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Characterizing a new species of *Nematoda* using genetic and morphological analyses

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ABSTRACT

Nematodes (Nematoda) are slim tubular worms ranging between 0.5 mm – 2 mm in length and 10 to 100 µm thick. They have effectively adapted to inhabit all regions of the Earth, but are most commonly found in soils, decomposing vegetation, and freshwater sources. *Ceanorhabditis elegans (C. elegans)*, an important member of this phylum, is a valuable model system. Owing to its small, fully sequenced genome, it is typically used to model the development of some diseases, such as neurodegenerative diseases. Nematodes are highly diverse, with over 30,000 species having not yet been described. While *C. elegans* will continue to be the primary model species, the classification of previously unknown species is valuable as it allows for study of the evolutionary pathway leading to each species, behavior and instincts, and how such animals behave as parasites. This diversity is exciting, and Drs. Kimberley Dej and Bhagwati Gupta work with students to document new species. In the laboratory, we use morphological analysis of the mouth, the pharynx, and the tail, combined with data generated by sequencing the 18S small ribosomal subunit rRNA gene to explore and document these new species. Here, we discuss how it was determined that a unique specimen collected from the Hamilton, Ontario area was found to have features of multiple genera: *Oscheius* and *Ceanoreabditis*.

Keywords: Nematode diversity, speciation, genetic analysis, sequencing, morphological analysis

INTRODUCTION

C. elegans and other nematodes continue to be major model organisms in biology, however, the known species of this family of invertebrates represents only 45% of an estimated 50,000 unique species[1]. Nematodes are diverse colonizers of microbe-rich habitats; rotting vegetation unites three of the most important lab model organisms in the same ecological niche; *S. cerevisiae, D. melanogaster, and C. elegans*. For example, analyses conducted in silico on the *C. elegans* genome have identified nearly a thousand G-protein coupled receptors which evolve at rapid rates, with evidence pointing to positive selection[1]. This great variation occurs as a result of nematodes seeking new ways to endure harsh conditions in unfamiliar environments, which is driven by natural selection[1]. *C. elegans* and other nematodes can be found to endure environments of hypoxia, osmotic stress, heat, cold, pathogens, and other toxins[1]. Logically, to understand how nematodes can successfully endure these conditions, new species need to be discovered and categorized into a

library. Currently, it is estimated that 30,000 species of nematode have yet to be described. The purpose of this study was to contribute to the building of this library by searching for a new species of nematode and using morphological and genetic analysis to verify its novelty.

LIFE CYCLE OF C. ELEGANS

It is important to first consider the life cycle of *C. elegans* and other nematodes. The life cycle of *C. elegans* begins with a 3.5 to 4-day embryonic developmental period, part of which occurs in the mother's uterus[2]. At this point, the nematodes exist as eggs[2]. Following this period, the eggs hatch and the resulting larvae- identical to adults with exception to their underdeveloped reproductive system- live through four stages of life, L1 to L4[2]. Each of these stages are separated by periods of lethargus and moulting. The natural environment of *C. elegans* has an influence on its development. When young larvae are exposed to environments of crowding by pheromone sensation, food depletion, and high temperature, they interrupt their developmental cycle and enter an alternative stage known as the dauer stage[1,2]. During the dauer stage, they possess reduced metabolism and increased stress resistance[2].

NEMATODE ANATOMY

Nematodes are slender creatures, and have a fairly linear morphology with several tubular tracts within them; one forms the digestive tract and the other forms the reproductive tract[1]. Morphological analysis in this study involved analysis of the mouth, pharynx, and tail regions.

Mouth

The mouth is found at the very front of the nematode and is a key characteristic involved in identifying the family of most nematodes[3]. Four distinct mouth families exist. *Rhabditidae* mouths have several protrusions at the opening of a long, narrow cylindrical tract, called a stoma[3]. *Diplogastridae*, a family which includes *Pristionchus pacificus*, contains a shorter stoma with characteristic teeth, providing it with the ability to consume live *C. elegans* when other food is scarce[3]. Finally, *Panagrolaimidae* and *Cephalobidae* have mouths where the stoma has been strengthened with hardened sclerotin[3] (Figure 1).

