Introduction

From 2012-2016, we made 6 collection trips (Table 1) to 70 sites (Table 2 and Figure 1) to the Big Thicket National Preserve, Marysee Prairie and Roy E. Larsen Sandyland Sanctuary to inventory the soil-dwelling nematodes of the BITH and its neighboring preserves and eventually to describe new nematode species, if discovered. We are very grateful that this two-part study, *Soil-dwelling Nematode Inventory of BITH and neighboring preserves* and *New Species Descriptions of Nematodes of the Family Criconematidae* had the generous support, time and constructive advice of The Thicket of Diversity (TOD), Big Thicket Association (BTA) and the National Park Service Staff at BITH. BITH and its neighboring preserves were a completely new territory for us and, as the following results indicate, for soil-dwelling nematode discovery.

The field collections and initial identifications were part of the first phase of an inventory of the soil-dwelling nematodes of Big Thicket National Preserve, Marysee Prairie and Roy E. Larsen Sandyland Sanctuary. A follow-up series of focal collections were part of Phase II, in which we attempted to collect enough material to describe the 7 new species of nematodes in the family Criconematidae, discovered in BITH and its neighboring preserves. To date, we have identified 1026 nematodes (Table 3), of which a smaller group of 614 nematodes, have digital images, morphometric measurements and DNA template vouchers. A subset of those, 136 Criconematidae (Table 4), have been COI DNA barcoded and are part of the current 1517 specimen COI DNA NJ Phylogenetic Tree.

Nematodes found in soil are microscopic allowing initial identification only after the soil collected is brought back to a lab. There the nematodes are extracted from the soil by a process of sieving and sugar centrifugation and then, examined microscopically. Many nematode species are also cryptic (organisms only distinguishable at the genetic level). Therefore, a further molecular identification is required. Over the last 15 years, the Thomas Powers Laboratory, at the University of Nebraska, has DNA sequenced a region of the mitochondrial gene, cytochrome oxidase I (COI), of many plant-parasitic nematodes from soils of cultivated and non-cultivated land. All DNA sequences (COI barcodes) of the nematode family Criconematidae, including those from BITH specimens, have contributed to the largest curated nematode COI reference database. Nematode sequence barcodes, images and associated metadata was submitted to and are archived on the Barcode of Life Database Systems (BOLDSystems[20,21]). In brief, our protocol entailed collecting 500cc of soil at 70 sites in two ways, either a composite of 5-10 soil cores taken within a 40mx40m grid or sampling the rhizosphere around a focal host plant. After processing and extracting from the soil, individual nematodes were examined microscopically and initially identified by morphometric diagnostic characters. When possible, each nematode of the family Criconematidae and a subset of other nematodes of interest, were digitally photographed, measured and the whole nematode body crushed in 18ul sterile double distilled water and frozen at -20°F until the DNA template could be identified molecularly using a mitochondrial gene, cytochrome oxidase I (COI).

Materials and Methods

Field Collections

Our field collections in the Big Thicket were conducted in two phases. Phase I was the initial survey of the soil-dwelling nematodes of the terrestrial units of BITH, Marysee Prairie and Roy E. Larsen Sandyland Sanctuary where we collected from sites representative of the each unit's plant

communities with the goal of at least one composite sample from a 40mx40m site from each BITH unit, Marysee Prairie and Roy E. Larsen Sandyland Sanctuary. For details and summary of our collection results see Table 1, and Table 2, and Figure 1. Once the initial soil samples had been processed and the nematodes extracted, tentatively identified and sorted as a candidate species new to the preserve, state, US or science, we returned to BITH and its environs to collect from previous sites or targeted specific host plants for focal soil samples for Phase II.

The standard protocol for composite samples is to establish a 40mx40m grid at each site. Soil samples are obtained using an Oakfield tube to extract cores from a maximum depth of 25cm. Cores of 2.5 cm diameter from 5-10 cores within a 40mx40m plot were combined together.

