEGA X. The sequences from our study are highlighted with blue boxes. Support values are given above branches. The D2-D3 of 28S rDNA sequence of *Coslenchus costatus* was used as an outgroup.

The neighbor-joining tree obtained from ITS of rDNA region revealed that, all six populations of *P. scribneri* clustered together into other *P. scribneri* populations. The four *Pratylenchus* sp. ND-2016 isolate HG51 populations were divided into three sub-groups and were clustered into closely related species *P. alleni* and *P. loosi*. The two *Pratylenchus* sp. ND-2017 populations clustered together separately and the two *P. neglectus* populations were clustered with other *P. neglectus* populations into two sub-groups (Fig. 3.8).

Fig. 3.8. Phylogenetic relationships of different *Pratylenchus* spp. found in North Dakota corn fields from ITS of rDNA region with other *Pratylenchus* species based on Neighbor-joining tree analysis using MEGA X. The sequences from our study are highlighted with blue boxes. Support values are given above branches. The ITS of rDNA sequence of *Caenorhabditis elegans* was used as an outgroup.

The neighbor-joining tree obtained from COI of mtDNA genomic regions showed that eight populations of *P. scribneri* clustered with other *P. scribneri* populations and *P. hexincisus* populations and divided into two sub groups. Four populations of *P. alleni* (*Pratylenchus* sp. ND-2016 isolate HG51) were clustered together with other *P. alleni* populations. Three populations of *Pratylenchus* sp. CD1302 (*Pratylenchus* sp. ND-2017) were clustered together. Single population of *P. neglectus* were clustered with other *P. neglectus* populations (Fig. 3.9).

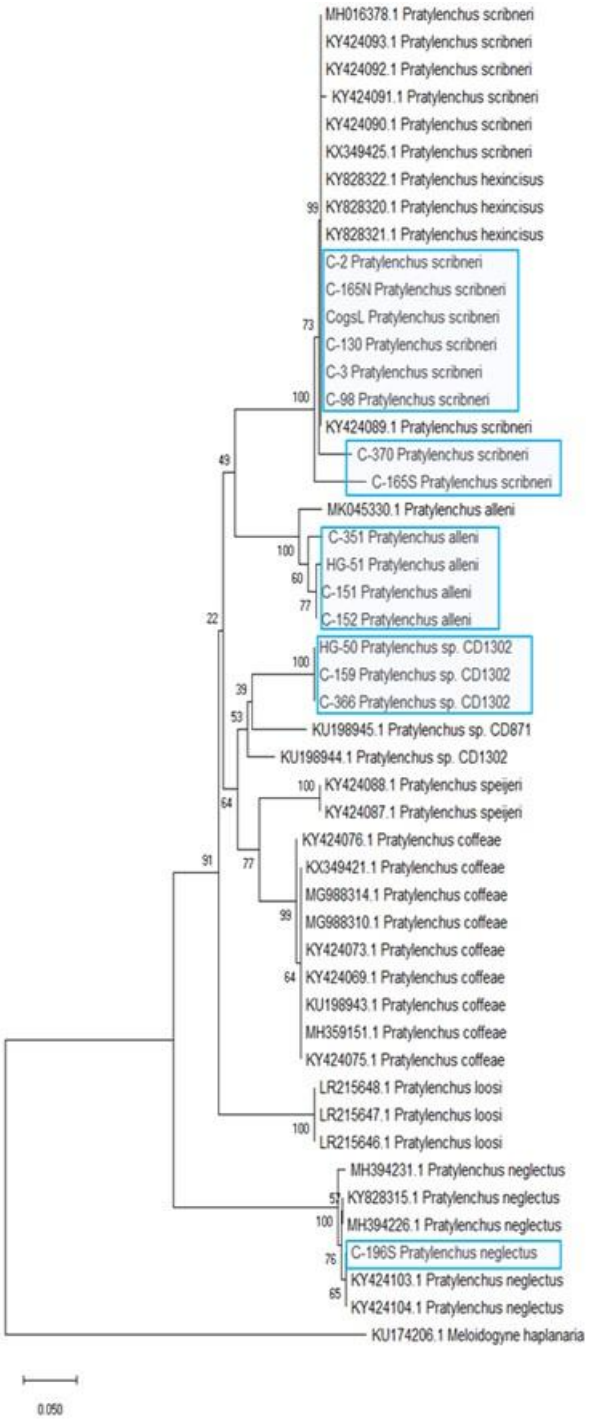


Fig. 3.9. Phylogenetic relationships of different *Pratylenchus* spp. found in North Dakota corn fields from partial cytochrome oxidase subunit I (COI) gene region with other *Pratylenchus* species based on Neighbor-joining tree analysis using MEGA X. The sequences from our study are highlighted with blue boxes. Support values are given above branches. The COI gene region of *Meloidogyne haplanaria* was used as an outgroup.

In the three phylogenetic trees, four species were clustered in four separate clades. Among the four species, *P. scribneri* and *Pratylenchus* sp. ND-2016 isolate HG51 were more closely associated with each other. The *Pratylenchus* sp. ND-2017 were also closely related to the *Pratylenchus* sp. ND-2016 isolate HG51. However, *P. neglectus* was not closely associated with other three species.

Discussion

This study is the first report of molecular characterization and phylogenetic relationships analysis of root-lesion nematodes in corn fields of North Dakota. This study evaluated the presence of root-lesion nematodes in corn fields of North Dakota and confirmed their species identity. The sequences of D2-D3 of 28S rDNA, ITS of rDNA, and COI of mtDNA genomic regions were used to characterize root-lesion nematode species and their phylogenetic relationships were established. The similarity in morphological features, the presence of morphotypes, along with a higher level of intraspecific and interspecific genetic variation in *Pratylenchus* spp., make the identification and separation of these species complicated. However, DNA sequencing and phylogenetic analyses using D2-D3 of 28S rDNA, ITS of rDNA, and COI of mtDNA regions have eased the analysis of variation among species including closely related species. According to Subbotin et al. (2008), the D2-D3 region is an efficient target for analyzing higher degrees of interspecific genetic variation. However, ITS region was reported to be a useful region in root-lesion nematode identification in spite of having high nucleotide variability (De Luca et al. 2011). COI of mtDNA regions was successfully used as molecular marker in specific detection of new *Pratylenchus* species (Nguyen et al. 2019). Therefore, the variation in sequences in this three regions was used to identify the nematodes at species level.

During 2017 and 2018, 17 of the samples (34 %) were found positive for root-lesion nematodes. Therefore, it could be considered as the second most prevalent plant-parasitic nematodes after spiral nematodes in North Dakota corn fields. According to Chowdhury et al. (2019), approximately 20 % of the samples collected from North Dakota corn fields were positive to root-lesion nematodes in 2015 and 2016. Therefore, this nematode has a potential to impact corn yield. Above finding have confirmed our results and it could be said that, root-lesion nematodes are present in North Dakota corn fields. Moreover, Smiley et al. (2005) described that the economic damage thresholds for *P. neglectus* were 2,000 nematodes/ kg soil in Oregon and Washington states. The densities of root-lesion nematodes obtained from our study was as high as 2,100 nematodes/ kg of soil which was higher than the damage thresholds described by Smiley et al. (2005). Chowdhury et al. (2019) also found highest root-lesion nematodes densities 2,125 nematodes/ kg of soil which exceeded the damage thresholds of *P. neglectus*. In Illinois, 500 *Pratylenchus*/ kg of soil on corn was considered to be of significant risk (Niblack 2009). Our study also revealed that, in North Dakota, out of 17 corn fields positive for *Pratylenchus* spp., 11 had *Pratylenchus* population density above the range mentioned by Niblack (2009). Therefore, root-lesion nematodes could be considered as an important constrain in agriculture in our region as a consequence the proper identification and characterization of root-lesion nematode is necessary.

The present study identified a total of four *Pratylenchus* species as *P. scribneri*, *P. neglectus*, *Pratylenchus* sp. ND-2016 isolate HG51, and *Pratylenchus* sp. ND-2017 by species-specific PCR and direct DNA sequencing. These four species were previously reported from other crop fields of North Dakota which supports our results (Yan et al. 2016a, 2016b, 2017a, 2017b). Species-specific PCR has been widely used to detect different *Pratylenchus* spp. such as

P. scribneri, *P. coffeae*, *P. loosi*, *P. neglectus*, *P. brachyurus*, *P. crenatus*, *P. zeae*, *P. thornei*, and *P. vulnus* (Huang and Yan 2017; Al-Banna et al. 2004; Machado et al. 2007; Yan et al. 2008; Yan et al. 2013; Momota et al. 1998; Waeyenberge et al. 2009; Yan et al. 2012). According to Yu et al. (2012), the PCR based method is a very powerful tool to detect, distinguish and identify root-lesion nematodes and widely used to identify nematodes with equivocal morphology. The *P. scribneri*-specific primers PsF7/PsR7 (Huang and Yan 2017) used in our study successfully separated *P. scribneri* from other *Pratylenchus* spp. as well as other plant-parasitic nematodes. Similarly, *P. neglectus*-specific primer sets PNEG-F1/D3B5 (Yan et al. 2008) and Pn-ITS-F2/Pn-ITS-R2 (Yan et al. 2013) used in our study were able to separate this species from *P. scribneri* and *P. penetrans* clearly. These three primer sets distinctly identified *P. scribneri* and *P. neglectus* in our study which was further confirmed by direct DNA sequencing.

The amplified sequences of all *Pratylenchus* spp. obtained from direct DNA sequencing of D2-D3 of 28S rDNA region varied both in amplicon length and nucleotide sequences. Approximately 700 bp sequence length were obtained from amplification of this region. The two *P. neglectus* populations showed high insertion-deletion variation (22.15 %) thus they were present in the separate clades along with other *P. neglectus* populations from different geographic regions in the phylogenetic tree. The four *Pratylenchus* sp. ND-2016 isolate HG51 populations were present in two separate clades in the phylogenetic tree. This was due to high variation found in insertion-deletion mutation (7.06 %), transition mutation (10.01 %) and transversion mutation (2.7 %) among the four populations. The total nucleotide variation of six *P. scribneri* populations and three *Pratylenchus* sp. ND-2017 populations were very low (1.92 % and 3.16 %, respectively). These results conform to the phylogenetic tree where *P. scribneri* populations and *Pratylenchus* sp. ND-2017 populations were present independently in single

clade (Table 3.5) (Fig. 3.7). Subbotin et al. (2008) amplified D2-D3 of 28S rDNA region of 31 different populations of *Pratylenchus* spp. and constructed phylogenetic tree which revealed that *Pratylenchus* spp. were divided into at least six distinct major clades. Therefore, it can be said that this region is a good marker for phylogenetic analysis of *Pratylenchus* spp.

The amplified sequences of all *Pratylenchus* spp. obtained from ITS of rDNA region varied both in amplicon length and nucleotide sequences. Approximately 580 bp sequence length were obtained from amplification of this region. The *P. scribneri* populations were present in single clade in the phylogenetic tree constructed from ITS of rDNA region despite of having high intra-species variation (13.59 %). All the *P. scribneri* populations in our study were present along with other *P. scribneri* populations from different geographic regions in the same clade. This result indicates that the *P. scribneri* populations from North Dakota might be closely related to other populations from different geographic regions. The four *Pratylenchus* sp. ND-2016 isolate HG51 populations were present in three separate clades in the phylogenetic tree. It can be said that *P. scribneri*, *P. alleni*, and *P. loosi* are the closely related species of *Pratylenchus* sp. ND-2016 isolate HG51. The total nucleotide variation of *Pratylenchus* sp. ND-2017 populations in our study were very low (2.76 %). In the phylogenetic tree, the two *Pratylenchus* sp. ND-2017 population were clustered in a single clade. The two *P. neglectus* populations showed high insertion-deletion variation (11.93 %) thus they were not present in the same clade in the phylogenetic tree (Table 3.5) (Fig. 3.8).

Different species of root-lesion nematodes were reported to have intraspecies nucleotide variation (Waeyenberge et al. 2000, 2009; De Luca et al. 2011). The intraspecies diversity of ITS region sequences was observed in *P. coffeae* (Duncan et al. 1999; Waeyenberge et al. 2000), *P. parazeae* (Wang et al. 2015), *P. agilis*, *P. brachyurus*, and *P. penetrans* (Waeyenberge et al.

