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DNA BARCODING OF PRATYLENCHUS FROM AGROECOSYSTEMS IN THE NORTHERN GREAT PLAINS OF NORTH AMERICA

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DNA BARCODING OF PRATYLENCHUS FROM AGROECOSYSTEMS IN THE NORTHERN GREAT PLAINS OF NORTH AMERICA

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Pratylenchus species are among the most common plant parasitic nematodes in the Great Plains Region. The objectives of this study were to barcode *Pratylenchus* specimens for species identification in the Great Plains region using mitochondrial CO1 DNA barcode. In order to (1) determine species boundaries, (2) assess the host associations of barcoded *Pratylenchus*, (3) to determine the distribution patterns across the Great Plains Region and, (4) to evaluate the species status of *P. scribneri* and *P. hexincisus* by a multivariate morphological analysis of haplotype groups identified by DNA barcoding. Soil samples, primarily associated with eight major crops, were collected from Colorado, Kansas, Montana, Nebraska, North Dakota, and Wyoming. A total of 439 infested field samples from 122 counties representing 11 states were selected for CO1 DNA barcoding. The CO1 region of each individual nematode was amplified by PCR resulting in a 727-739 CO1 nucleotide sequence. Maximum likelihood, neighbor-joining, and Bayesian phylogenetic trees each displayed 19 distinct haplotype groups that were well supported by bootstrap, genetic distances, and posterior probabilities, ages of lineages. Species delimitation analysis (ABGD-GMYC-TCS) revealed variation in detecting putative species number. Most of the tentatively labeled haplotype groups were not easily associated with a named species of *Pratylenchus* and ambiguous results were

especially evident for *P. scribneri* and *P. hexincisus*. The most common haplotype group was *P. neglectus* detected from 178 fields from 100 counties associated with potatoes, wheat, corn, barley, alfalfa, dry beans, vineyard, and sugar beet soils. The second most prevalent haplotype group was *P. scribneri* recovered from 104 fields from 45 counties. Mixed field populations were encountered of approximately 20% of infested fields, suggesting most often *P. neglectus* and *P. scribneri* together. Morphological assessment of Great Plains specimens of *P. hexincisus* and *P. scribneri* revealed difficulties in the morphological discrimination of this pair species, as evident by overlapping on plot of canonical discriminant analysis. Identification of *Pratylenchus* species by DNA barcoding should lead to specific, focused, and effective management strategies for lesion nematodes.

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General Introduction

1.1 *Pratylenchus* Root Lesion Nematode

The family Pratylenchidae Thorne, 1949 consists of 11 genera including *Achlysiella, Apratylenchoides, Apratylenchus, Hoplotylus, Hirschmanniella, Pratylenchus, Pratylenchoides, Radopholus,* and *Nacobbus, Zygotylenchus, Zygradus.* These nematodes characteristically penetrate and feed on internal root tissues and are capable of migrating within root tissues and between root and soil, with exception of *Nacobbus.* Four of these genera, *Pratylenchus* (root lesion nematodes), *Hirschmanniella* (rice root nematodes), *Radopholus* (burrowing nematodes), and *Nacobbus* (false root-knot nematodes), are of major economic importance in agricultural production (Mendoza and Lopez, 2012). The family is morphologically characterized by a small body size (under 1mm), low labial region, sclerotized cephalic framework, stout stylet (under 25 μm), overlapping pharyngeal glands ventrally or dorsally, moderate size of tails and position of vulva (Mendoza and Lopez, 2012; Siddiqi, 2000; Nickle, 1991). The genus *Pratylenchus* Filipjev, 1936 is distinguished from other genera in the family by pharyngeal glands extending over the intestine ventrally, one functional ovary, and a posterior position of vulva (Siddiqi, 2000). The species of genus *Pratylenchus* are characterized by a vermiform robust body, short stylet with rounded knobs (average size 16 μm), 4-6 lateral fields, pharyngeal glands overlapping intestine ventrally, a single anteriorly directed reproductive system, relatively short post-vulval uterine branch , vulva at 75%-85%, and a rounded tail (Castillo and Vovlas, 2007; Loof, 1978; Thorne and Malek, 1968).

1.2 Biology

Pratylenchus species are migratory endoparasites, but ectoparasitic behavior is characteristic of juvenile stages feeding on root hairs without entering the root tissue (Richard W Smiley, 2015). The life cycle of *Pratylenchus* consists of the basic egg stage followed by four molts leaded to the adult stage (Davis and MacGuidwin, 2005; Castillo and Vovlas, 2007). The species may complete multiple generations in a growing season (Mendoza and Lopez, 2012). Many *Pratylenchus* species reproduce by parthenogenesis although sexual reproduction occurs in species such as *P. penetrans* and *P. alleni* (Castillo and Vovlas, 2007). After eggs are laid in the root, corms, tubers, or adjacent soil, the first stage juveniles develop within the eggs and molt to the second stage juvenile that hatches from the egg. A gradual increase in size accompanies the third and fourth stages before reaching sexually mature adult stage. The life cycle duration may vary under field conditions and has been estimated in laboratory experiments (Castillo and Vovlas, 2007). Estimated life cycles may last three to nine weeks depending on the environmental conditions such as temperature, moisture, host plant, and species (Jones et al., 2013). For example, *P. neglectus* and *P. thornei* complete their life cycle six to eight weeks and the reproductive rate is highest between 20-25 °C (Richard W Smiley, 2015). All stages from the second juvenile stage to adult are vermiform and may enter and infect the host roots (Jones and Fosu-Nyarko, 2014), migrating either intra- and intercellularly (Siddiqi, 2000) and can leave the root and enter other host roots (Nickle, 1991). *Pratylenchus* species can survive under adverse conditions for several years at the egg stage and through cryptobiosis or anhydrobiosis until host plants are available and favorable environmental conditions occur (Castillo and Vovlas, 2007). The complete genome sequences of *Pratylenchus* have been reported for two species *Pratylenchus*

coffeae and *Pratylenchus vulnus*.(Burke et al., 2015; Sultana et al., 2013). Burke et al. (2015) reported the genome size of *P. coffeae* as 19.67 Mb with 6712 protein-encoding genes, which is the smallest gene number in any metazoan. However, Kikuchi et al (2017) suggested that researchers should be cautious in interpreting these results due to the lack of publicly available information regarding the assembly of the genome. Sultana et al. (2013) reported the complete mitochondrial genome size of *P. vulnus* as 21,656 bp. with 12 protein-coding genes, among the largest mitochondrial genomes in the class Chromadorea.

1.3 Distribution

As a genus *Pratylenchus* species have a global distribution. They occur in northern and southern temperate ecosystems as well as tropical equatorial ecosystems. Castillo and Vovlas (2007) reported *P. neglectus, P. penetrans, P. thornei, P. vulnus,* and *P. scribneri* from every continent, except Antarctica where a single species, *P. andinus,* is found. There are currently over 100 described species of *Pratylenchus* (Janssen et al., 2017 and Singh et al., 2018). Twenty-seven of this species were reported in North America by Castillo and Vovlas (2007). Following that report, *P. floridensis* and P. *parafloridensis* were described and the number of species from North America increased to twenty-nine (De Luca et al., 2010). Several studies suggest more new species await description (Yan et al, 2017a; Yan et al., 2017).

Of the 29 species, nine different *Pratylenchus* species reported from Great Plains region. (Huang and Yan, 2017; May et al., 2016; Norton, 1983; Orr and Dickerson., 1967; Smolik and Lewis, 1982; Thorne and Malek, 1968; Todd et al., 2014).

1.4 Host Range

Pratylenchus species are predominantly recognized as polyphagous and they can parasite a broad variety of plant species including cereals, vegetables, forage crops, industrial crops, cotton, coffee, and potatoes, and ornamental plant, as well as weed species (Bélair et al., 2007; Castillo and Vovlas, 2007; Pinochet et al., 1992; Zirakparvar, 1980). Host preferences, however, can differ significantly among species. For example, *P. penetrans* are able to parasite approximately 400 plant species and the species are significant parasites of horticultural plants. They are most commonly associated with fruit trees such as apple (*Malus* sp.) (Wallace and MacDonald, 1979), cherry (*Prunus avium*) and peach (*Prunus persica)* (Askar et al., 2012) but also recorded associated with potatoes (Morgan et al., 2002; Thorne, 1961) and corn (Dickerson et al., 1964). *Pratylenchus vulnus* Allen and Jensen, 1951 is also a common parasite of fruit trees associated with almond (*Prunus dulcis*) apple, grapevine (*Vitis* sp.), plum (*Prunus* subg. *prunus*), and walnut (*Junglans dulcis*) but seldomly associated with agronomic crops (Askary et al., 2012; Hammas et al., 2018; Pinochet et al., 1992). In contrast, *Pratylenchus scribneri* is commonly associated with agronomic crops: potatoes (Brown et al., 1980; Macguidwin and Stanger, 1991; Yan et al., 2015), corn (Smolik and Evenson) and soybean (Niblack, 1992; Reboish and Golden, 1985). *Pratylenchus thornei* is also reported on major agricultural crops such as wheat (Smiley et al., 2005), corn (Urek et al., 2003), and potatoes (Brown et al., 1980), but also on occasion recorded on pome and stone fruits (Sogut and Devran, 2011; Urek et al., 2003). Though *Pratylenchus zeae* is recorded on as pathogenic to sorghum, soybean, sugarcane, and corn the species has a relatively narrow host range among monocots (Castillo and Vovlas, 2007).

1.5 Economic Importance

Pratylenchus species are considered the third most important groups of plant parasitic nematodes following root-knot and cyst nematodes (Castillo and Vovlas, 2007; Jones et al., 2013). At the regional scale yield losses can approach 70% as in the Pacific Northwest where high abundances of *Pratylenchus neglectus* exist in fields cropped to wheat (Smiley et al., 2005). Vanstone et al (2008) reported *P. neglectus* can casuse a yield reduction up to 30% and Thompson et al. (2008) reported yield losses caused by *P. thornei* can be as high 70% for intolerant wheat cultivars in Australia. Estimated yield losses in wheat fields by *P. thornei* reaches up to 85% in Australia, 70% in Isreal, 37% in Mexico (May et al., 2015). In 2017, Kansas wheat disease loss due to *Pratylenchus* species was estimated to be 0.6% or 1.95 million bushels (Hollandbeck et al., 2017). Based on a regional survey, Todd and Powers (2018) estimated that yield losses caused by *Pratylenchus* species were 3-4% for corn and 1.5-2% for wheat in the Central Great Plains Region. Bird and Warner (2018) reported *P. penetrans* can decrease marketable carrrot yields by 50% in West Central Michigan. MacGuidwin and Bender (2016) estimated a yield reduction of 3.79% by *P. penetrans* in corn in Wisconsin. The severity of losses depends on a multitude of factors such as soil type, environmental and climatic conditions (Nicol et al., 2011), not the least of which is species identity and host association. For example, corn yield losses caused by *P. hexincisus* and *P. scribneri* are related to field conditions, suggesting *P. hexincisus* more damaging to dry land maize and *P. scribneri* to irrigated mazie (Smolik and Evenson, 1987). These yield loss estimates can be underestimates due to the complex nature of root diseases and the biotic interactions that underlie symptomatic nutrient deficiency (May et al., 2015).

1.6 Identification of *Pratylenchus* species

Traditionally, morphological characteristics has been used to identify and distinguish *Pratylenchus* species. Several of these characters are number of lip annuli, shape of labial region, stylet length, length of overlapping gland lobe, structure and number of lateral fields, position of vulva, presence and shape of spermatheca, length of the post-vulval uterine sac, shape of female tail and terminus, and presence or absence of males (Loof, 1978; Ryss, 2002; Nickle, 1991). Diagnostic keys and compendium have been prepared using these characters (Frederick and Tarjan, 1989; Handoo and Golden, 1989; Loof, 1978; Ryss, 2002a, 2002b; Castillo and Vovlas, 2007). However, identification of these species based on these characters is complicated due to the large number of described species within the genus, their morphological similarities, and overlapping morphological characteristics among species (Castillo and Vovlas, 2007). Notably, when rapid and accurate diagnosis of any species required, morphology may not deliver the required level of certainty, particularly in the availability of mixed populations (Ryss, 2002; Castillo and Vovlas, 2007; Mekete et al., 2011; Powers, 2004). Additionally, the issue of mixed populations further complicates morphology-based identification.

DNA-barcoding, using a small piece of 400-800bp DNA sequence, is a powerful tool for fast and precise identification of known species and an initial step in species discovery (Bhadury et al., 2006; Hebert et al., 2003; Kress and Erickson, 2008; Powers, 2004). A global standard of DNA barcoding was proposed by (Hebert et al., 2003a, 2003b) suggesting an approximately 600-650bp sequence of cytochrome c oxidase subunit 1 (COI) gene of mitochondrial DNA could be used as a suitable genetic marker for identification of all animal species. The COI gene has essential advantages for DNA

based identification. First, conserved primers for COI can amplify a wide range of taxa. Second, this gene appears to have an appropriate phylogenetic signal that provides nucleotide substitution data applicable for species and population level genetic discrimination. (Hebert et al., 2003; Mandal et al., 2014; Rach et al., 2017; Rodrigues et al., 2017).

Several molecular diagnostic tools have been applied to identify *Pratylenchus* species. The D3 expansion segment of 28S rRNA was employed in a phylogenetic analysis of 10 *Pratylenchus* species (Al-Banna et al., 1997). Subsequent analysis included the D2-D3 fragment of 28 (Araya et al., 2016; Subbotin et al., 2008; Troccoli et al., 2016), internal transcribed spacer (ITS) of rRNA (De Luca et al., 2011; Janssen et al., 2017) and 18S rDNA (Rius et al., 2014; Singh et al., 2018; Subbotin et al., 2008)was used for identifying *Pratylenchus* species. The COI gene region of mitochondrial DNA has been successfully used as a barcoding marker in many *Pratylenchus* studies and sequence results of those studies exist in the GENBANK database (Hammas et al., 2018; Janssen et al., 2017; Liu et al., 2017; Palomares-Rius et al., 2014; Qing et al., 2018; Singh et al., 2018; Sultana et al., 2013; Troccoli et al., 2016). Following the discovery of DNA sequence polymorphism in phylogenetic studies, species-specific primers targeting a specific gene have been developed to detect and quantify the to aid management decision (Baidoo et al., 2017; Huang and Yan, 2017).

1.7 *Pratylenchus* studies in the Great Plains Region

The Great Plains Region encompasses a broad area in central North America, extending from Canada to the Texas-Mexican border and from the Rocky Mountains to western Indiana (Samson and Knopf, 1994). This region blankets the states of Kansas,

Nebraska, North and South Dakota and comprises parts of the states of Texas, New Mexico, Oklahoma, Iowa, Illinois, Colorado, Minnesota, Montana, and Wyoming in North America (Hartman et al., 2011). The native vegetation of the Great Plains region was extensively covered by diverse grasslands up to approximately 200 years ago (Dornbush, 2004). By the time European settlers populated the region in the 19th century, most of the grassland was converted into agricultural fields due to high soil fertility (Dornbush, 2004; Fierer et al., 2013). This conversion resulted in the loss of both dry and wet grasslands throughout the region (Samson et al., 2004). The most substantial conversion occurred in tallgrass prairie biome, with less than one percent of the historical extent remaining (Samson and Knopf, 1994; Todd et al., 2006). Agricultural practices demonstrate considerable variation across the region due to the variation in rainfall and precipitation patterns. The growing season shrinks from Texas to Canada (Samson and Knopf, 1994) and the annual rainfall decreases by over 50% moving east to west.

Pratylenchus species are known to be one of the most common and important plant parasitic nematodes across the Great Plains Region. An early study of nematode community composition of the tallgrass prairie in Kansas recorded that the prairie soils contained 228 species from 80 genera, including 23 genera of plant parasitic nematodes and two *Pratylenchus* species, *P. coffee* (Zimmermann, 1898) Filipjev and Stehoven, 1941 and *P. penetrans* (Orr and Dickerson, 1967). Thorne and Malek (1968) reported four *Pratylenchus* species in the Northern Great Plains. These included P. *scribneri* and *Pratylenchus agilis* (later synonymized with *P. scribneri* by Subbotin et al., (2008)) with both species reportedly associated with prairie and cultivated potato fields in Nebraska. Two other species were collected in cultivated fields, *P. tenuis* Thorne and Malek, 1968

and *P. hexincisus* were associated with corn in South Dakota. Smolik and Lewis (1982) recorded *P. tenuis* and *P. scribneri* from the mixed shortgrass prairie ecosystems of western South Dakota. *Pratylenchus scribneri, P. hexincisus,* and *P. tenuis* were reported from corn fields in South Dakota (Smolik, 1977; Smolik and Evenson, 1987). *Pratylenchus alleni* was described from soybean fields in southeast Illinois (Ferris, 1961). Norton (1983) reported the occurrence of *P. agilis, P. alleni* Ferris, 1961, *P. flakensis* Seinhorst, 1968, *P. hexincisus, P. neglectus,* and *P. scribneri* in Iowa corn fields. Johnson (2007) and May et al. (2016) recorded the presence of *P. neglectus* in multiple counties of Montana cropped to wheat. Todd et al. (2014) reported *P. neglectus* and *P. thornei* in wheat fields of Kansas and Colorado as well as *P. penetrans* from eastern Kansas. Siddiqi (2000) reported *P. scribneri* and P. *alleni* as a common parasite of soybean in midwestern USA. Also, several molecular studies have reported presence of *Pratylenchus* species using molecular markers across the region. *P. penetrans* was reported from potatoes in Minnesota (Baidoo et al., 2017) and North Dakota (Yan et al., 2015). *P. neglectus* occurs in Montana wheat fields (Yan et al., 2013), and recently *P. scribneri* was found in soybean, barley, and corn fields in North Dakota (Huang and Yan, 2017). Moreover, the first report of the *Pratylenchus neglectus* on wheat, *Pratylenchus scribneri* on potato, and two unnamed *Pratylenchus* spp. on soybean reported from North Dakota using molecular markers from different gene regions, except COI (Yan et al., 2015; 2016; 2017a; 2017b)**.** This accounting of *Pratylenchus* species reported from the Great Plains region totals nine different species and two awaiting description, not including *P. agilis.*

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Chapter 2

A CO1 DNA Barcoding survey of *Pratylenchus* species in the Great Plains Region of North America

Introduction

Global estimates indicate that there are approximately 100 described species in the genus *Pratylenchus* Filipjev, 1936 (Janssen et al., 2017; Singh et al., 2018). Twenty-seven of these species have been reported from North America by Castillo and Vovlas, (2007). Following that report, the number recorded from North America increased to 29 with the descriptions of *P. floridensis* De Luca, Troccoli, Duncan, Subbotin, Waeyenberge, Moens and Inserra, 2010 and *P. parafloridensis* De Luca, Troccoli, Duncan, Subbotin, Waeyenberge, Moens and Inserra, 2010 (De Luca et al., 2010). Several studies suggest more species await description (Yan et al., 2017a; Yan et al., 2017b). Although most *Pratylenchus* species descriptions were originally based solely on morphological features, many species have now been placed within a phylogenetic context using molecular characters (Araya et al., 2016; Hammas et al.,2018; Fanelli et al., 2018; Flis et al., 2018; Inserra et al., 2007; Palomares-Rius et al., 2014; Singh et al., 2018; Subbotin et al., 2008). These phylogenetic trees provide a framework for species delimitation and establish testable species hypotheses for species discovery (De Luca et al., 2012, De Luca et al., 2010; Janssen et al., 2017; Qing et al., 2018).

Root lesion nematodes in the genus *Pratylenchus* are migratory, intercellular endoparasites that penetrate the root of the host plants, feed and reproduce within the root epidermis and cortex. This feeding behavior results in root lesions that enhance fungi and bacteria infection, secondarily contributing to yield and economic losses in agricultural

production (Jones et al., 2013; Smiley, 2015). Based on their pathogenic capability as reflected by estimates of global yield loss, they are ranked third after cyst (*Heterodera* spp.) and root-knot (*Meloidogyne* spp.) nematodes (Castillo & Vovlas, 2007; Jones et al., 2013). At the regional scale yield losses can approach 70% as in the Pacific Northwest where high abundances of *Pratylenchus neglectus* (Rensch, 1924) Filipjev and Schuurmans Stekhoven, 1941 exist in fields cropped to wheat (Smiley et al., 2005). These yield loss estimates can be underestimates due to the complex nature of root diseases and the biotic interactions that underlie symptomatic nutrient deficiency (May et al., 2015). The severity of losses depends on a multitude of factors, not only the least of which is species identity and host association.

