ISOLATION AND CHARACTERIZATION OF FREE-LIVING NEMATODES AND THEIR TROPHIC SYMBIONTS FROM KARTCHNER CAVERNS

By

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ABSTRACT

The focus of this project was to identify and characterize nematodes and their associated microbes from the Kartchner Caverns, a cave system near Benson, Arizona. Additionally, samples were also taken from desert soil above the cave to assess potential nematode diversity differences in the cave habitat. Two novel species of bacterivore nematode were identified and placed in the genus *Rhabditis* (Nematoda: Rhabditidae) based on morphological and molecular ribosomal DNA sequence evidence. These nematodes appear to be loosely associated with a number of both rare and common fungi and bacteria found in the environs of the cave, particularly in the guano habitat. Nematodes isolated from above the desert soil were also bacterivores but identified as members of a different family, Cephalobidae, specifically the genus *Acrobeloides*. Nematodes are currently maintained *in vitro* in agar media. Fungi associated to the recovered nematode isolates from the cave habitats were also isolated and cultured in PDA agar. Molecular diagnosis of these fungi is currently in progress.

INTRODUCTION

Understanding how microorganisms and their hosts interact is an extremely important ecological question for virtually all ecological systems; Symbioses between microbes and eukaryotic hosts occur at almost all trophic levels (Bordenstein, 2003). A large number of interactions are possible, from the most obligatory internal symbioses to loose, facultative associations (Margulis, 1998). Some of the key questions relevant to these interactions are how microbes move between host species, how host and microbe adapt to each other physiologically and genetically, and what evolutionary consequences result from microbial-host associations. One of the most common eukaryote-prokaryote interactions is that between nematodes and bacteria. The range of associations between nematodes and bacteria is incredibly broad, ranging from fortuitous to obligate and from beneficial to pathogenic. Moreover, this extensive spectrum of symbioses occurs in all possible habitats of our planet.

Among the most poorly studied environments are those considered "extreme", such as hot springs, deserts, tundras and caves. Bacteria from these environments are often extremely interesting and through their study useful tools have been developed. For example the widely used Taq polymerase, one of the key components in a PCR reaction, was originally found in the thermophilic bacterium, *Thermus aquaticus,* in a hot spring in Yellowstone National park (Chien *et al*, 1976). Furthermore, extreme environments are fertile ground for the search for novel microbes, as these unusual environments often spur the evolution of mechanisms to cope with these harsh conditions. These coping mechanisms are rare, and often can be both industrially and scientifically useful (Chien *et a*l, 1976, Lutz *et al*, 2007). .

One fascinating extreme environment is that of the Kartchner Caverns, located in the Whetstone

Mountains near Benson, Arizona. This cavern is widely acknowledged as a beautiful example of carbonate cave development and is one of the top ten caves in the world in terms of mineralogical and speleothem diversity. Recently this cavern has been depicted as a model laboratory to catalog the existing microbial diversity focusing mostly on bacteria and fungi which have long been suspected of playing a role in the creation or shaping of cave formations in conjunction with the known geochemical processes (R. Maier, pers comm.). So far the most commonly encountered bacteria are Firmicutes and Proteobacteria.

Until now, the only study addressing nematode diversity in this cavern only described their presence and trophic groups they belong to (i.e. bacterivores) (Welbourne, 1999). Indeed preliminary sampling conducted by P. Stock's laboratory indicated that bacterivore nematodes are very common in certain areas of this cave, particularly in the rooms where heavy bat guano deposits are found (P. Stock, pers. comm.). Studies of nematode-bacteria associations have yielded extensive insights in numerous fields of biology, including ecology (i.e. multitrophic interactions, trophic cascades), plant, animal and human pathology, genetics and genomics, among many others. For example, it has been shown that free-living nematodes help distribute bacteria through the soil and along roots by carrying live and dormant microbes on their surfaces and in their digestive systems (Knox *et al*, 2004).

In this study we investigated the diversity of free living bacterivore nematodes and associated microorganisms in Kartchner caverns to help understand interactions between eukaryote and prokaryote communities in extreme environments.

MATERIALS AND METHODS

1- Collection of samples

Samples were collected from the Kartchner Caverns in collaboration with the Maier Lab (Family and Consumers Sciences Department, University of Arizona) who periodically collect samples in the cavern. Three sampling sites were considered: mud, guano and soil above and around the cavern.