2009). The ITS sequences obtained in our study also showed sequence variation. According to Hall (1999), presence of multiple and highly divergent copies of the ITS region might be responsible for this heterogeneity, as suggested for *Meloidogyne* sp.

The amplified sequences of all *Pratylenchus* spp. obtained from COI of mtDNA region varied both in amplicon length and nucleotide sequences. Approximately 400 bp sequence length were obtained from amplification of this region. The *P. scribneri* populations were present in three different clades in the phylogenetic tree constructed from COI of mtDNA region because of having high intra-species variation (11.19 %). All the *P. scribneri* populations in our study were clustered with other *P. scribneri* and *P. hexincisus* populations from different geographic regions. This result indicates that the *P. scribneri* populations from North Dakota might be closely related to other *P. scribneri* populations from different geographic regions. The COI of mtDNA region sequence of new *Pratylenchus* species reported from North Dakota (Yan et al. 2017a) was not available in the GenBank. Therefore, the four *Pratylenchus* sp. ND-2016 isolate HG51 populations appeared as *P. alleni* in the phylogenetic tree and were present in two separate clades. They also had high intra-species variation (14.69 %). Moreover, in case of *Pratylenchus* sp. ND-2017 populations, due to absence of COI of mtDNA region sequence of new *Pratylenchus* species reported from North Dakota (Yan et al. 2017b) in the GenBank, this species identity was appeared as *Pratylenchus* sp. CD1302. They showed lowest intra-species variation (0.52 %) thus clustered in a single clade in the phylogenetic tree (Table 3.5) (Fig. 3.9).

The three regions used in our study to characterize *Pratylenchus* spp. has been previously used by Palomares-Rius et al. (2014) in characterization and evolutionary analysis of *Pratylenchus oleae*. Subbotin et al. (2008) demonstrated the phylogenetic framework for the genus *Pratylenchus* from D2-D3 of 28S rRNA and 18S rRNA genes. They used 31 populations

of 13 *Pratylenchus* spp. which were clustered into six distinct major clades in phylogenetic trees. Moreover, Palomares-Rius et al. (2014) constructed a phylogenetic tree from D2-D3 of 28S rRNA region of *Pratylenchus* spp. which was divide into seven major clades. In both of the study, *P. scribneri* belonged to the first major clade together with closely related species *P. hexincisus*. Moreover, *P. neglectus* belonged to the fifth major clade without any closely related species. The phylogenetic trees obtained from our study showed similarity with these finding where *P. scribneri* situated at the top of the tree together with closely related species *P. hexincisus. P. neglectus* was situated at the bottom of the tree without any closely related species (Fig. 3.7, 3.8, and 3.9). Taheri et al. (2013) amplified D2-D3 expansion segments of the 28S rRNA genes of 13 *Pratylenchus* spp. in which all *P. neglectus* populations were clustered together in a clade. *P. scribneri* was situated together *P. pseudocoffeae* and situated distantly from *P. neglectus*. Our study also showed congruence with these pattern in the phylogenetic trees (Fig. 3.7, 3.8, and 3.9).

In our study, two new putative *Pratylenchus* species has been characterized from ITS, D2-D3 and COI regions: *Pratylenchus* sp. ND-2016 isolate HG51 and *Pratylenchus* sp. ND-2017. Similarly, these regions were used to characterize new *Pratylenchus* species reported from different countries (Kim et al. 2016; Palomares-Rius et al. 2014; Troccoli et al. 2008; Nguyen et al. 2017, 2019). Moreover, we determined the relationship of these two new putative *Pratylenchus* spp. with other *Pratylenchus* species. Our study revealed that *Pratylenchus* sp. ND-2016 isolate HG51 was closely related to *P. alleni*, *P. loosi*, and *P. scribneri*. *Pratylenchus* sp. ND-2017 was closely associated with *Pratylenchus* sp. ND-2016 isolate HG51 (Fig. 3.7, 3.8, and 3.9). Palomares-Rius et al. (2014) revealed that *P. oleae* n. sp. was closely related to *P. dunensis*,

P. penetrans, and *P. pinguicaudatus.* Nguyen et al. (2017) revealed that a new *Pratylenchus* species, *Pratylenchus haiduongensis* had close relation with *P. parazeae, P. zeae*, and *P. bhattii*.

Subbotin et al. (2008) suggested that the shorter length of the D2-D3 region facilitates its amplification and is a better target for higher degrees of interspecific genetic variation analysis. According to Powers et al. (1997), the ITS region could be considered as useful marker in nematode diagnoses because of the versatility, specificity, and ease of experimental manipulation of this region. De Luca et al. (2011) also revealed that detection of *Pratylenchus* spp. by ITS sequence comparison and analysis is feasible where they characterized eighteen *Pratylenchus* spp. In our study, the sequences obtained from D2-D3 and ITS regions had longer amplicon length compared to the sequences obtained from COI region. Therefore, these two regions serve as useful marker in the identification and characterization of complex genus *Pratylenchus*. However, the COI region also had significant contribution to confirm the species identity.

The phylogenetic relationship among sequences were established using Neighbor-Joining method which allows tree reconstruction when variation exists in nucleotide bases (De Luca et al. 2004; Tan 2012). This method was previously used in phylogenetic relationship analysis of *Pratylenchus* spp. (De Luca et al. 2004, 2011). The outgroup taxa used in our phylogenetic tree from D2-D3 and COI regions were previously used by Subbotin et al. (2008) and Nguyen et al. (2017), respectively. The outgroup taxa used in our phylogenetic tree from ITS region was selected to determine the effect of outgroups in tree reconstruction.

To summarize the results obtained from three phylogenetic trees, four species were clustered in four separate clades indicating the divergence among species. Among the four species, *P. scribneri* and *Pratylenchus* sp. ND-2016 isolate HG51 populations were more closely associated with each other. The *Pratylenchus* sp. ND-2017 populations were also closely related

to the *Pratylenchus* sp. ND-2016 isolate HG51 populations. However, *P. neglectus* was not closely associated with other three species (Fig. 3.7, 3.8, and 3.9).

In conclusion, we revealed the molecular data of 17 populations of four *Pratylenchus* spp. including two new *Pratylenchus* spp. reported from North Dakota for the first time. Our study validated that these four species were clustered in four distinct clades indicating their individuality. Our study also demonstrated that despite of close relationship of the two new *Pratylenchus* spp. in phylogenetic analysis, they were found to be genetically distinct species in DNA sequencing analysis. The precise species identification and genetic variability analysis within species in corn fields of North Dakota is the crucial step in root-lesion nematode management. This will provide breeders useful information to develop effective management strategies. The extracted knowledge from this study is also useful to understand the relationship of these species with other *Pratylenchus* species.

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CHAPTER 4. EVALUATION OF CORN HYBRIDS FOR RESISTANCE TO THREE ROOT-LESION NEMATODE SPECIES, *PRATYLENCHUS SCRIBNERI, P. NEGLECTUS,* AND NEW PUTATIVE *PRATYLENCHUS* SP. FROM NORTH DAKOTA

Abstract

Root-lesion nematodes (RLN), *Pratylenchus* spp., are third major plant-parasitic nematodes which cause significant yield losses in corn. Nematode surveys conducted by the North Dakota State University revealed that *Pratylenchus scribneri, P. neglectus,* and new putative *Pratylenchus* sp. ND-2017 are present in North Dakota (ND) corn fields. Genetic resistance is the best environmentally friendly approach among sustainable management strategies. However, the resistance of corn hybrids in ND to these three RLN species has not been reported. The objective of this research was to evaluate ten corn hybrids used in ND for resistance against *P. scribneri, P. neglectus,* and new putative *Pratylenchus* sp. ND-2017. To achieve the objective, ten corn hybrids in ND accompanying one non-planted control each with five replications were used to set up the greenhouse experiments. The soil naturally infested with specific RLN species was used for each experiment. All three experiments were repeated to obtain a more precise result. The reproductive factor (RF) was calculated from the final population divided by the initial population after 15 weeks from planting. For resistance screening of three RLN species, the average RF of each hybrid was calculated from the mean RF of the first and second trials. The average RF of each hybrid was compared to the hybrid with the highest average RF and used to classify corn hybrids for resistance. *P. scribneri* reproduced least on L-2916 VT2PRO, 1392 VT2P, 4913 VT2RIB, GX89 VT2P, and LR-9487 VT2RIB thus considered as moderately resistant. DK 43-48 RIB, PFS74K89, and DKC 44-13 were moderately

susceptible and best reproduction found in X5B-8801 and DK 43-46 thus grouped as susceptible to *P. scribneri*. The hybrids moderately resistant to *P. neglectus* were 1392 VT2P, PFS74K89, and 4913 VT2RIB. L-2916 VT2PRO, GX89 VT2P, and X5B-8801 were grouped as moderately susceptible hybrids to *P. neglectus*. Corn hybrids susceptible to *P. neglectus* were DK 43-48 RIB, DKC 44-13, DK 43-46, and LR-9487 VT2RIB. Moreover, the new putative *Pratylenchus* sp. ND-2017 reproduced least on corn hybrids DK 43-48 RIB, 1392 VT2P, and PFS74K89 thus considered as moderately resistant. Corn hybrids LR-9487 VT2RIB, 4913 VT2RIB, and DK 43-46 were moderately susceptible and GX89 VT2P, X5B-8801, DKC 44-13, and L-2916 VT2PRO were susceptible to new putative *Pratylenchus* sp. ND-2017. The comparison of host ranking of three RLN species revealed that 1392 VT2P was moderately resistant to three RLN species and PFS74K89 and 4913 VT2RIB were moderately resistant to two of the three RLN species. X5B-8801, DK 43-46, and DKC 44-13 were found susceptible to two of the three RLN species. The knowledge from this study will be useful to accelerate the selection of suitable hybrids for growers and to develop effective and specific nematode management strategies.

Keywords: *Pratylenchus scribneri*, *P. neglectus*, new *Pratylenchus* sp. ND-2017, corn hybrids, reproductive factors, host ranking.

Introduction

Corn (*Zea mays* L.) is the third most important cereal crops in the world after wheat and rice and known as "King of grain crops" (Tollenaar and Dwyer 1999). The U.S. is the major producer of corn in the world, with crop value for corn $51.45 billion (USDA-NASS 2018). It is considered as one of the most important cereal crops used in human consumption, animal feeding, starch industry and oil production. Its importance also increases for its high content of carbohydrates, fats, proteins, some important vitamins and minerals (Prasanna et al. 2001). The

popularity of corn expended with the use of corn as feedstock for ethanol, a fuel additive for vehicles (USDA-ERS 2013). As a result, the corn producing areas are expanding with time. In North Dakota, the corn producing areas has nearly doubled in the last three decades. With the expansion of corn production, the concerns on diseases of corn are expanding. Plant-parasitic nematodes are one of the detrimental pathogens of corn which is threatening to the economy. Norton (1983) reported that about 120 species of plant-parasitic nematodes are able to infect corn worldwide of which more than 60 species are constraint of corn production in North America. The nematode survey from North Dakota State University revealed that plant-parasitic nematodes are prevalent in North Dakota corn fields.

Root-lesion nematodes (*Pratylenchus* spp.) are one of the major nematode pests in corn, distributed worldwide and cause significant yield losses in corn (Norton 1983, 1984; Castillo and Vovlas 2007). The fibrous root-system of corn is favorable for the growth and reproduction of *Pratylenchus* spp. (Norton 1983). De Waele et al. (1988) showed that corn roots influence *Pratylenchus* spp. egg hatch as well. Another interesting character of *Pratylenchus* spp. is that depending upon species, they can reproduce sexually and/or asexually by parthenogenesis (Agrios 2005). Furthermore, Norton (1983) reported that per gram dry root of an infected plant could contain as high as 10,000 to 40,000 *Pratylenchus* spp. which is menacing to corn production. The damage threshold estimated in Michigan for *Pratylenchus* spp. was 250 per gram of dry root on corn whereas in Midwest, the average *Pratylenchus* spp. population has been estimated to be around 1,000 per gram of dry root (Batista da Silva 2013). The population densities of *Pratylenchus* spp. have a positive correlation with yield losses of corn (McSorley and Dickson 1989; Tarte 1971). Out of more than 80 well-known species of *Pratylenchus* worldwide, 27 have been present in North America, and at least 5 of them have caused

significant damage to corn: *P. penetrans*, *P. hexincisus*, *P. scribneri*, *P. brachyurus*, and *P. zeae* (Castillo and Vovlas 2007; Windham and Edwards 1999). The first three of these five are the most damaging nematode pests in the Midwestern Corn belt creating estimated yield losses of 10 % to 26 % (Castillo and Vovlas 2007; Duncan and Moens 2013, Windham and Edwards 1999). In South Dakota, yield losses of corn due to *P. hexincisus* averaged 9.5 bushels/acre and *P. scribneri* averaged 4 bushels/acre (Smolik and Evenson 1987).