Pharynx

The pharynx extends from the mouth into the beginning of the digestive tract. Generally, for the most commonly studied nematode, *C. elegans*, the mouth has 4 characteristic portions: the procorpus, the bulb-like metacorpus, the isthmus, and the terminal bulb[4] (Figure 2). The functions of the metacorpus and terminal bulb are similar, in that they are responsible for transporting food, usually bacteria, into the digestive tract while also grinding it for more efficient digestion[4]. The pharynx varies between genuses, therefore it forms a reliable method of distinction between different samples that are isolated. When compared to another commonly studied soil nematode, *Oscheius tipulae*, it is found that rather than having a distinct metacorpus, the oesophageal muscles are spread out from the procorpus through to the terminal bulb[4].

Tail

The tail is unique in that it is the only source of sexual dimorphism occurring outside of the internal anatomy of nematodes[3]. Most *C. elegans* worms are born as hermaphrodites, which means that they have both male and female reproductive systems. Males develop as a result of non-disjunction of the sex chromosomes during meiosis, which is a rare event[3]. The male tail is different from a hermaphrodite's by having a fan-like projection at the end of the tail[3]. The tail is where the nematode's body tapers in an asymmetrical manner, and contains the rectum[1,3]. For morphological analysis, the tail can be used to distinguish between species by comparing the side which the rectum exits to[3].

MATERIALS AND METHODS

Collection and Decontamination

Samples of decomposing vegetation were collected from different regions of the McMaster University campus in Hamilton, Ontario. Most samples were collected from the McMaster community garden. The community garden contains many different ecological niches within which a diverse collection of nematode species may live. This, in turn, would improve the likelihood of finding a new species, and so therefore provided a preferred place of collection.

Portions of collected specimens were then transferred into 50 mm petri dishes with Nematode Growth Media (NGM) seeded with Escherichia coli. The animals were then kept at room temperature (20 °C to 25 °C) and serially transferred to new plates to decontaminate them from mites, molds, and fungi.

Clonal Colony Formation

Out of the 9 different samples, animals at adult stage were selected and placed as individuals into new NGM plates to grow into new clonal colonies. As known from their lifecycle, selection of adults allows for both gonochoristic and hermaphroditic animals to be bred, as the potential fertilization event has already occurred[5]. The worms were observed and kept at room temperature and success was determined if they produced a clonal colony with a high number of individuals (50+ per entire 50 mm dish).

Reproductive Methods

Out of 5 different clonal colony formation attempts, a single clonal colony was selected for further testing and work within the study: sample M.1.1.1. Several young animals, preferably between the L2 and L4 stage, were selected from the M.1.1.1 clonal colony and transferred to individual NGM plates and observed for clonal colony formation according to the previous criteria of 50+ individuals per 50 mm dish. Young worms were selected to eliminate the chance of a fertilization event from occurring, in the case that the species was gonochoristic. Formation of a clonal colony indicates that the species is not gonochoristic, which introduces the possibility for a hermaphroditic or parthenogenetic species[6].

Morphological Analysis

Nomarksi microscopy was used for the morphological analysis. To prepare the worms for observation, the WormAtlas agar pad protocol was followed[7]. A solution of 5% agarose in PBS was first melted, from which a drop was dispensed onto a glass microscope slide. A second slide was laid upon the drop of agarose solution to flatten it. Then, a drop of 10 mM NaN_3 solution was dispensed onto the agarose flat pad after the flattening slide was removed. 5-10 animals were placed onto the drop of NaN_3. Once the animals were motionless, a glass coverslip was added above the worms and the now prepared slide was observed at 20x, 40x and 60x objective magnification using Nomarski prisms.

Genetic Analysis

Adult worms were first digested in proteinase K. Polymerase chain reaction amplified the genomic content of the samples and was followed by separation of cell contents by gel electrophoresis to yield pure genomic DNA. A third-party DNA sequencing service (MOBIX Lab) returned the gene sequence obtained by Sanger-sequencing. The sequence was then compared to known sequences using the National Library of Medicine Blastn service.

