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Tropical nematode diversity: vertical stratification of nematode communities in a Costa Rican humid lowland rainforest

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Abstract

Comparisons of nematode communities among ecosystems have indicated that, unlike many organisms, nematode communities have less diversity in the tropics than in temperate ecosystems. There are, however, few studies of tropical nematode diversity on which to base conclusions of global patterns of diversity. This study reports an attempt to estimate nematode diversity in the lowland tropical rainforest of La Selva Biological Research Station in Costa Rica. We suggest one reason that previous estimates of tropical nematode diversity were low is because habitats above the mineral soil are seldom sampled. As much as 62% of the overall genetic diversity, measured by an 18S ribosomal barcode, existed in litter and understorey habitats and not in soil. A maximum-likelihood tree of barcodes from 360 individual nematodes indicated most major terrestrial nematode lineages were represented in the samples. Estimated 'species' richness ranged from 464 to 502 within the four 40 × 40 m plots. Directed sampling of insects and their associated nematodes produced a second set of barcodes that were not recovered by habitat sampling, yet may constitute a major class of tropical nematode diversity. While the generation of novel nematode barcodes proved relatively easy, their identity remains obscure due to deficiencies in existing taxonomic databases. Specimens of Criconematina, a monophyletic group of soil-dwelling plant-parasitic nematodes were examined in detail to assess the steps necessary for associating barcodes with nominal species. Our results highlight the difficulties associated with studying poorly understood organisms in an understudied ecosystem using a destructive (i.e. barcode) sampling method.

Keywords: biodiversity, La Selva Biological Research Station, Neotropical forests, DNA barcode, soil invertebrates

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Introduction

The humid lowland tropical rainforest of the Atlantic coast of Costa Rica is well-known for its diverse biota (Janzen 1983; McDade *et al*. 1994). Intensive studies at La Selva Biological Research Station, a 1536-hectare nature preserve administered by the Organization of Tropical Studies, have recorded a remarkable abundance and species richness including birds (> 400 species), amphibians (48 species), ferns (173 species), orchids (114 species), and trees (> 400 species) (McDade & Hartshorn 1994; http://www.ots.duke.edu/en/laselva/). Moths contribute an estimated 4000 species and ants 437 species to an insect community that must certainly contain 10^4 to 10^6 species (Mcdade & Hartshorn 1994; Longino *et al*. 2002). The biotic inventory of La Selva, which includes records extending back more than 40 years, also provides a valuable resource for monitoring biotic alterations associated with climate change and various forms of anthropogenic disturbance.

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Given the role of La Selva as an international laboratory for studies of biodiversity, it is surprising that little is known about the nematodes within its borders. The taxon Nematoda is often referred to as the most abundant and one of the most diverse groups of multicellular animals on the planet (May 1988; Groombridge 1992; Wilson 2000). Nematode communities are typically comprised of bacterial-, fungal- and algal-feeding species, predators of small invertebrates including other nematodes, and internal and external associates (e.g. parasitic, phoretic) of plants and animals. It is possible that each of the plant and animal species mentioned above has at least one specific nematode associate, existing in a parasitic or commensal relationship. Ironically, just outside La Selva Biological Research Station, the extensive banana and pineapple plantations that have replaced much of the native rainforest of the region are routinely treated with chemicals to suppress plant-parasitic nematode populations (Fernando 2006; De Waele & Elsen 2007).

Our lack of knowledge of tropical nematode communities extends beyond Neotropical ecosystems. Tropical nematode diversity generally has not been studied with the same intensity as temperate systems; only 10 of 134 published ecosystem surveys at the time represented tropical forests (Boag & Yeates 1998). These comparisons of nematode diversity across broad geographic regions have led to the impression that tropical ecosystems have less nematode diversity than temperate broadleaf forests, temperate grasslands, or even cultivated soils (Procter 1984; Groombridge 1992; Giller 1996; Maraun *et al*. 2007). Given the scarcity of surveys that represent tropical forests, it is difficult to determine whether reports of lower diversity of nematodes in the tropics compared to high diversity in temperate ecosystems is because of less-intensive sampling or genuine differences (Boag & Yeates 1998). Published nematode surveys range in sampling intensity from a single soil core taken at the base of a tree to a composite of soil cores as representative of a hectare-sized area. Among these ecosystem surveys, temperate broadleaf forests represented by 27 studies, recorded the greatest mean nematode species richness (61.7 species). This compares to a mean nematode species richness of 46.7 species for the 10 tropical rainforest ecosystems reported. While nematode diversity is thought to be correlated with plant diversity and the corresponding diversity of litter substrates for microbes and microbial-feeding nematodes, it is not clear how this relationship would account for differences in diversity among nematode communities of temperate and tropical forests. Several studies have reported relatively high tropical nematode diversity (Pradhan & Dash 1987; Bloemers *et al*. 1997). Observations across a disturbance gradient of tropical soils in the Mbalmayo Forest Reserve in Cameroon recorded 431 nematode species among approximately 5000 individuals from 24 1-ha sites, each site represented by a single 15- to 30-cm deep soil core (Bloemers *et al*. 1997). A second study of a tropical rainforest conducted in Korup National Park, Cameroon, listed 153 species from an unspecified total number of specimens associated with 22 tree species (Price & Siddiqi 1994).

We hypothesize that nematode diversity is high in tropical forests of Costa Rica when vertical assemblages and invertebrate hosts are included in the estimate. A direct relationship between biomass distribution and nematode diversity would predict remarkable nematode diversity among the tropical epiphytic community. Given the abundance of standing biomass, organic matter and high levels of moisture above the mineral soils, environmental conditions appear favorable for the existence of distinct aboveground nematode communities. Insects and their associated nematodes provide another possibly overlooked source of tropical nematode diversity.