Nematode surveys conducted by the nematology group of North Dakota State University revealed that root-lesion nematodes are present in North Dakota corn fields with 20 % samples positive in 2015 and 2016 (Chowdhury et al. 2019). Moreover, the surveys from potato, wheat, and soybean fields of North Dakota revealed the species name of these root-lesion nematodes as *Pratylenchus scribneri*, *P. neglectus*, and a new putative *Pratylenchus* spp. (new *Pratylenchus* sp. ND-2017), respectively (Yan et al. 2016a; Yan et al. 2016b; Yan et al. 2017).

Pratylenchus scribneri is one of the most economically important species in Midwestern Corn Belt with a wide host range including corn, barley, soybean, potato, sugar beet, broccoli, strawberry, onion, tomato, and peach (Castillo and Vovlas 2007). They reproduce by parthenogenesis as the adult male is absent in the life cycle (Roman and Hirschmann 1969). According to Waudo (1984) the reduction in root volume and weight and development of dark discrete lesions correlated with the increase in *P. scribneri* population. Thus they are responsible for great economic loss in corn (Smolik and Evenson 1987). *P. scribneri* is very common in Midwestern Corn Belt (Waudo and Norton 1983).

Pratylenchus neglectus is another economically important species in North America. Their wide host range includes corn, wheat, chickpea, vetch, barley, oat, alfalfa, sorghum, potato, peach, pear, apple, canola, annual medic, and wheat grass (Taylor 2000). It reproduces by

parthenogenesis as the adult male is rare or absent (Smiley 2010). The economic damage thresholds for *P. neglectus* are reported at 2,000 nematodes/kg dry soil in wheat in Oregon and Washington states (Smiley et al. 2005). It causes significant yield losses in wheat and other host crops in Israel, Mexico, and Oregon and Washington State in North America (Smiley 2010).

The new *Pratylenchus* sp. ND-2017 was identified as a new root-lesion nematode species based on morphometric measurements analysis and sequencing analysis of 28S D2-D3 and ITS rDNA genomic regions (Yan et al. 2017). This species was detected from a soybean field in Walcott, Richland County, North Dakota with a density of 2,000 nematodes/ kg of soil. One greenhouse experiment was conducted using naturally infested soil with an initial population of 350 nematodes/ kg of soil and was planted with soybean cultivar Barnes with four replicates. The reproductive factor was 5.02 after 15 weeks of planting which represents a good association of this species with soybean cultivar Barnes (Yan et al. 2017).

Although the common ways to manage *Pratylenchus* spp. are crop rotation and cultural practices, several nematicides such as terbufos and carbofuran were used by the growers in reducing *P. scribneri* (Todd and Oakley 1995). A broad-spectrum neurotoxic nematicide 'abamectin' is widely used in seed treatment in corn (Bai and Ogbourne 2016). Abamectin was very effective in reducing *P. zeae* penetration into corn roots up to 80 %, leading to increased yield (Cabrera et al. 2009; MacGuidwin and Bender 2016). Recently, the seed treatment with sedaxane fungicide along with abamectic nematicide has improved the seedling health and suppressed *Rizoctonia solani* and *P. penetrans* (da Silva et al. 2017). Identification of genetic resistance is the best environmentally friendly approach among sustainable management strategies. There is very less studies on resistance of corn hybrids to *P. scrbneri*. The wild corn species *Zea diploperennis* and *Z. mexicana* were reported to have resistance to *P. scribneri*

(Norton et al. 1985). Later on, many breeders crossed *Z. diploperennis* with corn to obtain fertile hybrids (De Waele and Elsen 2002). Corn inbreds B37Ht and B68Ht were reported to have some resistance against *P. scribneri* and corn inbreds C123Ht, C103, Mo17Ht, C123Ht x Mo17Ht, and C123Ht x C103 showed susceptibility to *P. scribneri* (Waudo and Norton 1983; Waudo 1984). Smolik and Wicks III (1987) reported that corn inbreds SD101, SD102, and SD103 were found resistant to *P. scribneri* among which, SD101 was selected as a source of resistance. Moreover, inbred A619Ht was found susceptible to both *P. hexincisus* and *P. scribneri* (Smolik and Wicks III 1987). Wicks et al. (1990) developed and registered *P. hexincisus* and *P. scribneri* resistant yellow corn line. Furthermore, Kimenju et al. (1998) indicated that the differences in susceptibility of corn cultivars depends on genotypes of *P. zeae.* They demonstrated that corn cultivars DLC1, H511, and H512 showed significant reduction of root-weight and H614, H625, and Pwani hybrid showed insignificant reduction in root weight while infecting with *P. zeae*. In Nigeria, *P. zeae* resistant corn inbreds were 9450, 5057, and Western Yellow and *P. zeae* susceptible inbreds were Gandajika 8022 and Rilemne 88 TZSR-Y-1 (Oyekanmi et al. 2007). In Uganda, *P. zeae* resistance was checked for 30 corn hybrids based on the reproduction factor where 24 hybrids were found susceptible and six hybrids were found resistant. The resistant hybrids were CML395/MP709, CML312/5057, CML312/CML206, CML312/CML444, CML395/CML312, and CML312/CML395 (Kagoda 2010). To our best knowledge, resistance of corn hybrids to *P. neglectus* has not been reported. However, wheat and barley cultivars showed some resistance to *P. neglectus*. Wild and cultivated barley accessions were found to be moderately resistant to *P. neglectus* (Keil et al. 2009). Sharma et al. (2011) mapped five quantitative trait loci associated with *P. neglectus* resistance in barley genome. May et al. (2016) revealed that barley cultivar Harrington showed resistance against *P. neglectus.* Vanstone et al.

(1998) demonstrated that wheat varieties showed resistance to *P. neglectus*. Smiley et al. (2014a) revealed that wheat cultivar Alpowa and Louise were susceptible, Perisa 20 was moderately susceptible, and AUS28451 was resistant to *P. neglectus* in field condition. The new *Pratylenchus* sp. ND-2017 was screened for reproducibility with soybean cultivar Barnes under greenhouse condition and reported to have greatly increased population after 15 weeks of growth (Yan et al. 2017). However, the resistance of *P. scribneri, P. neglectus* and the new *Pratylenchus* sp. ND-2017 populations to corn hybrids in North Dakota has not been reported. Hence, the objectives of this study were to evaluate ten corn hybrids used in North Dakota for resistance against *P. scribneri, P. neglectus* and the new *Pratylenchus* sp. ND-2017 using naturally infested field soil.

Materials and methods

Reproduction ability of *P. scribneri, P. neglectus,* and new *Pratylenchus* sp. ND-2017 using naturally infested field soil

Soil collection and processing

Three corn fields in North Dakota naturally infested with three different species of root-lesion nematodes (*P. scribneri, P. neglectus*, and new *Pratylenchus* sp. ND-2017) were selected based on previous survey data (Chowdhury et al. 2019). During 2017 and 2018 growing season, soil samples were collected from those three corn fields to determine the reproduction ability of these nematodes using ten corn hybrids in greenhouse conditions. A total of 60 kg of soil was collected from each field using 2.5 cm diameter soil probes (Gempler's model L Sampler, Madison, WI) and shovel (in case of large amount of soil core). Each soil core was taken following a zig-zag pattern, from 30 to 35 cm deep rhizosphere region (Fig. 4.1). The soil cores were subsequently collected in a Rubbermaid storage or cooler box and immediately transported

to the laboratory. Afterward, the entire soil was mixed thoroughly for two hours so that uniformity for initial nematode densities could be maintained in each experimental pot or unit. To determine the initial nematode density, three sub-samples of 0.2 kg were taken from the entire mixed soil. The rest of the soil was kept in a cold storage room at 4 ºC for seven to ten days until setting up the experiment.

Fig. 4.1. Soil sample collection from corn fields of ND using soil probes and transportation to the laboratory using cooler box.

Nematode extraction, identification, and quantification to determine initial population

Nematode extraction was done from those three sub-samples using Whitehead tray nematode extraction technique (Whitehead and Hemming 1965). The extracted nematodes were accumulated using a 20 µm sieve and then collected in a nematode suspension vial as a 20-25 ml suspension. Average of nematode density of three sub-samples was determined by counting the nematodes from the suspension under an inverted transmitted light microscope at 100X magnification (Zeiss Axiovert 25, Carl Zeiss Microscopy, NY). The average nematode densities per kg of soil were used as initial nematode population densities (Pi) for reproduction ability experiments (Fig. 4.2).

Fig. 4.2. White-head tray nematode extraction to determine the initial population of root-lesion nematodes before setting up Greenhouse bioassay experiments.

Root-lesion nematode species identification and confirmation

Before setting up experiments, the nematode suspensions were screened for species identity of root-lesion nematodes (*Pratylenchus* spp.). *Pratylenchus* spp. were identified primarily to genus level according to the morphological description provided by Fortuner (1988) and Mai et al. (1996) under an inverted transmitted light microscope at 100X magnification (Zeiss Axiovert 25, Carl Zeiss Microscopy, NY). The images of nematodes and its body parts were captured using a modular microscope (Zeiss Axio Scope.A1; Zeiss, Oberkochen, Germany) at different magnifications (200X, 400X, and 800X) depending upon the body parts.

Molecular examinations were performed to identify *Pratylenchus* spp. to species level. Six adult female *Pratylenchus* sp. from each nematode suspension were handpicked based on their morphological features (Fortuner 1988; Mai et al. 1996). DNA was extracted from a single individual nematode using Proteinase K method. A single nematode was chopped in a concave glass slide and nematode suspension (10 µl) was pipetted into a 0.5-ml sterile Eppendorf tube containing 2 µl of 10x PCR buffer, 2 µl of Proteinase K (600 µg/ml), and 6 µl of double-distilled water. Tubes containing chopped nematodes and lysis solution were incubated at -20 ºC for 20

minutes followed by 65 ºC for 1 hour to digest the cell proteins and then tubes were incubated at 95 ºC for 10 minutes to eliminate the activity of Proteinase K (Huang and Yan 2017).

The three species of root-lesion nematodes (*P. scribneri*, *P. neglectus*, and new *Pratylenchus* sp. ND-2017) were identified by species-specific PCR and directing sequencing using purified PCR products. The primers used in directing sequencing were: the forward D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and the reverse D3B (5′-TCGGAAGGAACCAGC TACTA-3′) primers (Subbotin et al. 2006) for amplification of the D2-D3 expansion segments of 28S rRNA gene. Moreover, the primers used for species-specific PCR were: the forward PsF7 (5′-AGTGTTGCTATAATTCATGTAAAGTTGC-3′) and the reverse PsR7 (5′-TGGCCAGATGCGATTCGAGAGGTGT-3′) primers (Huang and Yan 2017) for amplification of the ITS region of rDNA (*P. scribneri*-specific PCR); and the forward PNEG-F1 (5′-CGCAATGAAAGTGAACAATGTC-3′) and the reverse D3B5 (5′-AGTTCACCATCTTTCGGGTC-3′) primers (Yan et al. 2008) for amplification of the ITS region of rDNA (*P. neglectus*-specific PCR). After completion of PCR cycles in the Bio-Rad T100 Thermal Cycler (Hercules, CA, USA), agarose gel electrophoresis was performed at 100 V for 25 minutes where 2 µl of PCR product was mixed with 3 µl of 2x loading dye and a total of 5µl of the mixture was loaded in 2 % agarose gel. The gel was visualized under UV light and AlphaImager Gel Documentation System (Proteinsimple Inc., Santa Clara, CA) was used for documenting banding patterns of PCR products. In case of direct sequencing, amplified DNA was purified from the remaining PCR product using E.Z.N.A. Cycle Pure Kit (Omega BIO-TEK, Norcross, Georgia) and sent for DNA sequencing by GenScript (GenScript, Piscataway, NJ). Afterward, DNA sequences were aligned using the sequence alignment tool, ClustalX. The Basic Local Alignment Search Tool (BLAST) in NCBI (www.ncbi.nlm.nih.gov) was used to compare

and identify similarity with the known nematode species sequences previously deposited in the GenBank database.