Pratylenchus species, in general, are polyphagous parasitizing a broad variety of plants including cereals, fruits, vegetables, forage crops, industrial crops, cotton, coffee, and potatoes, and ornamentals plant, as well as weed species (Bélair et al.,2007; Castillo and Vovlas, 2007). Host preferences, however, can differ significantly among species. For example, although *Pratylencus penetrans* (Cobb, 1917) Filipjev and Schuurmans Stekhoven, 1941 has recorded associations with 400 different plant species, the species is most commonly associated with fruit trees such as apple (*Malus* sp.) (Wallace and MacDonald, 1979), cherry (*Prunus avium*), and peach (*Prunus persica*) (Askary et al., 2012) and regionally found on potatoes (Morgan et al., 2002) and corn. *Pratylenchus vulnus* Allen and Jensen, 1951 is also a parasite of fruit trees commonly associated with almond (*Prunus dulcis*), apple, grapevine (*Vitis* sp.), plum (*Prunus* subg. *prunus)*, and walnut (*Junglans nigra*), and seldom associated with agronomic crops (Askary et al., 2012; Hammas et al., 2018; Pinochet et al., 1992). In contrast, *Pratylenchus scribneri*

Sherbakoff and Stanley, 1943 is primarily associated with agronomic crops such as potatoes (Brown et al., 1980; Yan et al., 2015), corn and soybeans (Reboish and Golden, 1985). *Pratylenchus thornei* Shen and Allen, 1953 is also recorded on major agronomic crops; wheat (Smiley et al., 2005), corn (Urek et al., 2003), and potatoes (Brown et al., 1980), but also rarely recorded on pome and stone fruits (Sogut and Devran, 2011; Urek et al., 2003).

The geographical distribution of *Pratylenchus* extends from cold temperate and sub-alpine ecosystems to tropical, equatorial ecosystems around the world. The distribution and abundance of individual species may be influenced by temperature optima (Acosta and Malek, 1979; Dickerson, 1979) and soil properties (Thompson et al., 2010). Many *Pratylenchus* species exhibit a preference for sandy soils with a relatively high level of oxygen (Castillo and Vovlas, 2007; Olabiyi et al., 2009). Norton (1983) observed that increased percentage of silt or sand level in soil led to an increase in the population density of *Pratylenchus* species. Thompson et al. (2010) however, reported a positive correlation between population density of *P. thornei* and clay soil in contrast to *P. neglectus* preferring mostly sandy soils. Similarly, populations of *P. hexincisus* Taylor and Jenkins, 1957 were found to increase in clay soil cropped to dryland corn (Smolik and Evenson, 1987).

Postglacial history is a legacy effect that may have shaped *Pratylenchus* distribution in North America prior to the intensive cultivation brought by European settlers. Presently it is not clear which species were introduced to the region, and which might have existed on native grasses prior to European settlement. An early study of nematode community composition of the tallgrass prairie in Kansas recorded that the

prairie soils contained 228 species from 80 genera, including 23 genera of plant parasitic nematodes and two *Pratylenchus* species, *P. coffeae* (Zimmermann, 1898) Filipjev and Stehoven, 1941 and *P. penetrans* (Orr and Dickerson, 1967). Thorne and Malek (1968) reported four *Pratylenchus* species in the Northern Great Plains. These included P. *scribneri* and *Pratylenchus agilis* (later synonymized with *P. scribneri* by Subbotin et al., (2008)) with both species reportedly associated with prairie and cultivated potato fields in Nebraska. The other two species collected in cultivated fields were, *P. tenuis* Thorne and Malek, 1968 with host unspecified, and *P. hexincisus* associated with corn in South Dakota. *Pratylenchus alleni* Ferris, 1961 was described from soybean fields in southeast Illinois (Ferris, 1961). Smolik and Lewis (1982) recorded *P. tenuis* and *P. scribneri* from the mixed shortgrass prairie ecosystems of western South Dakota. *Pratylenchus scribneri, P. hexincisus,* and *P. tenuis* were reported from corn fields in South Dakota (Smolik, 1977; Smolik and Evenson, 1987). Norton (1983) reported the occurrence of *P. agilis, P. alleni, P. flakensis* Seinhorst, 1968, *P. hexincisus, P. neglectus,* and *P. scribneri* in Iowa corn fields. Johnson (2007), May et al. (2016) and (Yan et al., 2013) recorded the presence of *P. neglectus* in counties in Montana cropped to wheat. Todd et al. (2014) reported *P. neglectus* and *P. thornei* in wheat fields of Kansas and Colorado as well as *P. penetrans* from eastern Kansas. *P. penetrans* was also reported from potatoes in Minnesota (Baidoo et al., 2017) and North Dakota (Yan et al., 2015). Recently *P. scribneri* was reported in soybean, barley, and corn fields in North Dakota (Huang and Yan, 2017). This accounting of *Pratylenchus* species reported from the Great Plains region totals nine different species, not including *P. agilis*.

The objectives of this study are to (i) barcode *Pratylenchus* specimens for species identification across the Great Plains region using a cytochrome oxidase subunit 1 (CO1) DNA gene barcode (ii) determine the species boundaries of CO1 barcoded specimens (iii) assess the host associations of CO1 barcoded *Pratylenchus* species (iv) determine *Pratylenchus* species distribution patterns across the Great Plains region.

Materials and Methods

Sample Collection

Soil and root samples analyzed in this study were obtained from USDA Cooperative Agricultural Pest Survey Program (CAPS), Wheat and Corn Disease Surveys in Kansas and Nebraska, and field samples submitted to University of Nebraska-Lincoln Disease Diagnostic Clinic, representing different crops and geographic region primarily within the Northern Great Plains of North America. Five statewide surveys associated with the CAPS program were conducted by Departments of Agriculture in Kansas, Montana, Nebraska, North Dakota, and Wyoming. A minimal number of samples were acquired from the Great plains state of South Dakota and no samples from Iowa were included due to lack of available collaborators. Sample collection sites (locations), nematode identification (NID) numbers and host information on each analyzed specimen are presented in supplementary table 1 and all collection sites mapped in figure 1.

Nematode Extraction

Nematodes were extracted from 100 cm^3 field soil using a modified flotation-sieving and sugar centrifugation method (Jenkins, 1964) and from the host-root material

using root incubation (Russell, 1987; Todd and Oakley, 1996). A majority of the samples recovered from Kansas were obtained from root extracts.

Morphological Analysis and Vouchers

Nematodes extracted from soil and roots were first evaluated under a stereo dissecting microscope and select specimens belonging to the genus *Pratylenchus* were handpicked for light microscopy examination and DNA extraction. Following immobilization of live specimens by heating, a subset of specimens were mounted on the temporary glass slides, measured with a Leica DMLB light microscope with Differential Interference Contrast and photographed with a Leica DC300 video camera. Images were stored in the database system of the Nematology laboratory at the University of Nebraska-Lincoln. For PCR amplification, image vouchered specimens were removed from temporary slides and smashed in an 18 µl drop of sterile deionized distilled water utilizing the tip of a sterile transparent micropipette. Smashed specimens were transferred to PCR reaction microfuge tubes and stored at -20 °C until PCR amplification. At least five specimens from each sample were smashed for DNA analysis to assess the possibility of *Pratylenchus* species mixtures within fields.

PCR Primers and Amplification Conditions

The cytochrome c oxidase subunit 1(CO1) gene region of mitochondrial DNA was amplified by PCR using primer sets of CO1- F7bP (5′-GGDTGRACWTTHTAYCCNCC-3′), and CO1-JB5 (5′-AGCACCTAAACTTAAAACATAATGAAAATG-3′) Derycke et al. (2005) that resulted in 727-739bp of sequence for genetic analysis after trimming the primers from the amplified product. On occasion, forward primer JB3 (5′-

TTTTTTGGGCATCCTGAGGTTTAT-3′) of Derycke et al. (2005) was used in a combination with the JB5 primer. The D2-D3 region of 28S rDNA was amplified using the primer sets of De Ley et al. (1999) D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′). PCR was conducted in a total volume of 30 µl reaction mix consisting of 1.2 µl molecular biology grade water, 2.4 µl of each primer and 15 µl of 2x JumpStart REDTaq ReadyMix Reaction Mix (Sigma-Aldrich). Amplification conditions were: a hot-start at 104°C, initial denaturation treatment at 94°C for 5min followed by 45 cycles of denaturation for 30sec, annealing at 50°C for 30sec, extension at 72°C for 90sec and a final extension at 72°C for 5min. Annealing temperature was at 48°C for D2-D3 amplification. A check gel of 1% agarose using 0.5XTBE and ethidium bromide was used to visualize and evaluate the amplification products under UV light.

DNA cleaning and sequencing

High-quality PCR products in a 0.7% agarose TAE gel were extracted by the x-tracta agarose gel extraction tool (USA Scientific) and cleaned using Gel/PCR DNA Fragments Extraction Kit (IBI Scientific). All cleaned DNA templates were shipped to the University of California-Davis DNA Sequencing Facility for sequencing in both directions.

Phylogenetic analysis

Upon obtaining sequence results, the sequences were edited, and primers trimmed off using CodonCode Aligner version 8.0.1 (www.codoncode.com) and subjected to a BLAST search to assess quality and identity on GenBank. Sequences were submitted to repositories, GenBank and Barcode of Life Database system (BOLD). GenBank

accession number of each sequence for CO1 and 28S were represented in table1. Multiple sequence alignment was conducted using Muscle (Edgar, 2004), at a gap opening penalty -400 and a gap extension penalty -200, in MEGA version 7 (Kumar et al., 2016). The best DNA Model tool in MEGA 7 was used to determine a best-fit substitution model, general time reversible (GTR) with a gamma distribution (G) and proportion of invariable sites (I). The substitution model (GTR+GI) was used for maximum-likelihood analysis with 200 bootstrap replications using software MEGA 7 and for Bayesian inference analysis (BI) using the software MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). Bayesian analysis were conducted with four Markov chains of 5M generations with sampling at every 1000 generations. After discarding burn-in samples (25%), the remaining samples were used to generate a 50% majority rule tree. Posterior probabilities are given on appropriate clades. Bayesian trees were visualized by software Figtree version 1.4 (tree.bio.ed.ac.uk). A Neighbor-joining analysis was also conducted using the Kimura-2 Model with gamma distribution and 2000 bootstrap value under MEGA 7. Between and within group genetic distance matrices were computed by Kimura-2 Method with gamma-distributed rate, 2000 bootstrap selections, and data treatment of pairwise deletion. CO1 haplotypes were reduced by removal of redundant sequences using software Jalview.2.10 (Waterhouse et al,, 2009).

Species Delimitation/Delineation

Molecular species delimitation was assessed using Automatic Barcoding Gap Discovery (ABGD) (Puillandre et al., 2012), the Generalized Mixed Yule Coalescent (GMYC) method (Pons et al., 2006) and by statistical parsimony networks (TCS) (Clement et al., 2002), all applied to a non-redundant, 143 specimen CO1 data set.

ABGD method calculates all pairwise distances of any set of sequences and uses several prior thresholds a “barcode gap” in the pairwise distribution of pairwise differences, corresponding to the upper limit of intraspecific distance and lower limit of interspecific distances. ABGD splits sequences into putative groups based on the barcoding gap (initial partitioning) then this procedure recursively applied to each previously obtained groups of sequences to detect a second gap for recursive partition (Modica et al., 2014; Puillandre et al., 2012; Roy et al., 2013). A prior species boundary is not required for this method (Puillandre et al., 2012). ABGD analyses were performed at the online webserver (https://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) using Kimura (K80) distance model with defaults parameters.

GMYC is a model-based Maximum likelihood approach that uses an ultrametric tree to delimit species and determine diversification and coalescence events based on branching patterns. It calculates a single or multiple threshold for species delimitation and all nodes crossing the threshold value are considered species (Beatty et al., 2017; Fujisawa 2013; Pons et al., 2006). An ultrametric tree was constructed using Beast 2.5.2 (Bouckaert et al., 2014) with GTR model with Gamma distribution (G) and four gamma categories for nucleotide evolution. The tree was estimated with a strict clock model or a lognormal relaxed clock model assigned to a yule or a coalescence branching model. All other parameters were set as defaults and tree was not dated for GMYC analysis. Markov Chain Monte Carlo (MCMC) chain was run at 30-250M million generation at sampling every 3000^{th} -5000^{th} generation based on model selection. Tracer V1.7 (Rambaut et al., 2018) was used to visualize the convergences and evaluate effective sample size (ESS >200) of traces. A maximum clade credibility tree was produced by TreeAnnotator

V2.5.2 (Rambaut et al., 2018) with common ancestor node heights, after discarding 25% of the trees as burn-in. It was visualized using FigTree V.1.4 (tree.bio.ed.ac.uk). Beast analyses were run on the XSEDE server of the CIPRES Science Gateway (Miller et al., 2011). The GMYC analysis was conducted using the single threshold option (T. Fujisawa and Barraclough, 2013) using the SPLITS package (Ezard et al., 2014) and ape package (Paradis et al., 2004) available for R v3.5.2 (R Development core Team, 2018). After analysis all outputs (Estimated threshold time and the list of ML clusters and entities) was exported from R.

TCS is a clustering method that calculates a distance matrix for pairwise comparison of haplotypes to recognize species boundaries, while calculating mutational differences at an assigned cut-off probability. Parsimony criterion applies before the mutational differences reach the cut-off percentage (Templeton et al., 1992). In this study, we assigned two connection limits (90% and 95%) to delimit species.

Haplotype Network Analysis

A haplotype network analysis was conducted to visualize the relationships among CO1 haplotypes of *Pratylenchus* species based on their geographic and host information. Haplotype networks were calculated using TCS plug-in (Clement et al., 2002; Templeton et al., 1992) in the software PopART 1.7 (Leigh and Bryant, 2015) using 95% connection limit to delimit species.

Divergence Time Estimation Analysis

Molecular divergence time was estimated with a molecular clock analysis using the non-redundant CO1 dataset in BEAST v2.5.2 (Bouckaert et al., 2014). Because no fossil or geological calibration point are available for dating the *Pratylenchus*, we used

two mutation/clock rates from the literature, first a widely used substitutions/site/my/lineage clock rate of 0.0115 corresponding to a common invertebrate CO1 mitochondrial pairwise sequence divergence rate of 2.3% per site per million years (Brower, 1994) and second the mitochondrial substitution genome rate of 7.2×10^{-8} per site per generation calculated for the nematode *Caenorhabditis briggsae* (Howe et al., 2010). For *Pratylenchus* an assumption two generations per year was applied to the analyses. The BEAST analysis was carried out under a fixed strict clock model with a yule speciation model as tree prior. All other parameters were set as defaults. Markov Chain Monte Carlo (MCMC) chain was run at 30M million generation at sampling every 3000th generation. Tracer V1.7 (Rambaut et al., 2018) was used to visualize the convergences and evaluate effective sample size (ESS >200) of traces. A maximum clade credibility tree was produced by TreeAnnotator V2.5.2 (Rambaut et al., 2018) with common ancestor node heights, after discarding 25% of the trees as burn-in. MCC tree and node ages was visualized using FigTree V.1.4 (tree.bio.ed.ac.uk). Beast analysis were conducted on the XSEDE server of the CIPRES Science Gateway (Miller et al., 2011).

Results

Survey Results

A total of 860 soil samples were assayed during the growing season of 2017 and 2018. These samples represented statewide surveys of eight major agronomic crops in Colorado, Kansas, Montana, Nebraska, North Dakota, and Wyoming, as well as five other crops opportunistically sampled across the Great Plains Region (Figure 1). In total, *Pratylenchus* species were recovered from approximately 71% of all samples. Recovery

rates for cornfields in Kansas, Nebraska, and Montana were 94.9%, 86.4%, and 63.6%, respectively (Table 2). *Pratylenchus* was recovered in 80.3% of Kansas wheat fields, but only 37% of wheat fields in North Dakota (Table 1).

Phylogenetic analysis and nematode identification

There was a total of 439 infested field samples representing 122 counties from 11 states of Great Plains region, in the *Pratylenchus* dataset. From that dataset, 270 infested fields were selected for DNA barcoding that maximized geographic and host coverage across the region (Figure 2). An additional 24 cultures and 18 field samples from outside the Great Plains region (mostly from Arkansas) were included in the analyses. Topotype specimens of *P. alleni* were isolated from a soybean field five miles north of Eldorado city, in Saline County in Illinois. A total of 915 specimens of *Pratylenchus* were sequenced for a 727-739-bp of CO1 gene. The sequence length was 730-bp for most of the specimens, except *P. neglectus* specimens (727-bp) and *Pratylenchus* sp. 9 and *Pratylenchus* sp. 10 specimens (739-bp). A total of 915 *Pratylenchus* CO1 haplotypes were reduced to a 143 sequences dataset by the removal of redundant sequences. Maximum likelihood, neighbor-joining and Bayesian phylogenetic trees each identified 19 distinct CO1 haplotype groups that were well-supported by bootstrap values (BS: 96-100% based on tree construction method), intra vs. interspecific genetic distance and posterior probabilities (PP:100 for all groups) (Figure 3). These haplotype groups were tentatively labeled as *P. neglectus, P. scribneri, P. thornei, P. hexincisus, P. alleni, P. penetrans, P. zeae, P. crenatus, P. vulnus,* Pratylenchuıs sp. 1-10, plus one unnamed singleton. Binomial names were applied based on a combination of factors including distinctive morphological features, agreement with GenBank vouchers, congruence

between independent markers, and in the case of *P. alleni*, topotype specimens. Four haplotype groups in figure 3 consisted exclusively of specimens collected outside the Great Plains. These were *P. crenatus, P. vulnus,* and species 9 and 10, both exclusively represented by specimens from Arkansas. Among this larger 19 haplotype group dataset, males were recorded in 7 haplotype groups, *P.penetrans, P.alleni, P. crenatus* and unnamed haplotype groups species 1, 2, 9, and 10 (figure 3). In total 14 haplotype groups and one singleton were associated with agroecosystems of the Great Plains Region.

Sequences of the D2-D3 region were evaluated in a phylogenetic tree using 24 specimens previously sequenced by CO1 together with 70 GenBank accessions (Supplementary Figure 1). Ten of the 24 sequences were placed within well-supported clades designating named species of *Pratylenchus*. These include *P. alleni* (NIDs 10848, 10850,3717), *P. crenatus* (NID 8539), *P. thornei* (NIDs 7566, 7567), *P. neglectus* (NID 10756), and *P. penetrans* (NID 7091, 6260, 6261). Sequences of the D2-D3 region for the unknown *Pratylenchus* species 1, 2, 5, 8, and 9 were not situated together with named species within well-supported clades in NJ, ML, or Bayesian trees. A BLAST search of GenBank with *Pratylenchus* CO1 sequences generated in this study helped to determine species identity through near identical matches (99-100%) for seven of the CO1 haplotype groups (Table 2). These well-supported haplotype groups corresponded to *P. neglectus, P. penetrans, P. thornei, P.alleni*. *P. zeae*, *P. crenatus,* and *P. vulnus*. *Pratylenchus scribneri,* the second most abundant *Pratylenchus* species in this survey, provided conflicting results in GenBank BLAST searches. With NID 7839 as the query, the top 9 matches ranged from 98.05% match (*P. scribneri* KY424091) to 99.50% (*P. scribneri* MH016378) which bracket two closes matches of 98.75% (*P. hexincisus*

KY828320) and 98.71% (*P. hexincisus* KY828322). Similarly, for the D2D3 marker, the top 25 matches ranged from 99.73% to 98.12% for *P. scribneri* matches that included three *P. hexincisus* entries of 99.58%. Specimens hypothesized to be *P. hexincisus* had no close CO1 match identified as *P. hexincisus* (Table 2; Ozbayrak et al. in prep). For the D2-D3 marker, those specimens exhibited a relatively close match to accessions labeled as *P. hexincisus* (DQ498832-98% similarity) and *P. scribneri* (KX842632-99% similarity). Ten haplotype groups with no clear taxonomic affinities based on DNA sequence were labeled as "sp." These specimens generally had low identity values with CO1 BLAST scores and moderate or ambiguous similarity scores with D2-D3.