We chose termites and figs as specialized systems for sampling in La Selva to test whether there are host-specific relationships of nematode species with their invertebrate hosts. Cost and time make it prohibitive to systematically sample all invertebrates in an area. Therefore, we chose termites as representatives of an entire insect order (Isoptera). There are about 2800 nominal species (http://insects.tamu.edu/research/collection/hallan/Arthropoda/Insects/Insects.htm) of termites. They exhibit mutualism with protozoa or bacteria as gut symbionts that make them important decomposers of cellulose, especially in the moist tropics. Species diversity of termites is greatest near equatorial latitudes and they are considered the dominant invertebrate in tropical soils (Wood & Sands 1978; Collins 1989). Figs (*Ficus* species) represent another specialized type of association between insects, plants and nematodes that are expected to be more speciose near the equator (Harrison 2005). Although *Ficus* densities can be few per unit area, their species richness can be remarkable, especially in the lowland tropics. In fact, Harrison (2005) considers *Ficus* to be 'the only ubiquitously diverse genus in lowland rainforests' in the world. There are 16 species of *Ficus* present in La Selva, Costa Rica (making it the fifth most speciose dicotyledenous plant present; Hammel 1986; Harrison 2005) and 40 to 50 different species of *Ficus* occur in Costa Rica and Panama (Burger 1977).

To assess nematode diversity, we chose a molecular 'barcode' approach using a 635-bp 3′-end portion of the 18S ribosomal DNA gene. As an initial estimate of diversity, this particular barcode was selected because of the large number of 18S sequences in GenBank, the existence of an 18S-based phylogenetic tree, and the conserved nature of this gene to ensure complete phylogenetic coverage of the phylum (Blaxter *et al*. 1998; Floyd *et al*. 2002; Bhadury *et al*. 2006; Holterman *et al*. 2006). In general, the barcode approach permits a relatively rapid assessment and is not impaired by the high abundance of juvenile nematodes, a

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stage that typically does not possess the structural features necessary for morphological identification. In this project, barcodes were used for an initial appraisal of diversity and not an endpoint of taxonomic and ecological studies. Barcodes help to focus the species discovery steps that follow when we attempt to connect molecular operational taxonomic units (MOTUs) with morphological and ecological characteristics. A disadvantage of barcoding nematodes is the necessary destruction of the specimen. A subset of MOTUs in our sampling was derived from the monophyletic suborder Criconematina Siddiqi, 1980, an abundant group of plant-parasitic nematodes common in undisturbed tropical soils (Wouts 2006). These were compared with an in-house database of Criconematid MOTUs derived from North and Central American collections, which include data regarding morphological measurements, light and scanning electron microscopic images (http://nematode.unl.edu/crico_barcode_tree.htm). In this report, we present: (i) estimates of nematode diversity based on MOTU analysis, (ii) nematode distribution among soil, litter, and understorey habitats, (iii) a preliminary focal sampling of invertebrate-associated nematode diversity in termites and figs, and (iv) an attempt to connect a subset of MOTUs to nominal taxa in the suborder Criconematina.

Materials and methods

Study site

In March 2005, we sampled soil, litter, and understorey habitats at four discrete locations within La Selva Biological Station rainforest preserve in Costa Rica (10°25′52.513′N latitude, 84°00′12.959′W longitude, 60 m above sea level altitude), hereafter, referred to as 'La Selva'. These soils are classified as Andic Humitropepts (Sollins *et al*. 1994). Each plot was a circle of 1520 m^2 (22 m radius) at the 200-m, 300-m, 400-m and 500-m markers on the Sendero Suroeste (SSO) trail. Soils at the site were relatively acidic (water pH 4.3 to 4.6), contained 6.3 to 6.8% organic carbon (Walkley & Black 1934), and mineral content was 42 to 50% clay and 36 to 46% sand.

Sampling design

We employed a systematic sampling design to reduce bias. The identified centre point of each location was discerned by a preselected random number of paces from the trail. From each centre point, we marked boundaries at each of four cardinal directions, creating four sampling quadrants per plot. One canopy and one understorey tree were selected within each of the four quadrants (within a plot). Within a plot, three habitats (understorey, litter, and soil) and three insect-associations (termites, fig wasps, and soil) were sampled. All samples were stored in insulated containers until shipped to the laboratory at Universidad Nacional Autónoma (Heredia, Costa Rica) for processing.

Understorey sampling. Three 225-cm^2 areas of epiphytic material (lichens, moss, vascular plants, and algae) were removed from both types of trees in a stratified random design with 2.5 to 2.5 m from the soil surface to represent an understorey sample. The 2.5-m height was divided into three equal strata. A 15 × 15-cm area was sampled in each of three vertical strata. A total of 24 subsamples were pooled to form a composite sample for each plot (4 areas, 2 trees per area, and 3 heights per tree).

Litter and soil sampling. Soil (15 cm depth) and litter overlying soil (entire depth to surface of mineral soil) were collected within a 15 × 15-cm area within 1 to 2-m of the four canopy trees, to avoid major roots, and within 1 to 2-m distance for a neighbouring understorey tree. Eight subsamples of soil or litter were pooled to generate one composite (4 areas and 2 trees per area) soil and one composite litter sample per plot.

Termite sampling. Within the same plot areas described above, a random centre point for a 5-m-radius circle (78.5 m^2) was established within each of the four quadrants. In each quadrant, two scientists searched 20 min for termite niches by chopping wood and turning over rocks and wood in the leaf litter and soil. In sum, 314 m^2 were searched for a total of 160 min per plot. Each time a termite colony was discovered within the sampling area, 10 to 50 termites were collected with an aspirator and stored in a labelled conical tube.