RESULTS AND DISCUSSION

MORPHOLOGICAL ANALYSIS

Mouth

When the mouth of sample M.1.1.1 was compared to common mouth types of different nematode families, it was found that it showed very strong resemblance to the mouths of the *Rhabditidae*: a long and narrow stoma, preceded by protrusions at the front of the mouth which form the opening/closing mechanism of the mouth (Figure 3).

Pharynx

Knowing that the sample M.1.1.1 is likely a *Rhabditid*, the study of pharynxes was narrowed to members of that family. Comparison with *C. elegans* was performed initially as it was the most common. It was found that the pharynx of M.1.1.1 clearly resembles those of the *Oscheius genus,* as it lacks the metacorpus of a *Caenorhabditid* (Figure 4).

Tail

The final criterion for the morphological analysis of M.1.1.1 was comparing its tail to that of two other species, specifically *Ceanorhabditis elegans* and *Oscheius tipulae*. These two were chosen from insights generated from the pharyngeal analysis, as it was not anticipated that the tail would provide specific categorization abilities earlier in the study. When compared, it was found that M.1.1.1 had two features of its tail that were common to that of *C. elegans*. The tail of M.1.1.1 was moderately long (80 µm), with two faces; one linear and the other curved. The rectum and its opening were on the straight/linear face of the tail, a feature that is found in *C. elegans*. When examining

images of the tail of *O. tipulae*[8], it was clear that the rectum's position on the tail was reversed, which discounted support for M.1.1.1's inclusion into the *Oscheius genus* (Figure 5).

REPRODUCTIVE METHODS & GENETIC ANALYSIS

When individuals of the M.1.1.1 sample were placed independently on new seeded NGM dishes, it was found that they consistently produce full and healthy clonal colonies with nearly 50+ individuals by 72 hours (over 7 attempts). This provides evidence for the claim that M.1.1.1 is likely hermaphroditic. When the 18S small ribosomal subunit rRNA gene was sequenced and alignment generated, results returned very high (99%) query coverage with the 18S small ribosomal subunit rRNA gene of *C. briggsae* (Figure 6).

CONCLUSION

The purpose of this study was to use morphological and genetic analysis to characterize an unknown specimen of nematode from Hamilton, Ontario. Examination of the mouth indicated support for a *Rhabtididae* family. The genuses *Ceanorhabditis* and *Oscheius* were contenders from examination of the rectum and pharynx respectively. Considering the genetic similarity to *C. briggsae* and the hermaphroditic nature of M.1.1.1, we believe this sample is a variant of the *Oscheius* genus with deep influence from the *Ceanorhabitis* genus, a novel finding when consulting known phylogenies. However, additional studies into this unknown specimen is necessary, in order to conclude that it is indeed novel and not a consequence of genetic variability.

ACKNOWLEDGEMENTS

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Refer to next page for appendix