Intragroup and intergroup genetic distance matrices are presented in Tables 3 and 4. Estimated mean genetic Kimura-2 distances (K80) for all haplotype pairs was 0.35 (SE 0.03). Pairwise genetic distances within most haplotype groups ranged from 0 to 0.088. Haplotypes groups *Pratylenchus* sp. 2 and *Pratylenchus* sp. 9 exhibited the highest intraspecific diversity with K80 distance values of 0.088 and 0.053 respectively. Also, *P. hexincisus*, *P. penetrans*, and *Pratylenchus sp.* 10 had relatively high level of genetic variability with K80 distance values of 0.026, 0.048 and 0.036, respectively. Interspecific variability among haplotype groups was exceptionally high, ranging from a low of 0.092 (between haplotypes *Pratylenchus* sp. 5 and *Pratylenchus* sp. 6) to 0.90 (between haplotypes *Pratylenchus penetrans* and *Pratylenchus hexincisus*).

Species Delimitation

Molecular species delimitation using ABGD suggested 19-61 haplotype groups in the recursive partitioning based on differing prior intraspecific divergences (Ps) (Figure 4). The last three partitions settled on 19 groups, assuming P values of 0.0359, 0.0599

and 0.100, respectively. ABGD results were congruent with the haplotype numbers on phylogenetic trees at the last three partitions. The number of haplotype groups was 21 among P values (0.008-0.022), subdividing *P. hexincisus* and *P. penetrans* into two separate group.

GMYC result did not differ substantially based on the tree prior or clock model selected. The single threshold GMYC analysis revealed 22 ML cluster (CI 22-23) and 39 ML entities (CI 39-42) based on using a strict molecular clock and yule tree prior (Figure 4). The likelihood of null model was 972.166 and maximum likelihood of the GMYC model was 998.4352. The likelihood ratio (52.5385) test rejected the null hypothesis for the models tested, the assumption that all sequences belonged to the same species (LR test: $P < 0.001$). The threshold time was found to be -0.00914, indicating at that point a steep upturn in branching rates continuing to the present.

TCS analysis resulted in 36 and 32 haplotype networks at connection limits of 95% and 90%, respectively for the complete data set (Figure 4). Four distinct haplotype networks labeled A, B, C, D, were recognized for *P. neglectus* at both 95% and 90% connection limit. These four *P. neglectus* subgroups were also detected in GMYC delimitation methods. Network A consisted of eight unique haplotypes, occurring in 10 states of North America and Canada (Figure 5). The most abundant haplotypes were neg1, neg2, neg3 within network A. Haplotype neg 1 were found in six states and were most often associated with corn, but also associated with dry bean, wheat, alfalfa, potatoes, and cereal rye. Neg 2 and neg 3 were found respectively in ten and nine states plus Canada and were most frequently recovered from wheat, but also associated with other crops (see Figure 5-host). A total six haplotypes from networks B, C, D were

located in western and northwestern states of Colorado, Wyoming, Idaho, Montana, and North Dakota with only a few specimens from Nebraska and Kansas included in these networks. Specimens in networks B C, and D were associated with barley, potatoes, alfalfa, beans, corn, wheat, and sugar beet.

Pratylenchus scribneri appeared as a single TCS network comprised of 17 closely related haplotypes (Figure 6). *Pratylenchus thornei* was comprised of a single haplotype distributed across six different states (Figure 7). *Pratylenchus hexincisus* specimens were split into two separate groups in TCS networks and GMYC methods (Figure 7), but ABGD only recognized a second network at P values between 0.008-0.022. Four haplotype groups with male specimens, *P. penetrans*, *Pratylenchus* species 2, 9, and 10, displayed diverse TCS networks and multiple haplotype subgroups in GMYC methods. The final partition in ABGD however, recognized these four sexual groups as four distinct entities. For *Pratylenchus penetrans* ABGD revealed two distinct group at P values of 0.0359, 0.0599 and 0.100. Six haplotypes were detected, suggesting 3 distinct networks in TCS analyses at both 90 and 95% cutoff values and three entities were revealed in GMYC analysis. Eleven *Pratylenchus* sp. 2 haplotypes comprised six networks at 95% and 90% connection limits, respectively and GMYC methods exhibited nine entities. *Pratylenchus* sp. 9 and sp. 10 had four networks at 95% cutoff and two at 90% cutoff, respectively. GMYC analysis revealed four entities for both groups. Species delimitation results for other groups exhibited one network/haplotype/entity due to the existence of few specimens tin these groups.

Estimated divergence time was calculated using two different molecular clock rates, first a commonly used CO1 mitochondrial DNA substation rate of 0.0115 per site

per million years and second the mitochondrial substitution genome rate of 7.2 x 10^-8 per site per generation calculated for nematode *Caenorhabditis briggsae.* The nematode molecular clock rate with an assumption of two *Pratylenchus* generations per year, exhibited approximately ten-fold younger node ages than the insect substitution rate. Divergence time estimation based on the clock rate of the nematode revealed early diversification events of *Pratylenchus* clades occurred at estimated time 6.28 Mya (CI: 5.12 -7.59 Mya) in the late Miocene epoch, splitting haplotype groups in this study into two main clades. Other *Pratylenchus* species lineages were splitting in the Pliocene, prior to the onset of the ice ages approximately 2.7 Mya. *Pratylenchus neglectus* may have diverged from *P. penetrans* as early as 4.73 Mya (CI: 3.56-5.61 Mya) and *P. thornei* split from *P. zeae* an estimated 5.39 Mya (CI: 3.38-5.47 Mya). The most recent common ancestor (MRCA) of *P. hexincisus* and *P. scribneri* emerged early in the Pleistocene approximately1.46 Mya (CI: 1.24-1.69 Mya). The MRCA of *P. scribneri,* youngest clade, emerged in the late Pleistocene epoch at 0.65 Mya (0.48-0.73 Mya). Three lineages of sexually reproducing species, Pratylenchus sp. 9 *Pratylenchus* sp. 10, and *Pratylenchus alleni,* all diversified during early to mid-stages of the ice ages between 2 million and 500,000 years ago (Figure 8).

CO1 haplotype group host associations and distribution

Pratylenchus neglectus was the most frequently sampled haplotype group in the Great Plains, comprising 53% of all specimens on the 915 specimen phylogenetic trees. It was detected in 178 fields from 100 counties and was associated with potatoes, wheat, corn, barley, alfalfa, dry beans, vineyard, and sugar beet soils. *Pratylenchus neglectus* was identified from 96%, 90%, and 83% of all wheat, potatoes, and dry beans fields,

respectively. *Pratylenchus neglectus* was encountered in corn fields less frequently at 42% of corn fields across the Great Plains (Figure 9 and 10).

The second most abundant haplotype group in the Great Plains, comprising 30% of all specimens, was *P. scribneri*. It was recovered from 104 fields from 45 counties and was associated most often with corn fields in 4 states; 78 in Nebraska, 14 in Kansas, two in South Dakota, and one in Montana. Also, it was recorded from four potato fields in Nebraska, one wheat field from Nebraska and Texas, and one sugar beet field in Colorado (Figure 9 and 10).

Pratylenchus thornei was primarily associated with wheat in Kansas but was also collected from corn and alfalfa in Montana, corn in Oklahoma, a single plot in a cover crop (cereal rye) experiment in Nebraska, in a vineyard in California, and a sugar beet field rotated with barley in Colorado.

Pratylenchus penetrans was not common in the agronomic crops sampled in this Great Plains dataset. It was recovered from one south central cornfield in Nebraska and an apple orchard in eastern Nebraska, two cornfields in Montana. Infrequently encountered species in this Great Plains survey included *P. zeae* and *P. alleni*, both collected from single corn fields in Nebraska. *P. hexincisus* was primarily associated with corn fields in eastern Kansas, Nebraska, and South Dakota, as well as from dry beans in Wyoming and wheat in North Dakota (Figure 9 and 11).

Nearly all of the unnamed haplotypes were associated with corn, with the exception of one wheat, one soybean, and one cotton field. Barcoding also revealed that 44 of the 439 *Pratylenchus* infested fields had a mixed population of at least two *Pratylenchus* species. Mixtures were recorded in 19.5%, 10.6%. and 27.8% of the corn,

wheat, and dry beans fields, respectively. Five corn fields in Kansas and 23 corn field in Nebraska had a mixture of different haplotype groups. The most common combination of species was *P.neglectus* and *P. scribneri*, which were recovered together from approximately 55% of all mixed fields. Distribution maps and host associations table of haplotype groups are displayed in Figure 9,10, and 11.

Discussion

This study is the first comprehensive *Pratylenchus* species survey using CO1 DNA barcode to determine *Pratylenchus* haplotypes associated with agroecosystems of the Great Plains region of North America. This study also provides details on phylogenetic membership in haplotype groups, relationships among *Pratylenchus* haplotype groups, their geographic distribution and host associations with agronomically important crops from 11 states across the region. Earlier studies reported the presence of nine described *Pratylenchus* species associated with prairie and agricultural fields (Al-Khafaji, 2018; Orr and Dickerson, 1967; Smolik and Evenson, 1987; Thorne and Malek, 1968; Yan et al., 2016). This DNA barcoding survey demonstrated the presence of 7 distinct haplotype groups that coincide with binominal species *P. neglectus, P. scribneri, P. thornei, P. hexincisus, P. alleni, P. penetrans, P. zeae,* and eight unnamed haplotype groups and 1 unnamed singleton, all associated with agroecosystems of Great Plains. Posterior probability, bootstrap values, genetic distances, and calculations of lineage age strongly supported the genetic distinction of the haplotype groups. Group membership remained constant with different tree-building methods, although relationships at deeper nodes in the tree varied slightly. The amount of intraspecific genetic divergence was found to be low for most of the haplotype groups, although genetic substructure with the

haplotype groups was recognized by GMYC and TCS delineation approaches. Four haplotype groups with males (*P. penetrans*, *Pratylenchus* sp. 2, 10, and 11) had relatively high within group genetic variability. The overall mean intergroup divergence was high, suggesting a relatively long period since the *Pratylenchus* lineages diverged. Divergence time of *Pratylenchus* species in this study were estimated using a higher evolutionary rate than is commonly used in CO1 mitochondrial DNA studies (Brower, 1994; Howe et al., 2010). The higher substitution rate was derived from studies with *Caenorhabitis briggsae* and resulted in a rate approximately ten-fold higher than the standard rate derived from insect. Still the calculated rate of evolution supported divergence of the major species lineages present in the Great Plains at approximately 1-5 million years ago. Older divergence times in these analyzes, not surprisingly, are associated with lineages that are more readily differentiated using morphological characteristics.

Species delimitation analysis displayed variation in determining the number of putative species. GMYC and TCS methods generally yielded similar results and supported the recognition of subgroups as species within the haplotype groups in figure 1. The ABGD method generally mirrored the tree topologies at P value of 0.0359, 0.0599 and 0.100. GMYC and TCS retained P. *scribneri, P. thornei, P. alleni, P. cerenatus,* and most of the unknown groups as an independent lineages or evolutionary entities but split *P. neglectus, P. penetrans, Pratylenchus* sp. 2, *Pratylenchus* sp. 9, and *Pratylenchus* sp. 10 into two or more putative species.

Some *Pratylenchus* species reportedly found in the Great Plains were not observed in this study. Early reports of *P.tenuis* from cultivated fields and prairie ecosystems were derived from two publications. Following Thorne and Malek's (1968)

original description, Hando and Golden (1989) re-described the species, based on two female type specimens. The distinctive characters of this species were high, narrow tulip-shaped stylet knobs and an unusually elongate esophageal lobe three times the body width. Presently it is not possible to assign any of the haplotype groups to this species based on those characters.

There are several key conclusions that can be made from this DNA barcode-based survey of *Pratylenchus* specimens in the Great Plains. First, *Pratylenchus neglectus* is the most wide-spread and abundant lesion nematode across the region. It was recorded from field soils currently producing alfalfa, barley, corn, dry beans, potato, wheat, and sugar beet. Although presence in field soil is not direct evidence of parasitism on the current crop, and most agronomic crops in the region are grown in rotation with other plants, it is a safe assumption that active *Pratylenchus* populations found around the roots during a growing season are feeding on those roots. This observation complements the findings of Al-Khafaji (2018)and May et al. (2016) concerning the widespread presence *P. neglectus* on wheat in Montana, as well as the studies of Todd et al., (2014) and Todd and Oakley, (1996) that documented the high incidence of *P. neglectus* in Kansas wheat and corn fields, respectively. *Pratylenchus neglectus* was also observed as a frequent member of mixed species populations, most commonly associated with *P. scribneri*. It is not known to what extend a mixed species *Pratylenchus* population will complicate management strategies, but it accentuates the need to understand the host relationships and damage potential of both species for the implementation of predictive models of pest management.

Pratylenchus scribneri is the second most abundant nematode in the region, and the lesion species most likely to be recovered in the corn-soybean cropping rotations of Nebraska and Kansas. In contrast, it was not recovered frequently from wheat, suggesting a reduced reproductive capacity or an inability to successfully compete with *P. neglectus* on wheat. Another species known to reproduce on wheat, but not frequently encountered in the Great Plains Region is *P. thornei*. This species was infrequently encountered in Kansas wheat samples, collected from cereal rye in a single experimental plot in central Nebraska, in Colorado in a mixed planting of oats and alfalfa, and was not recovered at all in North Dakota wheat fields in spite of widespread sampling in the state. Smiley et al (2006) found *P. thornei* exclusively in 6% of wheat fields in the Pacific Northwest, and in combination with *P. neglectus* in 30% of the soils. In the Great Plains *P. thornei* may be limited by soil factors as suggested by Thompson et al. (2010) or there has been insufficient time for the species to spread across the region since its introduction. *Pratylenchus penetrans* is another species that is surprisingly limited in its distribution within the Great Plains. Among Great Plains agronomic crops, *P. penetrans* was only recovered from a single field of corn in Nebraska. Two other species with highly localized distributions were *P. zeae* and *P. alleni*. *Pratylenchus zeae* was collected from a single corn field in Keith County, Nebraska and outside the Great Plains region it occurred in Arkansas corn fields. *Pratylenchus alleni* was collected from a soybean field in Illinois on its type host at its type locality in Saline County. In spite of extensive production of corn and soybeans grown in rotation throughout the region, outside of the type locality *P. alleni* was only found in a single corn field in Madison County, Nebraska.

It is possible that a focused survey on soybeans and potato will increase the documented distribution of both *P. alleni* and *P. penetrans* within the Great Plains.

Some of the CO1 haplotype groups revealed by the phylogenetic analysis were not easily associated with a named species of *Pratylenchus*. For example, the species we tentatively identified as *P. hexincisus*, had no close match for CO1 in GenBank, and D2-D3 sequences provide moderately close matches to both *P. scribneri* and *P. hexincisus*. This haplotype group was collected from six states in the Great Plains associated with beans, corn, and wheat. A more extensive taxonomic analysis of this species is underway (Ozbayrak et al. in prep). At least eight other unnamed haplotypes groups and singletons were represented in this dataset, most with too few specimens for a taxonomic analysis. One haplotype group represented by 14 specimens collected from corn in Sumner and Shawnee Counties in Kansas and Buffalo County in Nebraska, was characterized by the relatively frequent presence of males. These specimens superficially resemble *P. penetrans* and may be the species recognized (Orr and Dickerson 1967; Todd et al., 2014)

This mosaic of *Pratylenchus* species distributed across the Great Plains raises the question about the necessity of identifying the species composition of agricultural fields. Management options of *Pratylenchus* species generally fall into four main categories: fallow, crop rotation, genetic resistance, and genetic tolerance (Smiley, 2015). If all these haplotype groups responded in a similar fashion to environmental and physiological conditions, then a common management strategy could be applied for lesion nematodes. Evidence, however, suggests these haplotype groups may differ in their host preferences, environmental tolerances, and possibly their competitive interactions. The frequency of *P. scribneri* in corn or the relative lack of that species in wheat indirectly suggests the

existence of host preferences (Smiley et al., 2005; Smiley, 2015; Todd and Powers, 2018). A necessary next step in the characterization of these haplotype groups is the establishment of pure cultures and an analysis of reproductive capabilities on hosts grown in the Great Plains Region.

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Appendix 1 - Terminology

DNA Barcoding: Use of specific DNA sequence to identify species

Haplotypes: A haplotype is a sequence differing from other sequences by the difference of at least one base pair.

Haplotype groups: A name for a group of similar haplotypes that have closely related sequences

Haplotype Networks: Represents the genealogical relationships among individual genotypes at intraspecific level and make inference about biogeography and history of population.

Lineages: A metapopulation extended through time (Equal to species in this study)

Clade: A clade is a group that includes a common ancestor and all the descendants (living and extinct) of that ancestor. (Clade is a branch (at any level) of a phylogenetic tree)

Singleton: A haplotype group with one specimen

Cryptic species: Species are phenotypically identical, but genetically distinct

Parthenogenetic: A type of asexual form of reproduction without fertilization

Table 1. Percentage and Prevalence of *Pratylenchus* Haplotypes in the Great Plains Region

Pratylenchus Prevalence (%)								
States	Potatoes	Corn	Alfalfa	Barley	Wheat	Dry Beans	Sugar beet	Soybean
NE	13/43 (56.50)	190/220 (86.40)		13/13 (100)	8/8 (100)			
MT		7/11 (63.60)	8/9 (88.90	17/25 (68.00)	5/25 (20.00)			
ND	5/21 (23.80)				40/108 (37.00)	0/1	3/25 (12.00)	3/15 (20.00)
WY	15/16 (93.75)		0/1	3/5 (60.00)	4/5 (80.00)	24/30 (80.00)	4/5 (80.00)	
KS		167/176 (94.90)			61/76 (80.30)			
CO		7/8 (87.50)	2/2 (100)	1/2 (50.00)		2/2 (100.00)	5/6 (83.3)	2/2 (100.00)
Total	33/80 (41.25)	371/415 (89.30)	10/12 (83.3)	34/45 (75.60)	118/222 (53.15)	26/33 (78.80)	12/36 (33.30)	5/17 (29.5)

Table 2. GenBank Best Matches and Accession numbers of *Pratylenchus* haplotype groups of Great Plains Region.

Haplotype Group (This study)	Blasted NID# (This study)	CO1 Best Match Accession#	Corresponding GenBank Name	Identity %	D2-D3 Best Match Accession#	Corresponding GenBank Name	Identity %
P. neglectus	N10756	KY424103	*P. neglectus*	100%	MG906766	*P. neglectus*	100%
P. penetrans	N6260	KY816936	*P. penetrans*	100%	JX046986	*P. penetrans*	99%
P. thornei	N7566	KY828316	*P. thornei*	100%	KYT213559	*P. thornei*	100%
P. alleni	N3717	MK045330	*P. alleni*	99%	MK037385	*P. alleni*	100%
P. zeae	N8934	KY424056	*P. zeae*	100%			
P. crenatus	N8539	KY816943	*P. crenatus*	100%	KY468865	*P. crenatus*	98%
P. vulnus	N7113	GQ332425	*P. vulnus*	99%			
P. scribneri	N7839	MH016378 KY828320	*P. scribneri* *P. hexincisus*	99% 98%	MK209593 KY828290 EU130841 KT175531	*P. scribneri* *P. hexincisus* *P. agilis* *P.pseudocoffeae*	99.73% 99.72% 99.73% 97.98%
P. hexincisus	N7726	No Match	--	--	KX842632 EU130841 KT175531 KY828291	*P. scribneri* *P. agilis* *P. pseudocoffeae* *P. hexincisus*	100% 98.78% 97.57% 98.87%
Pratylenchus sp. 1	N6350	KU198944	*Pratylenchus sp.*	83%	KX889989	*Pratylenchus* sp.	100%
Pratylenchus sp. 2	N8139	No match	--	--	KX842632 KT175531	*P. scribneri* *P.pseudocoffeae*	97% 97%
Pratylenchus sp. 3	N3908	MH394241	*P.gutierrezi*	76%			
Pratylenchus sp. 4	N6402	KY424092	*P. scribneri*	89%			
Pratylenchus sp. 5	N10767	KY424092	*P. scribneri*	87%	MH730449	*P. scribneri*	97%
Pratylenchus sp. 6	N6245	KY424092	*P. scribneri*	89%			
Pratylenchus sp. 7	N10770	KY424099	*P. hippeastri*	79%			
Pratylenchus sp. 8	N10685	KY424092	*P. scribneri*	89%	MK209593	*P. scribneri*	96%
Pratylenchus sp. 9 (1) *	N10841	No match	--	--	KT175531	*P. pseudocoffeae*	96%
Pratylenchus sp. 9 (2) *	N8930	KU522440	*P. zeae*	81%	KT175531	*P. pseudocoffeae*	96%
Pratylenchus sp. 10 (1) *	N10856	No Match	--	--			
Pratylenchus sp. 10 (2) *	N10853	KY424087	*P. speijeri*	75%			

Table 3. Estimates of Average Kimura 2 Evolutionary Divergence (Distance) across Sequence Pairs within Groups

Species	**Distances**
P.alleni	0
P.crenatus	0.000686813
P.hexincisus	0.02613078
P.neglectus	0.008998339
P.penetrans	0.047844971
P.scribneri	0.002883778
sp. 1	0.00386802
sp. 2	0.088149168
sp. 3	0.003693444
sp. 4	0
sp. 5	0
sp. 6	0
sp. 7	0
sp. 8	0
sp. 9	0.05367786
sp. 10	0.036133133
P.thornei	0
P.vulnus	0.011204833
P.zeae	0.001582905

Table 4. Estimates of Evolutionary Kimura-2 mean Divergence (Distance) across Sequence Pairs between Groups.