Greenhouse experiments

During 2017, three experiments were conducted independently with three different root-lesion nematode species *P. scribneri*, *P. neglectus*, and new *Pratylenchus* sp. ND-2017, respectively in the greenhouse of North Dakota State University. The soil used in these experiments were naturally infested with corresponding nematode species. The greenhouse experiments were conducted under 16 hours of day light and average temperature of 22 ºC which is the optimum condition for *Pratylenchus* spp. reproduction. Ten corn hybrids in North Dakota were used in each experiment (Table 4.1).

Table 4.1. Corn hybrids used in this study.

Name of hybrid	Originator[a]	Relative maturity[b]
DKC 44-13	DEKALB	94
LR-9487 VT2RIB	Legend Seeds	87
L-2916 VT2PRO	Legacy Seeds	88
DK 43-48 RIB	DEKALB	93
X5B-8801	NuTech	88
GX89 VT2P	Proseed	89
DK 43-46	DEKALB	93
PFS74K89	Peterson Farms Seed	89
1392 VT2P	Proseed	92
4913 VT2RIB	Producers Hybrids	89

[a]Originator refers to the developer of those corn hybrids.
[b]Relative maturity as given by industry. These data were obtained from different varietal trial extension bulletins from North Dakota State University (Ransom et al. 2018).

Each experiment was conducted using same experimental design: ten corn hybrids and non-planted control each with five replicates. Seeds of these hybrids were pre-germinated for 4-5 days by placing them in petri-dishes with wet paper so that it developed adequate roots for nematodes to feed on after planting at the greenhouse conditions. Each plastic pot was filled with

1 kg of naturally infested soil and fertilized with one tea spoon of slow release formulation 14-14-16 NPK and mixed thoroughly. Each pot (except non-planted pot) was subsequently planted with a single pre-germinated seed. Experiments were completely randomized in blocks and placed in greenhouse benches. The moisture needed for plant growth and *Pratylenchus* spp. reproduction was maintained by adequately watering the pots every day. At 15 weeks of plating, plant tops were cut near to soil surface, the soils with roots were placed in plastic bags and stored at 4 ºC until nematode extractions. In 2018, all three experiments were repeated following the same procedure (Table 4.2) (Fig. 4.3).

Table 4.2. Experimental details (species identity, county from where soil was collected, initial population densities, and experimental period) of greenhouse experiments for root-lesion nematode species[a].

Species ID	County[b]	Repetition (trial)	Pi/ kg of soil[c]	Planting date	Harvesting date
Pratylenchus scribneri	Sargent	1	430	6/27/2017	10/4/2017
		2	430	9/12/2018	12/28/2018
P. neglectus	Grand Forks	1	700	10/6/2017	2/9/2018
		2	690	7/28/2018	11/8/2018
New *Pratylenchus* sp. ND-2017	Richland	1	1,030	10/20/2017	2/9/2018
		2	2,065	6/21/2018	10/4/2018

[a]In all experiments, the reproduction ability of root-lesion nematode was determined by the reproductive factor (final population/ initial population) of *Pratylenchus* spp. The final nematode populations were recorded after harvest.
[b]The name of county of North Dakota from which the target inoculum containing soil was collected for the experiment.
[c]Pi refers to initial population of each *Pratylenchus* species at the time of planting.

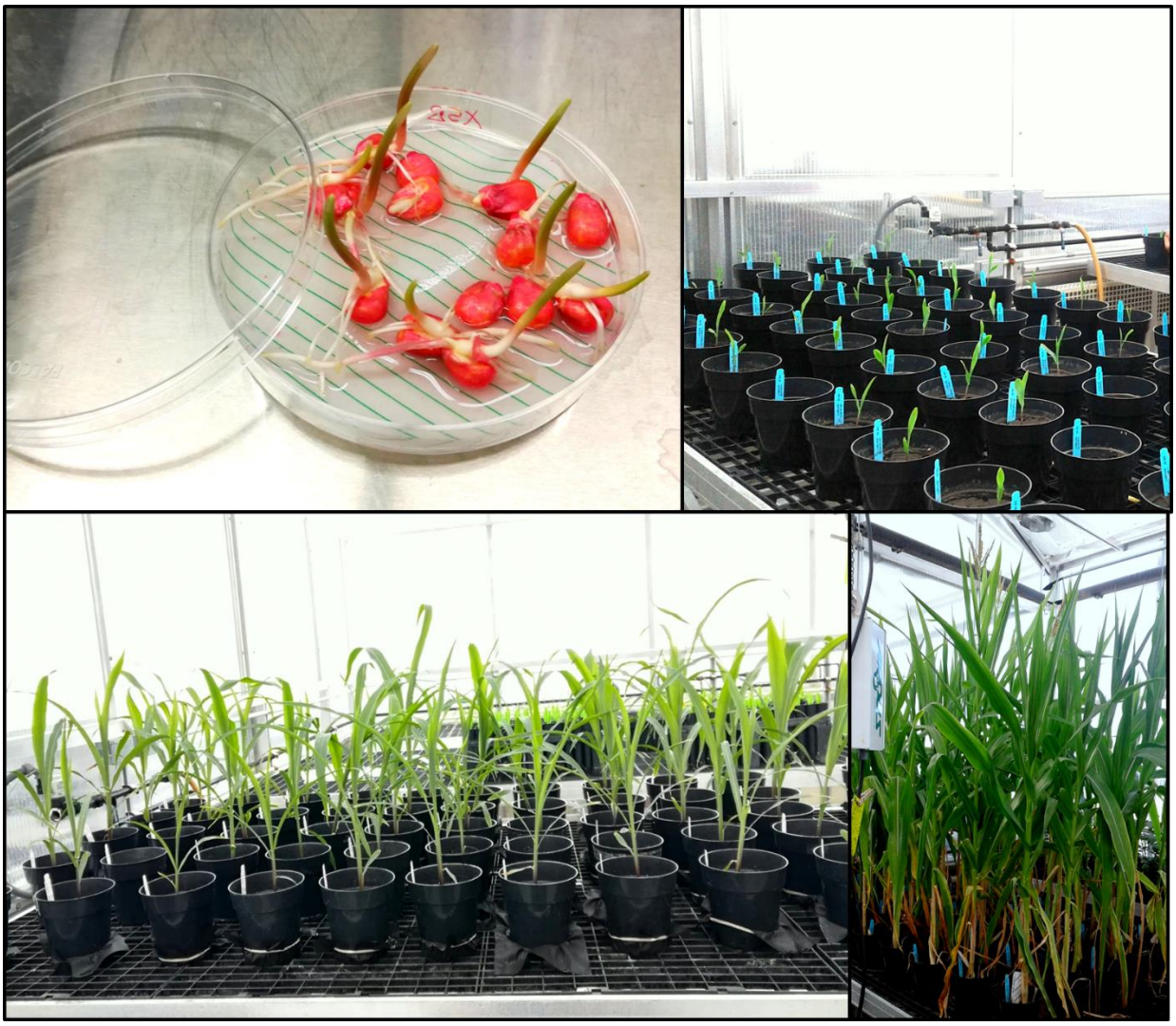

Fig. 4.3. Seed germination and different growth stages of corn plants in the greenhouse bioassay experiments.

Nematode extraction, identification, and quantification to determine final population

After harvesting, the soil with roots obtained from each pot were placed in a plastic tray (36 cm x 27 cm) and the roots were separated from the soil by hand. The roots were cut into small pieces (approximately 1 cm lengths) with a sharp scissor and mixed with the soil thoroughly. A sub-sample of 0.2 kg was taken from each root-soil mixture from which the nematodes were extracted over 48 hours using Whitehead tray nematode extraction method (Whitehead and Hemming 1965) in a room at a constant temperature of 22 ºC. The extracted

nematodes were accumulated using a 20 μm sieve and then collected in a nematode suspension vial. All the life stages of *Pratylenchus* spp. were counted under an inverted transmitted light microscope at 100X magnification (Zeiss Axiovert 25, Carl Zeiss Microscopy, NY). Finally, the estimate was multiplied five times to obtain the final population density (Pf) per kg of soil and root.

Reproductive factors and resistance ratings

The reproductive factor (RF) for each experimental unit was calculated by dividing the final population (Pf) by initial population (Pi). The mean RF of a hybrid was obtained from the five replicates of each hybrid. The average RF of each hybrid was calculated from the mean RF of the first and second trial. Ten corn hybrids were classified for resistance to *Pratylenchus* spp. based on the average RF of the hybrids. The hybrid with highest average RF was considers as the most susceptible hybrid and that was compared with average RF other hybrids to get the percent difference. The hybrids were categorized into four classes according to Smiley et al. (2014a): Susceptible = S (≥ 76 % of the most susceptible hybrid), moderately susceptible = MS (51-75 % of the most susceptible hybrid), moderately resistant = MR (26-50 % of the most susceptible hybrid), and resistant = R (≤ 25 % of the most susceptible hybrid).

Data analysis

The statistical analysis was performed using statistical software SAS 9.4 (PROC GLM of SAS 9.4; SAS Institute Inc., Cary, NC). An *F*-protected least significant difference (LSD) at $P < 0.05$ was used to separate means across hybrids and to investigate significant differences in reproductive factors across ten corn hybrids. According to *F*-protected least significant difference test ($P < 0.05$), RF values with same letters are not significantly different.

Results

Reproduction ability and resistance ratings of *P. scribneri* on ten corn hybrids using naturally infested soil

Pratylenchus spp. was identified primarily up to genus level by observing following characteristics at adult stage: flat dark lip region, short dark stylet with basal knobs (stomatostylet), dorsal overlapping esophagus, vulva at 85 % of the body length in adult stage, and tail region not pointed (Fig. 4.4) (Fortuner 1988; Mai et al. 1996). No adult males were observed in the population. These characters separated them from other plant-parasitic nematodes. The species-specific PCR revealed that the samples showed band with *P. scribneri*-specific primers and did not show band with *P. neglectus*-specific primers. The DNA sequencing of D2-D3 expansion segments of 28S rRNA region revealed that the sequence (752 bp) was 100 % identical with other *P. scribneri* populations such as from North Dakota (KX842628, KX842627, and KX842625) and China (MK209593, KY424300, and JX047001). Moreover, this sequence shared 93 % sequence identity with another species *Pratylenchus hexincisus* (KY828290) from Belgium. Therefore, above evidences confirmed the species identity of this root-lesion nematode population from North Dakota corn field as *P. scribneri*.

