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ARTICLE

Nematode biodiversity assessments need vouchered databases: A BOLD reference library for plant-parasitic nematodes in the superfamily Criconematoidea[1]

Thomas O. Powers, Timothy S. Harris, Rebecca S. Higgins, Peter G. Mullin, and Kirsten S. Powers

Abstract: Nematodes are frequently cited as underrepresented in faunistic surveys using DNA barcoding with COI. This underrepresentation is generally attributed to a limited presence of nematodes in DNA databases which, in turn, is often ascribed to structural variability and high evolutionary rates in nematode mitochondrial genomes. Empirical evidence, however, indicates that many taxa are readily amplified with primer sets specifically targeted to different nematode families. Here we report the development of a COI reference library of 1726 specimens in the terrestrial plant parasitic nematode superfamily Criconematoidea. Specimens collected during an ecoregion survey of North America were individually photographed, measured, and PCR amplified to produce a 721 bp region of COI for taxonomic analysis. A neighbor-joining tree structured the dataset into 179 haplotype groups that generally conformed to morphospecies in traditional analysis or Barcode Index Numbers (BINs) in the BOLD system, although absent formal BIN membership due to insufficient overlap with the Folmer region of COI. Approximately one-third of the haplotype groups could be associated with previously described species. The geographic distribution of criconematid nematode species suggests a structure influenced by the major habitat types in the United States and Canada. All sequences collected in the ecoregion survey are deposited in BOLD.

Key words: Nematoda, DNA barcoding, species distribution.

Résumé : Les nématodes sont souvent cités comme étant sous-représentés dans les inventaires faunistiques réalisés à l'aide de codes à barres de l'ADN avec le gène COI. Cette sous-représentation est généralement attribuée à la présence limitée des nématodes dans les bases de données ce qui, à son tour, est souvent imputée à la variabilité structurale et à un taux d'évolution élevé chez les génomes mitochondriaux des nématodes. Les évidences empiriques, cependant, indiquent que plusieurs taxons sont aisément amplifiés avec des jeux d'amorces ciblant spécifiquement les différentes familles de nématodes. Ici, les auteurs rapportent le développement d'une librairie de référence de séquences COI pour 1726 spécimens appartenant à la superfamille des Criconematoidea, des nématodes parasitant des plantes terrestres. Les spécimens ont été recueillis dans le cadre d'un inventaire de l'écorégion de l'Amérique du Nord. Ils ont été photographiés, mesurés et amplifiés individuellement pour obtenir une région de 721 pb du gène COI pour des fins d'analyse taxonomique. Un arbre de type neighbor-joining a séparé ces spécimens en 179 groupes haplotypiques qui étaient généralement conformes aux espèces morphologiques définies par une analyse traditionnelle ou encore sur la base des codes numériques (BIN de « Barcode Index Numbers ») du répertoire BOLD, bien qu'ils étaient absents de la description des BIN en raison d'un chevauchement insuffisant au sein de la région Folmer du gène COI. Environ le tiers des groupes haplotypiques ont pu être associés à des espèces déjà décrites. La distribution géographique des espèces de nématodes criconématides suggère une structure influencée par les principaux types d'habitats aux États-Unis et au Canada. Toutes les séquences obtenues au terme de cet inventaire de l'écorégion ont été déposées dans le répertoire BOLD. [Traduit par la Rédaction]

Mots-clés : Nematoda, codage à barres de l'ADN, distribution des espèces.