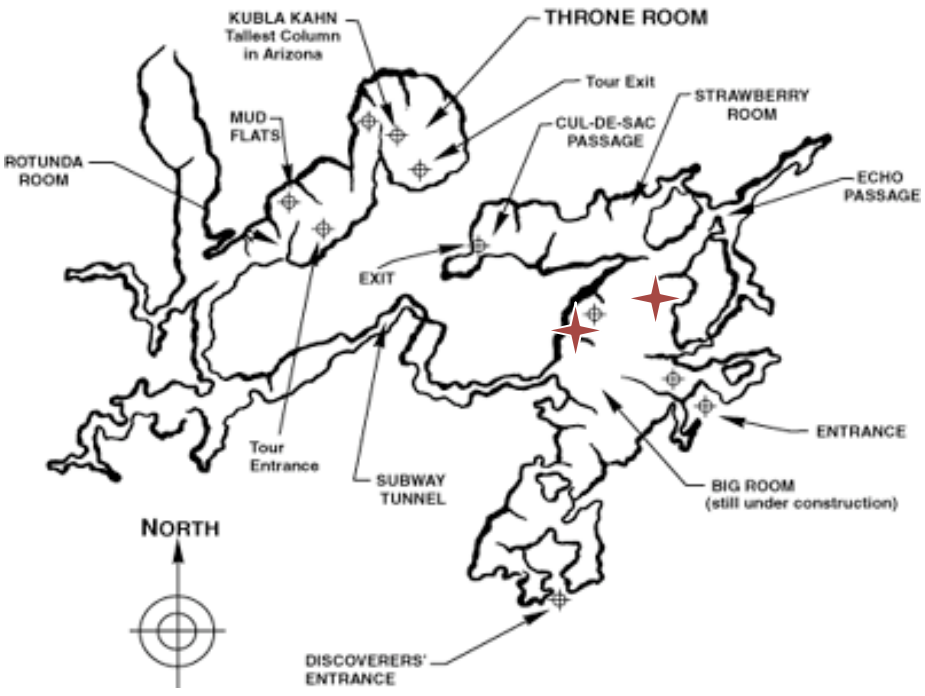

Figure 1: Schematic representation of Kartchner cavern chambers all sampling in the cave was done in the Big Room (red stars), which is also the seasonal roost of the bat community in the caverns. Map from http://www.pr.state.az.us/Images/parkmaps/kartchner_map.html.

Samples were maintained at 15 ˚C during transit to the laboratory. At each sampling site, factors such as soil characteristics (texture, pH, organic matter content), temperature and moisture were analyzed and related to nematode trophic groups encountered in the sampling sites.

2- Nematode Extraction from Samples

Nematodes were extracted using the modified Seinhorst mist extractor (Barker, 1985). This procedure helps in isolating nematodes from soil samples by making use of their positive hydrotropic behavior. Briefly, a soil sample of approximately 100 g was placed on top of tissue paper that lays on a wire mesh filter. The mesh containing the sample was then placed in a funnel with a collection flask attached to it. The apparatus was then placed in the mist chamber, where a fine water mist was applied at 15 min intervals. The water flew through the funnel and the water containing nematodes was collected for examination of nematodes recovered in each sample.

3- Nematode Culturing

Nematodes were first sorted out by placing them into different trophic groups (bacterivore, fungivores, predators, plant parasites). For this purpose the morphology of the stoma and anterior portion of the esophagus were examined following Barker (1985). Based on these observations, only bacterivore nematodes were depicted from the soil samples. Nematodes were then cultured on baby food agar according to procedures described by Stock *et al. (*2001). They were maintained by the periodic addition of LB (Luria Broth) (to induce a population boom so that mature adults could be obtained for microscopy and molecular studies) and also for subsequent subculturing for maintenance of cultures.

4- Fungi Isolation and Culturing

Fungi were isolated by excision of growing colonies from the nematode rearing baby food agar. Fungi were then subcultured on PDA (potato dextrose agar) following standard procedures. Plates were sealed to prevent dessication and potential contamination with parafilm and maintained at room temperature (22 ± 3 ˚C). Fungal cultures were verified as fungi-only by the addition of ampicillin and kanamycin to the original culture plates and light microscopic visual verification of nematode absence, though subsequent cultures were antibiotic free.

5- Nematode Diagnosis

5.1. Morphological Characterization

Differential Interference Contrast (DIC) microscopy was considered for 'rough' identification of nematodes and placement into family/genus levels. Nematodes were heat-killed and relaxed in M9 buffer in a water bath heated to 60 °C. Heat-killed specimens were fixed in triethanolamine formalin (TAF) at 50-60 °C (Courtney *et al*,. 1965), slowly dehydrated and processed to anhydrous glycerine (Seinhorst, 1959). Specimens were mounted on glass slides with Pliobond® industrial contact cement as a seal and to provide cover-glass support to avoid flattening the specimen. Quantitative measurements of each nematode were made using an Olympus BX51 microscope equipped with differential interference contrast optics and digital image software Olympus Microsuite v.7.0 (Soft Imaging System Corp., CA, USA).