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APPENDIX

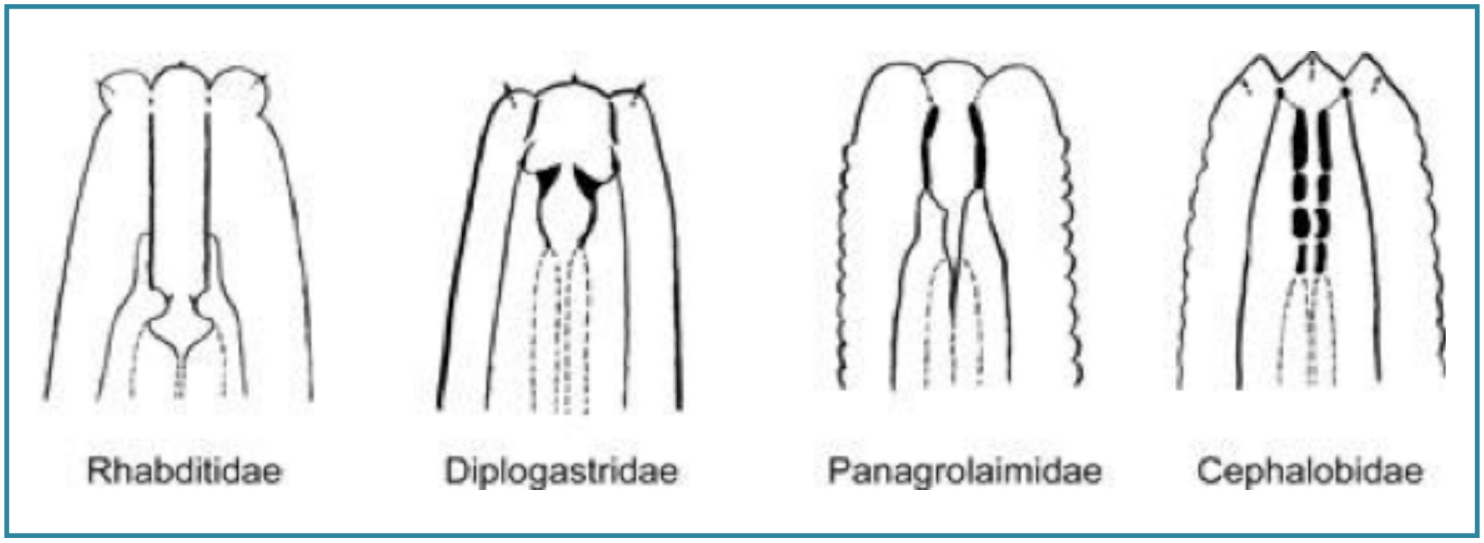

Figure 1 - Four nematode mouth families that form as a guide to examination of the mouth[3].