	P. alleni	*P. crenatus*	*P.hexincisus*	*P. neglectus*	*P.penetras*	*P.scribneri*	sp. 1	sp. 2	sp. 3	sp. 4	sp. 5	sp. 6	sp. 7	sp. 8	sp. 9	sp. 9	*P. thornei*	*P. vulnus*
P. alleni																		
P. crenatus	0.560																	
P. hexincisus	0.313	0.721																
P. neglectus	0.468	0.611	0.734															
P. penetrans	0.567	0.669	0.895	0.514														
P. scribneri	0.225	0.610	0.283	0.567	0.655													
sp.1	0.294	0.521	0.468	0.520	0.596	0.339												
sp.2	0.342	0.732	0.337	0.790	0.856	0.354	0.509											
sp.3	0.246	0.706	0.351	0.591	0.678	0.261	0.324	0.307										
sp.4	0.229	0.579	0.298	0.565	0.600	0.145	0.314	0.357	0.255									
sp.5	0.271	0.628	0.247	0.633	0.671	0.154	0.393	0.299	0.280	0.165								
sp.6	0.252	0.630	0.277	0.562	0.673	0.129	0.349	0.339	0.265	0.134	0.092							
sp.7	0.224	0.530	0.305	0.521	0.600	0.196	0.274	0.363	0.250	0.208	0.214	0.205						
sp.8	0.213	0.543	0.330	0.510	0.640	0.148	0.283	0.414	0.273	0.158	0.207	0.163	0.169					
sp.9	0.301	0.610	0.385	0.636	0.674	0.301	0.358	0.390	0.335	0.282	0.289	0.289	0.308	0.310				
sp.10	0.286	0.665	0.327	0.677	0.671	0.316	0.392	0.316	0.352	0.300	0.306	0.311	0.334	0.339	0.208			
P. thornei	0.639	0.706	0.890	0.482	0.548	0.725	0.596	0.848	0.716	0.684	0.731	0.710	0.614	0.603	0.660	0.686		
P. vulnus	0.352	0.602	0.590	0.561	0.572	0.483	0.416	0.656	0.480	0.437	0.531	0.496	0.400	0.453	0.436	0.439	0.581	
P. zeae	0.625	0.773	0.884	0.621	0.683	0.744	0.584	0.919	0.655	0.689	0.727	0.730	0.656	0.709	0.688	0.727	0.585	0.657

Figure 1. All sample collection sites by county.

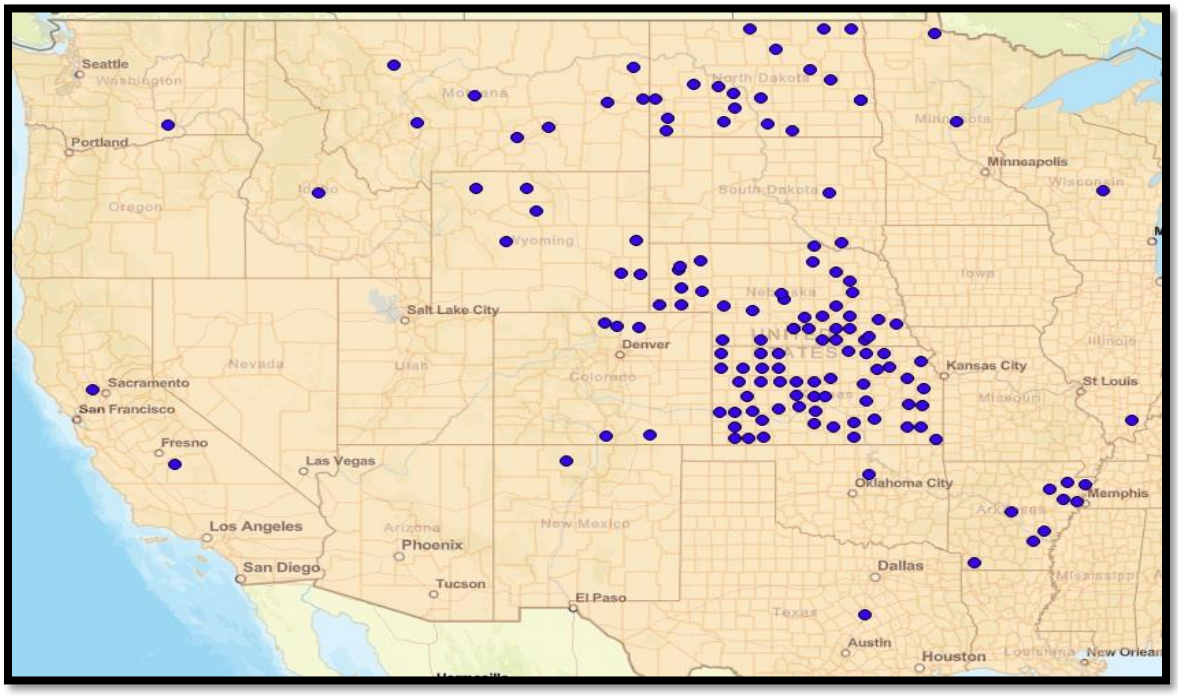


Figure 2. Number of Fields Used in DNA Barcoding Study. The number in the circle represents the number of fields by state and host.

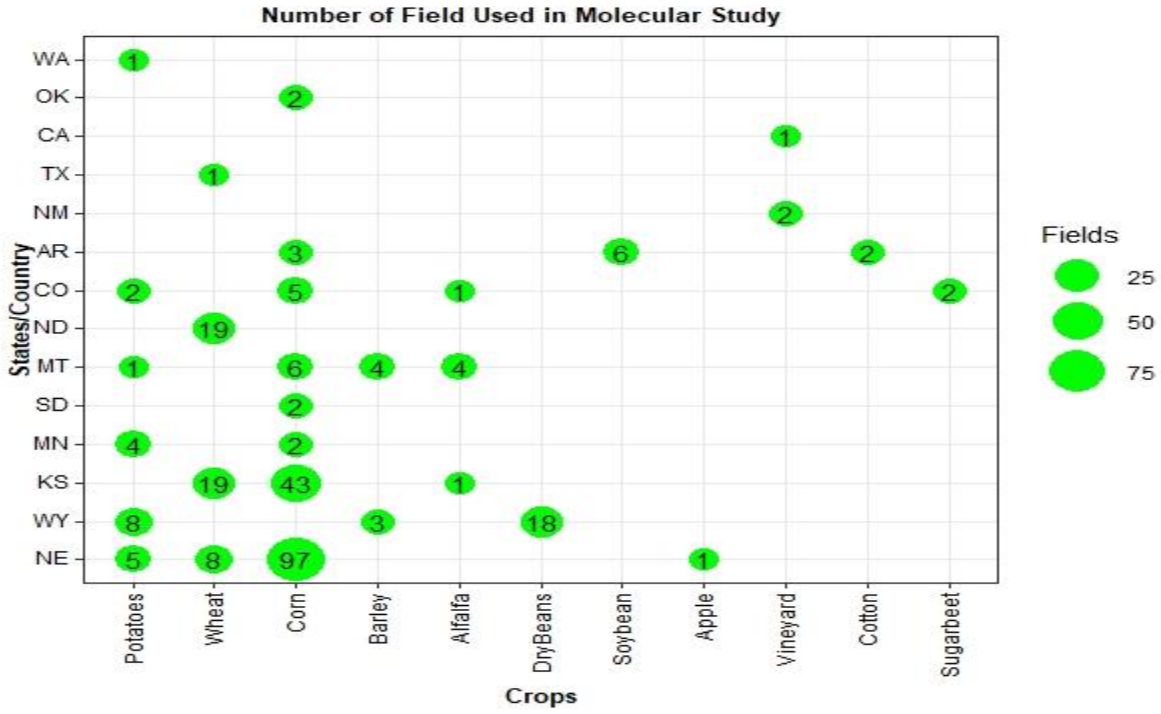


Figure 3. Phylogenetic relationship of Pratylenchus haplotypes in Great Plains Region as inferred from Bayesian Analysis of CO1 gene sequences. Posterior probabilities and bootstrap values are represented by different color based on tree construction methods.

Figure 4. Species Delimitation Tree of Pratylenchus haplotypes of Great Plains Region. Tree was constructed after dropping tips and keeping one specimen for each subgroup. The results of each delimitation methods are visualized by different colored bars on an ultrametric tree of CO1 gene. * represent the number of haplotypes at TCS 90% cut off.

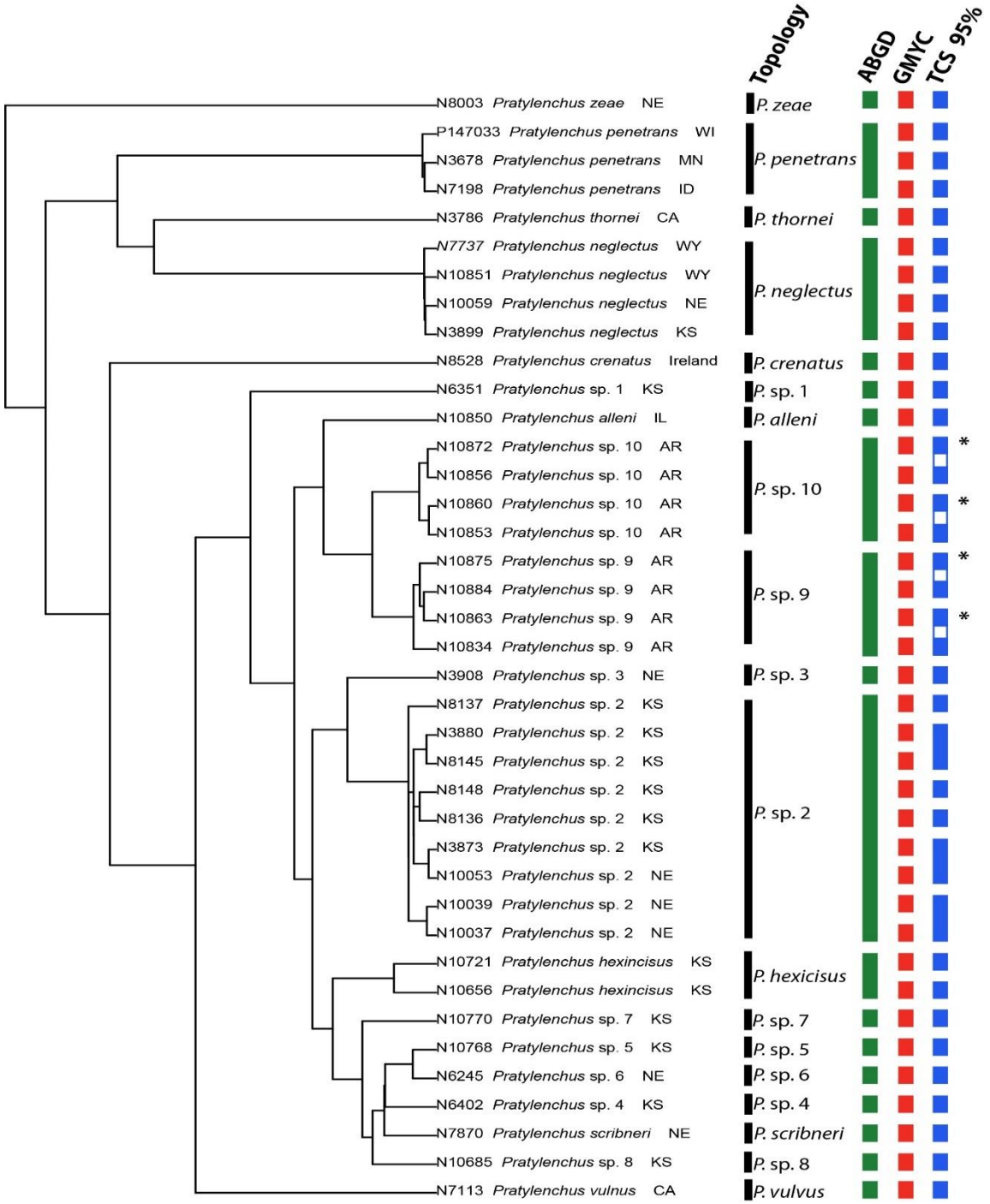


Figure 5. CO1 Haplotype networks of *P. neglectus* in the Great Plains region. Different networks are delimited by a dashed circle and labeled by a letter. Circle size are equal to the number of specimens that have identical sequences. Dashes on branch length represents the number of mutations between haplotypes. Geographic origin and host of each haplotypes is displayed by different colors.

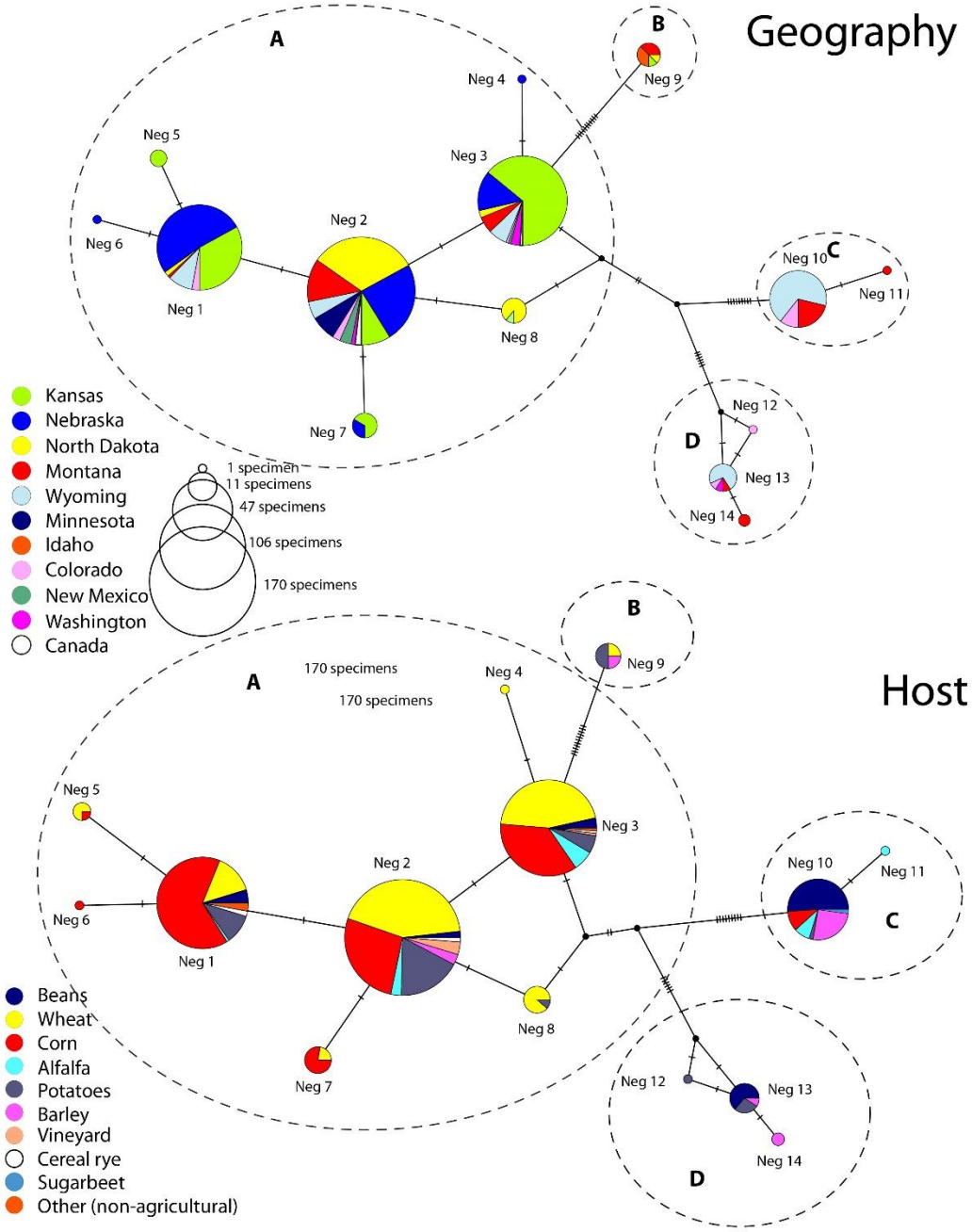


Figure 6. CO1 Haplotype network of *P. scribneri* in the Great Plains Region. Circle size are equal to the number of specimens that have identical sequences. Dashes on branch length represents the number of mutations between haplotypes. Geographic origin and host of each haplotypes is displayed by different colors.

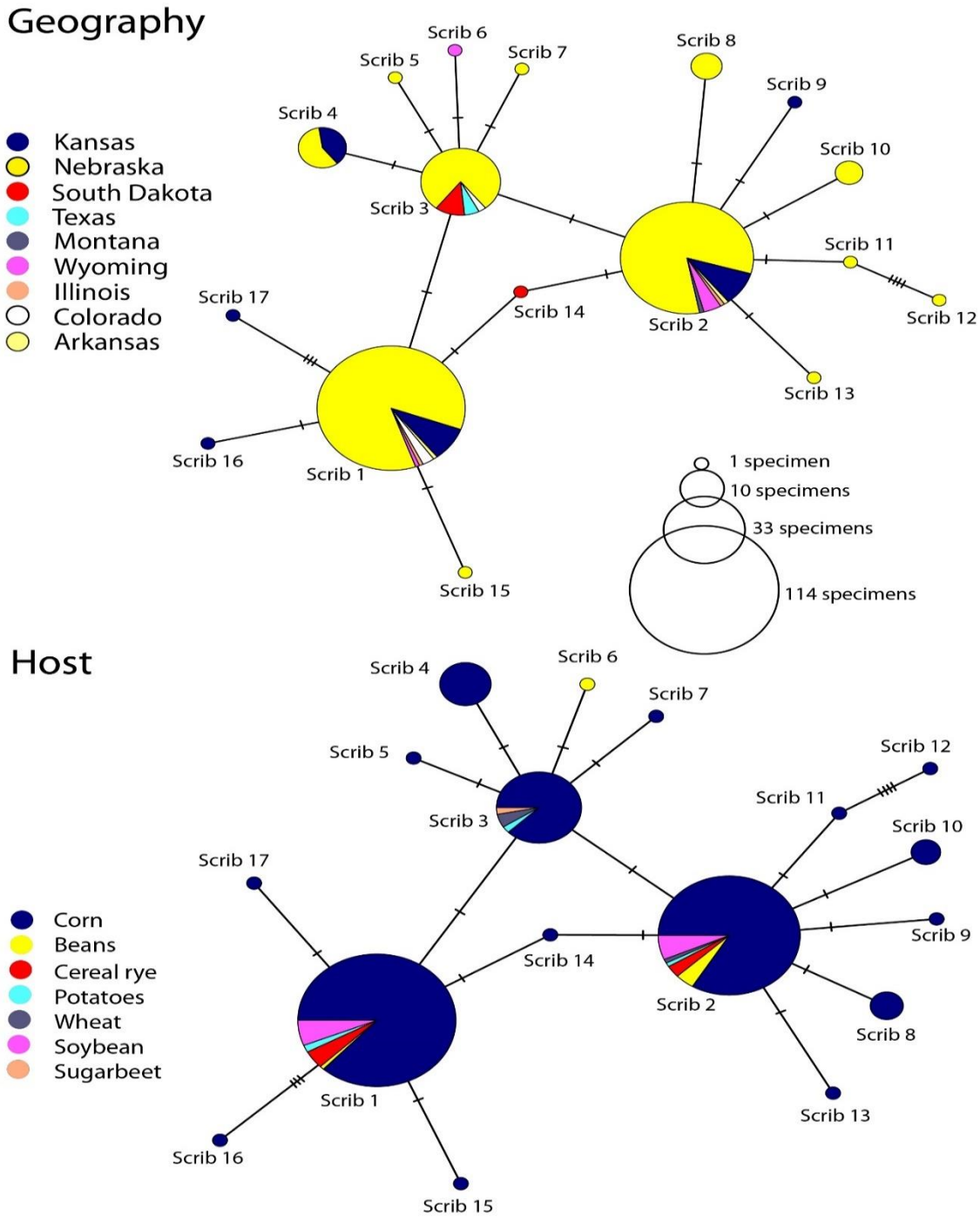


Figure 7. CO1 haplotype networks of *P. thornei* and *P. hexincisus* in the Great Plains region. Different networks for *P. hexincisus* are delimited by a dashed circle. Circle size are equal to the number of specimens that have identical sequences. Dashes on branch length represents the number of mutations between haplotypes. Geographic origin of haplotype groups is displayed by different colors

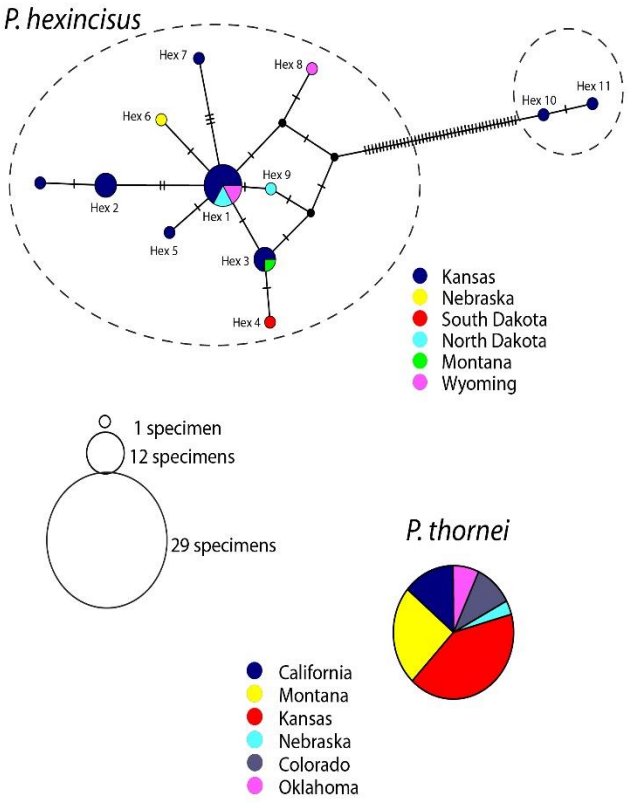


Figure 8A) Lineage time through plot (LTT) yielded by GMYC analysis represents the threshold time. B) Molecular divergence time estimation tree of Great Plains Pratylenchus haplotypes based on CO1 gene. The time interval on the nodes represents confidence interval of node ages. A timescale in million years (Mya) is provided below the tree.