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Introduction

A taxonomic reference library can provide essential information for studies of diversity, distribution, and taxon identity. Now, as investigations using environmental and community DNA are generating vast quantities of nucleotide sequences from prokaryotes and eukaryotes alike, reference libraries will help establish taxonomic context for meaningful interpretation of the sequence data (Miller et al. 2016; Richards et al. 2018; DeSalle and Goldstein 2019; Ekrem et al. 2007). Nematodes are frequently considered to be neglected in "all-taxa" surveys and are underrepresented in searchable nucleotide sequence databases (McGee et al. 2019; Brunbjerg et al. 2019; Rodrigues da Silva et al. 2010; Ahmed et al. 2019; Creer et al. 2010; Holovachov et al. 2017; Schenk and Fontaneto 2019). In particular, COI representation in GenBank and Barcode of Life Data Systems (BOLD) lags far behind other metazoans, especially considering estimates of total species in the phylum, which may exceed 5 million (Van den Hoogen et al. 2019; Schenk and Fontaneto 2019; Larsen et al. 2017). Their abundance in soil typically range from 1–8 million per m^2 and may include over 200 species in the top 20 cm of soil (Yeates 2010). A reference library that links nematode morphology with diagnostic genetic markers and environmental variables should accelerate studies of comparative morphology, stimulate the neglected fields of nematode biogeography, and serve as a preliminary outline of taxon diversity (Zaharias et al. 2020). The urgency of documentation is underscored by the diminishing number of museums with dedicated curators that currently maintain classical specimen repositories for nematodes. These repositories typically catalogue formalin-fixed nematodes preserved on slides or in vials. Unfortunately, despite the best efforts to preserve type and voucher species, fixed nematodes mounted in glycerin on microscope slides lose detail with time, eventually obscuring key morphological characters. Linking genetic and morphological data of individual specimens, while retaining a physically intact specimen, is not practical due to the small size and hydrostatic pressure that causes nematodes to collapse when punctured. However, nematodes are excellent candidates for digital imaging given their small size, transparent cuticle, and their limited number of organ systems (De Ley and Bert 2002). Once imaged, unfixed nematode specimens can be removed from a slide, crushed, and DNA barcoded. BOLD provides an excellent platform and repository for image vouchers and their associated sequence plus metadata. Here we report on the development of a reference library of mitochondrial COI sequences obtained from Criconematoidea, a globally distributed superfamily of plant-parasitic nematodes.

Our goals are to (*i*) illustrate the challenges associated with creating a reference library for a terrestrial nematode taxon with a cosmopolitan distribution and many undescribed species, and (*ii*) populate a nematode reference library with entries sufficient in geographic range and numbers to establish a framework to address questions of nematode identity, diversity, and distribution. Evidence already suggests that COI barcodes can recognize the taxonomic boundaries of traditionally described morpho-species, and that geographically these species exhibit nonrandom patterns of distribution (Powers et al. 2014, 2016*a*, 2016*b*; Olson et al. 2017; Van den Berg et al. 2018). Herein, we use the BOLD platform to organize records of approximately 1700 nematode specimens in the plant-parasitic superfamily Criconematoidea.