5.2. Nematode Molecular Diagnosis

5.2.1. DNA extraction

Plates of nematode culture were washed with M9 buffer, then centrifuged to pellet out the nematodes. Proteinase K was used to digest the cuticle of the nematode and debris that came with them, and then a DNA extraction was performed following a standard phenol-chloroform protocol. 10 microliters of RNAse was added to the sample prior to DNA extraction, and incubated at 37°C for 1 hour. The samples were centrifuged at 4000 R.P.M. for 2 min, the supernatant was then transferred to a new tube. Phenol was added in an equal in volume to the supernatant. Samples were vortexed and spun at 13000 r.p.m. for 5 min. in a centrifuge. The supernatant was transferred to a new tube, and an equal volume of 24:1 chloroform/isoamyl alcohol was added to the mixture. The mixture was vortexed and spun at 13000 r.p.m. for 5 minutes, then the supernatant was transferred to a new tube. The samples were centrifuged again, and then sodium acetate was added in 1:10 volume increments relative to the volume of supernatant. 100% ethanol was added to cover the volume of the tube and then the entire mix was shaken by hand. Tubes were placed in the freezer overnight. They were then spun in a centrifuge at 4°C for 10 min at 13000 r.p.m.. Liquid was then discarded and the tubes were left to dry in a dessicator. The resulting DNA was washed with 100% ethanol and re-suspended in 50 µl of MQ water. DNA was quantified using a spectrophotometer (Biospec Mini, Shimadzu Corp.).

5.2.2. PCR Amplification and Sequencing

PCR amplifications were performed using primers detailed in Table 1. Primers were chosen to cover both the invariant regions of the 18s gene and the ITS "internal transcribed spacer" region, or rDNA

which are considered useful markers for nematode barcoding and for discriminating between closely related species, respectively.

Table 1: Primers used for PCR amplification

Primer	**Orientation**	**Oligonucleotide composition (5'-3)**	**Reference**
18S	forward	TTGATTACGTCCCTGCCCTTT	Floyd *et al*., 2002
26S	reverse	TTTCACTCGCCGTTACTAAGG	Floyd *et al*., 2002
18S550	forward	GCAGCCGCGGTAATTCCAGCT	Mullins *et al*.,2005
18S977R	reverse	TTTACGGTTAGAACTAGGGCG	Mullins *et al*.,2005
18S 4A	forward	GGCGATCAGATACCGCCCTAGTT	De Ley *et al.,* 2002
18S 2B	reverse	TACAAAGGGCAGGGACGTAAT	De Ley *et al*., 2002
SSUF04	forward	GCTTGTCTCAAAGATTAAGCC	Vrain *et al.,* 1992
SSUR09	reverse	AGCTGGAATTACCGCGGCTG	Vrain *et al.,* 1992

Volume of DNA sample was adjusted to 100 ng/µl based concentration readings from the spectrophotometer. A typical PRC mix contained 1.0 µl of DNA, 9.5µl of MQ H20, 1.0 µl of each primer and 12.5µl of PCR Red Taq. All PCRs were performed on a BioRad-Mini thermolcycler. PCR parameters were the following: initial denaturation at 94°C for 7 min, followed by 33 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, followed by a final elongation step ran at 72°C for 10 min and hold at 4°C..Positive and negative controls were included in each amplification series.

A 1% agarose gel was considered for electrophoresis of PCR products. To make a 1% gel the following reagents were considered: 0.45g of agarose, mixed with 45ml of 1X TAE (Tris-acetate-EDTA) buffer. Ingredients were poured into a beaker and heated until boiling. After cooling down,

the agarose solution was poured into the molding set up, and allowed to solidify. The gel was then loaded with a ladder (1Kb), the PCR samples and the negative and positive controls as well. SYBR green I dye was used instead of Et-Br, because it allows gels to be reused. The SYBR green is added to the samples and not the gel. Finally, the gel was run at 80V for 20 min, and then visualize on gel box using SYBR Green filter.