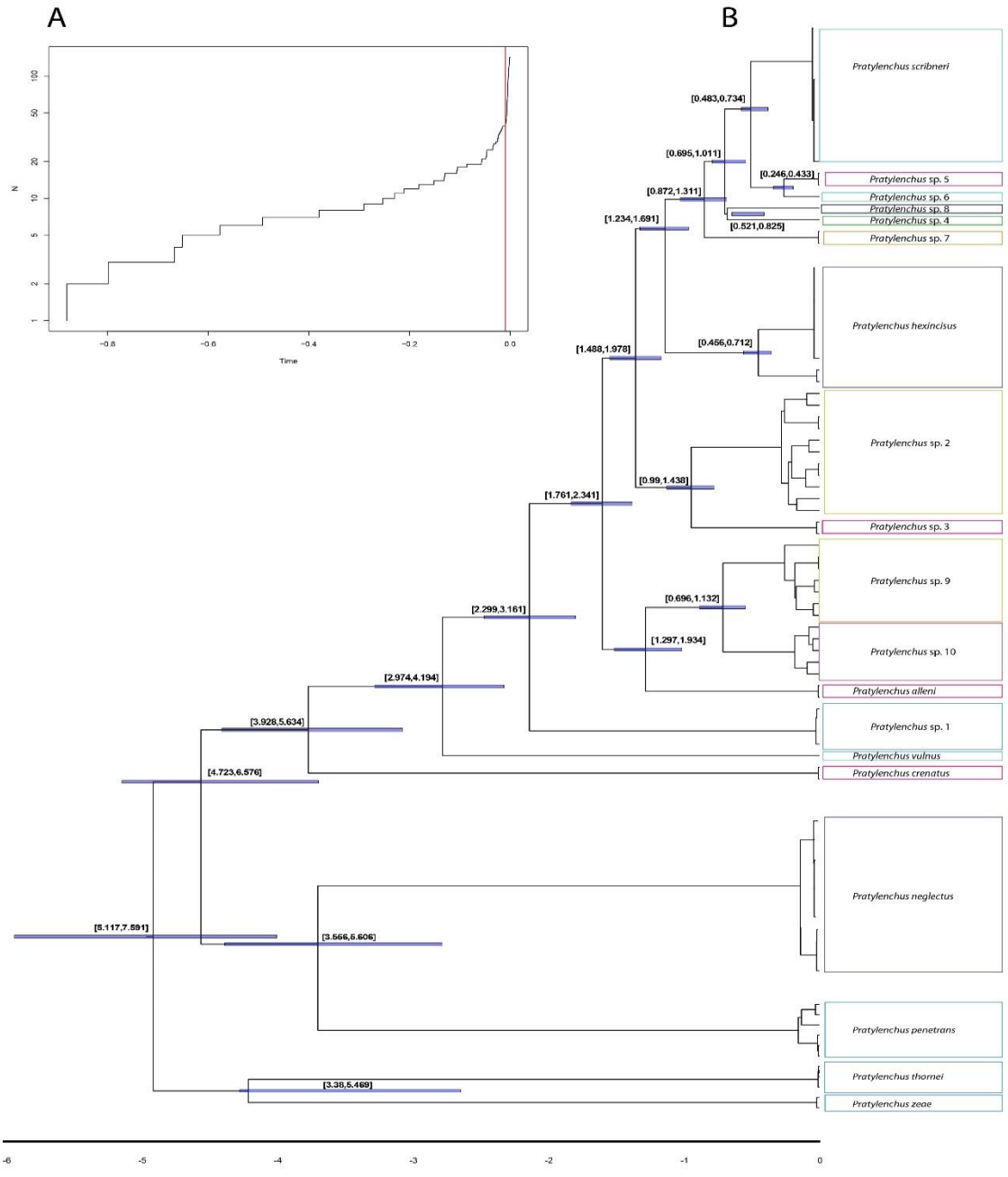


Figure 9. Host Association and Infested Field Number of Pratylenchus haplotypes of Great Plains.

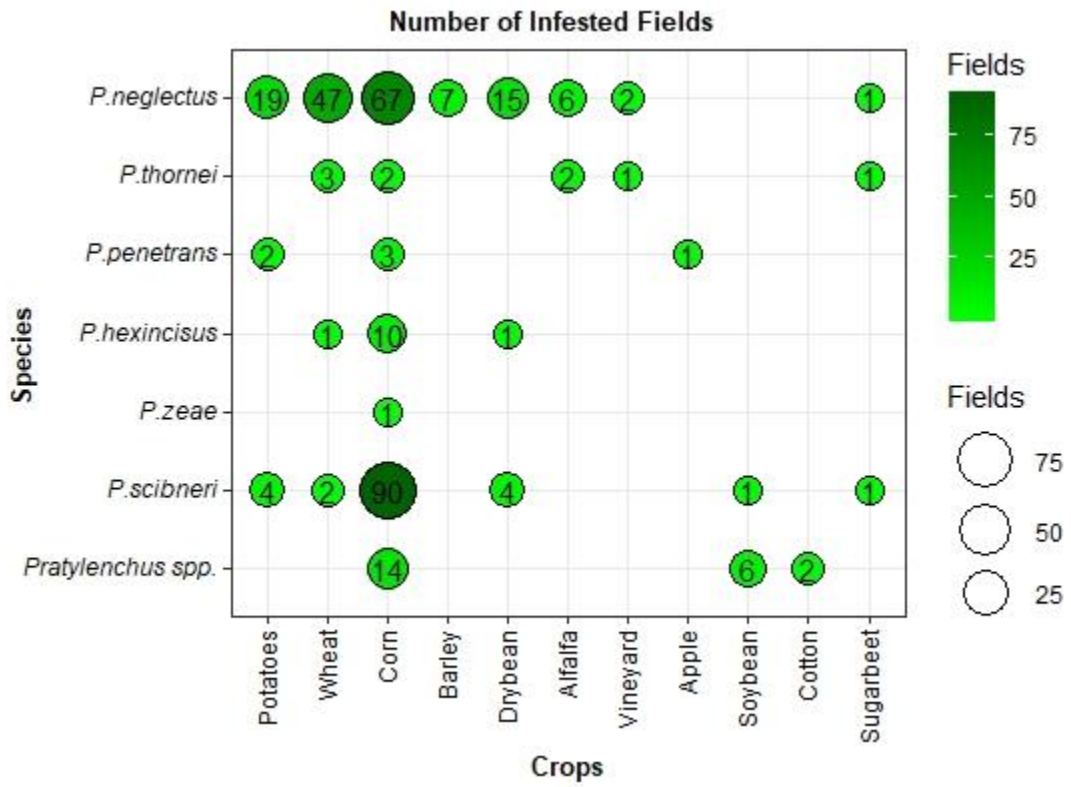


Figure 10. Distribution map of *P. neglectus*, *P. scribneri*, and mixed fields of both haplotypes.

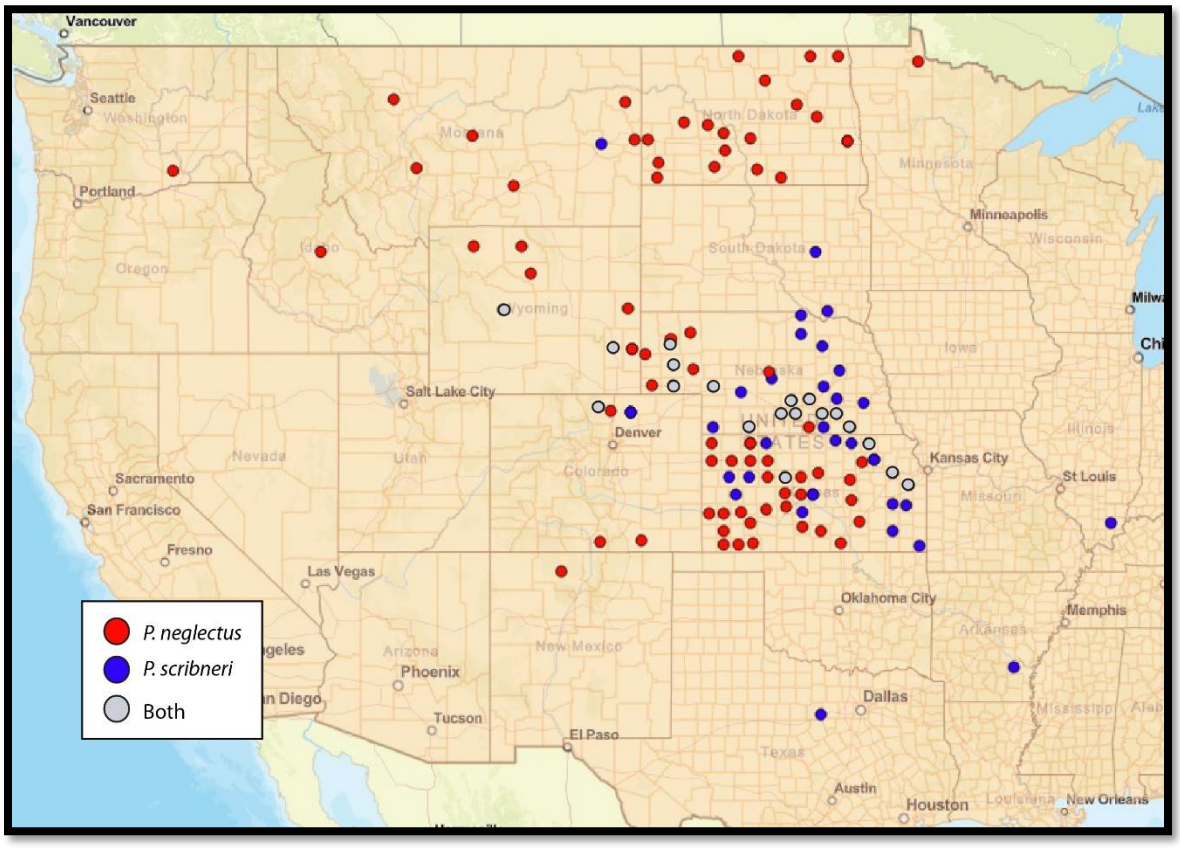
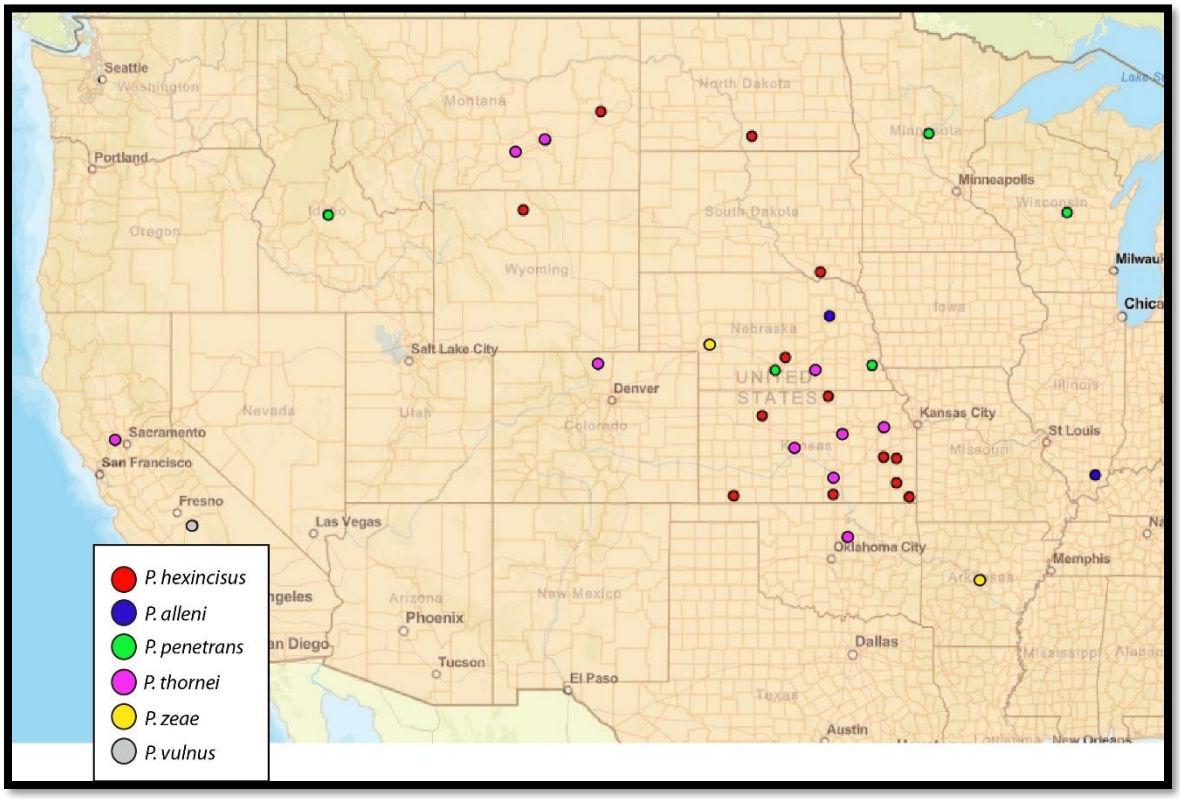


Figure 11. Distribution map of other haplotype groups of Great Plains Region.



Chapter 3

Morphological Assessment of *Pratylenchus* species in the Great Plains Region

Introduction

The Great Plains Region of North America is dominated by several *Pratylenchus* species associated with economically important crops (Chapter 2). Nine *Pratylenchus* species have been reported from previous studies across the region (May et al., 2016; Norton, 1983; Smolik and Evenson, 1987; Thorne and Malek, 1968; Todd et al., 2014). These species are *Pratylenchus coffeae, P. scribneri, P. neglectus, P. thornei, P. hexincisus, P. alleni, P. penetrans, P. flakensis,* and *P. tenuis.* In a DNA-based survey of *Pratylenchus* species from the Great Plains Region of North America, 15 distinct haplotype groups were identified using the COI mitochondrial gene in phylogenetic and species delimitation analyses (Chapter 2). Four of these haplotype groups identified as *P. neglectus, P. thornei, P. hexincisus, and P. scribneri,* are most often encountred within the region and have broad host associations that include wheat, corn, drybean, sugar beet, potato, and alfalfa across the region. Five of the haplotype groups corresponded to distinct, described species in molecular analyses. However, two haplotype groups, tentatively identified and P. *scribneri* and *P. hexincisus,* provided conflicted results when DNA sequence of molecular markers were submitted to a BLAST analysis in GenBank. For CO1, the results of the BLAST analysis for putative *P. scribneri* identified strong matches for both *P scribneri* and *P. hexincisus*. For putative *P. hexincisus* there was no strong match in the database. For the D2-D3 fragment of 28S rDNA both species provided strong matches to each other as well as species labeled as *P. pseudocoffeae*. The potential for mislabeling these two species is high when considering their morphology.

Inserra et al. (2007) have discussed their morphological similarity and overlapping morphometrics. They suggest that two characters, a longer stylet length in *P. hexincisus* (14.7 vs 15.5 μm) and a higher "a" ratio in *P. scribneri* (25.6 vs. 23.8 μm) may aid in their discrimination. Furthermore, they mentioned that *P. scribneri* typically had a hemispherical or subhemispherical tail with a smooth terminus whereas *P. hexincisus* tended to possess a truncate tails or subdigitate termini althought they commented that the same tail types were observed in *P. hexincisus*. In the Great Plains region, these two species have been the subjects of numerous studies in native prairies and agricultural ecosystems (Huang and Yan, 2017; Norton, 1983; Siddiqi, 2000; Smolik, 1977; Smolik and Evenson, 1987; Smolik and Lewis, 1982; Thorne, 1961; Thorne and Malek, 1968; Hando and Golden, 1989). The objective of this study is to reexamine morphological traits of *P. scribneri* and *P. hexincisus* and perform a multivariate morphological analysis of four primary species of *Pratylenchus* previously identifield by DNA barcoding.

Material and Methods

Morphological Analysis and Vouchers

Nematodes extracted from soil and roots were first evaluated under a stereo dissecting microscope and select specimens belonging to the genus *Pratylenchus* were handpicked for light microscopy examination and DNA extraction. Following immobilization of live specimens by heating, a portion of the specimens was mounted on the temporary glass slides, measured with a Leica DMLB light microscope with Differential Interference Contrast and photographed with a Leica DC300 video camera. Images are stored in the database system of the Nematology laboratory at the University of Nebraska-Lincoln. Measurements taken from adult females included the following

morphometric parameters: body length (L), length of esophagus (pharynx), position of median bulb from anterior end, distance of excretory pore (Ex. Pore) from anterior end, stylet length, number of lip annuli, number of lateral fields, position of vulva from anterior end and from tail, % distance of vulva from anterior (V%), position of intestinal valve (junction) from anterior end, tail length, post vulval-uterine sac (PUS), mid-body width (MBW), vulval body width (VBW), anal body width (ABW), and distance between vulva and anus plus the ratios a (body length/maximum body diameter) ,b (body length/ distance from anterior to phyrangeal intestinal junction), b' (body length/distance from anterior to base of phyrangeal gland lobe) ,c (bodt length/ tail length) , c' (tail length / anal vulva width (ABW), and MB (% distance from anterior to median bulb/length of esophagus).