Materials and methods

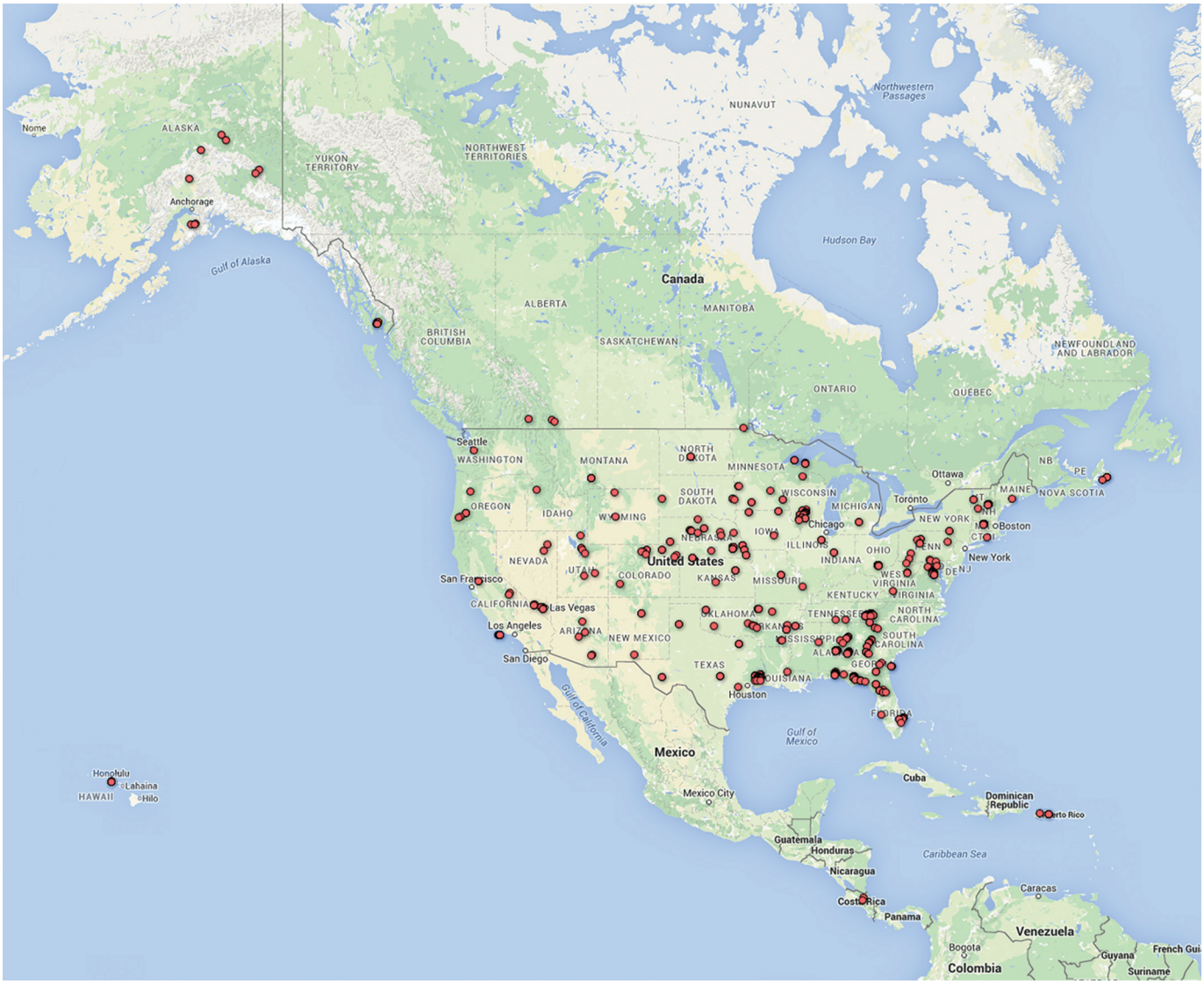
Collection data

North American collection sites are indicated on Fig. 1. A majority of the specimens in this 1726-sequence dataset were obtained from soil within a 40 m × 40 m grid in which each of the corners were georeferenced. Field notes and digital imaging recorded the vegetation and topography of the grid. These metadata can be found in two separate complementary archives. Table S1[2] (also available at University of Nebraska-Lincoln Data Repository, https://doi.org/10.32873/unl.dr.20200330) provides the individual specimen's ID number, collection location information, GenBank accession number, and other genetic marker accession numbers associated with that specimen. On the public BOLD site (http://www.barcodinglife.org/), the project CRICO (dx.doi.org/10.5883/DS-NEMACRIC) yields much of the same information as above, as well as specimen images, habitat images, morphometrics, and DNA sequence data. One-hundred and five GenBank sequences of specimens not observed by the authors were included in the neighbor-joining tree of the superfamily (Fig. 2; Fig. S1[2], also available at University of Nebraska-Lincoln Data Repository, https://doi.org/10.32873/unl.dr.20200330). Ninety-six of these GenBank sequences were reported in recent taxonomic studies of two genera, *Hemicycliophora* and *Hemicriconemoides*, which employed the COI-F5/COI-R9 primer set discussed below (Maria et al. 2019; Van den Berg et al. 2018). These GenBank accessions were not added to the dataset of COI sequences submitted to BOLD, but they are listed in Table S2[2] (also available at University of Nebraska-Lincoln Data Repository, https://doi.org/10.32873/unl.dr.20200330).

To obtain nematode samples, a standard soil corer with a 3 cm diameter was used to extract cores to a depth of 20 cm if possible. Depending on the depth of the soil core, 15–25 cores would constitute a single sample. The

[2]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/gen-2019-0196.

Fig. 1. Map of North America indicating collection sites for criconematid nematode samples obtained during an ongoing ecoregion survey. Map data: Google Fusion.