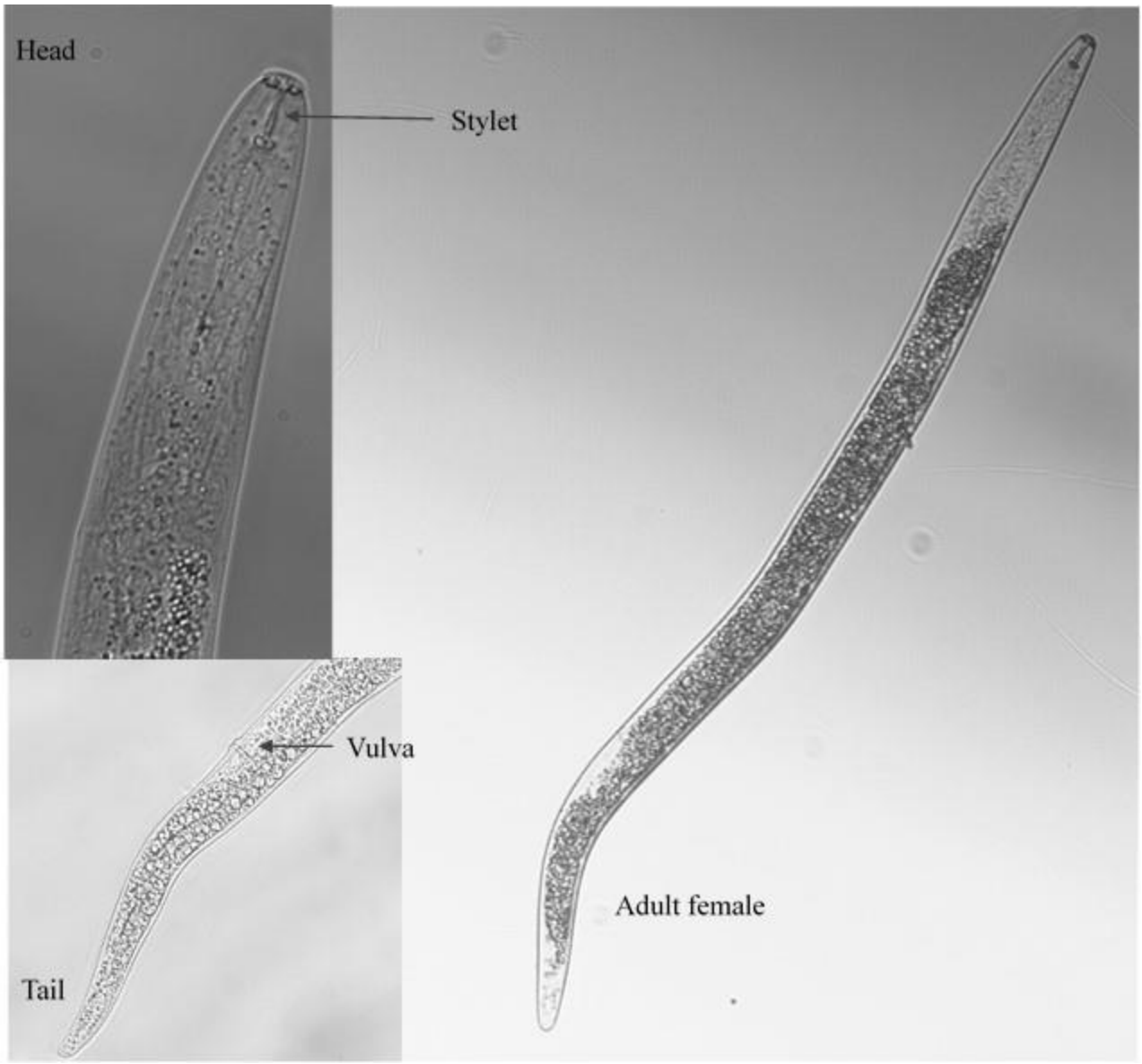

Fig. 4.4. Microscopic image of *Pratylenchus scribneri* adult female.

The greenhouse experiments for evaluation of corn hybrids revealed that the mean reproductive factors (RF) of *P. scribneri* ranging from 0.00 to 6.40 for first trial and from 0.00 to 2.00 for second trial. The *F*-protected least significant difference (LSD) at $P < 0.05$ revealed that no significant differences were seen in RF among hybrids in the first trial whereas significant difference were evident in RF across ten corn hybrids in the second trials. In the first trial, *P. scribneri* reproduced best on corn hybrid X5B-8801 (RF= 6.40) at initial population density of

430 *P. scribneri* per kg of soil (Fig. 4.5A). Moreover, the RF ≥ 4.00 was observed in corn hybrids DK 43-48 RIB, DK 43-46, PFS74K89, and L-2916 VT2PRO (Fig. 4.5A). Least reproduction found in corn hybrids DKC 44-13, 1392 VT2P, 4913 VT2RIB, GX89 VT2P, and LR-9487 VT2RIB (4.00 > RF ≥ 2.1) (Fig. 4.5A). However, no significant difference were observed in RF among hybrids (F test, *P*= 0.14) as there was a higher RF variability among the replicates of each hybrids in the first trial. Corresponding resistance reaction was observed in the second trial where *P. scribneri* reproduced best on corn hybrid X5B-8801 followed by DK 43-46 and DKC 44-13 (Fig. 4.5B) at initial population density of 430 *P. scribneri* per kg of soil. Moreover, corn hybrids DK 43-48 RIB, PFS74K89, and 4913 VT2RIB showed reproductive factors more than 1.00 (Fig. 4.5B). Least reproduction found in corn hybrids 1392 VT2P, GX89 VT2P, LR-9487 VT2RIB, and L-2916 VT2PRO (RF < 1.00) (Fig. 4.5B). Statistical analysis showed that corn hybrid X5B-8801 had significantly higher RF than GX89 VT2P, LR-9487 VT2RIB, and L-2916 VT2PRO. Moreover, L-2916 VT2PRO had significantly lower RF than X5B-8801, DK 43-46, and DKC 44-13 (F test, *P*= 0.03).

Interestingly, corn hybrids X5B-8801, DK 43-46, and DK 43-48 RIB showed consistent susceptibility in both trials. Moreover, GX89 VT2P and LR-9487 VT2RIB showed consistence resistance in both trials (Fig. 4.5A, 4.5B). Although the initial population in two trials were same, the ranges of mean RF were different in both trials. Therefore, hybrids were classified for resistance to *P. scribneri* based on the average mean RF of the first and second trials. X5B-8801 was considered as most susceptible hybrid. Thus, DK 43-46 was determined as susceptible (≥ 76 % of the most susceptible hybrid). DK 43-48 RIB, PFS74K89, and DKC 44-13 were grouped as moderately susceptible hybrids (51-75 % of the most susceptible hybrid). Lastly, L-2916 VT2PRO, 1392 VT2P, 4913 VT2RIB, GX89 VT2P, and LR-9487 VT2RIB were grouped as

moderately resistant hybrids (26-50 % of the most susceptible hybrid) (Smiley et al. 2014a). No hybrid was found resistant to *P. scribneri*. The host ranking of *P. scribneri* has shown in Table 4.3.

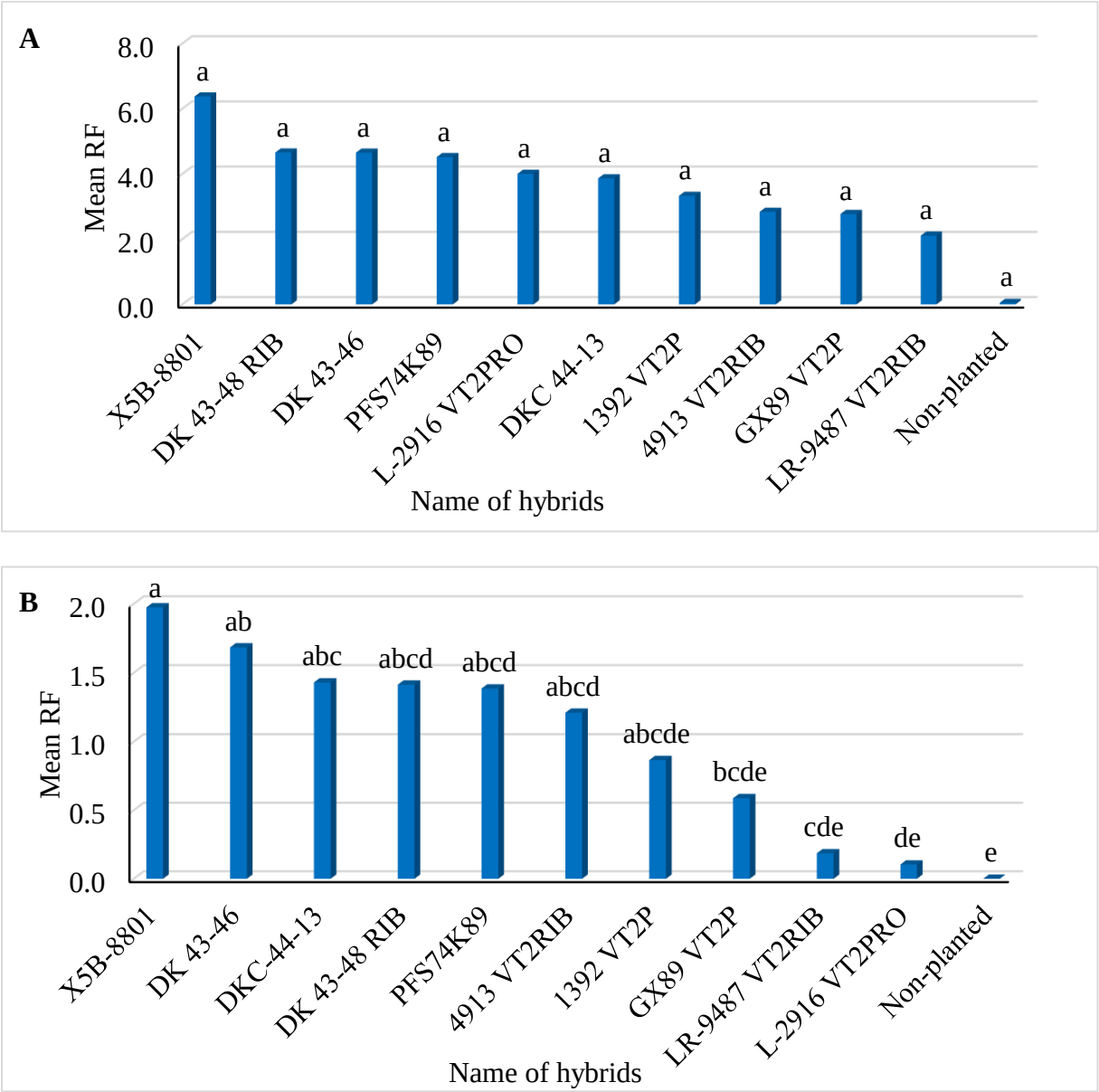

Fig. 4.5. Reproductive factor (RF) values of root-lesion nematode (*P. scribneri*) in the first trial (A) and second trial (B) using inoculum from a field of Sargent County, ND on ten corn hybrids used in ND. Final nematode density per kg of soil and root was determined after harvesting the trials on 15th week after planting. The *P*-value in the first trial (A) was 0.14 thus there is no significant difference among the hybrids. The *P*-value in the second trial (B) was 0.03 thus *F*-protected LSD test ($P < 0.05$) result was shown. Mean RF values with same letter are not significantly different according to *F*-protected LSD test ($P < 0.05$).

Reproduction ability and resistance ratings of *P. neglectus* on ten corn hybrids using naturally infested soil

Pratylenchus spp. was identified primarily up to genus level by observing following characteristics at adult stage: flat dark lip region, short dark stylet with basal knobs (stomatostylet), dorsal overlapping esophagus, vulva at 85 % of the body length in adult stage, and tail region not pointed (Fig. 4.6) (Fortuner 1988; Mai et al. 1996). No adult male observed in the population. These characters separated them from other plant-parasitic nematodes. The species-specific PCR revealed that the samples showed band with *P. neglectus*-specific primers and did not show band with *P. scribneri*-specific primers. The DNA sequencing of D2-D3 expansion segments of 28S rRNA region revealed that the sequence (601 bp) was 99.49 % identical with two *P. neglectus* populations from Iran (JX261951 and JX261947). Moreover, this population was 99.0 % identical with a *P. neglectus* population from China (MG906766), 98.98 % identical with a *P. neglectus* population from California (EU130854), and 98.83 % identical with another *P. neglectus* population from China (KY424316). Therefore, above evidences confirmed the species identity of this root-lesion nematode population from North Dakota corn field as *P. neglectus*.