Statistical Analysis

A total of 96 female specimens were included for multivariate analysis. We utilized from a reduced morphological dataset of 15 morphological characters: body length (L), length of esophagus (pharynx), position of median bulb from anterior end, distance of excretory pore from anterior end, stylet length, position of vulva from anterior end and from tail, % distance of vulva from anterior, position of intestinal valve (Junction) from anterior end, tail length, post vulval-uterine sac, mid-body width, vulval body width, anal body width, and distance between vulva and anus. Missing values were placed with mean values from their corresponded haplotype groups. Morphometric characters (variables) with correlation coefficients under 0.80 were selected from within the 15 characters. A stepwise variable selection was applied to determine the best combination of the characters that separate four haplotype groups, reducing the number

of variables to ten. Following that, linear (LDA) discriminant analysis was employed to determine the most accurate model, including assesment of the effect of equal prior probabilities. Disciriminant analysis is a statistical method that seeks a set of prediction function based on the independent variables and categorizes specimens or individuals into their respective groups (Hardle and Simar, 2007; Tatsuoka and Tiedeman, 2008). Multivariate statistical analysis was conducted using the packages, MASS (Venables and Ripley, 2010), and candisc in software R version 3.5.2 (R Core Team 2019)

Results

Measurements of *Pratylenchus* species of Great Plains Region are presented in Table 1. Morphometrics of *P. neglectus* conform to the values in the tabular key in Castillo and Vovlas (2007), except for a more posterior position of the vulva (77.0-85.0 vs 75.0-79.9 μm) and a longer posterior uterine sac (mean 17 vs < 16 μm). The Great Plains specimen morphometrics also agree with the original description of the species provided by Rensch (1924) (as provided Castillo and Volvas, 2007), except for a higher range of body length (380-657.5 vs 0.31-0.58 μm), and a sligthly lower range of "a" (18.8-29.2 vs 16.5-32.2 μm) and a higher mean of "c" (22.5 vs 20.0 μm) ratios. All morphometric values of *P. thornei* agree with the original description given by Sher and Allen (1953) (as provided by Castillo and Volvas, 2007). Great Plains *P. thornei* differed from their morphometrics by a slightly larger range of stylet length (15.0-18.0 vs 17-19 μm), a slightly higher c ratio (16.8-26.8 vs 18.0-22.0 μm) and a slightly more posterior vulva position (74.8-81.0 vs 73-80 μm). The mean value of *P. thornei* morphometrics were within the ranges of the tabular key provided by (Castillo and Vovlas, 2007).

Morphometrics of *P. scribneri* in this study of Great Plains specimens conform to the previous descriptions (Roman and Hirschmann, 1969; Inserra et al., 2007; Sherbakoff and Stanley, 1943; Thorne and Malek, 1968) and is characterized by the following traits (Table 1): body length 526.5 ± 52.9 (385-610 μm), vulva position 78.1 ± 1.5 (74.9-82.2 μm), stylet length 15.6 ± 0.8 (14.0-17.0 μm), tail length 27.5 ± 3.1(20.0-35.0 μm) and PUS length 21.6 ± 6.0 (12.0-40.0 μm). Comparison of *P. scribneri* with previous descriptive studies are presented in table 2. Compared with the first description of *P. scribneri* (Sherbakoff and Stanley, 1943), *P. scribneri* in this study have slightly longer body length (385-610 vs 280-590 μm), a higher a ratio (19.8-30.9 vs 17-23 μm), a higher c ratio (13.7-26.8 vs 16.9-22.7 μm), slightly more posterior position of vulva (74.9-82.2 vs 75.7-80.5 μm) and slightly smaller b ratio (4.6-6.7 vs 5.6-8.0). *P. scribneri* in this study differ from the report given by Thorne and Malek (1968) by a slightly smaller body length (vs 500-700 μm), a slightly longer a ratio (vs 17-26 μm), c ratio (vs 16-18), and slightly anterior position of the vulva (vs 79%). They differed from Inserra et al. (2007) by a slightly longer body length (vs 450-530 μm), position of vulva (vs 77-79%), stylet length (vs 14.0-15.5 μm) and a smaller a ratio (vs 22.1-29.8 μm). Great Plains morphometrics of *P. scribneri* were quite similar to those of Inserra et al. (2007), except for a relatively longer stylet length, relatively smaller pharyngeal overlap, and slightly smaller PUS (Table 2). The mean value of morphometrics and qualitative morphology matched with the tabular key of Castillo and Vovlas (2007) but differed from Roman and Hirschmann (1969) with a slightly larger range of length (385.0-610 vs 436.8-553.2), a relatively higher range of c ratio (13.7-26.8 vs 16.9-20.6), and a higher PUS value (12.0-40.0 vs 13.8-31.2). Compared with a recent *P. scribneri* description study (Yan et al.,

2015), our *P. scribneri* had relatively higher mean morphometrics, except for a slightly smaller b, MBW, and PUS value (Table 2).

As in shown in Table 1, *P. hexincisus* from the Great Plains are characterized by body length 513.4 ± 43.5 (457-575 μm), posterior position of the vulva 79.0% ± 1.5 (77.4-81.8), stylet length 14. 9 ± 0.3 (14.0-15.0 μm), tail length 24.8 ± 5.0 (18.0-30.0 μm), and PUS length 17.1 ± 4.1 (11.0-25.0 μm). They differ from the original description of Taylor and Jenkins (1957) by a greater range of body length (vs 340-540 μm), a smaller mean a ratio (24.9 vs 26 μm) but within ranges, a slightly more posterior position of the vulva (79.0 vs 78.0 μm), smaller mean b ratio (5.3 vs 7.2 μm) but within ranges and a larger mean c ratio (21.3 vs 19.0 μm) and ranges (15.7-25.4 vs 16.0-22.7 μm). Range values of stylet length (vs 13.0-15.9 μm), position of vulva (vs 75.0-79.9 μm), PUS (vs 16.19.9 μm), and pharyngeal overlap (vs < 30) were higher than the tabular key of Castillo and Vovlas (2007) but all mean values are within the ranges, except for a higher pharyngeal overlap value. *P. hexincisus* in this study had a relatively higher mean body length (513 vs 500 μm), stylet length (14.9 vs 14.0 μm), a ratio (24.9 vs 18.0 μm) , c ratio (21.3 vs 20.0 μm) and slightly more anterior position of the vulva (79 vs 80%) than the report of Thorne and Malek (1968). Compared with the report of Inserra et al. (2007) the Great Plains *P.hexincisus* had higher ranges and mean values for morphometrics, except for a slightly smaller stylet length (vs 14.5-15.5 μm) (Table 2).

Qualitative morphological characters of *P. scribneri* in this study are similar to those of *P. scribneri* as described by Castillo and Vovlas (2007), Sherbakoff and Stanley (1943), and Thorne and Malek (1968). Great Plains specimens of *P. scribneri* had slightly higher or offset lip region, with 2 lip annuli. The stylet was short with mostly rounded

knobs, with anterior indentions (cup-shaped anteriorly) observed in some specimens. The median bulb was massive and generally rounded. The valve of the median bulb most often situated centrally but an anterior position was observed in some specimens (Figure 1). The lateral field most often consisted of four lines although a fifth, and sixth lines were observed in some specimens, particularly in the vulval region (Figure 2). The posterior uterine sac length was approximately equal to mid-body width (Figure 3). Tail shapes varied from slightly tapering (subhemispherical) to broad (hemispherical) with a smooth terminus (Figure 4).

There was no differentiation in the qualitative morphology of *P. hexincisus* when compared to the conspecific description of (Castillo and Vovlas, 2007; Taylor and Jenkins, 1957; Thorne and Malek, 1968). Lip region of *P. hexincisus* was slightly low with rounded or spherical knobs, bearing two lip annuli. The median bulb was rounded or oval shape, with a valve most often anterior to center (Figure 5). The lateral field was usually composed of six lateral lines, often reducing in number towards the tail. Some specimens occasionally displayed five lines (Figure 6) The post uterine sac was short, less than the vulva body width (figure 7). The tail tapered near to end with a smoothly rounded terminus (Figure 8). In the overall comparison between the Great Plains specimens of *P. scribneri* and *P. hexincisus*, morphometric values generally overlapped and morphological characters such as stylet knobs and tail shapes displayed strong similarity, indicating an insufficiently distinct morphological signal necessary to distinguish these two species.

The stepwise variable selection targeted ten morphological variables as the best subset that explains haplotype group membership among the four Great Plains species.

These variables are stylet length, the position of the median bulb from anterior, length of the esophagus, length of pharyngeal overlap, the position of the pharyngeal intestinal junction, mid-body width (MBW), anal body width (ABW), length of the posterior uterine sac, vulva-anus distance, and tail length (Figure 9). Linear discriminant analysis (LDA) correctly classified 91.6% of the specimens with equal prior probabilities into their correct genetic groups. *P. neglectus* and *P. thornei* specimens were classified with 97% and 91% accuracy by LDA with equal prior probabilities, respectively. *P. scribneri* specimens and *P. hexincisus* specimens were classified with 85% and 89% accuracy by LDA equal prior probabilities into their correct haplotype groups, respectively. The classification plot of the specimens is illustrated in Figure 10 based on the canonical scores. Of the three generated canonical variables, Canonical 1 explained 79.7% of the variation and Canonical 2 accounts for 15.2% of the variation, together explaining 94.9 of the total variation in haplotype group classification. Separation of *P. neglectus* and *P. thornei* was nearly complete, but *P. scribneri* and *P. hexincisus* overlapped and grouped closely together. The largest negative standardized canonical coefficients for the morphological characters that separate haplotype groups in canonical 1 were vulva-anus distance (-0.82), position of anterior intestinal junctional (-0.81), and mid-body width (0.52) and in canonical 2 were stylet length (0.70) and length of pharyngeal gland overlap (0.59).

Discussion

The first description of *P. scribneri* was published by Sherbakoff and Stanley (1943) based on Steiner's original description. Taylor and Jenkins (1957) reported that *P. hexincisus* closely resembles *P. scribneri* and *P. neglectus* and can be differentiated from

P. scribneri by its smaller size and longer lateral field and from *P. neglectus* by a the more anterior position of vulva. Most of the *P. hexincisus* specimens in this study were indistinguishable from *P. scribneri* and *P. neglectus* based on the above three characters. (Loof, 1960) reported *P. scribneri* differed from *P. hexincisus* by its larger body size, longer tail length, and four incisures in the lateral field, but also noted that the body length of *P. hexincisus* matched with Steiner's *P. scribneri*. Also, Loof (1964) indicated the existence of third lip annulus and 5-6 lateral grooves for *P.hexincisus* whereas four, five, and six lines were reported by Loof (1985) for *P. scribneri* as reported by Inserra et al., 2007). The Great Plains *P. scribneri* had a longer tail length than *P. hexincisus* as described by (Loof, 1960) and we observed 4-6 lateral field for both haplotype groups as described in Loof (1964,1978). Thorne and Malek, (1968) reported additional data for *P. scribneri* from cultivated and native prairie within the Great Plains, mentioning four incisures, a massive basal bulb, a longer posterior uterine sac and a preferences for lighter soils. For *P. hexincisus* collected from corn fields, they mentioned six incisures, somewhat rounded stylet knobs, the valve of median bulb anterior to center, PUS as long as body diameter and a preference for heavier soils. Handoo and Golden, (1989) reported *P. hexincisus* had 4-6 lateral incisures on most of the body (usually six), rounded stylet knobs and the valve of median bulb anterior to center. In their account, *P. scribneri* and *P. hexincisus* displayed the diagnostic characters of the Thorne and Malek (1968) report, but some characteristics were observed in both haplotype groups such as the number of lateral fields and anterior position of the valve of the median bulb. Based on the tabular key prepared by Castillo and Vovlas (2007), only four morphological characters differentiate these two species. These characters are the shape of spermatheca, length of

posterior uterine sac, the length of the pharyngeal overlap, and number of lateral fields. Applying these characters to *P. scribneri* and *P. hexincisus* to the Great Plains specimens, *P. hexincisus* had a shorter PUS length than *P. scribneri* as was suggested in the tabular key. The spermatheca, however, was not sufficiently clear in the Great Plains specimens for characterization. The *P. hexincisus* specimens had a slightly longer pharyngeal overlap than *P. scribneri*, but the range of pharyngeal overlap length in *P. scribneri* was larger than *P. hexincisus*. Also, four to six lateral lines were observed in both species..

In our multivariate analysis, a stepwise variable selection indicated the best ten morphological characters for the discrimination of the four major *Pratylenchus* species in the Great Plains Region. Linear discriminant analysis with these ten characters showed that *P. neglectus* and *P. thornei* specimens can be correctly classified into their haplotype groups. Classification accuracy of *P. scribneri* and *P. hexincisus*, however, was low, classifying some of *P. scribneri* specimens as *P. hexincisus* and vice versa. Canonical plots further indicated that two of *Pratylenchus* haplotype groups (*P. neglectus* and *P. thornei*) were distinguishable, but *P.hexincisus* and *P. scribneri* specimens largely overlapped, in spite of the genetic distance between two these haplotype groups. The morphological characters that contributed most to canonical 1 were vulva-anus distance and position of anterior intestinal junction (valve) and for canonical 2 it was stylet length and length of the pharyngeal overlap.

Based on the existance of the other unknown groups in our DNA barcoding analysis in chapter 2, the question could be raised, "Is it possible that a different haplotype group could be *P. hexincisus*?" Historically in the Great Plains region, both *P. hexincisus* and *P. scribneri* were considered to be broadly distributed and observed most

frequently in corn fields (Norton, 1983; Smolik, 1977; Thorne and Malek, 1968). We chose as *P. hexincisus*, a haplotype group that was, in phylogenentic studies, relatively closely related to *Pratylenchus scribneri* and like *P. scribneri* it was geographically spread across multiple states in the region and most frequently associated with corn. Five smaller haplotype groups, labeled *Pratylenchus* sp 4-8, were also closely related to *P. scribneri*, but they were geographically limited to a single county or single state. Also, like Thorne and Malek (1968), Smolik and Evenson (1987) reported a soil preference for *P. hexincisus*, suggesting the species was more often associated with silty clay loams, compared to sandy loams reportedly prefered by *P. scribneri*. Although a soil type association was not explicitly tested in this study, this observation matches the situation in Kansas (Todd, pers. obs.).

One factor that may have complicated historical records, is the recovery of *P. hexicisus* and *P. scribneri*, and often *P. neglectus* all in the same field. From our survey data, mixed species populations are common in Great Plains fields. Mixed species populations may have contributed to a blurring of species boundaries in earlier morphological studies. Other haplotypes groups in the Great Plains Region were characterized by the presence of males. Populations of *Pratylenchus alleni* and *Pratylenchus* species 1 and 2 had males and females often had sperm in the spermatheca. Therefore, by the process of elimination, plus assumptions about the geographic distribution, host and soil-type prefences, and a morphological similarity to *P. scribneri*, we hypothesize that *P. hexincisus* is an extant species, widespread in the Great Plains, frequently associated with corn grown in the heaveir soils of the region. This hypothesis can be tested by greenhouse and field studies.

Globally the genus *Pratylenchus* contains over 100 described species. DNA barcoding with COI has revealed the existence of probable new species. Historically, *Pratylenchus* species descriptions were based on morphological characters with special attention paid to characters such as number of lip annuli, body length, stylet length, form of stylet knobs and posterior position of vulva (Castillo and Vovlas, 2007; Loof, 1978; Ryss, 2002a, 2002b; Thorne, 1961; Thorne & Malek, 1968). Several diagnostic keys and compendium have been published based on these morphological characters (Frederick and Tarjan, 1989; Handoo and Golden, 1989; Loof, 1978; Ryss, 2002a). It is undeniable that species discovery will increase and human activities will continue to introduce species into new areas. Given the morphological similarity of these species, *Pratylenchus* diagnosis will become increasingly reliant on molecular characterization. It is critical that the DNA databases are accurate and populated with validated sequences. This also includes the need for a reexamination of sequences that currently exist in repositories like GenBank. Other investigators have recently made the same points in comparitive studies with *Pratylenchus goodeyi*, *Pratylenchus pratensis*, and *Pratylenchus flakkensis* (Janssen et al., 2017). Here we have highlighted problematic issues in the identification of *P. scribneri* and *P. hexincisus.*

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Figure 1. Anterior region of Great Plains *P. scribneri*.

Figure 12. Lateral fields of Great Plains *P. scribneri.*

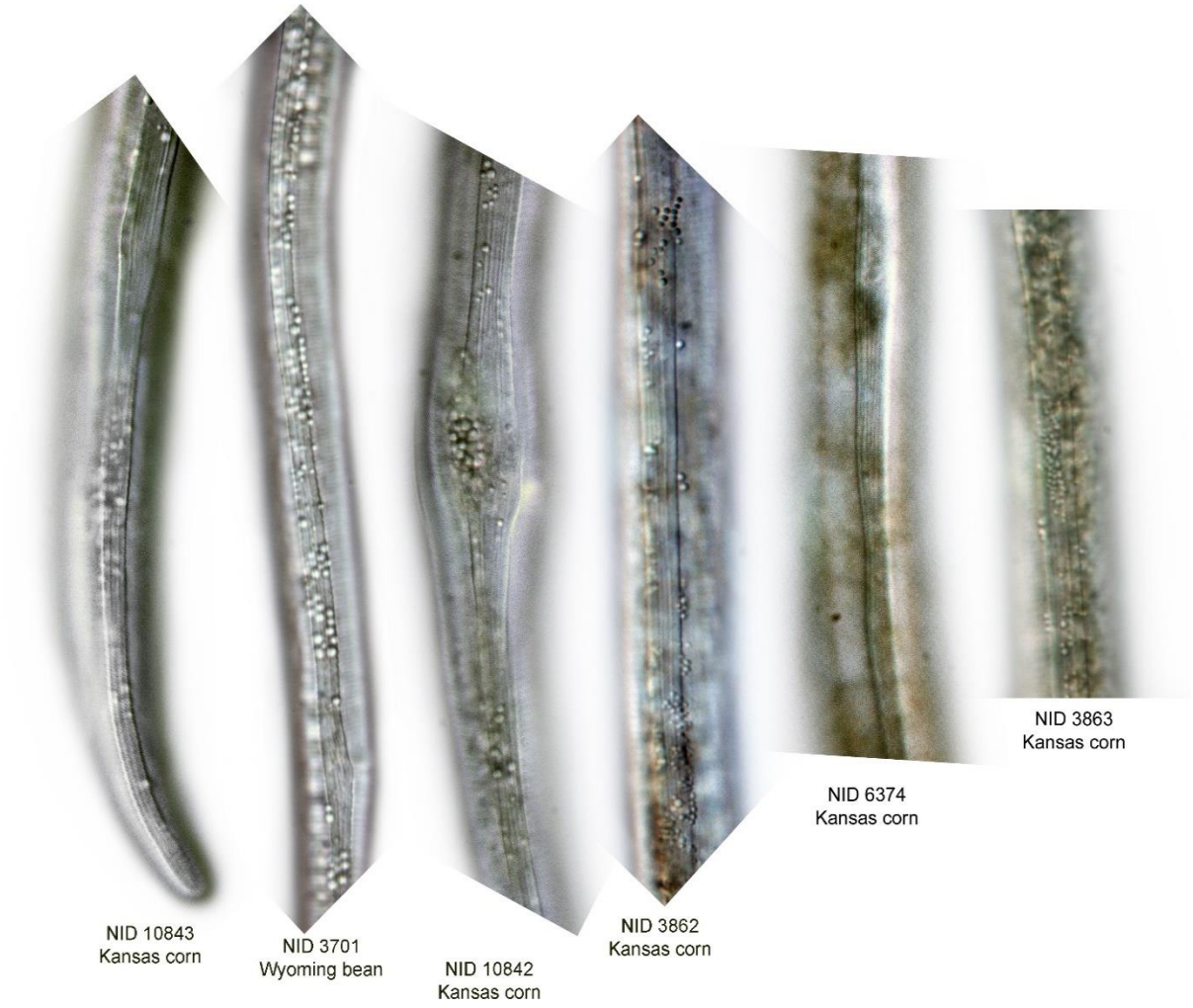

Figure 13. Vulva region of Great Plains *P. scribneri*.

Figure 14. Tail shapes and tips of Great Plains *P. scribneri*.