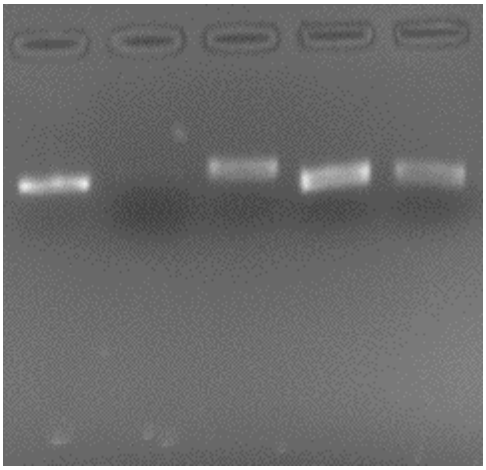

Figure 2: Image of an agarose gel, assaying efficacy of PCR for the 18s rDNA region. Each well, from the left: Positive control, Negative control, Guano isolate, Mud isolate, Soil isolate.

PCR products were treated enzymatically using ExoSAP-IT (USB Corporation). Five microliters of PCR product were transferred to a 0.5 ml tube to which 2µl of ExoSAP-IT were added. The mixture was placed in a thermal cycler and incubated at 37°C for 15 min, to activate the enzymes. A second heating phase at 80°C for 15 min was followed to 'kill" enzymes. PCR products were then submitted to DNA sequencing.

Sequencing of amplified products was carried out under contract by the Laboratory of Molecular Systematics and Evolution (LMSE, University of Arizona). Successfully sequenced samples were carefully edited and ambiguity resolution was performed with the aid of Seq Edit and SeqMan software

v.7.2 (Lasergene, DNAStar Corp.) Sequence segments corresponding to the PCR amplification primers were removed prior to multiple sequence alignment. Sequences were aligned initially using CLUSTAL X version 1.53b (Thompson et al., 1997), and the resulting output was adjusted manually to improve homology statement considering MacClade software (Madison and Madison, 2000).

RESULTS

1. Nematode diagnosis

1.2. Morphological Studies

1.2.1. Cave Isolates:

Three distinct isolates of nematodes were obtained from the cave environs, one from a guano pile in the Big Room, one from the mud sample in the same room, and one from soil outside the cave.

1.2.1.1. Guano Isolates

Nematodes isolated from Guano were identified as members of the family Rhabditidae Örley, 1880, specifically to the genus *Rhabditis* Dujardin, 1845. Members of this family are characterized by the presence of closed lips, not forming an open cup. Esophageal collar is always present. Stoma with metarhabdions with 5 or 3 tubercules in place of teeth, often anisoglottid, sometimes anisomorphic. Females with Vulva located in the midbdy, ovaries paired, oppposed reflexed. Female tail of various shapes. Male tail usually leptoderan, peloderan in one subgenus. Spicules separate, gubernaculums present.

Key diagnostic traits of this isolate include; a straw-like buccal tube (stoma) which is more than three times as long as it is wide, with no ornamented or serrated lips. Nerve ring is situated in the middle of the isthmus or right above the basal bulb. Live nematodes under the DIC scope were directly observed feeding on bacteria.

Females of this isolate possess two ovaries, with a vulva slightly below the midline of the body. Females tail is acute with a long whip-like end. Males possess a pseudopeloderan bursa (a portion of the tail-tip is free) and have nine pairs of bursal rays (genital papillae) arranged as follows: 3 () 3 3,

with pair 3 adcloacal and pairs 4-6 7-8 postcloacal. (Figure 3).