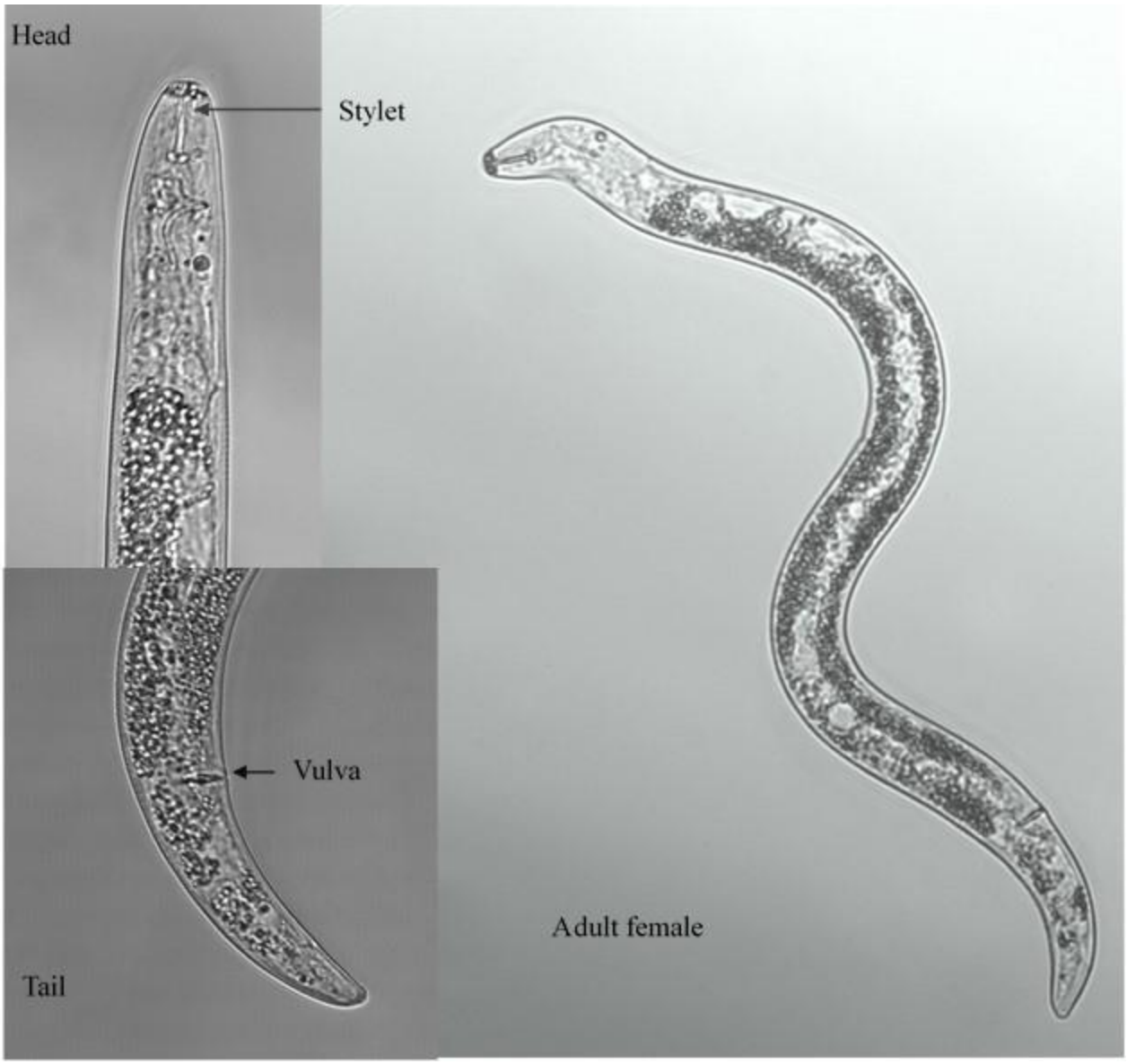

Fig. 4.6. Microscopic image of *Pratylenchus neglectus* adult female.

The greenhouse experiments for evaluation of corn hybrids revealed that the reproductive factors (RF) of *P. neglectus* ranging from 0.06 to 3.27 for first trial and from 0.26 to 3.71 for second trial. The *F*-protected least significant difference (LSD) at $P < 0.05$ revealed that significant difference were evident in RF across ten corn hybrids in both trials. In the first trial, *P. neglectus* reproduced best on corn hybrid DK 43-48 RIB followed by DK 43-46 (Fig. 4.7A) at initial population density of 700 *P. neglectus* per kg of soil. Moreover, corn hybrids LR 9487

VT2PRIB, GX89 VT2P, X5B-8801, 1392 VT2P, and L-2916 VT2PRO showed reproductive factors more than 1.00 (Fig. 4.7A). Least reproduction found in corn hybrids DKC 44-13, PFS74K89, and 4913 VT2RIB (RF < 1.00) (Fig. 4.7A). Statistical analysis showed that the RF of *P. neglectus* with DK 43-48 RIB and DK 43-46 were significantly higher than DKC 44-13, PFS74K89, and 4913 VT2RIB (Fig. 4.7A). Almost similar resistance reaction was observed in the second trial where *P. neglectus* reproduced best on corn hybrid DKC 44-13 followed by LR-9487 VT2RIB and L-2916 VT2PRO (Fig. 4.7B) at initial population density of 690 *P. neglectus* per kg of soil. Moreover, corn hybrids DK 43-48 RIB, X5B-8801, GX89 VT2P, DK 43-46, PFS74K89, and 1392 VT2P showed reproductive factors more than 1.00 (Fig. 4.7B). *P. neglectus* reproduced least on 4913 VT2RIB in the second trial (Fig. 4.7B).

It was fascinating that corn hybrid 4913 VT2RIB showed the least reproduction in both trials whereas no hybrid showed consistent susceptibility in both trials (Fig. 4.7A, 4.7B). Therefore, hybrids were classified for resistance to *P. neglectus* based on the average mean RF of the first and second trials. DK 43-48 RIB was considered as most susceptible hybrid. Thus, DKC 44-13, DK 43-46, and LR-9487 VT2RIB were grouped as susceptible hybrids (≥ 76 % of the most susceptible hybrid). L-2916 VT2PRO, GX89 VT2P, and X5B-8801 were grouped as moderately susceptible hybrids (51-75 % of the most susceptible hybrid). Lastly, 1392 VT2P, PFS74K89, and 4913 VT2RIB were grouped as moderately resistant hybrids (26-50 % of the most susceptible hybrid) (Smiley et al. 2014a). No hybrid was found resistant to *P. neglectus*. The host ranking of *P. neglectus* has shown in Table 4.3.

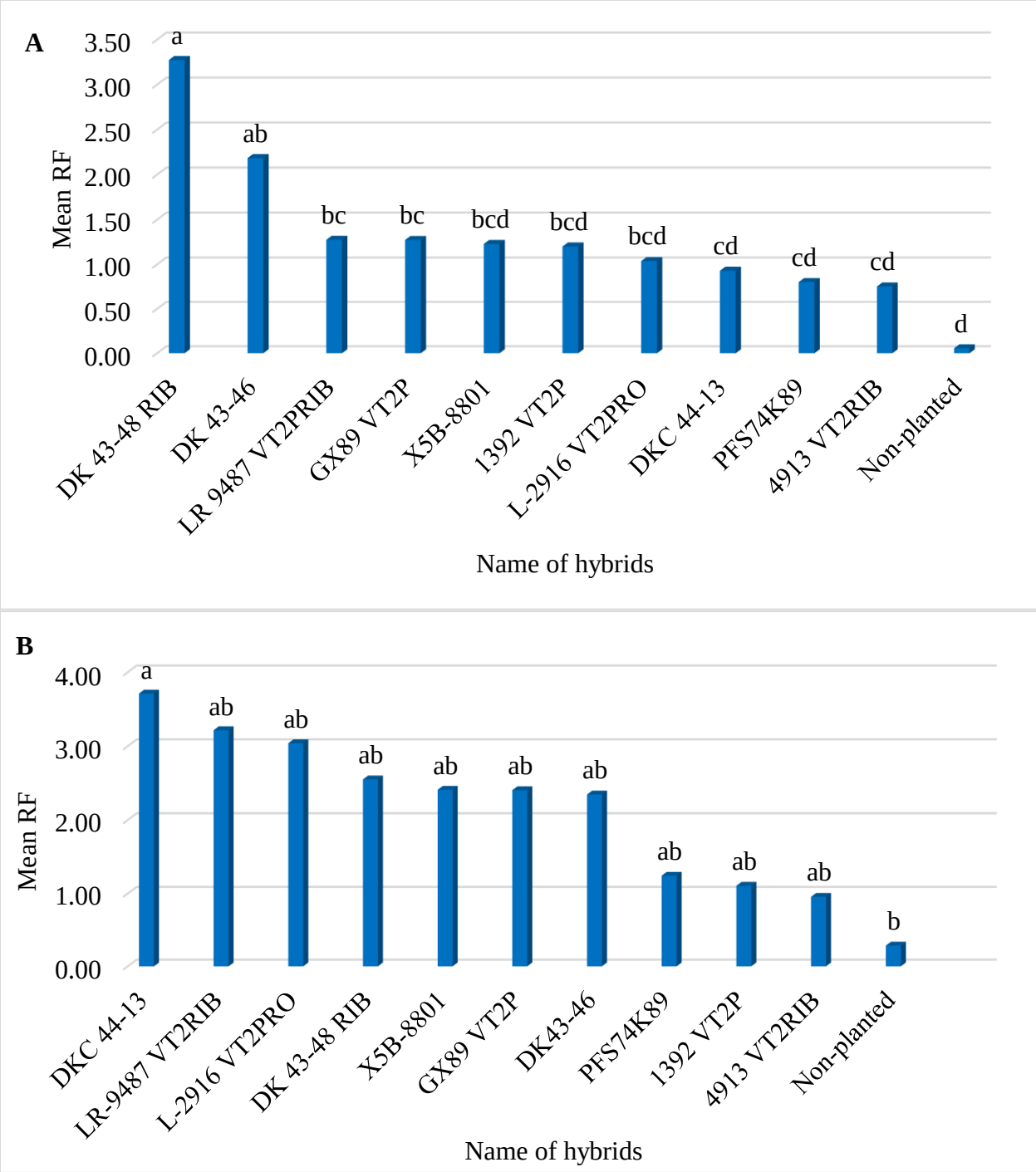

Fig. 4.7. Reproductive factor (RF) values of root-lesion nematode (*P. neglectus*) in the first trial (A) and second trial (B) using inoculum from a field in Grand Forks County, ND on ten corn hybrids used in ND. Final nematode density per kg of soil and root was determined after harvesting the trial on 15th week after planting. Mean RF values with same letter are not significantly different according to *F*-protected least significant different test ($P < 0.05$). The *P*-values in the first and second trials were 0.0006 and < 0.0001, respectively.

Reproduction ability and resistance ratings of new *Pratylenchus* sp. ND-2017 on ten corn hybrids using naturally infested soil

Pratylenchus spp. was identified primarily up to genus level by observing following characteristics at adult stage: flat dark lip region, short dark stylet with basal knobs (stomatostylet), dorsal overlapping esophagus, vulva at 85 % of the body length in adult stage, and tail region not pointed (Fig. 4.8) (Fortuner 1988; Mai et al. 1996). Interestingly, adult male observed in this population. These characters separated them from other plant-parasitic nematodes. The species-specific PCR revealed that the samples did not show band with *P. scribneri*-specific primers and *P. neglectus*-specific primers. The DNA sequencing of D2-D3 expansion segments of 28S rRNA region revealed that the sequence (754 bp) was 100 % identical with a population reported from soybean field in North Dakota (new *Pratylenchus* sp. ND-2017) (KX889989) and a population reported from Wisconsin (MN251269). Moreover, this sequence shared 98.59 % sequence identity with another species *Pratylenchus scribneri* (MG925218) from Ohio and 94.31 % sequence identity with another species *Pratylenchus loosi* (JN091970) from China. Therefore, above evidences confirmed the species identity of this root-lesion nematode population from North Dakota corn field as new *Pratylenchus* sp. ND-2017.

Fig. 4.8. Microscopic image of an adult female and an adult male of the new *Pratylenchus* sp. ND-2017 (Yan et al. 2017).

The greenhouse experiments for evaluation of corn hybrids revealed that the reproductive factors (RF) of new *Pratylenchus* sp. ND-2017 ranging from 1.16 to 4.43 for first trial and from 0.24 to 1.82 for second trial. The *F*-protected least significant difference (LSD) at $P < 0.05$ revealed that significant difference were evident in RF across ten corn hybrids in both trials. In the first trial, new *Pratylenchus* sp. ND-2017 reproduced best on GX89 VT2P followed by L-2916 VT2PRO (Fig. 4.9A) at initial population density of 1,030 new *Pratylenchus* sp. ND-2017/ kg of soil. Moreover, corn hybrids 4913 VT2RIB, LR 9487 VT2PRIB, DKC 44-13, and X5B-8801 showed reproductive factors more than 2.00 (Fig. 4.9A). Least reproduction found in corn

hybrids DK 43-48 RIB, DK 43-46, 1392 VT2P, and PFS74K89 (RF < 2.00) (Fig. 4.9A). Statistical analysis showed that the RF of new *Pratylenchus* sp. ND-2017 with GX89 VT2P and L-2916 VT2PRO were significantly higher ($P < 0.05$) than DK 43-48 RIB, DK 43-46, 1392 VT2P, and PFS74K89 (Fig. 4.9A). In the second trial, new *Pratylenchus* sp. ND-2017 reproduction was the highest on hybrid X5B-8801 with significantly high ($P < 0.05$) RF compared to other tested corn hybrids at initial population density of 2,065 new *Pratylenchus* sp. ND-2017/ kg of soil (Fig. 4.9B). Moreover, the second highest reproduction was found in corn hybrid DKC 44-13 with significantly high ($P < 0.05$) RF than PFS74K89, GX89 VT2P, 1392 VT2P, 4913 VT2RIB, and L-2916 VT2PRO (Fig. 4.9B). Least reproduction found in corn hybrids PFS74K89, GX89 VT2P, 1392 VT2P, 4913 VT2RIB, and L-2916 VT2PRO (RF < 0.50) (Fig. 4.9B).