Fig wasp sampling and processing. Only two species of the 16 *Ficus* species identified at La Selva (O. Vargas, personal communication) had fig fruits for sampling in March 2005, i.e. *F. colubrinae* and *F. pertusa*. Because *F. pertusa* figs were all phase A or E (i.e. too young or too old for fig wasps and nematodes), our sampling was, thus, restricted to two *F. colubrinae* trees with phase C (interfloral phase) fruit which was harvested from near the ALAS project laboratory (within 10 m) and kept cool until dissected (within 3 days) for fig wasp hosts. Dissections were performed in a small volume of water (Giblin-Davis *et al*. 1995, 2007a).

Sample processing

Soil, litter, understorey subsampling. Composite samples of litter and understorey material were chopped with hand clippers into pieces 2 cm or smaller in diameter and mixed thoroughly within each bag. Homogenized litter or understorey samples were subsampled to provide approximately half for entomopathogenic nematodes (145 to 372 g) and half for nematode (103 to 313 g) isolation, respectively.

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Bulk substrate nematodes. Bulk soil samples of each plot were homogenized by breaking up clumps and shaking soil within a large plastic bag. A subsample of 100 g was soaked in tap water for 30 min and processed by Oostenbrink elutriator (s'Jacob & van Bezooijen 1984). Nematodes were extracted from litter and understorey debris by cotton wool filters, pooling subsamples removed after 24 h and 48 h of incubation at room temperature. Four filter trays were used per composite sample to allow material to be no more than 1.5 cm in depth above the filter (s'Jacob & van Bezooijen 1984).

One hundred nematodes per composite sample were observed under a stereomicroscope (40×), hand-picked and placed into 15 µL of distilled water, smashed with a pipette tip, transferred to a 250-µL microfuge tube, and stored at –20 °C for subsequent polymerase chain reaction (PCR) amplification and DNA sequencing.

Insect-baiting for entomopathogenic nematodes. Nematodes were recovered from soil and litter samples using a modified version of the insect-baiting method described by Bedding & Akhurst (1975). Nematode baits (10 last-instar *Galleria mellonella* (L.) and/or *Tenebrio molitor* (L.) larvae) were placed in 250-mL plastic containers (five containers per sample) with moistened soil (approximately 50 g) obtained from each substrate sample. Containers were covered with a lid, turned upside down and kept at room temperature (25 °C). Insect larvae were checked every 2 to 3 days and dead larvae were replaced by fresh ones. After 7 days, *G. mellonella* larvae were recovered, and parasitized cadavers, recognized by a change in colour (usually red\purple for heterorhabditids, and ochre\brown\black for steinernematids), were placed in modified White traps (Kaya & Stock 1997) to allow the infective-stage juveniles to emerge. Emerging nematodes were pooled from the modified White traps and rinsed thoroughly to remove any debris. These nematodes were used to infect fresh *G. mellonella* and/or *T. molitor* larvae to confirm Koch's postulates for pathogenicity. A second round of baiting was performed by placing fresh *G. mellonella* and/or *T. molitor* into the same soil. Nematode cultures were stored in 250-mL tissue culture flasks at 10 to 15 °C following procedures described by Kaya & Stock (1997).

Termites. Within 24 h, samples were examined and soldiers and workers (3 to 15) were placed into 85% ethanol as vouchers for identification. The remaining termites of each sample were placed into a drop of deionized water to check for externally contaminating nematodes, and then dissected and examined for entomophilic nematodes. If a termite was positive for nematode(s), the location, number, stage and general morphology were recorded and at least one nematode for each morphotype was placed into a tube with digestion buffer and stored at –20 °C prior to DNA extraction and amplification. Usually, at least 20 termites per colony were dissected. The remaining termites were smashed to release any nematodes onto 2% water agar plates to check for nematode development to adults and reproduction.

Barcode determinations

DNA for each nematode specimen was extracted from single individuals (Powers & Harris 1993). Small subunit (18S) rDNA amplifications were performed in a 50-µL reaction containing: 31.4 µL distilled water, 5 µL 10× PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin), 8 µL dNTP mixture (1.25 mM each of dATP, dCTP, dGTP, and dTTP), 1.5 µL 50 mM $MgCl_2$, 0.8 µL of each primer (20 µM), 0.5 µL of JumpStart *Taq* polymerase (Sigma; 2.5 U/µL), and 2 µL of DNA template. All PCRs were performed on a DNA Engine PTC-200 Peltier thermal cycler (MJ Research) with the following run parameters: one initial denaturation cycle at 94 °C for 2 min, followed by 45 cycles at 94 °C for 30 s, 53 °C for 30 s, ramped increase at 0.5 °C/s to 72 °C for 1 min. A final elongation step was run at 72 °C for 4 min. Positive and negative controls were included for each amplification series. The following primers were used in this study: 18S 965: 5′-GGCGATCAGATA-CCGCCCTAGTT-3′ (forward) 18S 1573R: 5′-TACAAAGGG-CAGGGACGTAAT-3′ (reverse). Primer 18S 965 (positions 879 to 901) was designed using consensus GenBank arthropod sequences (A. Szalanski, T.S. Harris and T.O. Powers, unpublished), while primer 18S 1573R (positions 1567 to 1547) was the reverse compliment of primer rDNA2 from Vrain *et al*. (1992).

DNA sequences

PCR products were purified and concentrated with Microcon-100 centrifugal filter units (Millipore Inc.). Purified DNA was sent to Davis Sequencing (Davis, California) and DNA Sequencing Laboratory (University of Arkansas for Medical Sciences) for direct sequencing in both directions, using the amplification primers as sequencing primers. Small subunit sequences were edited and assembled using Sequence Navigator (Applied Biosystems) and CodonCode Aligner (CodonCode Corp.). Sequences that could not be confirmed in both directions or contained numerous base ambiguities were discarded prior to phylogenetic analyses. Eight amplification products were discarded on this basis and seven additional products were determined to be non-nematode upon DNA sequencing. DNA alignment was by MUSCLE 3.7 (Edgar 2004) and maximum-likelihood analysis was carried out by PHYML 3.0 using approximate likelihood-ratio tests for the estimation of branch support (Anisimova & Gascuel 2006).