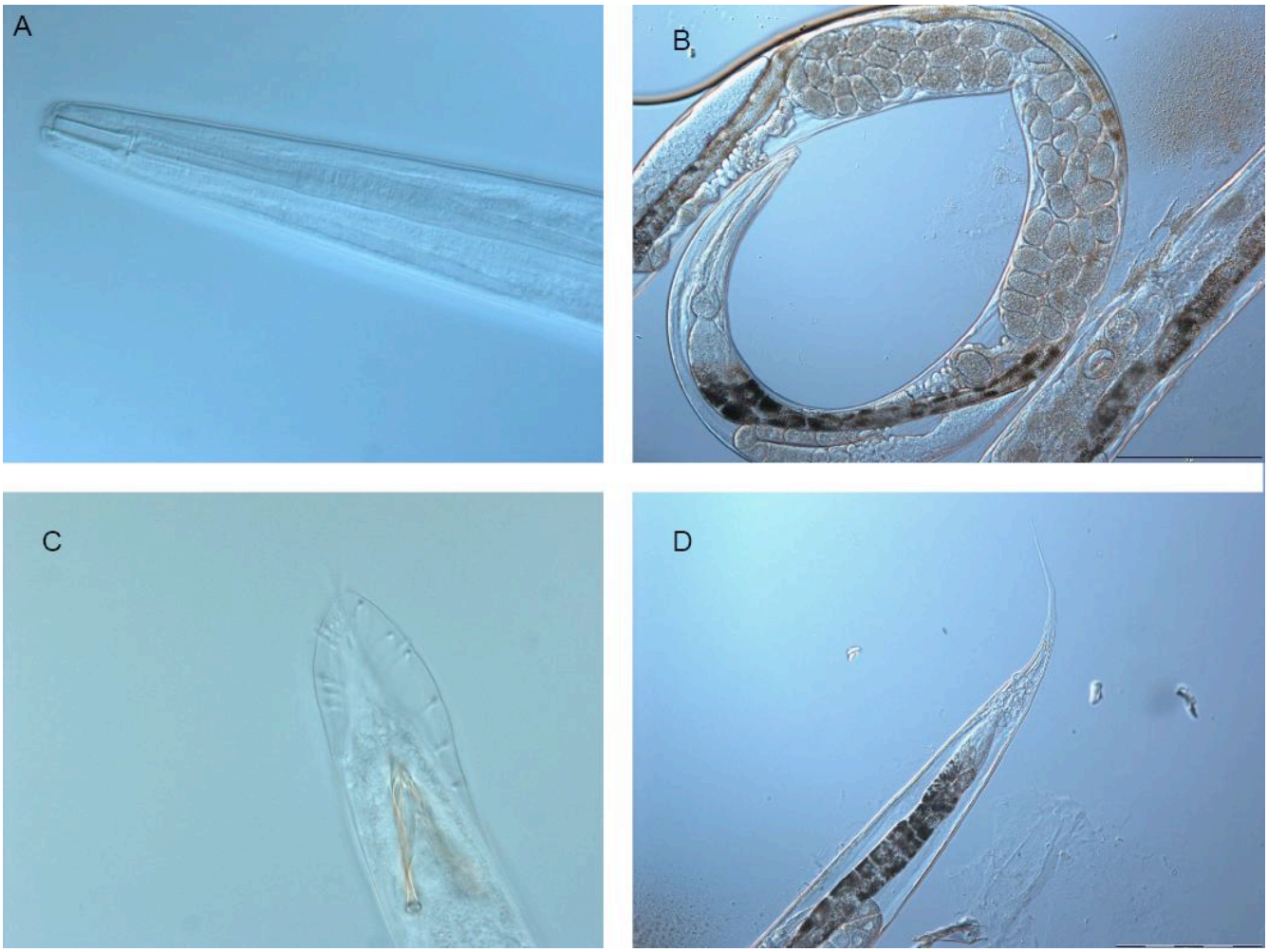

Figure 3: Morphological characteristics of the nematodes of the "guano isolate". A) Anterior portion of male, lateral view, showing buccal tube, B) Gravid adult female, lateral, view showing uterus filled with eggs. C) Posterior end of male, ventral view, showing spicules and bursa, D) Posterior end of female, lateral view showing acute tail.

1.2.1.2. Mud Isolate:

Nematodes isolated from the mud sample were also identified as members of the family Rhabditidae Örley, 1880 and genus *Rhabditis* but belonging to the subgenus *Oscheius.*. The *Oscheius* subgenus has been divided in two groups: *Dolichura* and *Insectivora* (Sudhaus and Hooper, 1994). The mud isolate belongs to the "insectivore-group".

.Adults are characterized by a long and narrow stoma but significantly shorter than the guano isolate. Length of stoma is about, about twice as long as it is wide. Esophagus has the typical rhabditoid morphology. Nerve ring is situated in the middle of the isthmus or right above the basal bulb. Females are amphidelphic, with vulva positioned near the midbody. Females of this isolate were observed to , produce far fewer eggs than those females from the guano isolate. Endotokia matricidia was frequently observed. Female tail is conical but shorter that that of the females from the guano isolate. A postanal swelling is present. Males have a peloderan bursa sustained by 7 pairs of bursal rays (Figure 4).