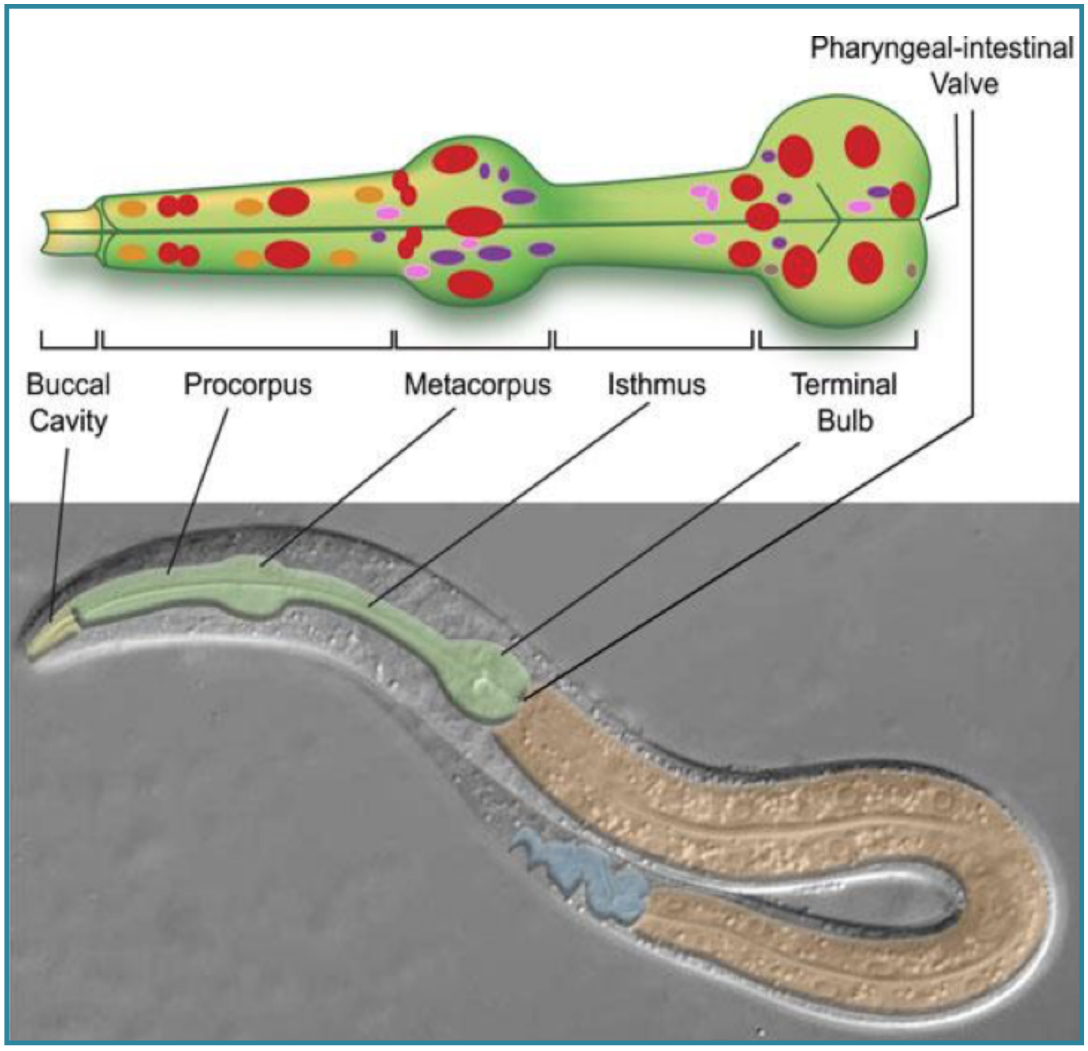

Figure 2 - The pharynx of the commonly studied nematode, *C. elegans*. There are four distinct regions of the pharynx, each of which are essential to transportation of food into the digestive tract[4].