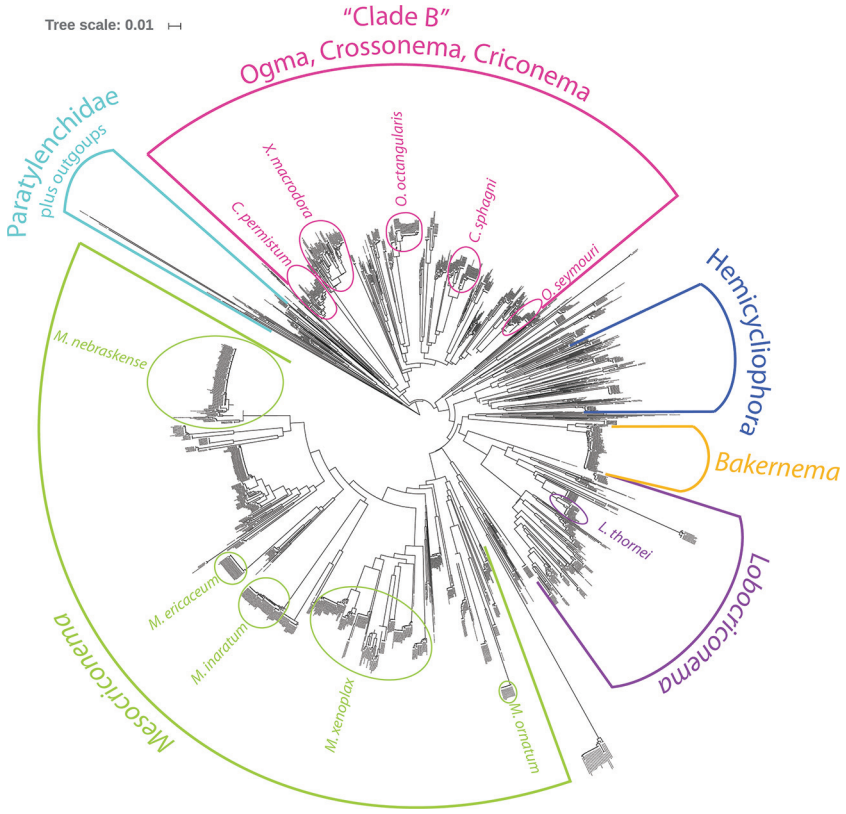
soil cores were mixed and stored in plastic bags at 15 °C prior to processing. A second sampling strategy consisted of focal samples in which all cores were centered on a single plant species, in an effort to determine nematode – host plant associations. In other cases, approximately 9% of specimens for this dataset were obtained from agricultural surveys of plant pests or sent by colleagues and clients requesting specific identification.

Sampling locations were guided by a goal to sample a majority of the ecoregions representing the major habitat types in North America north of Mexico (Olson et al. 2001). Several ecoregions were sampled intensely. For example, Central Tall Grasslands, which have been largely converted to agricultural production, required sampling numerous small remnant prairies across the former native range of this plant community. National Parks and wildlife areas were sampled intensely due to their preservation of specific plant communities and the desire to establish baseline measures of soil nematode diversity within nature preserves and all-taxa biotic survey sites (Nichols and Langdon 2007; Watson 2006).

Soil processing to isolate nematodes was conducted by an initial flotation and sieving step, followed by sugar centrifugation (Jenkins 1964). Importantly, criconematid nematodes are not well-represented in soil extractions that require active movement of the nematode for isolation (e.g., Baermann funnel, pie pans). These nematodes do not move vigorously and tend to remain in the substrate.

Once isolated, nematodes were hand-picked into a 20 μL drop of water on a glass slide, overlaid with a coverslip, and observed through a microscope. Gentle heating was used to immobilize nematodes for imaging and measurements. Following measurement, the coverslip was removed, the nematode was added to a fresh 20 μL drop of water on another coverslip, and crushed

Fig. 2. Neighbor-joining tree of 1726 Criconematid COI DNA sequences. Major genera and prominent species in the superfamily Criconematoidea are identified.



with a transparent micropipette tip, without subsequent DNA extraction. Crushed nematodes were stored in PCR tubes at –20 °C prior to PCR.