Focal samples are obtained using an Oakfield tube to extract cores from a maximum depth of 25cm from the rhizosphere around the target plant host. For example, when sampling around a loblolly pine (ie. sample TX20150614007BITH, see Table 3.), 5-10 cores were taken from the rhizosphere around the trunk in a circle defined by the tree's canopy.

All samples were stored in an 8°C cold room until a 200 cc subsample could be processed for nematode isolation. In the laboratory, nematodes are extracted from soil using a combination of sieves and sugar centrifugation. The extracted nematodes are then observed, measured, and photographed with a light microscope in the following manner. Nematodes are picked out from a 200cc soil extract using a dissecting scope. When possible, five individuals from each morphotype in the extract were individually mounted on glass slides, measured, digitally imaged with a Leica DC 300 videocamera on a Leica DMLB microscope using Differential Interference Contrast optics. After documentation the slide was dismantled, the nematode crushed in 18 µl of water with a transparent micropipette tip, and added to PCR microfuge tube which is placed in a -20F Freezer to be processed for DNA barcoding.

PCR amplification and DNA sequencing.

Methods for PCR amplification and DNA sequencing have been previously described (Powers et al., 2014). We attempt to DNA barcode using the COI mitochondrial genetic marker all Criconematidae but also specimens from the following families Aphelenchoididae, Heteroderidae, Meloidogynidae and Pratylenchidae, all nematode groups with which we have established protocols for PCR amplification and phylogenetic trees developed from the sequenced COI DNA. For new species descriptions, our molecular data are from multiple genes, nuclear ribosomal DNA (18S rDNA and the internal transcribed spacer (ITS1) and mitochondrial (cytochrome oxidase subunit I (COI)).

PCR amplification reactions for DNA barcoding using the COI mitochondrial genetic marker for the Criconematidae produced an approximately 790-bp amplification product that after removal of primers from consideration, provided 721 bp of sequence for genetic analysis. The PCR amplification protocol, conducted in a 30.0-µl total volume within 0.6-ml reaction tubes, consisted of 9.0 µl of template from the ruptured nematode specimen, 2.4 µl of each 20µM primer solution for a 1.6-µM final primer concentration, 1.2 µl ddH20, and 15 µl of 2x taq polymerase for a 0.03U/µl final enzyme concentration. PCR conditions included a hotstart and 5-minute treatment at 94°C followed by 40 cycles of 30 seconds at 94°C denaturation, 30 seconds at 48°C annealing, and 1.5 minutes at 72°C with a ramping rate of 0.5°C/second for the elongation step. A final 5:00 minute extension at 72°C completed the process.

DNA Sequencing. Following amplification, an initial check gel was run followed by cleaning of the PCR product by gel fragment extraction from a 0.7% agarose gel, using Gel/PCR DNA Fragment Extraction Kit (IBI Scientific). DNA templates were sequenced by the UCDavis sequencing center. Nearly all amplification products are sequenced in both directions, then edited and aligned.

Scanning Electron Microscopy and Voucher Documentation.

We use the Scanning Electron Microscope at the UNL Morrison Microscopy Core Research Facility**.** Training in the use of microscope and recordkeeping of images is required for all users. Preparation of SEM specimens requires an alcohol dehydration phase, critical point drying and mounting and gold coating. We typically get 50-60 photos of 10 specimens in 3-4 hours. An undergraduate student in our lab and postgraduate technical staff member and Tom Powers are trained in electron microscopy.

All nematodes submitted to the Big Thicket National Preserve NPS catalog as vouchers have a unique individual specimen identifier (NID) in the Powers Lab at the University of Nebraska, Lincoln. Every nematode voucher may consist of any or all of the following with the same NID: a morphometric record, a digital image, a microcentrifuge tube with DNA template, and a 15 ml tube of extracted nematodes bulk fixed in 8:2 Formalin:Glycerol. DNA sequences are submitted to the National Center for Biotechnology Information Database (GenBank) with the associated sample GPS coordinates. Both females and juveniles were used in molecular analyses. Only adult females were used in the morphological analyses.