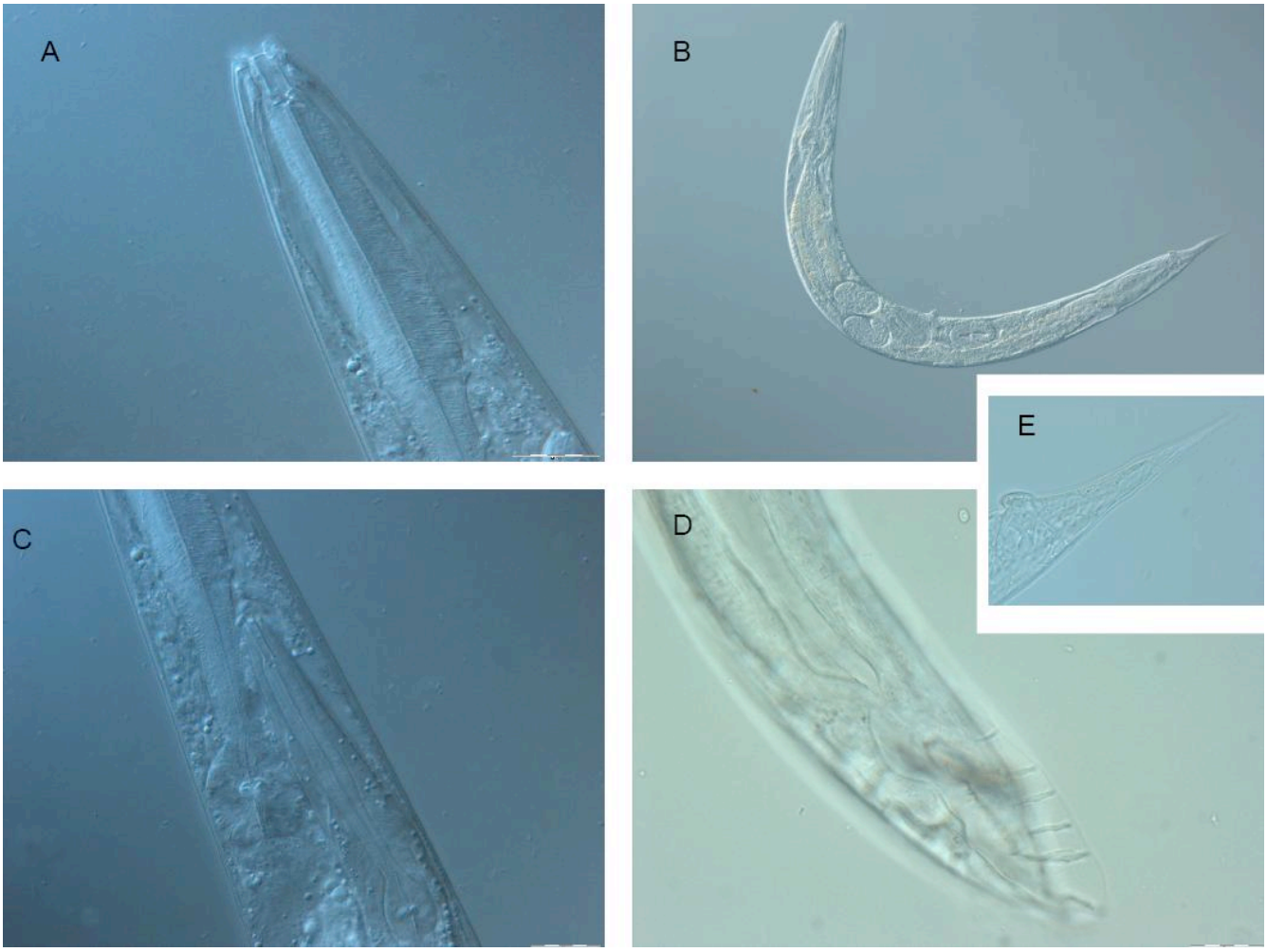

Figure 4: Morphological characteristics of the "mud isolate" nematodes. A) Anterior portion of adult female showing buccal tube and esophagus; B) Gravid adult female *in toto*.; . C) Adult female with a small offspring in body cavity (endotokia matricidia); D) Posterior end of adult male showing tail and bursal rays in ventrolateral view.

1.2.2 Soil isolate:

The nematodes retrieved from the soil outside of the cave were identified as members of the family Cephalobidae Filpijev, 1934 and belonging to the genus *Acrobeloides* (Thorne, 1937). Members

of the family Cephalobidae (Nematoda) are among the most common and morphologically striking soil nematodes. Many members of Cephalobidae have extensive lip elaborations called probolae, but two taxonomically problematic genera, *Acrobeloides* and *Cephalobus*, have simple, low probolae.