Aligned and edited sequences that displayed any single-base difference (substitution, insertion, or deletion), were

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Table 1 Mean (± 1 standard deviation) abundance of nematodes (per gram of dry substrate) and the number of MOTUs identified per habitat

Habitat	Mean§	Standard deviation	# MOTUs	Unique* MOTUs	% Unique MOTUs
Understory	39.7[a]	17.52	57	38	66
Litter	51.4[b]	14.52	73	52	71
Soil	18.7[c]	9.78	63	55	87

§Contrasting letters present statistically significant differences ($P < 0.05$) determined by least-squared means.
*Refers to MOTUs found exclusively in a habitat, either soil, litter, or understorey.

defined as a new MOTU (molecular operational taxonomic unit). As a point of reference, *Caenorhabditis elegans* (AY268117) and *C. briggsae* (U13929), two congeneric nematodes that are believed to have diverged from a common ancestor about 18 million years ago (Cutter 2008), differ by three nucleotides across this region of the 18S gene. *Meloidogyne hapla* and *M. incognita*, two common species of sedentary endoparasitic plant-parasites, differ by 13 nucleotides (T. Powers, unpublished). Two sibling species of potato cyst nematode, *Globodera pallida* (AY284620) and *G. rostochiensis* (AY593877), are identical for this region.

All MOTUs were compared with existing sequences in GenBank via BLAST pairwise searches to determine the nearest match which provided the taxon label for terminal branches on the likelihood tree (Fig. 1; Table S1, Supporting Information). All 167 unique MOTU sequences were deposited in GenBank (Accessions EU879989–EU880155).

Sequence data reported in this study are results from 30 of the 100 nematodes from each composite sample, totalling 120 PCR amplifications from each of the three habitats, and 360 nematodes analysed by DNA sequence.

Estimation of species richness and habitat similarity

Estimates of asymptotic species richness were computed for each habitat as sample-based rarefaction curves (Gotelli & Collwell 2001). Frequency of MOTU occurrence was summed as rank order abundance (Fig. 2). Bray–Curtis similarity and dissimilarity were computed on nontransformed abundance data for each pairwise combination of samples (Tietjen 1980). A one-way analysis of similarities was performed to compare the similarity among and within vertical strata as the single factor. R-statistic values were computed as a quantitative measure of similarity among vertical stratum. An $R = 1$ represents the greatest similarity possible among replicates within a stratum. Statistical significance was computed by 999 permutations. Statistical significance was defined as $\alpha = 0.05/3 = 0.0167$ with 3 representing the number of pairwise tests to allow for a conservative Bonferonni adjustment. Rarefaction curves were computed using the ICE value in EstimateS 7.5 software (Colwell 2005). Statistical procedures were performed using the ANOSIM and CLUSTER modules of Primer-E version 5.2.9 software (Clarke & Gorley 2001).

Connecting MOTUs with nominal taxa

In 2007, the La Selva plots were resampled to establish a connection between morphologically discrete nematode taxa and MOTUs derived from 2005 samples. Nematodes were photographed and measured using an Olympus BX50 compound microscope with differential interference contrast prior to dismantling the glass slides and crushing the specimens for PCR amplification. Nematodes in the group Criconematina were initially selected for detailed analysis because their unique morphology and presumed monophyletic status would facilitate recognition and identification (Wouts 2006). All samples were added to an in-house MOTU database and used together with GenBank species in the construction of a maximum-likelihood criconematid tree consisting of 135 sequences (Fig. 3). Eighty-four new sequences of Criconematina specimens were added to GenBank in this study (Accessions FJ489517–FJ489599).

Results

Taxonomic comparisons

PCR amplification and 18S barcode sequencing of 360 hand-picked nematodes yielded 167 distinct MOTUs (Fig. 1; Table S1, Supporting Information). Only six MOTUs exactly matched an 18S sequence in GenBank, and 45% were 10 nucleotides (98% identity) or more removed from the closest match in the database. The mean distance from the nearest match was 12 nucleotides. Litter had the most MOTUs and soil had the most unique MOTUs (those found exclusively in a habitat) (Table 1). The number of unique MOTUs was related directly to abundance of total nematodes per gram of dry substrate (Table 1). There was a slight difference among plots (designated by trail markers 200, 300, 400 or 500) with respect to total number of MOTUs. Specifically, marker 300 had the most MOTUs with 74, followed by marker 200 with 61, marker 400 with