For new species descriptions, we need approximately 30 specimens of each candidate species to be picked out from the nematodes extracted from processed soil. A microscopic confirmation by taxonomists, Tom Powers, Ph.D. or Peter Mullin, Ph.D., on individual nematodes complete with morphometric record and digital image voucher archived at our nematode@unl.edu server and DNA template stored at -20F lab freezer while the project is ongoing and to the Harold W. Manter Laboratory of Parasitology upon project completion**.** The remainder of the extracted nematodes are bulk fixed with an 8:2 Formalin:glycerin fixative. Selected nematodes are then processed for PCR amplification and DNA sequencing to provide a DNA sequence voucher and if there remain enough specimens, the remainder are glycerine infiltrated for permanent slides to be sent to the USDA Nematology Laboratory in Beltsville and Harold W. Manter Laboratory of Parasitology.

Results

Included is a list of the 9 species we had considered as candidates for new species descriptions (see Table 5, same as Table 1. from the initial proposal for Phase II). We are in the process of describing the first 7 listed, all Criconematidae, but do not have enough material for Opistodorylaimus or Sclerotylus species. Examples of plates, all partials, are in Figure 2. The Meloidygne paper, see #3 in list of publications below has the first record of the species, *Meloidogyne partityla* found in non-cultivated land in Texas.

The following are publications based on specimens or including specimens from BITH and its neighboring Preserves.

1. Powers TO, Harris T, Higgins R, Olson M, Matczyszyn J, Mullin P, Powers KS. New species of plant-parasitic nematodes in the family Criconematidae from Big Thicket National Preserve and the Roy E Larsen Sandyland Sanctuary (in prep) 2019.

2. Matczyszyn J, Everhart S, Powers KS, Powers TO. Phylogenetic and population structure of Mesocriconema xenoplax across the United States. (in prep) in partial completion of Ph.D. 2018

3. Powers TO, Harris TS, Higgins RS and Powers, KS. Discovery and identification of Meloidogyne species using COI DNA barcoding. DOI:10.21307/jofnem-2018-037.

4. Munawar M, Powers TO, Zhongliang T, Harris T, Higgins R, Zheng J . Description and Distribution of Three Criconematid Nematodes from Hangzhou, Zhejiang Province, China. DOI: 0.21307/jofnem-2018-010.

5. Powers, T. O., Bernard, E. C., Harris, T., Higgins, R., Olson, M., Olson, S., Lodema, M., Matczyszyn, J., Mullin, P., Sutton, L., Powers. K. S. 2016. Species discovery and diversity in *Lobocriconema* (Criconematidae: Nematoda) and related plant-parasitic nematodes from North American ecoregions. Zootaxa 4085 (3): 301–344. http://doi.org/10.11646/zootaxa.4085.3.1

6. Powers, T.O., Mullin, P., Higgins, R., Harris, T. and Powers, K.S., 2016. Description of Mesocriconema ericaceum n. sp.(Nematoda: Criconematidae) and notes on other nematode species discovered in an ericaceous heath bald community in Great Smoky Mountains National Park, USA. Nematology, 18(8), pp.879-903. http://dx.doi.org/10.1163/15685411-00003001 Accepted for publication: 31 May 2016

7. Olson, M., Harris, T., Higgins, R., Mullin, P., Powers, K., Olson, S. and Powers, T.O., 2017. Species Delimitation and Description of Mesocriconema nebraskense n. sp.(Nematoda: Criconematidae), a Morphologically Cryptic, Parthenogenetic Species from North American Grasslands. Journal of Nematology, 49(1) 42-68.

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10. Powers T.O., Harris T, Higgins R, Olson M, Lodema M, Mullin P, Sutton L, Powers KS. Checklist of the soil-dwelling nematodes of the Big thicket National Preserve, Marysee Prairie and Roy E. Larsen Sandyland Sanctuary (in Prep).

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2. Jörger KM, Schrödl M. How to describe a cryptic species? Practical challenges of molecular taxonomy. Front Zool. 2013 Sep 27;10(1):59. doi: 10.1186/1742-9994-10-59.
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