Females of this isolate are characterized by the presence of low rounded or conical labial probolae (lips), Stoma distinctly jointed; cheilorhabdion wide, rest of stoma narrower.Esophagus with cylindrical procorpus, isthmus barely seen, basal bulb with a valve.Female gonad prodelphic, reflexed down body, usually with an additional double flexure towards the end of the ovary. Vulva is far from anus, with vulva lips symmetrical and protuberant. Tail is convex-conoid (except for the species *A. clavicaudatus*). Males are very rare and were not observed in our samples (Figure 5).

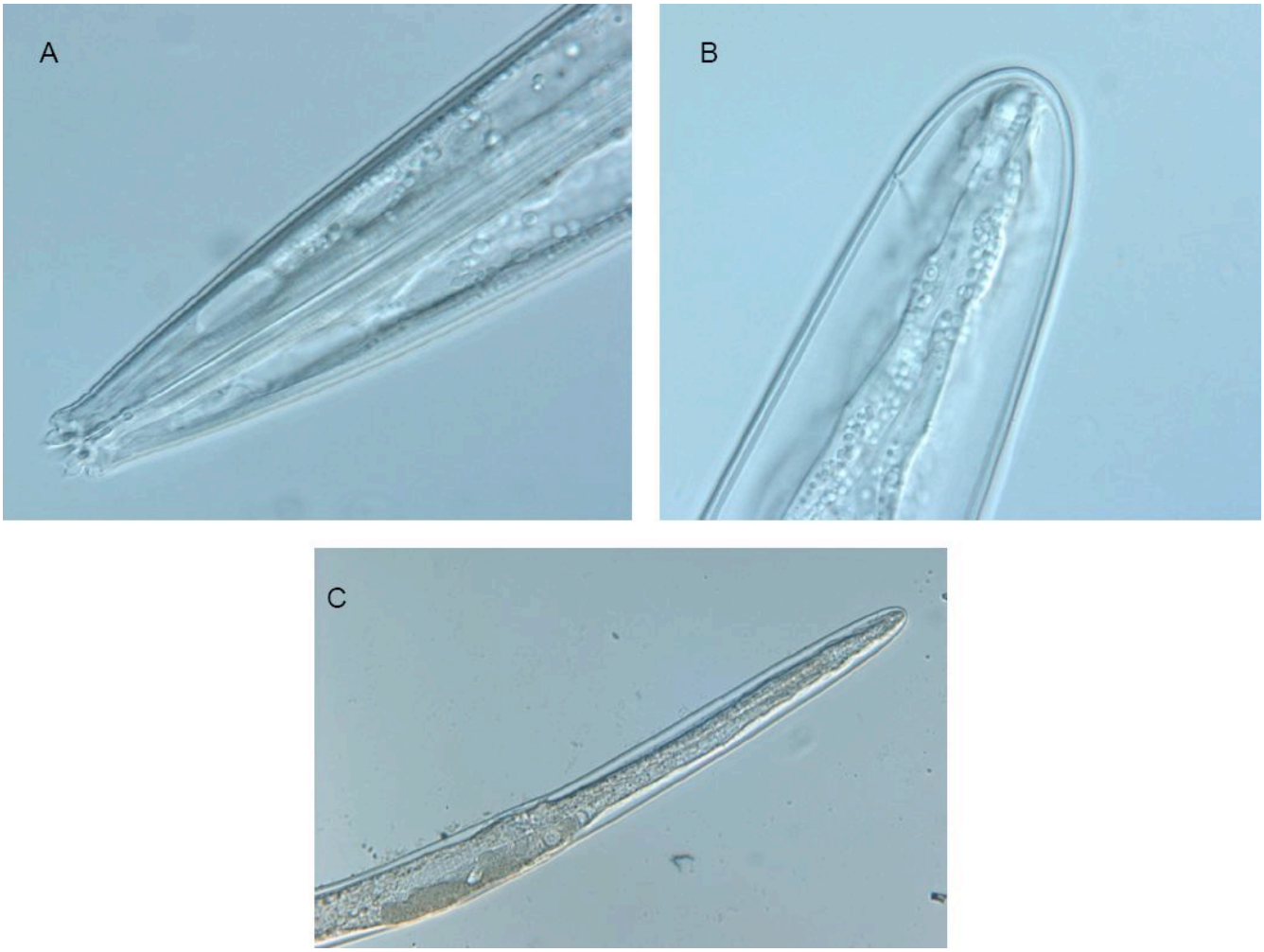

Figure 5: Morphology of nematodes retrieved from the soil outside the caverns. A) Anterior portion adult female, showing buccal tube and lips; B) Posterior end of adult female, lateral view showing typical rounded tail; C) Posterior end of adult female showing the ovary .