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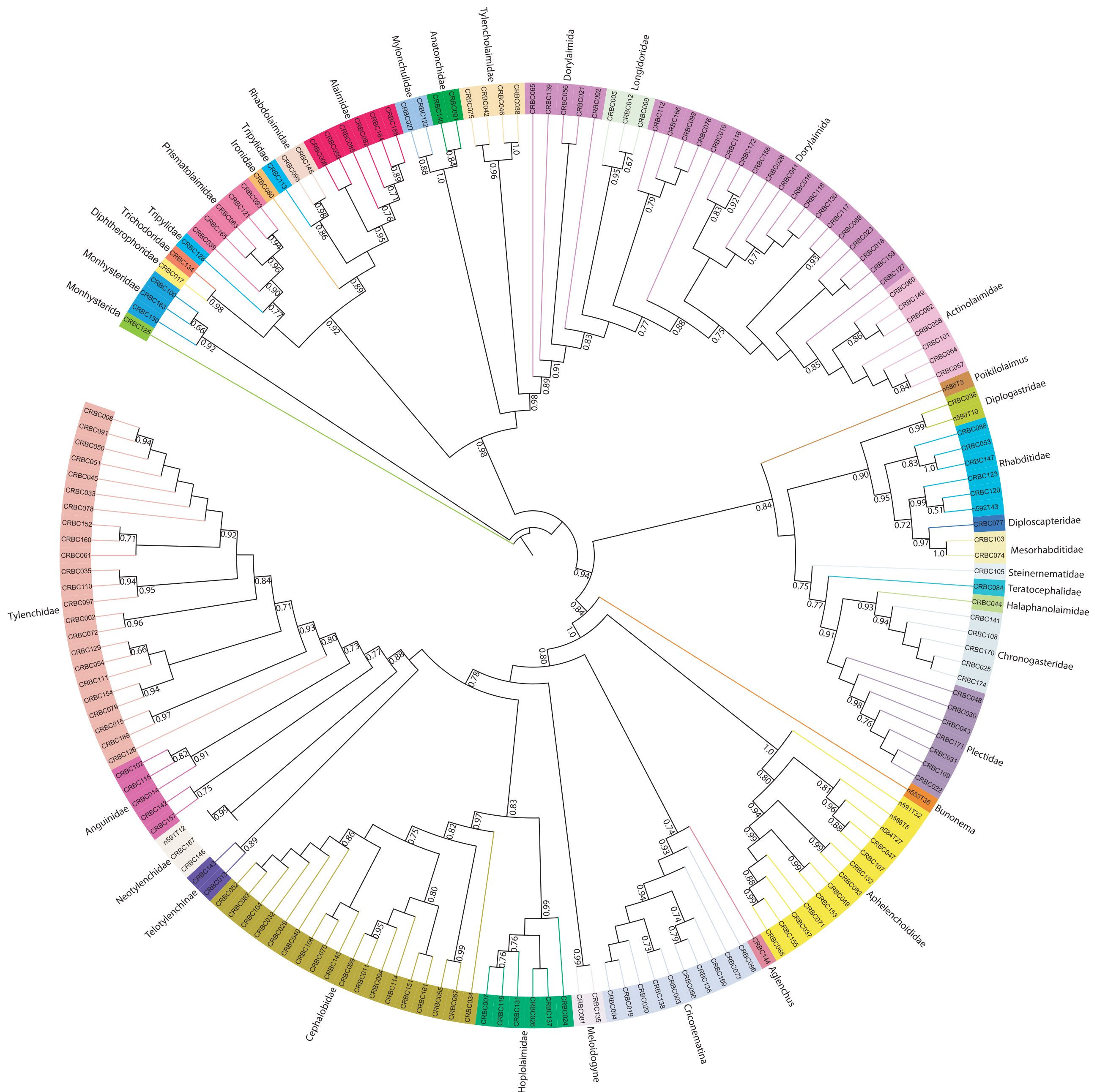

Fig. 1 Maximum-likelihood tree of 167 MOTUs isolated from the March 2005 survey of La Selva. Numbers at branch nodes are approximate likelihood ratio support values. Membership in a higher taxon was estimated based on the classification of the closest BLAST score to the MOTU and is arranged by colour on the tree. Terminal nodes are identified by the prefix CRBC (Costa Rica Barcode) and followed by a number corresponding to individual specimens (see Table S1 for additional information on CRBC accessions). Terminal nodes with an 'n' prefix identify taxa collected in termite and fig samples.

56, and marker 500 with 54. Four MOTUs were common to all plots, whereas 118 MOTUs were only recovered from a single site (Fig. 2). Nineteen MOTUs were found five or more times among the 360 specimens (Table 2). The most commonly encountered MOTU was an identical match for the GenBank sequence of *Labronemella ruttneri* (Schneider 1937) Andrássy, 1985. It was recovered 13 times, from all four plots including soil, litter and understorey habitats. *Discocriconemella* sp. A was identified 11 times at marker 200 and *Discocriconemella* sp. D, also represented by 11 specimens, was spread among the remaining three plots, i.e. markers 300, 400, and 500.

Sampling efficiency curves estimate that 22 to 30% of the nematode species have been recovered from the soil, litter,

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Table 2 All MOTUs recovered five or more times from 360 total specimens

MOTU no.	BLAST identification*	Similarity	Soil	Litter	Understorey	Total abundance
016	*Labronemella ruttneri*	634/634	2	10	1	13
004	*Discocriconemella* sp. B	620/635	11			11
020	*Discocriconemella* sp. D	619/635	11			11
026	*Helicotylenchus* sp. B	633/635	6	2	3	11
030	*Anaplectus* sp.	623/632		9	2	11
018	*Coomansinema* sp.	633/634	1	3	5	9
049	*Aphelenchoides* sp. A	576/603		2	6	8
067	Cephalobinae	615/634		1	7	8
009	*Xiphinema variegatum*	637/637	7			7
013	Telotylenchinae sp. A	624/636	7			7
027	*Mylonchulus* sp. A	629/635	1	4	2	7
038	*Tylencholaimus* sp. A	609/635		6	1	7
064	*Laimydorus* sp.	628/634			7	7
012	*Xiphinema brasiliense*	638/638	3		2	5
033	Tylenchinae sp. C	608/638		1	4	5
037	*Aphelenchoides* sp. B	628/630		4	1	5
041	*Labronemella* sp.	634/635		2	3	5
077	Diploscapteridae sp.	627/638			5	5
121	*Prismatolaimus* sp.	627/634			5	5

*BLAST identification based on the closest match in GenBank except in the case of *Discocriconemella* where the distinct morphology allowed genus identification prior to processing for PCR.