The observation of both trials revealed that no hybrid showed consistent resistance or susceptibility in both trials (Fig. 4.9A, 4.9B). Therefore, hybrids were classified for resistance to new *Pratylenchus* sp. ND-2017 based on the average RF of the first and second trial. GX89 VT2P was considered as most susceptible hybrid. Thus, X5B-8801, DKC 44-13, and L-2916 VT2PRO were grouped as susceptible hybrids (≥ 76 % of the most susceptible hybrid). LR-9487 VT2RIB, 4913 VT2RIB, and DK 43-46 were grouped as moderately susceptible hybrids (51-75 % of the most susceptible hybrid). Lastly, DK 43-48 RIB, 1392 VT2P, and PFS74K89 were grouped as moderately resistant hybrids (26-50 % of the most susceptible hybrid) (Smiley et al. 2014a). No hybrid was found resistant to new *Pratylenchus* sp. ND-2017. The host ranking of new *Pratylenchus* sp. ND-2017 has shown in Table 4.3.

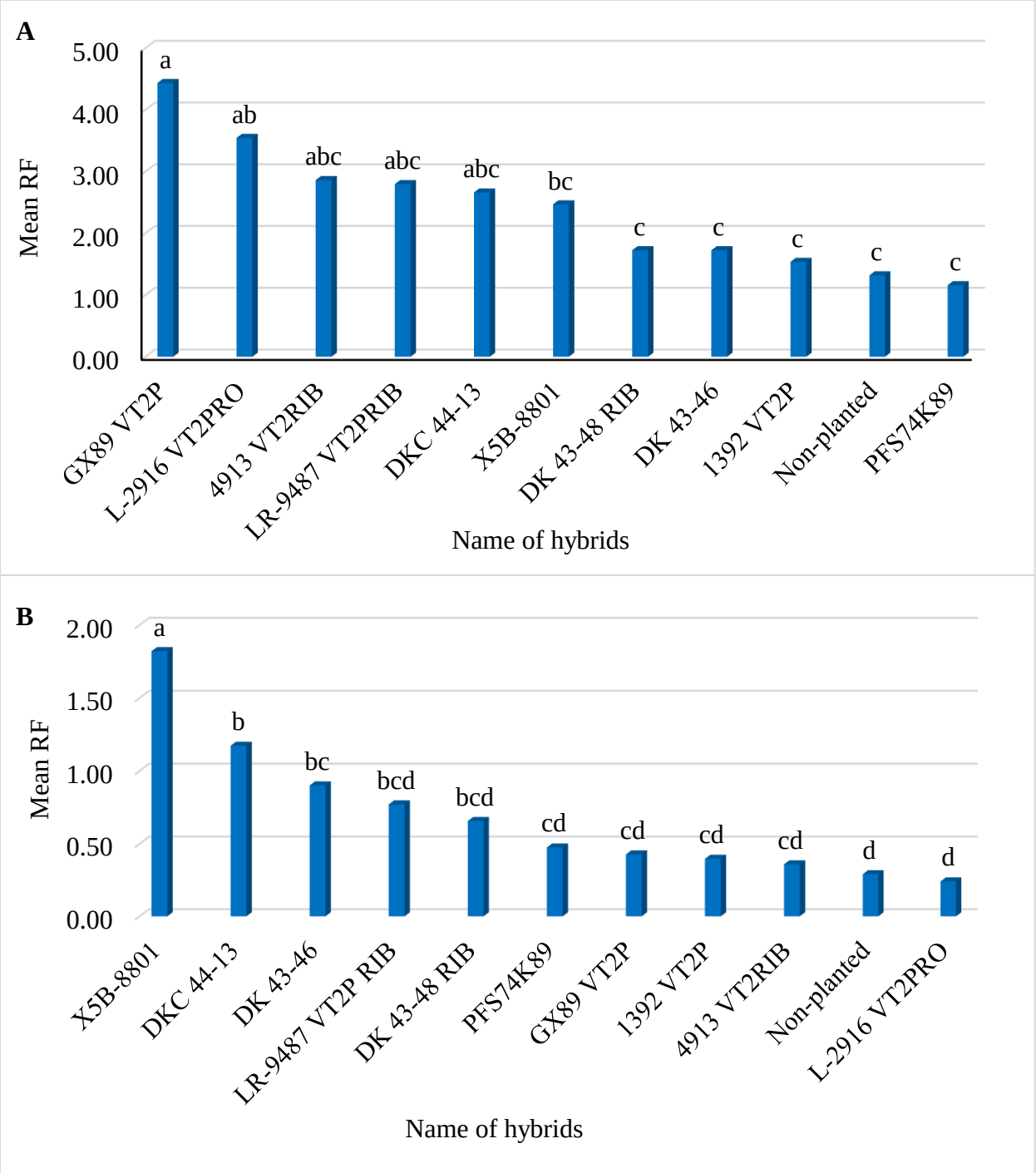

Fig. 4.9. Reproductive factor (RF) values of new *Pratylenchus* sp. ND-2017 in the first trial (A) and second trial (B) using inoculum from a field of Richland County, ND on ten corn hybrids used in ND. Final nematode density per kg of soil and root was determined after harvesting the trial on 15th week after planting. Mean RF values with same letter are not significantly different according to *F*-protected least significant different test ($P < 0.05$). The *P*-values in the first and second trials were 0.015 and < 0.0001, respectively.

Comparison of resistance of three species of root-lesion nematodes, *P. scribneri*, *P. neglectus*, and new *Pratylenchus* sp. ND-2017 on corn hybrids of North Dakota

Considering the host ranking of three *Pratylenchus* species, a more or less similar susceptibility pattern was observed among the tested corn hybrids. Corn hybrid 1392 VT2P was found moderately resistant with all three root-lesion nematode species. Corn hybrids PFS74K89 and 4913 VT2RIB were found moderately resistant with two of the three root-lesion nematode species. PFS74K89 was moderately resistant to *P. neglectus* and new *Pratylenchus* sp. ND-2017 and 4913 VT2RIB was moderately resistant to *P. scribneri* and *P. neglectus*. Moreover, DK 43-48 RIB, L-2916 VT2PRO, GX89 VT2P, and LR-9487 VT2RIB showed variable responses (susceptible, moderately susceptible, and moderately resistant) to three root-lesion nematode species. Corn hybrids X5B-8801, DK 43-46, and DKC 44-13 were found susceptible to two of the three root-lesion nematode species. X5B-8801 was found susceptible to *P. scribneri* and new *Pratylenchus* sp. ND-2017, DK 43-46 was susceptible to *P. scribneri* and *P. neglectus*, and DKC 44-13 was susceptible to *P. neglectus* and new *Pratylenchus* sp. ND-2017. No hybrid was found resistant to new *Pratylenchus* sp. ND-2017 or *P. scribneri* or *P. neglectus*. The host ranking with elaborate information of ten corn hybrids to three *Pratylenchus* species were shown in Table 4.3.

Table 4.3. Host ranking of ten corn hybrids to three species of root-lesion nematodes, *P. scribneri*, *P. neglectus*, and new *Pratylenchus* sp. ND-2017.

Hybrid	*P. scribneri*[a]		*P. neglectus*[a]		New *Pratylenchus* sp. ND-2017[a]	
	Ratio (%)[b]	Host Ranking[c]	Ratio (%)[b]	Host Ranking[c]	Ratio (%)[b]	Host Ranking[c]
X5B-8801	100.00	S	62.54	MS	88.07	S
DK 43-48 RIB	71.42	MS	100.00	S	48.97	MR
DK 43-46	76.19	S	77.66	S	53.91	MS
PFS74K89	71.42	MS	35.05	MR	33.74	MR
L-2916 VT2PRO	50.00	MR	70.10	MS	77.77	S
DKC 44-13	61.90	MS	79.73	S	78.19	S
1392 VT2P	50.00	MR	39.52	MR	39.91	MR
4913 VT2RIB	49.50	MR	28.52	MR	66.25	MS
GX89 VT2P	40.47	MR	63.23	MS	100.00	S
LR-9487 VT2RIB	26.19	MR	77.32	S	73.25	MS

[a]The resistance evaluation experiment of ten corn hybrids to *P. scribneri*, *P. neglectus* and new *Pratylenchus* sp. ND-2017.
[b]Ratio= (RF in the test line/maximum RF) x 100.
[c]Host ranking based on RF (RF in a test line/maximum RF) categorized into four classes: Susceptible = S (≥ 76 % of the most susceptible hybrid), Moderately susceptible = MS (51-75 % of the most susceptible hybrid), Moderately resistant = MR (26-50 % of the most susceptible hybrid), and Resistant = R (≤ 25 % of the most susceptible hybrid) (Smiley et al. 2014a).

Discussion

To our knowledge, this is the first report of the evaluation of resistance of corn hybrids to root-lesion nematode species *P. scribneri*, *P. neglectus*, and new putative *Pratylenchus* sp. ND-2017 in North Dakota. This study described the preference of *P. scribneri*, *P. neglectus*, and new *Pratylenchus* sp. ND-2017 reproduction on ten corn hybrids commonly used by the growers of North Dakota. The soil texture used in our study was sandy loam. Toktay et al. (2012) revealed that sandy soil is suitable to obtain more accurate and stable results in all replicates. In this research, the experiments were harvested from greenhouse after 15 weeks of planting. MacGuidwin and Stanger (1991) reported that *P. scribneri* reproduced highest at 15 to 16 weeks

of planting corn which supports our work. The 16 weeks duration of planting was also used in the study of Smiley et al. (2014b).

The field soil naturally infested with specific root-lesion nematode species was used in our experiments. This allows the nematodes to act and reproduce similarly to natural field soil conditions. The results will have a resemblance to the field condition because all those corn hybrids are grown in the natural field conditions in our region. In South Dakota, the dent corn was tested for resistance in the greenhouse where soil naturally infested with *P. hexincisus* was used (Smolik and Wicks III 1987). They maintained the nematode population on corn for four years in the greenhouse.

Usually, the reproductive factor RF (Pf/Pi) is used as a quantitative value to determine resistance. Toktay et al. (2012) described that the initial population density of 400 nematodes/plant was suitable in case of *P. thornei*. However, they did not mention the quantity of soil per pot. In our study, one kg of soil was used and one seedling was planted in each pot with an initial population density more than 400/pot. Keil et al. (2009) used 0.5 kg of sand per pot planted with one seedling and showed that there is no significant difference in reproduction with inoculum between 400 and 600 *P. neglectus*/pot.

Toktay et al. (2012) recommended that root-lesion nematodes should be extracted from both soil and roots in resistance screening experiments because of their migratory nature. They extracted the nematodes from the soil by mister extraction method and from the roots by modified Baermann funnel method. However, they did not mention the quantity of soil and roots they used for extraction. Previously, MacGuidwin and Stanger (1991) extracted *P. scribneri* from both soil and roots of corn and potato. They used sugar centrifugal floatation method to extract nematodes from 0.1 kg of soil and Baermann funnel method to extract nematodes from

the roots recovered from 0.1 kg of soil. Afterword, the sum of these two extraction results was considered as nematode abundance. In our study, after cutting the roots into small pieces (approximately 1 cm lengths) and mixing with the whole soil (1 kg) thoroughly, a sub-sample of 0.2 kg was taken from each root-soil mixture and nematodes were extracted using Whitehead tray nematode extraction method. Finally, the estimate was multiplied five times to obtain the final population density (Pf) in each pot. Identical procedure of our study was used by Smiley et al. (2014b) to determine the final population density (Pf) in hosting ability rating experiment of *P. neglectus* and *P. thoreni*.