The COI primer sequences COI-F5 (5′-AATWTW GGTGTTGGAACTTCTTGAAC-3′) and COI-R9 (5′-CTTAAA ACATAATGRAAATGWGCWACWACATAATAAGTATC-3′) resulted in an approximately 790-base pair (bp) amplification product (Powers et al. 2014). The 5′ primer, COI-F5 provides a 351 nucleotide overlap with the Folmer primer set that is approximately 150 nucleotides short of the required 500 nucleotide overlap required to generate a BIN designation in the BOLD system (Ratnasingham and Hebert 2013). Following removal of primer sequences, 721 bp of sequence were used in genetic analyses. Primer set JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB5 (5′-AGCACCTAAACTTAAAACATAATGAAAATG-3′) (Derycke et al. 2005, 2010), which produces an approximately 400 bp amplicon and is internal to the COI-F5/COI-R9 set, was used for 27 sequence entries as part of a separate study comparing taxonomic content from a smaller amplicon. The JB3/JB5 primer set amplifies a region that is being evaluated for metabarcoding of nematode communities (Ahmed et al. 2019; Macheriotou et al. 2019; Tytgat et al. 2019).

PCR amplification reactions, 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 20 μM primer solution for a 1.6 μM final primer concentration, 1.2 μL ddH20, and 15 μL of 2× JumpStart REDTaq ReadyMix (Sigma–Aldrich) for a 0.03 U/μL final enzyme concentration. PCR conditions included a hotstart and 5 min treatment at 94 °C followed by 40 cycles of 30 s at 94 °C denaturation, 30 s at 48 °C annealing, and 1.5 min at 72 °C, with a ramping rate of 0.5 °C/s for the elongation step. A final 5 min extension at 72 °C completed the process. 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 in both directions by the University of California-Davis Sequencing Services, with final editing using CodonCode Aligner Version 4.2 (http://www.codoncode.com). Each trace in a pair was aligned and, in the case of heteroplasmic sites, the appropriate base degeneracy letter was assigned. All sequences were screened for stop codons. A neighbor-joining phylogram of 1726 Criconematid COI DNA sequences was constructed using MEGA7.0.26 and K2P with 500 boot strap repetitions in an effort to maximize comparisons with trees generated in the BOLD System. Gap treatment was pairwise deletion with gap opening (–400) and gap extend (–200) parameters.

Nematode classification and species assignment

Given that COI does not provide well-supported nodes at deeper positions in phylogenetic trees of Nematoda,

genus membership in this study was guided by a near full-length 18S tree that addressed subfamily and genus classification in the suborder Criconematoidea (Powers et al. 2017). The 18S tree supported many of the traditional and formally described taxa in the superfamily (Geraert 2010). Substrate from specimens used in the 18S study provided template for 166 COI amplifications in the present study. Nevertheless, both 18S and COI provide evidence of paraphyly in the genera *Criconemoides*, *Discocriconemella*, and *Hemicriconemoides*. For species identification, an initial morphological analysis was conducted with each specimen, recording the images, measurements, and character analysis typically applied to the suborder (Powers et al. 2010, 2014, 2016*a*, 2016*b*; Yu et al. 2016; Olson et al. 2017; Maria et al. 2018, 2019). The descriptions, keys, and identification charts found in Geraert (2010) and the tables of Brzeski et al. (2002*a*, 2002*b*) were an invaluable source for species discrimination and formed an initial morphological-based hypothesis about species identity. A neighbor-joining, COI tree-based taxonomy assignment approach (Holovachov et al. 2017) followed the morphological analysis and resulted in the designation of haplotype groups (Fig. S1[2], also available at University of Nebraska-Lincoln Data Repository, https://doi.org/10.32873/unl.dr.20200330). Haplotypes were grouped if they consisted of more than a single specimen, had bootstrap values above 97, exhibited mean K2P within-group distance of less than 5%, and the distance to the nearest neighbor was not exceeded by the maximum within-group distance. The Barcode Gap Analysis Tool in the BOLD workbench allowed for a comparison of the morphologically identified species with Latin binomials deposited in BOLD and haplotype group taxonomic assignments (Table S3[2], also available at University of Nebraska-Lincoln Data Repository, https://doi.org/10.32873/unl.dr.20200330). These haplotype groups serve as primary species hypotheses for taxonomic studies prior to analyses of species delimitation (Jörger et al. 2012).