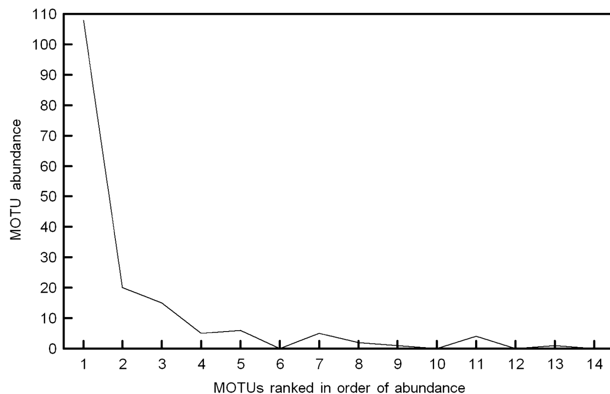

Fig. 2 MOTUs ranked by abundance. The majority of MOTUs were only encountered once in the 360-specimen data set. Only 19 MOTUs were observed five or more times (see Table 2).

and understorey habitats (Table 3). Estimated 'species' richness ranges from 464 to 502 among the four plots. Furthermore, 87% of the MOTUs were confined to a single habitat in the rainforest (Table 1). Replicates within habitats were more similar than among habitats ($R = 0.91$, $P = 0.001$), suggesting that discrete nematode communities exist in vertically stratified habitats.

The structure of the MOTU tree resembles the full-length 18S maximum-likelihood tree of Blaxter (2004). Groupings on the MOTU tree correspond to major lineages of the full-length 18S tree (Fig. 1). Based on BLAST results from GenBank comparisons, the families Tylenchidae and Cephalobidae, represented by 23 and 18 MOTUs, respectively, had the greatest diversity. Both families were encountered in all three habitats. Some taxa were predominantly encountered in a single habitat. MOTUs with BLAST scores most similar to predaceous dorylaimids, in the genera *Paractinolaimus*, *Prodorylaimus*, *Laimydorus* and *Talanema,* were commonly encountered in the understorey habitat. Members of the plant-parasitic groups Criconematina and Hoplolaimidae, with few exceptions, were confined to soil habitats. However, the plant-parasite *Tylenchocriconema*, a member of the Criconematina, was associated exclusively with the understorey habitat. Approximately 18% of the understorey community was comprised by large predaceous dorylaimids. Similarly, 18% of the litter community was comprised of predaceous dorylaimids, although there was little taxonomic overlap between these two groups of predators. The soil habitat had the highest percentage of presumed plant-parasitic taxa based on tree position (21 of 23 MOTUs) and several of the most abundant taxa in the plots (Table 4).

Entomopathogenic nematodes

There were six species of entomopathogenic nematodes recovered through insect-baiting of soil and litter strata with different insect (lepidopteran and coleopteran) baits, yielding two unique MOTUs. Three *Steinernema* species, i.e. *S. puntauvense* (FJ381666), *S. feltiae* (FJ381667) and *S.*

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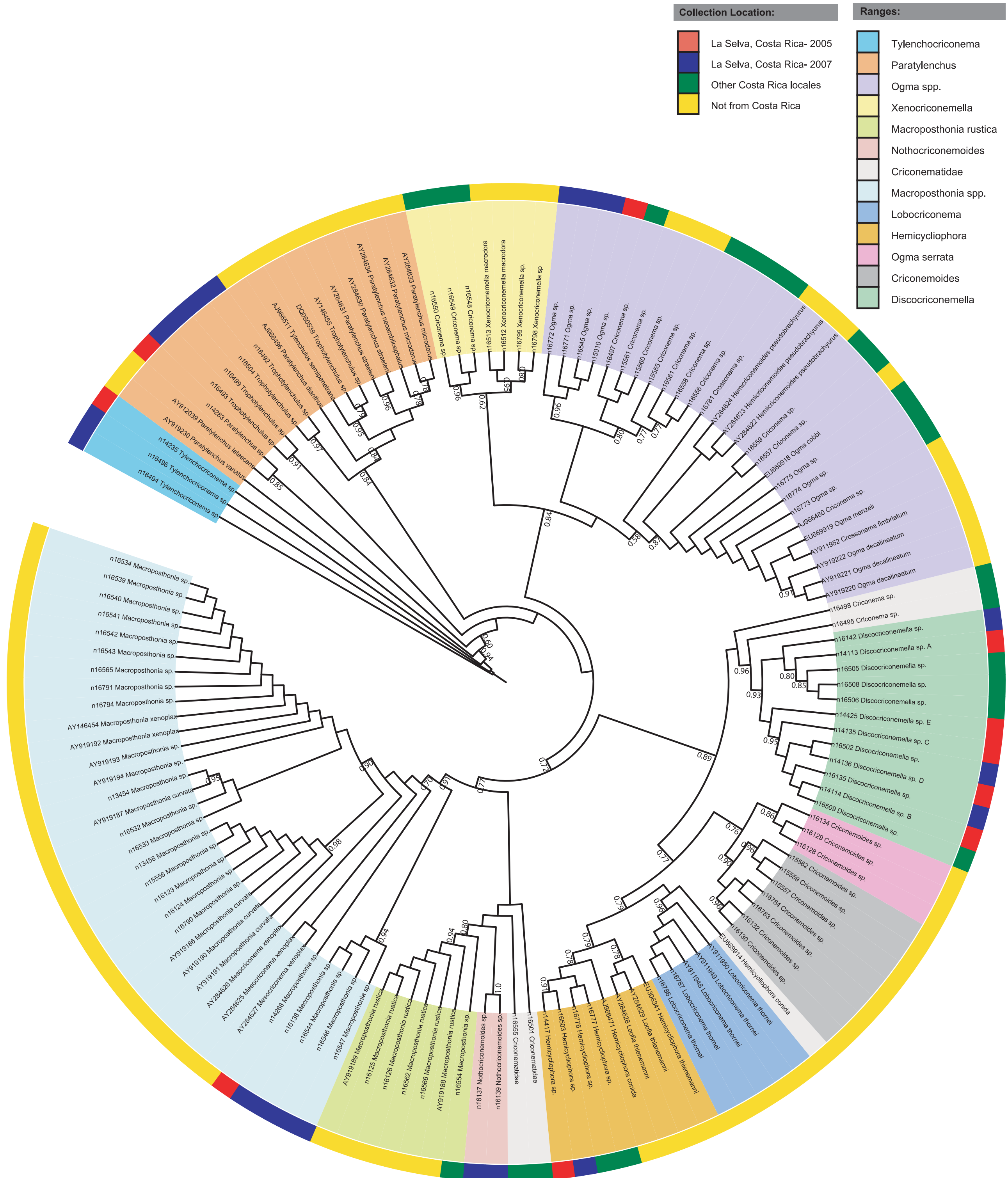