2. Molecular diagnostics and phylogenetic relationships of the recovered nematode isolates

Obtained 18S rDNA sequences were compared with published nematode sequences in Genbank by doing a "rough" BLAST search. Sequences were aligned with existing *Rhabditis* sequences in GenBank. Criteria for phylogenetic interpretation of data considered maximum parsimony (MP) jack-knife (1000 replicates), weighting transversions twice (Figure 6). The guano isolate resembled most closely (98% identical over the first ~1kb of the 18s ribosomal DNA) *Rhabditis blumi* (provide here accession no. from GenBank). ITS rDNA sequences of the same isolate were also blasted in GenBank. Results from these comparisons placed the guano isolate as closely related to *Butlerius* sp (66%) and *R. blumi* (40%). Similarly, 18S rDNA sequences of the mud isolate were depicted as closely related to *Rhabditis. rainai* (95 and 99% homology).

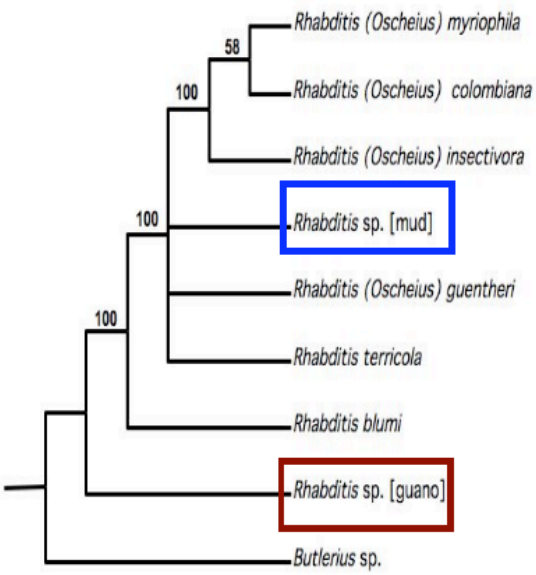

Figure 6: Phylogenetic tree containing all relevant *Rhabditis* species in relation to the cave isolate nematodes. Sequences considered in the analysis included those from R. *(Oscheius) myrophila* (Genbank Accession Number U81588.1), *R. (Oscheius) colombiana* (AY751546.1), R. *(Oscheius)*

insectivora (AF083019.1), *R. (Oscheius) guentheri* (EU196022.1), *Rhabditis blumi* (U13935.1), and *Butlerius* sp.(EF192135.1).

DISCUSSION

Because the ecosystem of Kartchner Caverns is entirely dependent on the nutrients brought in by bats during their summer inhabitation of the cave, it stands to reason that any organisms in the cave will either digest the guano directly, or the microorganisms that live in it. This premise seems to hold for the nematodes of the cave, as they have several classical bacterivore nematode characteristics, most notably the straw-like mouth parts. The fact that adding Luria broth to the culture plates stimulates a population boom for the nematodes also supports this notion, as does the nematodes' failure to thrive in culture plates with antibiotics. On several occasions I also observed the nematodes feeding on bacteria while under the microscope.

I hypothesize that the guano nematodes may be associated with the bats that live in this cavern. Bats may play an important role in disseminating these nematodes. External phoresis of nematodes by mammals or other animals has been widely document for other nematode groups.

When collected, the population of nematodes from guano samples were larger than those from the mud sample. This insinuates that the guano nematodes may be more prominent in the caverns given that the guano environment may harbor more food (bacteria and/or fungi) that stimulate growth of this nematode's populations

An interesting follow up to this experiment would be to examine a sample of guano and mud from the cavern immediately after the bats have left, to determine if the guano nematodes are more prevalent following the bats.

Based on the sequence comparisons to known nematode species, it is likely that both the guano and the mud-derived nematodes are as-yet un-described species. I will not know for sure until

phylogenic analysis is done, but the differences in the 18S rDNA gene sequences are large enough that it is a fairly good guess, especially in the case of the guano nematodes.

The ecology of the caves is interesting; why is there one species of bacterivore nematode in the guano, but a completely different species in the mud? The guano nematodes may out-compete the mud nematodes in guano, or there may be something toxic about guano that the mud nematodes cannot tolerate, but guano is clearly the richest environment in the cave; the only other source of nutrients would be that carried in through the water that flows from an underground aquifer, which is almost certainly very nutrient-poor. Raina Meyer's lab is investigating if bacteria may play a role in the formation of certain interesting mineral formations within the cave—if this is the case, the mud nematodes may feed on these bacteria, rather than those brought in with the guano.

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