Results

Dataset description

A collection of 1621 COI sequences, largely collected as part of a North American ecoregion survey, plus an additional 105 sequences obtained from GenBank were combined to create a 1726-specimen cohort. Individual criconematid nematodes were collected from 409 locations (Fig. 1). The collections included 377 sites from North America, and 32 sites from locations outside the continent (Table S1[2]). Non-North American collections came from 12 countries represented by 131 specimens, ranging from a single specimen from Greece to 47 specimens obtained in Costa Rica. The majority of collection sites were in native plant communities. Seventy percent of the criconematid nematodes collected from agricultural soils belonged to a haplotype group that included members collected from native plant communities.

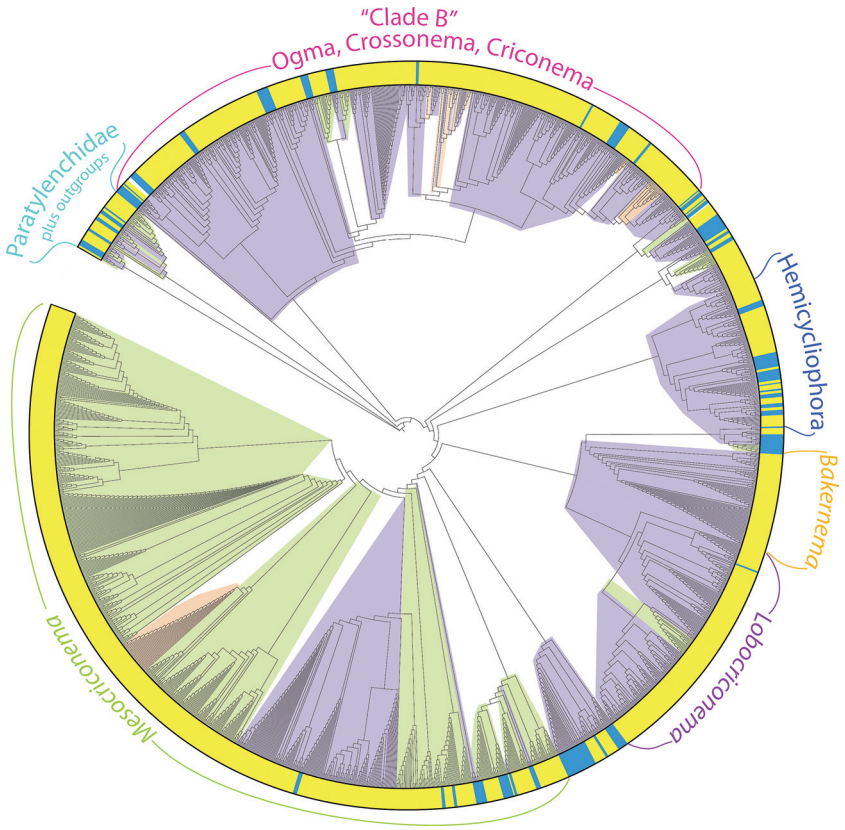
A neighbor-joining tree of the 1726 sequences clustered the criconematid specimens into 179 haplotype groups, with an additional 71 specimens recognized as singletons (Fig. 2; Fig. S1[2]). The largest haplotype group analyzed was comprised of 102 specimens of *Mesocriconema nebraskense*, a largely native grassland species that ranges from New Mexico to Tennessee. Other well-populated haplotype groups included *Mesocriconema* HG 24, with 98 specimens also found in the central grasslands of North America; *Bakernema inaequale* (76 specimens), a monotypic genus from eastern North American deciduous forests; and *Xenocriconemella macrodora* (63 specimens), a species most often associated with *Quercus* species.

One quarter of the singletons were from collections outside North America, in locations that were not exhaustively surveyed. The proportion of singletons within a genus may provide an indication of how complete the taxonomic coverage is within that genus. For example, the genus *Mesocriconema* was represented by 678 specimens, 45 haplotype groups, and 12 singletons that comprised 1.7% of the total *Mesocriconema* specimens. By comparison, 115 specimens of *Hemicycliophora* included 24 haplotype groups and 15 singletons comprising 13% of the total *Hemicycliophora* specimens. The relatively large percentage of *Hemicycliophora* singletons suggests considerable genetic variation remains to be characterized within the genus.