Fig. 3 Maximum-likelihood tree of nematodes from the suborder Criconematina. Colours on the outer and inner circles designate origin of the sample and taxonomic grouping, respectively. Red specimens designate criconematid nematodes collected in the original 2005 samples. Taxa at terminal nodes with an 'n' prefix refer to nematodes collected by the authors and were added to GenBank in this study (Accessions FJ489517-FJ489599). Taxa with a GenBank Accession number were in the database at the time of BLAST analyses. Numbers at branch nodes are approximate likelihood ratio support values. This tree was rooted with 18S sequence from *Tylenchocriconema*.

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Table 3 Sampling efficiency* based on four samples (= location) per habitat

Habitat	Estimated MOTUs sampled*	Estimated total MOTUs†	% of species sampled
Soil	66	260	25.4
Litter	73	323	22.6
Understorey	61	200	30.5

*Mao tau (expected species richness) values computed in EstimateS software version 7.5 (R.K. Colwell, viceroy.eeb.uconn.edu/EstimateS).
†Mean ICE values computed in EstimateS software.

Table 4 Robust* plant-parasitic nematodes identified from the soil

Taxon	MOTUs per taxon	No. of specimens per taxon	% of total soil nematodes
Discocriconemella	5	29	24.0
Xiphinema	3	12	10.0
Helicotylenchus	5	13	10.8
Trophurus	2	8	6.7
Meloidogyne	2	5	4.1
Other criconematids	4	4	3.3
Totals	21	71	59.1

*Robust refers to nematodes known to feed on plant roots and not fungal mycelia.

websteri (FJ381665), were recovered from soil samples. Three *Heterorhabditis* species (*H. indica, H. bacteriophora* and an undescribed *Heterorhabditis* species) were recovered from litter samples.

Termite-associated nematodes

Focal sampling of nematode associates of termites revealed additional nematode diversity among the four plots. Nine species of termites (1 Kalotermitidae, 1 Rhinotermitidae, and 7 Termitidae) were recovered from the focal sampling and were associated with seven unique nematode MOTUs for an association rate of 0.8 unique MOTUs per termite species. These MOTUs represented nematode clades that have not been reported previously as associates of termites (i.e. Thelostomatidae, putative *Aphelenchoides*, *Bunonema,* putative *Poikilolaimus* and putative *Howardula*), or have only recently been reported to be associated with termites (i.e. *Halicephalobus*; Fürst von Lieven & Sudhaus 2008). None of these unique MOTUs matched those recovered in the soil, litter or canopy habitats of the same plots. About 38% of the colonies sampled ($n = 26$) had termites with nematodes, while only about 21% ($n = 140$) of the termites from infested colonies had nematodes. The most common infestation was a single nematode associate per termite; however, termites typically had fewer than five nematodes present. In one case, a single worker rhinotermatid (*Coptotermes testaceus*) contained more than 100 parasitic juveniles of a nematode that was identified tentatively (morphologically and molecularly) as *Howardula*.

Fig-associated nematodes

An unusual tylenchid was discovered in early dissections of phase C sycones from *Ficus colubrinae*. This nematode occupied the area under the bracts of the figs and was not found inside the syconia as was a new species each of *Parasitodiplogaster* and *Schistonchus*. Thus, we discovered three unique MOTUs from one of 16 *Ficus* species present in La Selva that happened to have the appropriate phase of figs for harbouring fig wasps (Giblin-Davis *et al*. 2007a). Since March 2005, we have resampled La Selva several times in 2007 and 2008 and found two other species of *Ficus* with the appropriate phase fruit, *Ficus cahuitensis* (one unique MOTU of *Schistonchus*) and *F. tonduzii* (one unique MOTU each of *Schistonchus* and *Parasitodiplogaster*). This suggests an association rate of two unique nematode MOTUs per fig species.

Connecting MOTUs with nominal taxa

One major goal of this project was connecting MOTUs derived from this diversity study to known nematode taxa. Ten MOTUs, apparently belonging to the suborder Criconematina (Fig. 1) were selected to evaluate the feasibility of placing the MOTUs within a taxonomic framework of finer resolution. Prior to this analysis, we were limited to only 17 GenBank accessions available for 18S comparisons, and each of those accessions were from nematodes of temperate climates. Therefore, it was necessary to construct a reference system for Criconematina that included nominal

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taxa. Eighty-four specimens from North and Central America were added to the database which included morphological and 18S sequence information (http://nematode.unl.edu/crico_barcode_tree.htm). A maximum-likelihood tree illustrates sample origin and taxonomic grouping (Fig. 3). The 10 MOTUs sampled from the plots in 2005 (Fig. 3, marked in red), represented at least six genera, spread broadly across the phylogenetic tree. One-half of the MOTUs belonged to the genus *Discocriconemella*, a pantropical group most often associated with native trees. The recovery of *Tylenchocriconema* Raski & Siddiqui 1975 is only the second report of this arboreal nematode, an inhabitant of bromeliads, and the first from a native habitat (Raski & Siddiqui 1975). Repeated sampling from the plots in 2007 only recovered four identical matches with the 10 MOTUs belonging to Criconematina, although eight novel criconematid MOTUs were added to the faunal list.