According to Taylor et al. (2000), the final population density of *P. neglectus* could be used for hybrid resistance evaluation. Bacetty et al. (2009) used reproductive factor to evaluate the host range of *P. scribneri*. The host ranking in our study was also obtained based on reproductive factors. Interestingly, in the study with *P. scribneri*, the range of mean reproductive factors (RF) in the first trial (mean RF= 0.00 to 6.4) were much higher compared to the range in the second trial (mean RF= 0.00 to 2.0) despite of same initial population density in both trials (Pi= 430 *P. scribneri*/ kg of soil). Moreover, the variation of RF within the replicates were observed in the first trial which results in non-significant mean separation test. This inconsistency might be due to the environmental variation within the greenhouse such as fluctuating temperatures, air currents, and pests from outside the building. It is important to note that the first trial was conducted in summer 2017 and the second trial was conducted in fall 2018. This variation in growing season might be responsible for low mean RF in the second trial. Moreover, in case of *P. neglectus* study, a similar range of mean RF of *P. neglectus* in the first (mean RF= 0.06 to 3.27) and second (mean RF= 0.26 to 3.71) greenhouse trials were observed which might be as a consequence of similar initial population density of *P. neglectus* in both

trials (Pi= 700 and 690/ kg of soil in the first and second trials, respectively). Despite the similarity in range of mean RF in both trials, the reproducibility of *P. neglectus* on ten corn hybrids was inconsistent in two trials. Furthermore, in the reproduction ability experiment of new *Pratylenchus* sp. ND-2017, the first and second trials did not show consistent reproducibility on ten corn hybrids. The difference in growing season or pot position (pots were placed by following completely randomized designs) might be responsible for this variability. May et al. (2016) revealed that the reproductive success of *P. neglectus* was significantly variable among seven trials which corresponded to our results.

In our study, the initial new *Pratylenchus* sp. ND-2017 population density in the first trial was lower than the second trial (Pi= 1,030 and 2,065/ kg of soil in the first and second trials, respectively). Despite lower initial density than the second trial, the first trial showed significantly higher RF (mean RF= 1.16 to 4.43). In this case, competition has been observed with high initial population density. According to Seinhorst (1967), the higher the initial population density of sedentary nematodes the smaller the proportion of juveniles that penetrated roots and became adults resulting in less multiplication. The rating of varieties within each trial mostly evaluated by multiplication rate. However, sometimes the presence of interaction between initial density and crop tolerance influences the multiplication rate. Low initial density exhibited less plant damage influencing nematode multiplication (Taylor 2000). Moreover, the variation in the RF range with initial density could also be due to more intraspecific competition for food at the high starting density than at the low density. According to Duncan and Ferris 1983, the competition for feeding sites can limit their reproduction. Brinkman et al. (2005) observed the intraspecific competition of *Pratylenchus penetrans* leading to lower females than males at high inoculation densities. Toktay et al. (2012) found that the highest *Pratylenchus*

thornei reproduction factor was determined at a lower inoculum density. Taking the above findings and our results under consideration, it could be concluded that new *Pratylenchus* sp. ND-2017 has potential to reproduce quickly at lower initial densities compared to higher initial densities.

The reproduction ability experiment of *P. scribneri* with ten corn hybrids revealed that 100 % of the tested hybrids in the first trial and 60 % of the tested hybrids in the second trial showed increased population density. Moreover, *P. neglectus* population density increased in 90 % of the corn hybrids tested in the first trial and 70 % in the second trial. Therefore, this study showed that *P. scribneri* and *P. neglectus* to be able to successfully develop and reproduce on corn hybrids under controlled greenhouse conditions. This comes in agreement with the association of *P. scribneri* and *P. neglectus* with corn (Castillo and Vovlas 2007; Taylor 2000). Our study also evaluated the resistance of corn hybrids to new *Pratylenchus* sp. ND-2017 which was first reported as a new root-lesion nematode species in North Dakota crop fields (Yan et al. 2017). This was the first elaborate examination of resistance of corn hybrids to new *Pratylenchus* sp. ND-2017 where 100 % of the tested hybrids showed increased population density in the first trial. This result demonstrated that new *Pratylenchus* sp. ND-2017 to be able to successfully develop and reproduce on corn hybrids under controlled greenhouse conditions and confirmed the presence of association with corn. Yan et al. (2017) demonstrated the association of this nematode species with soybean cultivar Barnes in which the RF was as high as 5.02.

Very few studies on variability in resistance of *P. scribneri* and *P. neglectus* in crop hybrids were conducted. The reproduction ability experiment using naturally infested soil showed that *P. scribneri*, *P. neglectus*, and new *Pratylenchus* sp. ND-2017 reproduction potential varied with hybrid type and there was significant variation ($P < 0.05$) in reproductive

factor among the hybrids. Griffin and Jensen 1997 showed a divergent reproducibility in *P. neglectus* in wheat. May et al. (2016) also found that *P. neglectus* showed significant variation in resistance among different barley lines.

Among the tested corn hybrids, 50 % acted as moderately resistant hosts and 50 % acted as susceptible and moderately susceptible hosts to *P. scribneri.* Moreover, in case of *P. neglectus,* 30 % of the tested hybrids acted as moderately resistant hosts and 70 % acted as susceptible and moderately susceptible hosts. The experiment with new *Pratylenchus* sp. ND-2017, 30 % of the tested hybrids acted as moderately resistant hosts, 30 % acted as moderately susceptible and 40 % acted as susceptible hosts (Table 4.3). This result suggests that the specific corn hybrids have a significant role in determining the reproducibility of *P. scribneri* and *P. neglectus,* and new *Pratylenchus* sp. ND-2017.

In the resistance ratings of ten corn hybrids, the hybrids were categorized into four classes: susceptible, moderately susceptible, moderately resistant, and resistant based on the host ranking proposed by Smiley et al. (2014a). In case of *P. scribneri,* LR-9487 VT2RIB hybrid showed the least RF in both trials indicating the moderately resistance of *P. scribneri*. Susceptibility of *P. scribneri* found in X5B-8801 and DK 43-46 hybrids by having increased population density (Table 4.3). The resistance ratings of *P. neglectus* revealed that 4913 VT2RIB hybrid showed potential in *P. neglectus* resistance by decreasing the population density in pots planted with this hybrid in both trials. Conversely, DK 43-48 RIB, DK 43-46, DKC 44-13, and LR-9487 VT2RIB supported the *P. neglectus* reproduction. A modest increase of *P. neglectus* density was observed in L-2916 VT2PRO, GX89 VT2P, and X5B-8801 hybrids (Table 4.3). Furthermore, in the resistance ratings of new *Pratylenchus* sp. ND-2017, PFS74K89, 1392 VT2P, and DK 43-48 RIB hybrids showed potential in resistance by decreasing the new

Pratylenchus sp. ND-2017 population density in pots planted with these hybrids while X5B-8801, L-2916 VT2PRO, DKC 44-13, and GX89 VT2P supported the multiplication (Table 4.3). This finding will be valuable for the growers and future studies with this new species. Identical research work was done by Smiley et al. (2014a) under field condition where they used the identical resistance rating scale of our study to classify the wheat hybrids for resistance to *P. neglectus*. Moreover, Taylor et al. (2000) tested *P. neglectus* for host suitability evaluation of eighty-one hybrids from 12 field crop species. They were grouped as good host, moderate host, and poor host based on multiplication rate. Smiley et al. (2014b) tested thirty crop species and hybrids for the hosting ability test of *P. neglectus* and *P. thornei.* They classified the crops as nonhost, poor host, minor host, good host, and very good host based on RF.

From the comparative analysis of resistance ranking of ten corn hybrids to three root-lesion nematode species *P. scribneri*, *P. neglectus,* and new *Pratylenchus* sp. ND-2017, it can be summarized that corn hybrids PFS74K89 and 4913 VT2RIB showed moderately resistance to two of the three species. Moreover, X5B-8801, DK 43-46, and DKC 44-13 hybrids were susceptible to two of the three species. Interestingly, 1392 VT2P showed moderately resistance to all three species. Therefore, corn hybrids 1392 VT2P, PFS74K89, and 4913 VT2RIB can be considered as a source of resistance and will help in future studies to determine the mode of inheritance of resistance to root-lesion nematodes. This comparison of resistance evaluation for three root-lesion nematode species is a novel finding in the literature. Previously, Smolik and Wicks III (1987) showed a comparison of reproduction of *P. hexincisus* and *P. scribneri* in corn inbreds where test with *P. hexincisus* was conducted in the greenhouse with naturally infested soil and test with *P. scribneri* was conducted in the field condition. The resistance of wheat hybrids to *P. neglectus* and *P. thornei* were compared where the experiments were conducted in

fields naturally infested with these nematodes (Smiley et al. 2014a). Smiley et al. (2014b) also showed the comparison of the hosting ability of thirty crop species and hybrids to *P. neglectus* and *P. thoreni* where the experiments were conducted in the glasshouse.

To conclude, our study revealed that the root-lesion nematode species of North Dakota reproduced least on some of the corn hybrids such as 1392 VT2P, PFS74K89, and 4913 VT2RIB. Whereas, X5B-8801, DK 43-46, and DKC 44-13 hybrids supported *P. scribneri*, *P. neglectus*, and new *Pratylenchus* sp. ND-2017 reproduction. This finding provides very exclusive information regarding the resistance of corn hybrids of North Dakota. However, the reproduction rate of these three species was dependent upon initial density at the time of planting. Hence, further research may be required to confirm this differential rate of reproduction in corn hybrids. Moreover, the use of sterilized soil and artificial inoculation may be required to re-evaluate the resistance of corn hybrids and to measure yield losses caused by these nematodes infection in corn. These findings provide useful information to farmers of our region to choose appropriate corn hybrids identified in our study using naturally infested soil because all those hybrids are grown in the natural field conditions in our region. Moreover, this research will be beneficial in corn germplasm resistance screening and enhance genetic engineering studies for the incorporation of resistance genes against these root-lesion nematode species into corn.

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CHAPTER 5. SUMMARY

Out of 50 samples collected from North Dakota corn fields, 17 samples (34 %) were found positive for root-lesion nematodes. Of the positive samples, six were identified as *P. scribneri*, four were new putative *Pratylenchus* sp. ND-2016 isolate HG51, three were new putative *Pratylenchus* sp. ND-2017, and one was *P. neglectus*. Interestingly, three of the fields were found to contain a mixed population of *P. neglectus* and *P. scribneri*. The lowest intra-species variation (0.52 to 3.16 %) was found in the *Pratylenchus* sp. ND-2017 population whereas the highest intra-species variation (11.93 to 24.21 %) was found in the *P. neglectus* population. In the three phylogenetic trees, four species were clustered in four separate clades indicating the divergence among species. Among the four species, *P. scribneri* and *Pratylenchus* sp. ND-2016 isolate HG51 were more closely associated with each other. The *Pratylenchus* sp. ND-2017 was also closely related to the *Pratylenchus* sp. ND-2016 isolate HG51. However, *P. neglectus* was not closely associated with the other three species.

The resistance screenings of three root-lesion nematode species were carried out in controlled greenhouse condition. For *Pratylenchus scribneri*, corn hybrids L-2916 VT2PRO, 1392 VT2P, 4913 VT2RIB, GX89 VT2P, and LR-9487 VT2RIB were considered as moderately resistant; DK 43-48 RIB, PFS74K89, and DKC 44-13 were moderately susceptible; and X5B-8801 and DK 43-46 were susceptible. Moreover, corn hybrids 1392 VT2P, PFS74K89, and 4913 VT2RIB were moderately resistant; L-2916 VT2PRO, GX89 VT2P, and X5B-8801 were moderately susceptible; and DK 43-48 RIB, DKC 44-13, DK 43-46, and LR-9487 VT2RIB were susceptible to *P. neglectus*. Furthermore, corn hybrids DK 43-48 RIB, 1392 VT2P, and PFS74K89 were moderately resistant; LR-9487 VT2RIB, 4913 VT2RIB, and DK 43-46 were moderately susceptible; and GX89 VT2P, X5B-8801, DKC 44-13, and L-2916 VT2PRO were

susceptible to new *Pratylenchus* sp. ND-2017. The comparison of the host ranking of three root-lesion nematode species revealed that 1392 VT2P was moderately resistant with all three root-lesion nematode species and PFS74K89 and 4913 VT2RIB were moderately resistant with two of the three root-lesion nematode species. Moreover, DK 43-48 RIB, L-2916 VT2PRO, GX89 VT2P, and LR-9487 VT2RIB showed variable responses (susceptible, moderately susceptible, and moderately resistant) to three root-lesion nematode species. Corn hybrids X5B-8801, DK 43-46, and DKC 44-13 were found susceptible to two of the three root-lesion nematode species.