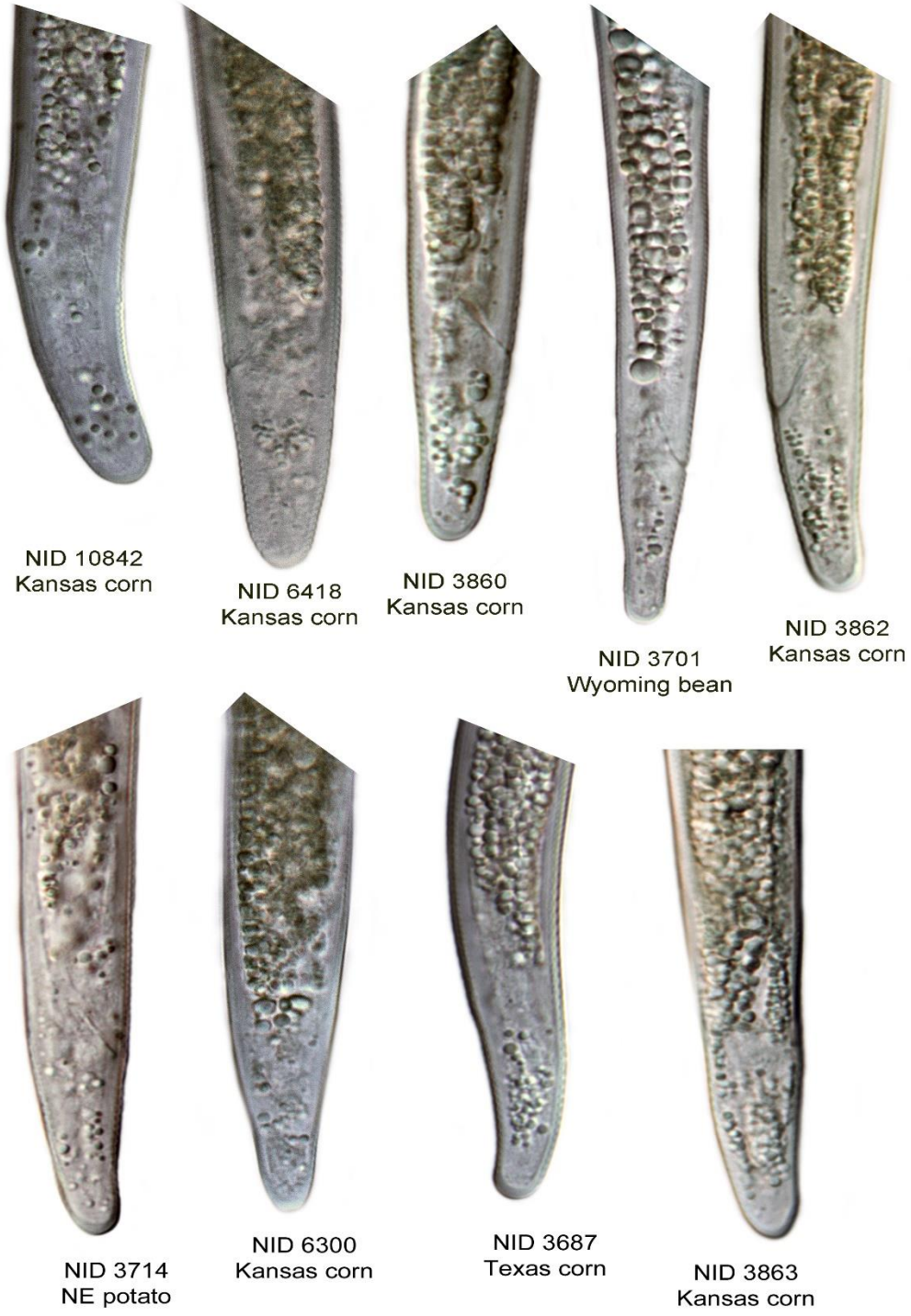

Figure 15. Anterior region of Great Plains *P. hexincisus.*

Figure 16. Lateral fields of Great Plains *P. hexincisus*.

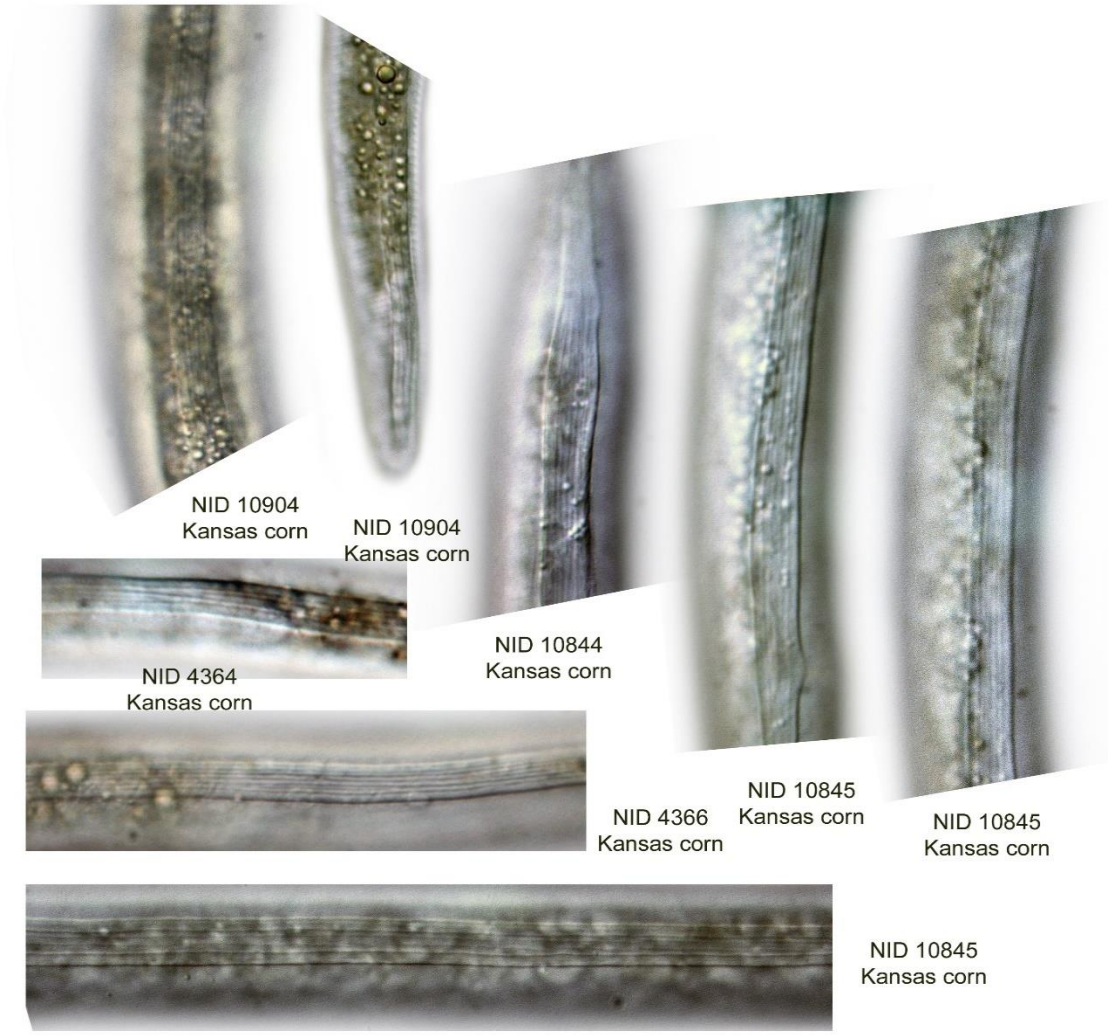

Figure 17. Vulva region of Great Plains *P. hexincisus.*

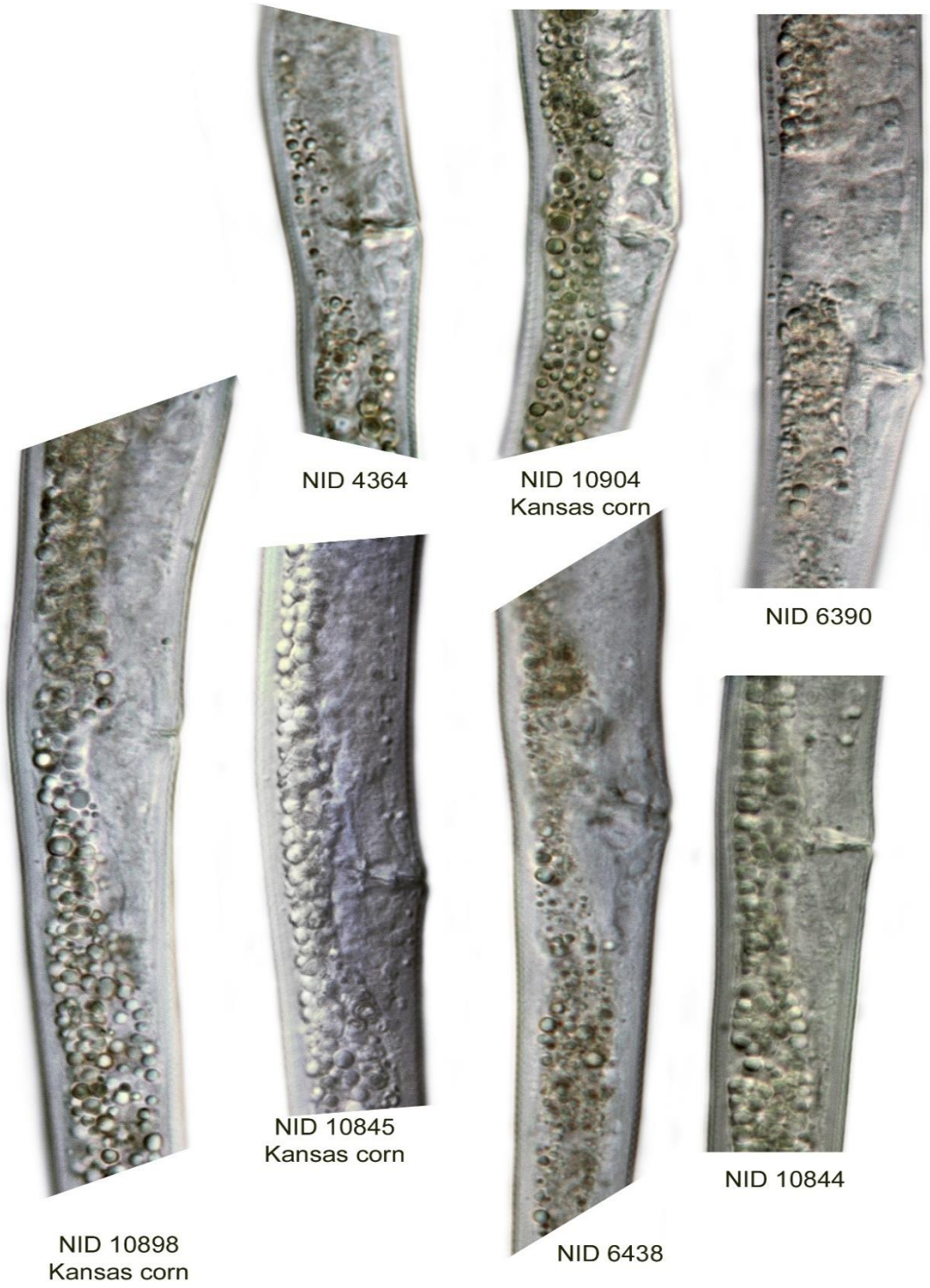

Figure 18. Tail shapes and tips of Great Plains *P. hexincisus*.

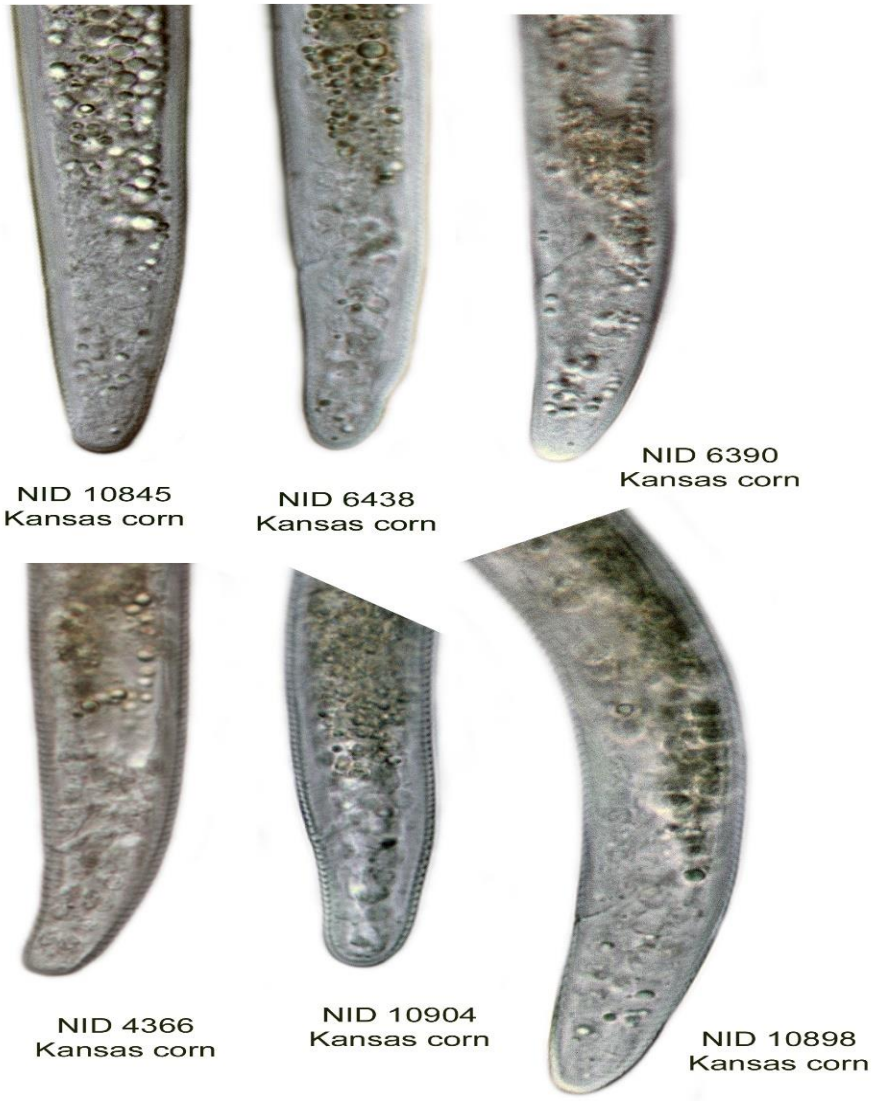

Figure 19. Best subset of morphological characters for classification derived from stepwise variable selection.

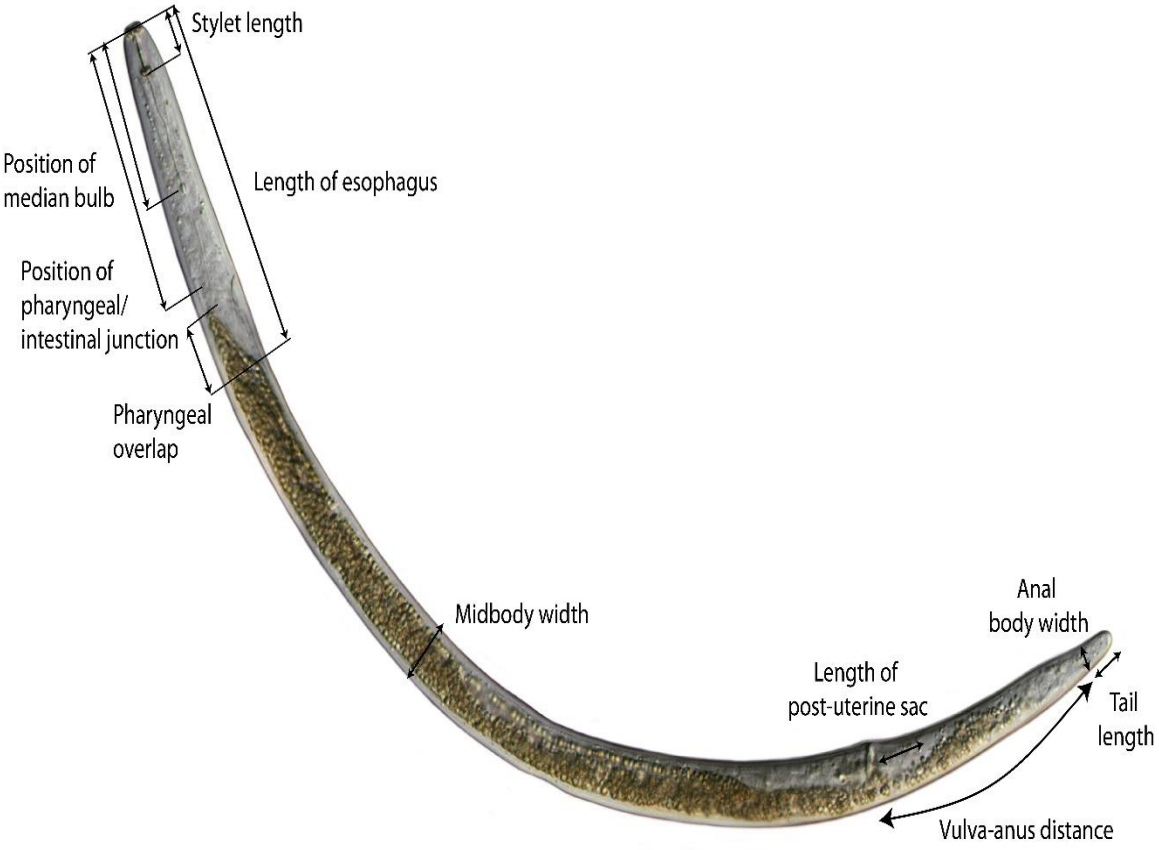

Figure 20. Discriminant Function Analysis canonical plot including ten variables. Vectors display the independent variables. Inner ellipse represents 95% confidence interval and outer ellipse represents the region that contains 50% of the specimens for each haplotype groups.

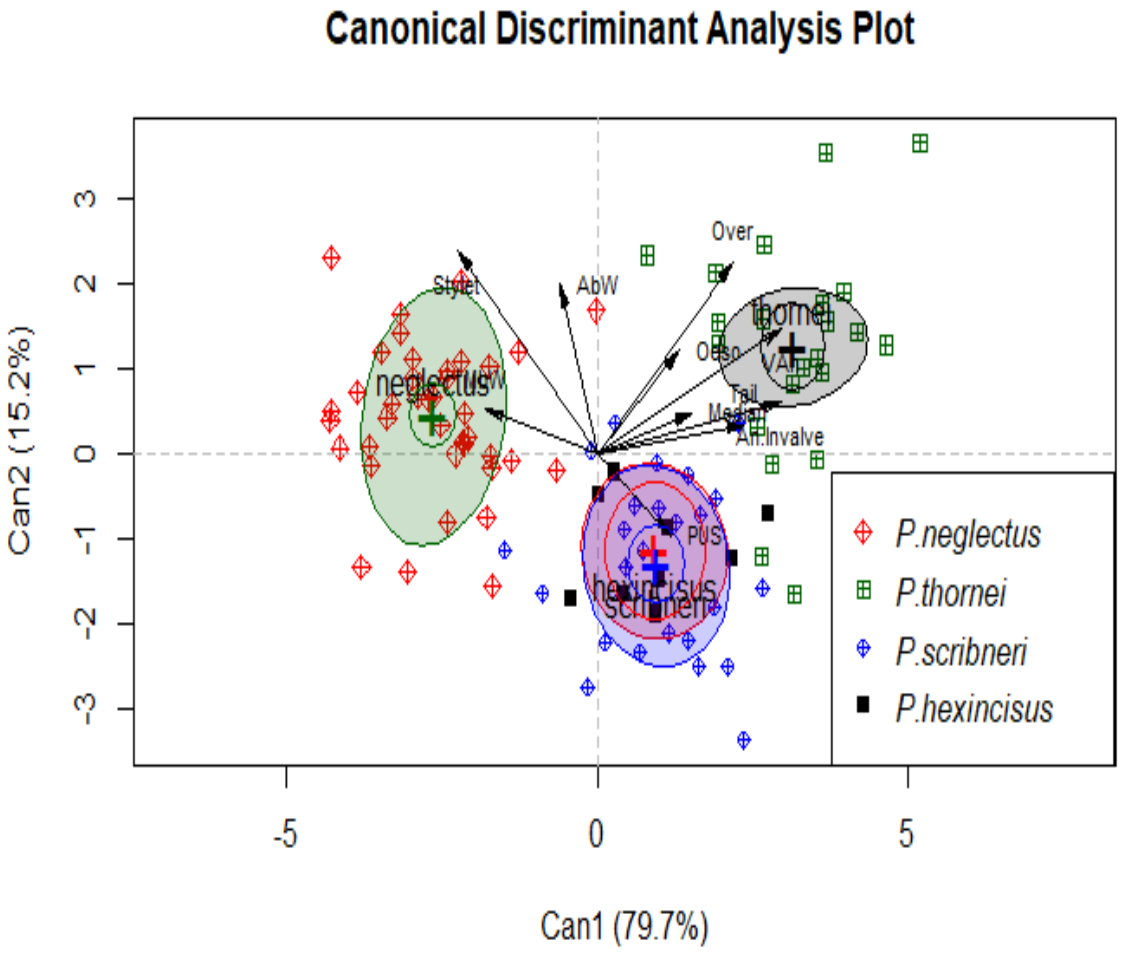

Table 1. Measurements of all *Pratylenchus* named haplotypes groups.