Based on morphological examination, DNA sequence, and collections from type localities, Latin binomial names were assigned to 70 of the 177 haplotype groups or singletons. Seventeen of the species with assigned Latin binomials were in the genus *Hemicycliophora* of which 11 were represented by a single specimen. Whether these sparsely populated groups and singletons represent rare species, a lower sampling effort, or the use of exemplars in a taxonomic study, they present challenges for species delimitation. Cryptic species also present difficulties for assignment of taxonomic names. For example, *Mesocriconema xenoplax* is a species commonly found in eastern US forests and feeds on agricultural host plants such as walnut (*Juglans nigra*), peach (*Prunus persica*), and domesticated grape (*Vitis vinifera*). This monophyletic species has seven subgroups with mean pairwise intergroup distance values (p-distance) of 9.1% (Powers et al. 2014). Further research on the subgroups has revealed additional evidence of incipient speciation in the form of ecological differentiation and host preference (J. Matczyszyn in preparation).

Many other genera are clearly in need of taxonomic characterization. For example, *Lobocriconema*, a genus of robust-bodied nematodes common in southeastern conifer and coastal lowland forests, has three haplotype groups with Latin binomials based on COI barcoding of specimens from type localities. Another 22 *Lobocriconema*

Fig. 3. Neighbor-joining tree of 1726 Criconematid COI DNA sequences. Inner color blocks indicate major biomes associated with collection habitats: purple– forest, green– grassland, pink– heathland. Outer colored ring indicates collection region: yellow– sample collected from the North American continent, blue– sample collected from outside the North American continent.



haplotype groups in the dataset remain to be characterized and named.

The geographic distribution of haplotype groups

On a continental scale from graphical inspection, the distribution of criconematid taxa does not appear random. Criconematid genera may provide evidence of biome conservation, where ancestral taxa evolved within a specific biome and subsequent shifts between biomes are relatively rare (Wiens and Graham 2005; Crisp et al. 2009; Cavender-Bares et al. 2016) (Fig. 3). *Mesocriconema* is predominately a grassland taxon with 38 of 45 haplotype groups collected from North American grasslands. The *Mesocriconema* haplotype groups not associated with grassland include *M. ericaceum*, an endemic species from heath balds in Great Smoky Mountains National Park, and six of the subgroups in *M. xenoplax*. The one *M. xenoplax* haplotype group found in the central grasslands of North America is associated with woody plant incursions into grasslands, such as wild plum and chokecherry (*Prunus* sp.), sumac (*Rhus* sp.), and dogwood (*Cornus* sp.). Conversely, the genera *Lobocriconema*, *Criconema*, *Ogma*, *Crossonema*, *Hemicycliophora*, and *Bakernema* are taxa typically associated with, and most genetically diverse in, North American forests. *Lobocriconema*, *Criconema*, and *Ogma* each have a single haplotype group found in North American grasslands. A correlated morphological trait of many criconematid species existing in a forest habitat are long stylets, often in excess of 100 μm in length, necessary for root penetration. Grass-feeding species typically have a stylet one-half that length, presumably due to a thinner root epidermis.

Great Smoky Mountains National Park (GRSM) and Big Thicket National Preserve (BITH) were studied intensively due to the presence of all-taxa biotic inventories within their borders and the possibility that both locations served as refugia during Pleistocene glaciations (Pielou 1991). COI barcodes were obtained from 301 GRSM and 139 BITH specimens. In spite of the disparity in specimen numbers, BITH specimens were distributed across 33 haplotype groups or singletons, whereas GRSM specimens were distributed across 29 haplotype groups or singletons. Surprisingly, only three haplotype groups were shared by both GRSM and BITH. Two of these groups, *Xenocriconemella macrodora* from oak species and *Criconema loofi* from *Rhododendron* sp. and *Vaccinium* sp., feed on widespread hosts. The extent to which host range or geographic legacy determines geographic population structure of nematodes is an open but testable question that may be addressed using COI and 18S barcodes in a combined phylogeographic and phylogenetic analysis.