Discussion

It is clear that a complete understanding of nematode diversity in the tropics requires examination of habitats above the mineral soil. Our molecular approach indicates that nearly 66% of the nematode diversity is associated with litter or understorey habitats. A maximum-likelihood tree suggests that certain taxa may include species adapted specifically to arboreal life, e.g. Actinolaimidae, *Prodorylaimus*, and *Tylenchocriconema*.

Lawton *et al*. (1996) independently analysed five near-primary forest sites in the Mbalmayo, Cameroon study described in Bloemers *et al*. (1997). They recorded 204 morphospecies from 1009 individuals, and estimated an average sample of 200 individuals would contain 72 species. We recovered 167 MOTUs from 360 specimens and estimated approximately 500 MOTUs could be recovered from our plots. MOTUs based on 18S and morphospecies share a rough equivalence and both probably underestimate true nematode diversity. The significance of this study is that the majority of MOTUs were recovered from habitats above the mineral soil. We suggest that assessments of tropical diversity that exclude non-soil habitats are missing a significant portion of the nematode diversity. Furthermore, while focusing on soil habitat is most likely responsible for the impression of a relatively low tropical nematode diversity (Procter 1984, 1990; Groombridge 1992; Giller 1996), it is also likely that estimates of nematode diversity from temperate forests will increase with detailed examinations of arboreal microhabitats.

The distribution of nematode diversity in aboveground habitats is yet to be determined. Our bulk sampling approach mixed plant material from microhabitats including epiphytic plants, lichens, mosses, and organic detritus on understorey tree limbs. The upper canopy was not investigated as part of this study. A more fine-grained approach to sampling, combining morphological and molecular methods, will be necessary to characterize aboveground community structure. Some questions are yet to be answered. Are there several discrete arboreal nematode communities? How do the nematodes colonize aboveground habitats? How do they survive dry periods? Answers to some of these questions will be aided by the addition of well-described, vouchered specimens to DNA databases. The observation that only 6 of 167 MOTUs matched a GenBank entry, even while using a conserved portion of 18S as a barcode, provides an indication of the taxonomic work that remains. The high percentage of rare and cryptic species in these habitats will further complicate species descriptions. For example, the plant-parasite *Discocriconemella* is recognized easily because of its short, thickened body and an unusually large labial disc. This taxon was represented by five MOTUs in the 2005 collection and six additional MOTUs in 2007. Subsequent examination of fixed specimens by light and SEM microscopy has yet to reveal any morphological basis for subgroups in the genus. Resolution of this apparent cryptic species complex will obviously require additional morphological and molecular characterization, and should help us refine our measure of nematode diversity.

A larger challenge in refining our estimate of tropical nematode diversity lies in the incorporation of invertebrate-associated nematodes, potentially a major source of nematode diversity, into the assessment. In this study, we included examples of three different nematode–insect associations. Termites are an example of a strictly commensal or parasitic relationship. Not one of the seven MOTUs derived from termite dissection was detected in habitat sampling. Likely, the brief period nematodes spend outside termite hosts and the exclusion of termites (and other microinvertebrates) during the processing of soil and organic substrates in litter and understorey samples, precludes the detection of these internal commensals or parasites. The ratio of approximately 0.8 unique nematode MOTUs per termite species suggests this is a significant component of overall nematode diversity, and tightly links global patterns of nematode diversity to their invertebrate hosts. Other surveys of termite–nematode associations from Central and North America support this linkage. In Panama, 45 species of termites generated 33 nematode MOTUs (Giblin-Davis *et al*. 2007b); in southern Florida (25 to 26°N), nine species of termites produced seven nematode MOTUs; and in Nebraska (*c*. 41°N), a single species of termite (*Reticulitermes flavipes*) yielded one nematode MOTU (Giblin-Davis *et al*. 2007a). A second form of nematode–insect associations is displayed by entomopathogenic nematodes. Essentially, this group of nematodes can be characterized as bacterivores because they feed on bacteria that they inject into their larval hosts. They are rarely detected in soil surveys without the aid of larval 'baits', typically a

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permissive host such as wax-worm larvae (*Galleria mellonella*). Our habitat sampling only detected a single entomopathogenic nematode, whereas baiting recovered six species. Finally, highly specialized associations, such as the mutualism between fig and fig wasp reveal linear to exponential increases in species richness. These symbiotic relationships are host-specific and coordinated precisely with the development of the hosts. Although specialized associations are common in tropical rainforests, they are often missed in general surveys because of their patchy distribution in space and time.

In this study, we based our estimate of tropical nematode diversity on a vertically stratified examination of soil, litter, and understorey habitats including three prominent nematode–insect associations. An accurate assessment of nematode diversity will require further partitioning of the environment and a systematic examination of unique microhabitats found in the tropics.

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TO Powers research interests include nematode systematics, biogeography, and molecular diagnostics. DA Neher is a soil ecologist with interest in using nematodes and microarthropods as environmental indicators for terrestrial and wetland soils. P Mullin participated in the project as a doctoral student, with interests in nematode systematics. A Esquivel has interests in nematode biodiversity. RM Giblin-Davis and N Kanzaki are interested in understanding the diversity and abundance of invertebrate-nematode association, as well as the taxonomy and phylogeny of the players. SP Stock conducts research on the evolution diversity and systematics of insect-parasites and pathogens. Her interests are the ecology and molecular mechanisms involved in nematode-bacterium mutualisms. MM Mora has research interests in bioprospection, biotechnology, and biodiversity of microorganisms in extreme environments. L Uribe-Lorio is interested in microbial diversity, including bacteria from extreme environments and those in symbiosis with entomopathogenic nematodes and plant-pathogenic bacteria.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Listing of all 167 MOTUs recovered from 2005 sampling of La Selva lowland rainforest plots

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