Character	*P. neglectus*	*P. scribneri*	*P. hexincisus*	*P. thornei*	*P. penetrans*	*P. crenatus*	*P. zeae*	*P. alleni*
n	39	26	9	22	3	3	5	2
L	527.5 ± 56.1 (380-657.5)	526.5 ± 52.9 (385-610)	513.4 ± 43.5 (457-575)	613.7 ± 79.9 (447.5-725)	609.8 ± 34.8 (582.5-649)	454 ±73.7 (373-517)	476.4 ±72.9 (426-602)	426.3 ±79.5 (370-482)
a	23.0 ± 2.4 (18.8-29.2)	25.6 ±2.9 (19.8-30.9)	24.9 ± 2.08 (21.6-27.3)	30.2 ± 2.5 (24.2-34.9)	28.9 ± 4.2 (24.3-32.5)	26.6 ± 1.96 (24.9-28.7)	26.1 ± 2.25 (24.9-30.1)	21.8 ± 1.7 (20.6-23.0)
b	6.2 ± 0.62 (4.6-7.7)	5.7 ± 0.5 (4.6-6.7)	5.3 ± 0.5 (4.6-6.4)	6.3 ± 1 (4.2-7.9)	5.8 ± 0.9 (4.94-6.7)	5 ± 0.77 (4.24-5.81)	5.2 ± 0.9 (4.1-6.6)	5.1 ± 0.5 (4.7-5.5)
b'	4.1 ± 0.5 (3.2-5.6)	4.1 ± 0.4 (3.4-4.9)	3.8 ± 0.3 (3.3-4.3)	4.4 ± 0.5 (3.3-5.1)	4.2 ± 0.4 (3.7-4.5)	4.16 ± 1.19 (2.85-5.17)	4.2 ± 0.8 (3.2-5.1)	3.7 ± 0.2 (3.6-3.9)
c	22.5 ± 2.9 (18.1-31.3)	19.4 ± 2.7 (13.7-26.8)	21.3 ± 3.5 (15.7-25.4)	20.6 ± 2.1 (16.8-26.8)	17 ±0.5 (16.6-17.6)	21.2 ± 0.5 (20.7-21.5)	15 ± 0.4 (14.5-15.4)	19.3
c'	1.8 ± 0.3 (1.3-2.2)	2.3 ± 0.4 (1.7-3.5)	1.9 ± 0.3 (1.5-2.3)	2.3 ± 0.4 (1.4-3.0)	2.5 ± 0.4 (2.3-3.0)	1.9 ± 0.1 (1.8-2.0)	2.8 ± 0.3 (2.5-3.3)	2.08
V	81.3 ± 1.7 (77.0-85.0)	78.1 ± 1.5 (74.9-82.2)	79.0 ± 1.5 (77.4-81.8)	76.9 ± 1.7 (74.8-81.0)	81.7 ± 3.1 (79.4-85.2)	82.6 ± 0.8 (81.8-83.4)	71.5 ± 1.1 (70.2-72.8)	79.4 ± 0.5 (79.0-79.7)
MB%	41.4 ± 5.5 (16.7-49.8)	43.5 ± 3.6 (36.9-50.0)	41.3 ± 2.4 (38.0-45.0)	40.3 ± 3.2 (31.8-45.6)	43.2 ± 1.3 (42.0-44.4)	42.1 ± 8.7 (32.1-47.2)	41.6 ± 4.8 (33.3-45.0)	43.4 ± 4.8 (40.0-46.8)
Stylet L.	17.2 ±1.0 (14.0-19.0)	15.6 ± 0.8 (14.0-17.0)	14.9 ± 0.3 (14.0-15.0)	16.1 ± 0.9 (15.0-18.0)	16.3 ± 1.5 (15.0-18.0)	16.3 ± 1.2 (15.0-17.0)	15.8 ± 0.8 (15.0-17.0)	14.5 ± 0.7 (14.0-15.0)
Ex. Pore	88.1 ± 5.8 (75.0-100.0)	87.0 ± 7.2 (71.0-100.0)	82.1 ± 5.4 (76.0-90.0)	89.1 ± 7.6 (75.0-102.5)	92.0 ± 5.29 (88.0-98.0)	74.7 ± 9.29 (64.0-81.0)	80.0 ± 11.1 (65.0-95.0)	72.5 ± 14.1 (62.5-82.5)
Position of Median Bulb	52.8 ± 6.2 (23.0-62.0)	54.8 ± 3.8 (46.0-61.0)	55.2 ± 2.3 (52.0-59.0)	57.7 ± 5.6 (48.0-73.0)	63.0 ± 6.1 (59.0-70.0)	46.3 ± 4.0 (42.0-50.0)	47.2 ± 4.6 (40.0-52.0)	49.0 ± 1.4 (48.0-50.0)
MBW	23.1 ± 2.8 (18.0-30.0)	20.8 ± 2.7 (14.0-25.0)	20.7 ± 1.4 (18.0-23.0)	20.3 ± 2.8 (15.0-25.0)	21.3 ± 2.3 (20.0-24.0)	17.0 ± 1.8 (15.0-18.0)	18.2 ± 1.3 (17.0-20.0)	19.5 ± 2.1 (18.0-21.0)
VBW	20.9 ± 2.6 (16.0-28.0)	20.4 ± 2.2 (16.0-25.0)	19.6 ± 1.2 (18.0-21.0)	20.5 ± 2.7 (16.0-27.0)	19.7 ± 0.6 (19.0-20.0)	17.0 ± 1.7 (15.0-18.0)	17.4 ± 1.5 (16.0-19.0)	16.5 ± 0.7 (16.0-17.0)
ABW	13.6 ± 1.3 (12.0-16.0)	12.0 ± 1.6 (10.0-15.0)	12.9 ± 0.9 (11.0-14.0)	13.5 ± 2.4 (10.0-18.0)	14.3 ± 1.2 (13.0-15.0)	11.3 ± 1.2 (10.0-12.0)	11.4 ± 1.1 (10.0-13.0)	11.5 ± 0.7 (11.0-12.0)

Vulva-Anus Distance	74.0 ± 9.9 (53.0-95.5)	86.6 ± 14.7 (61.0-115.0)	83.3 ± 12.6 (66.0-103.0)	111.5 ± 20.0 (73.0-145.0)	75.0 ± 19.1 (53.0-87.0)	64.0	104.2 ± 20.7 (90.0-140.0)	76.0
Tail Length	23.8 ± 3.4 (16.0-30.0)	27.5 ± 3.1 (20.0-35.0)	24.8 ± 5.0 (18.0-30.0)	29.8 ± 4.1 (21.0-37.0)	36 ± 2.7 (34.0-39.0)	21.3 ± 3.1 (18.0-24.0)	31.8 ± 4.3 (28.0-39.0)	25.0
PUS	17.0 ± 2.7 (10.0-25.0)	21.6 ± 6.0 (12.0-40.0)	17.1 ± 4.1 (11.0-25.0)	19.7± 5.8 (9.0-28.0)	18.0 ± 4.2 (15.0-21.0)	16.7 ± 6.4 (12.0-24.0)	21.0 ± 9.5 (12.0-37.0)	11.8 ± 2.6 (10.0-13.6)
Pharyngeal Overlap	29.7 ± 5.3 (19.0-40.0)	30.5 ± 7.5 (20.0-48.0)	31.6 ± 4.1 (24.0-37.0)	43.2 ± 12.5 (16.0-59.0)	41.0 ± 5.6 (35.0-46.0)	21.3 ± 18.8 (10.0-43.0)	23.0 ± 8.6 (10.0-34.0)	43.0
Pharyngeal gland lobe	127.4 ± 11.6 (100.0-155.0)	127.2 ± 15.6 (100.0-162.5)	134.0 ± 6.9 (127.5-150.0)	141.1 ± 17.6 (100.0-170.0)	145.8 ± 10.5 (137.0-157.5)	112.3 ± 16.4 (100.0-131.0)	115.0 ± 18.4 (89.0-141.0)	113.8 ± 15.9 (102.5-125.0)
Pharyngeal Intestinal Junction	85.4 ± 6.0 (75.0-106.0)	91.4 ± 8.0 (75.0-108.0)	97.7 ± 3.8 (90.0-103.0)	100.9 ± 9.7 (85.0-113.0)	105.7 ± 11.0 (97.0-118.0)	90.7 ± 3.8 (88.0-95.0)	91.8 ± 10.8 (78.0-108.0)	83 ± 7.1 (78.0-88.0)

Table 1. Measurements of all *Pratylenchus* haplotypes groups without names (Continue)

Character	*Pratylenchus* sp. 1	*Pratylenchus* sp. 2	*Pratylenchus* sp. 4	*Pratylenchus* sp. 5	*Pratylenchus* sp. 6	*Pratylenchus* sp. 7	*Pratylenchus* sp. 9	*Pratylenchus* sp. 10
n	2	5	1	2	1	2	10	5
L	598 ±97.2 (530-667.5)	581.8 ± 57.6 (520-656)	550	475.0 ±35.4 (450-500)	512.5	572.5 ±3.54 (570-575)	466.9 ± 48.2 (402-570)	493.0 ±57.8 (432.5-575)
a	23.9 ± 1.19 (23-24.7)	23.9 ± 3.46 (20.5-28.9)	21.2	22.6 ±1.68 (21.4-23.8)	22.3	26.7 ± 1.0 (25.9-27.4)	24.8 ±1.7 (22.5-28.5)	23.6 ±2.8 (21.0-27.6)
b	6.7 ± 1.2 (5.9-7.6)	5.7 ± 0.42 (5.24-6.22)	6.9	5.3 ± 0.05 (5.3-5.4)	5.8	6.7 ±0.12 (6.6-6.8)	5.6 ± 1 (4.4-6.6)	6.0 ±0.4 (5.8-6.8)
b'	4.3 ± 1.1 (3.5-5.1)	4.4 ± 0.46 (3.94-5.09)	4	3.5 ±0.3 (3.3-3.8)	4.1	4.2 ±0.08 (4.2-4.3)	4.0 ± 0.5 (3.2-4.9)	4.3 ±0.5 (3.6-4.9)
c	20 ± 3.24 (17.7-22.3)	20.5 ± 0.4 (19.9-20.8)	27.5	20.9 ±4.11 (19-23.9)	NA	25.1 ± 3.0 (23.0-27.1)	19.8 ± 2 .1 (17.0-22.5)	22.8 ±5.5 (18.8-32.2)
c'	1.9 ± 0.1 (1.9-2.0)	1.9 ± 0.2 (1.7-2.2)	1.3	1.7 ±0.3 (1.5-1.9)	NA	1.9 ±0.3 (1.8-2.1)	2.1 ± 0.3 (1.8-2.6)	1.9 ±0.3 (1.5-2.2)
V	76 ± 2.6 (74.2-77.8)	78.9 ± 0.6 (78.2-79.6)	77.2	79.5 ±0.8 (78.9-80.0)	79.5	80.8 ±0.5 (80.4-81.1)	79.7 ± 1.0 (78.6-81.1)	76.9 ±1.02 (75.7-78.2)
MB%	41.9 ± 2.7 (40.0-43.9)	41.0 ± 2.9 (38.3-45.1)	45.1	37.1 ±53 (33.3-408)	41.2	40.0	44.4 ± 23 (416-50.0)	48.8 ±7.7 (40.0-59.0)
Stylet L.	18.5 ± 0.7 (18.0-19.0)	14.4 ± 3.2 (14.0-15.0)	16.0	15.0	16.0	14.5 ±0.7 (14.0-15.0)	15.5 ± 0.5 (15.0-16.0)	16.2 ± 0.5 (16.0-17.0)
Ex. Pore	88.8 ± 1.8 (87.5-90.0)	83.80 ± 3.56 (81.0-89.0)	85.0	82.5 ±3.5 (80.0-85.0)	90.00	87.5	79.3 ± 6.6 (71.0-90.0)	86.0 ±9.6 (72.5-97.5)
Position of Median Bulb	58.5 ± 2.12 (57.0-60.0)	53.6 ± 1.7 (51.0-55.0)	62.0	49.5 ±0.7 (49.0-50.0)	58.0	54.0 ±1.4 (53.0-55.0)	51.8 ± 2.8 (45.0-56.0)	56.0 ±4.6 (50.0-62.0)
MBW	25.0 ± 2.8 (23.0-27.0)	24.8 ± 4.8 (20.0-32.0)	26.0	21.0	23.0	21.5 ±0.7 (21.0-22.0)	18.8 ± 1.1 (17.0-20.0)	21.0
VBW	24.0 ± 1.4 (23.0-25.0)	21.8 ± 2.4 (19.0-25.0)	22.0	21.5± 0.7 (21.0-22.0)	NA	20.5±0.7 (20.0-21.0)	17.2± 1.7 (15.0-20.0)	20.5±2.5 (16.0-22.0)

ABW	15.5 ± 0.7 (15.0-16.0)	14.8 ± 2.0 (13.0-18.0)	15.0	13.5±0.7 (13.0-14.0)	NA	12.0	11.4 ± 1.0 (10.0-13.0)	11.4±1.1 (10.0-13.0)
Vulva-Anus Distance	115.0 ± 38.9 (87.5-142.5)	94.2 ± 11.9 (80.0-110.0)	105.0	74.5±6.4 (70.0-79.0)	NA	87.0±0.7 (86.5-87.5)	77.4 ± 10.9 (62.5-90.0)	91.6±14.7 (74.5-105.0)
Tail Length	30.0	28.4 ± 3.2 (25.0-33.0)	20.0	23.0±2.8 (21.0-25.0)	NA	23.0±2.8 (21.0-25.0)	23.8 ± 3.4 (18.0-29.0)	22.4±4.9 (15.0-28.0)
PUS	21.5 ± 2.12 (20.0-23.0)	19.2 ± 4.8 (14.0-26.0)	NA	14.0±1.4 (13.0-15.0)	NA	13.5±0.7 (13.0-14.0)	14.8 ± 2.8 (12.0-21.0)	18.5±8.0 (12.0-30.0)
Pharyngeal Overlap	45.0 ± 7.1 (40.0-50.0)	28.8 ± 12.3 (13.0-44.0)	30.0	26.0	37.0	33.5±5.0 (30.0-37.0)	33.3± 4.8 (27.0-40.0)	26.3±4.5 (20.0-30.0)
Pharyngeal gland lobe	140.0 ± 14.1 (130.0-150.0)	131.2 ± 6.8 (122.0-141.0)	137.5	135.0±21.2 (120.0-150.0)	125.00	135.0±3.54 (132.5-137.5)	117.0 ± 8.6 (100.0-125.0)	116.0±11.0 (105.0-130.0)
Pharyngeal Intestinal Junction	89.0 ± 1.4 (88.0-90.0)	102.6 ± 7.7 (97.0-116.0)	80.0	89.0±5.7 (85.0-93.0)	88.0	85.5±2.1 (84.0-87.0)	82.9± 6.7 (71.0-92.0)	81.4±4.7 (75.0-86.0)

Table 2. Morphometrics of *P. scribneri* and *P. hexincisus* measurements in this study and previous studies.

Character	*P. scribneri* in this study	*P. scribneri* Steiner's 1943	*P. scribneri* Thorne and Malek.1968	*P. scribneri* *Roman and Hirshmann. 1969*	*P. scribneri* Inserra *et al.*, 2007	*P. scribneri* *Yan et al. 2016*	*P. hexincisus* in this study	*P. hexincisus* Taylor and Jenkins, 1957	*P. hexincisus* Thorne and Malek, 1968	*P. hexincisus* Inserra *et al.*, 2007
n	26			50	28	10	9	96		20
L	526.5 ± 52.9 (385-610)	(280-592)	(500-700)	504.4 (436.8-553.2)	525 ± 36.7 (432-582)	476.5 (407.0-532.0)	513.4 ± 43.5 (457-575)	436 ± 44 (342-540)	500	517 ± 40.6 (429-574)
a	25.6 ±2.9 (19.8-30.9)	(17-23)	(17-26)	26.3 (21.4-29.0)	25.6 ± 2.0 (20.8-29.0)	20.4 (17.7-24.6)	24.9 ± 2.08 (21.6-27.3)	22.6 ± 2.5 (18.2-28.8)	18	23.8 ± 1.8 (20.1-26.8)
b	5.7 ± 0.5 (4.6-6.7)	(5.6-8.0)	(5.4-6.6)	6.32 (5.7-7.0)	5.7 ± 0.4 (5.0-6.4)	4.6 (4.0-5.2)	5.3 ± 0.5 (4.6-6.4)	7.2 ± 0.6 (5.9-8.4)	5.4	5.6 ± 0.3 (5.1-6.0)
b'	4.1 ± 0.4 (3.4-4.9)				3.8 ± 0.2 (1.9-2.6)		3.8 ± 0.3 (3.3-4.3)			3.7 ± 0.2 (3.2-4.1)
c	19.4 ± 2.7 (13.7-26.8)	(16.9-22.7)	(16-18)	18.4 (16.9-20.6)	19.6 ± 1.3 (17.5-22.3)	18.5 (16.2-22.4)	21.3 ± 3.5 (15.7-25.4)	18.6 ±1.3 (16.1-22.7)	20	17.9 ± 1.3 (15.1-20.5)
c'	2.3 ± 0.4 (1.7-3.5)				2.2 ± 0.2 (1.9-2.6)		1.9 ± 0.3 (1.5-2.3)			2.3 ± 0.2 (2.0-2.7)
V%	78.1 ± 1.5 (74.9-82.2)	(75.7-80.5)	79	77.4 (75.0-82.0)	78.2 ± 1.0 (76.2-79.9)	77.2 (75.5-78.7)	79.0 ± 1.5 (77.4-81.8)	78.0 ± 1.9 (75.3-82.2)	80	77.8 ± 0.8 (76.3-79.3)
MB%	43.5 ± 3.6 (36.9-50.0)			15.3 (14.4-16.8)			41.3 ± 2.4 (38.0-45.0)			

Stylet L.	15.6 ± 0.8 (14.0-17.0)	(14-15)	(15-17)	15.03 (14.4-16.8)	14.7 ± 0.1 (14.2-14.9)	15.0 (15.0-15.5)	14.9 ± 0.3 (14.0-15.0)	15.0 ± 0.0 (14.5-15.5)	14	15.5 ± 0.3 (14.5-15.5)
Ex. Pore	87.0 ± 7.2 (71.0-100.0)			80.9 (74.0-86.0)	89.4 ± 5.6 (78.4-99.9)		82.1 ± 5.4 (76.0-90.0)			89.4 ± 5.8 (73.5-98)
Position of Median Bulb	54.8 ± 3.8 (46.0-61.0)						55.2 ± 2.3 (52.0-59.0)			
MBW	20.8 ± 2.7 (14.0-25.0)			19.3 (16.8-23.4)	20.5 ± 2.3 (15.6-26.4)	23.5 (21.1-28.8)	20.7 ± 1.4 (18.0-23.0)			21.8 ±2.0 (18.6-26.4)
VBW	20.4 ± 2.2 (16.0-25.0)				19.7 ± 2.2 (15.6-24.9)		19.6 ± 1.2 (18.0-21.0)			20.2 ± 2.0 (15.1-24.5)
ABW	12.0 ± 1.6 (10.0-15.0)				11.9 ± 1.1 (9.8-14.2)		12.9 ± 0.9 (11.0-14.0)			12.6 ± 0.7 (11.2-14.7)
Vulva-Anus Distance	86.6 ± 14.7 (61.0-115.0)			84.2 (57.6-100.4)	87.9 ±5.8 (77.4-98)		83.3 ± 12.6 (66.0-103.0)			85.0 ± 7.6 (70.5-99.0)
Tail Length	27.5 ± 3.1 (20.0-35.0)			27.3 (24.0-30.6)	26.7 ± 2.1 (21.5-29.6)	25.6 (25-28.0)	24.8 ± 5.0 (18.0-30.0)			28.9 ±1.7 (24.5-32.3)
PUS	21.6 ± 6.0 (12.0-40.0)			(13.8-31.2)	25.5 ±3.0 (19.6-30.3)	23	17.1 ± 4.1 (11.0-25.0)			26.8 ± 3.5 (23.5-31.3)
Pharyngeal Overlap	30.5 ± 7.5 (20.0-48.0)				45.4 ± 5.9 (33.3-53.9)		31.6 ± 4.1 (24.0-37.0)			43.8 ± 4.9 (34.3-53.9)

Pharyngeal gland lobe	127.2 ± 15.6 (100.0-162.5)					101.4 (98.0-107.0)	134.0 ± 6.9 (127.5-150.0)			
Pharyngeal Intestinal Junction	91.4 ± 8.0 (75.0-108.0)						97.7 ± 3.8 (90.0-103.0)			