Fig. 4. Morphological variation among major genera of criconematid nematodes. (A) *Paratylenchus* sp. NID 4238, (B) *Hemicriconemoides wessoni* NID 2076, (C) *Criconema permistum* NID 3602, (D) *Xenocriconemella macrodora* NID 3497, (E) *Ogma octangularis* NID 1466, (F) *Crossonema menzeli* NID 898, (G) *Hemicycliophora* sp. NID 4179, (H) *Bakernema inaequale* NID 1460, (I) *Criconemoides annulatus* NID 1456, (J) *Lobocriconema thornei* NID 3366, (K) *Mesocriconema sphaerocephalum* NID 1456, (L) *Mesocriconema ericaceum* NID 5958.

Discussion

DNA barcoding of terrestrial nematodes by COI has lagged in development, in part because COI was viewed as too variable for broad taxonomic coverage using a single primer set (Holterman et al. 2006; Ahmed et al. 2019) and early studies reported a lack of amplification success with the Folmer primer set (Blouin et al. 1998). Instead, nematode barcoding historically focused on 18S

and 28S ribosomal sequences (Blaxter et al. 1998; Floyd et al. 2002; Powers 2004). Now multiple primer sets are available for amplification of COI, although few meet the requirements of the Barcode of Life Data Systems in terms of length and positioning for recognition with a formal Barcode Index Number (BIN) assessment (Ratnasingham and Hebert 2013). Currently there are 6173 nematode records with DNA sequence on BOLD, representing 1116 species. This number is far from the 30 000 described species of nematodes or the estimated 1–5 million species believed to represent total global diversity. From a COI barcode database perspective, nematodes are still among the most poorly represented animal taxa (Curry et al. 2018). Efforts to narrow the barcoding gap (Schenk and Fontaneto 2019) between estimated nematode species diversity and nematodes presently represented in sequence databases are currently underway, but not as a component of a coordinated group activity, as exists for other taxa such as FISHBOL (Ward et al. 2009).

COI barcoding of criconematid nematodes can provide a framework for studies of nematode diversity and distribution. In this project, a single primer set was used to amplify a region of COI across a relatively wide taxonomic range. In the classification of Siddiqi (2000), Criconematoidea is one of nine superfamilies in the largely plant-parasitic order Tylenchida. The nematode superfamily Criconematoidea is believed to be monophyletic based on ribosomal DNA phylogenies and distinctive characteristics of the pharynx, vulva location, cuticular ornamentation, and lack of key sensory structures in the lateral fields of the cuticle (Fig. 4) (Siddiqi 2000; Subbotin et al. 2005; Andrassy 2007). The superfamily is distributed globally, although some genera are confined to specific geographic regions (Geraert 2010; Wouts 2006). Geraert (2010) lists 18 valid genera in the largest family, Criconematidae, and suggests there are approximately 400 "good species" in the family. An earlier taxonomic assessment of the family lists 44 genera (Siddiqi 2000). Criconematid nematodes are abundant in many native plant communities, and in some cases, the dominant group of plant-parasitic nematodes (Olson et al. 2017). For example, it is common to observe 6–7 criconematid genera in a single 100 cm^3 soil sample in deciduous hardwood forests. In native grasslands and forests, criconematid abundance may exceed 200 individuals per 100 cm^3 of soil and can represent up to 50% of the specimens in undisturbed soils.

As is common for many soil-dwelling, microscopic invertebrates, nematode taxonomy is plagued by convergent morphological characters, intraspecific variability, and an abundance of asexual species that confound species identification. DNA barcoding adds a high degree of accuracy for identifications in groups where species delimitations have been completed, but the large number of undescribed nematode species compounds the difficulties in conducting faunistic surveys of nematode diversity.

For most DNA-based studies of nematode diversity, ribosomal DNA sequences have served as taxonomic units in evaluating community composition. Although these rDNA comparisons may sacrifice taxonomic resolution to attain broad taxonomic coverage using a single PCR primer set, this concern is considered to be offset by relatively well-populated ribosomal databases (Ahmed et al. 2019). However, by adding COI barcodes to surveys of nematode diversity, we may reveal critical ecological information such as host associations, which might otherwise be missed with markers of lower taxonomic resolution. We believe that a linkage between rDNA and well-populated mitochondrial databases, together with morphologically characterized specimens, will accelerate studies of nematode diversity and an understanding of the factors that drive that diversity. To this end we have deposited in BOLD 1621 criconematid sequences, including 1511 specimens with GPS location data and 596 specimens with images.

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