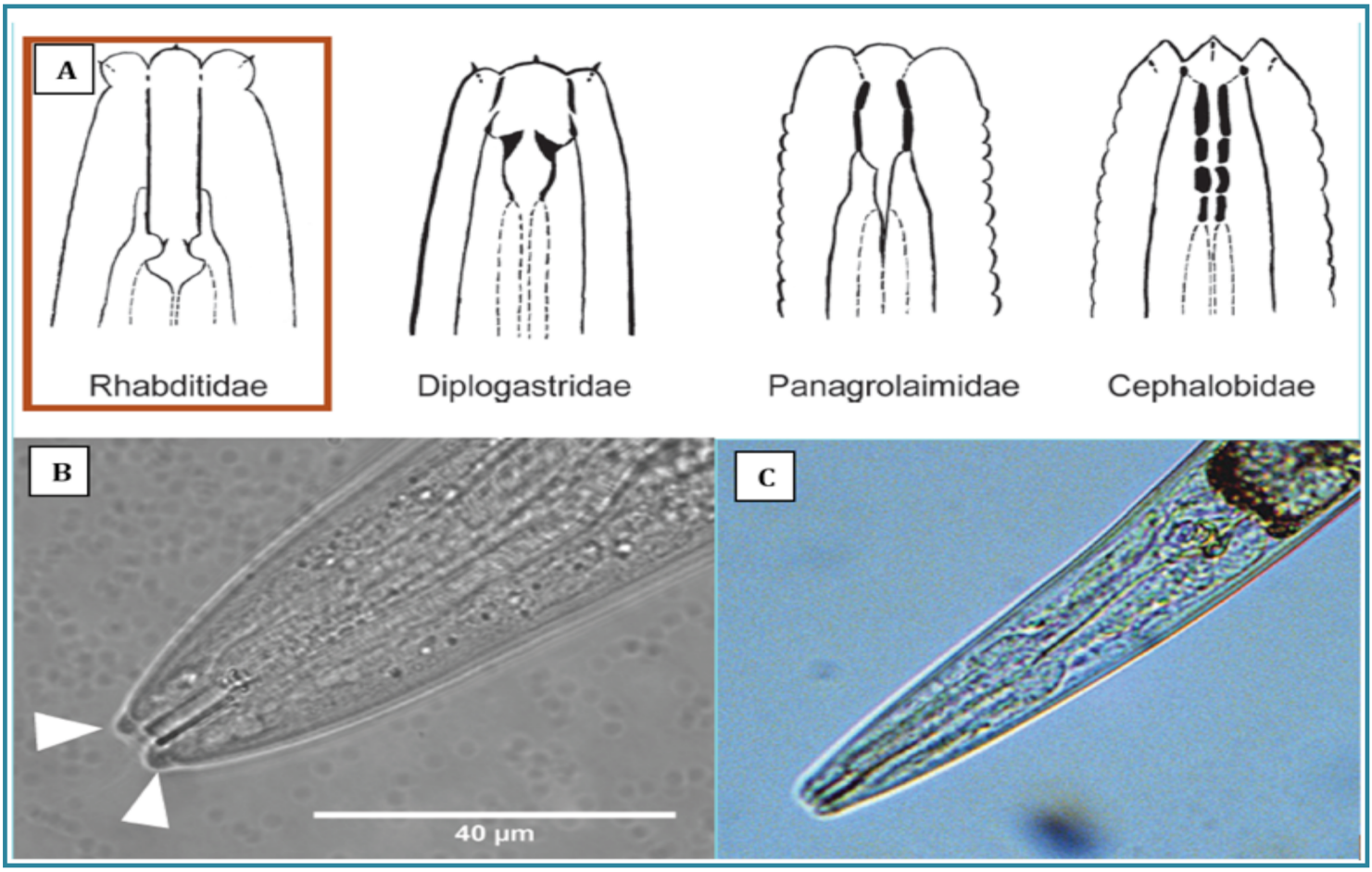

Figure 3 - Morphological Analysis of the mouth. (A): The four different mouth families of nematodes. (B) The mouth of sample M.1.1.1 at 60x oil immersion objective, with arrowheads highlighting the front mouth protrusions. (C) The mouth of *C. elegans,* which is very similar in structure to that of M.1.1.1.[3].

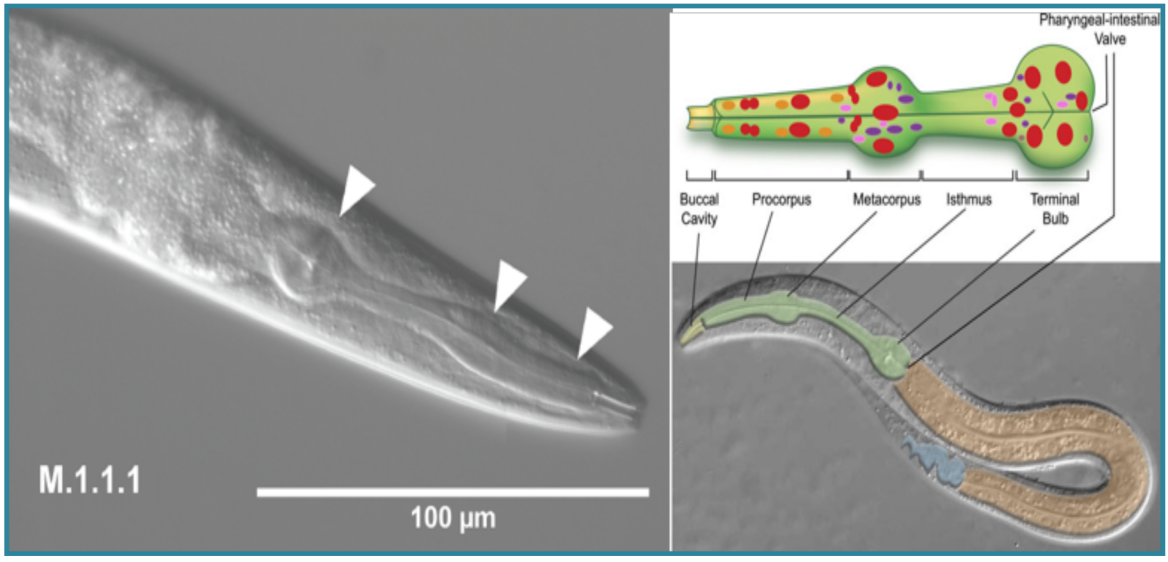

Figure 4 - Comparison of the pharynx of sample M.1.1.1. to the pharynx of *C. elegans*, a member of the same family. It is clear that the sample M.1.1.1. lacks the metacorpus which would have been found at the middle arrowhead. Right image[4].

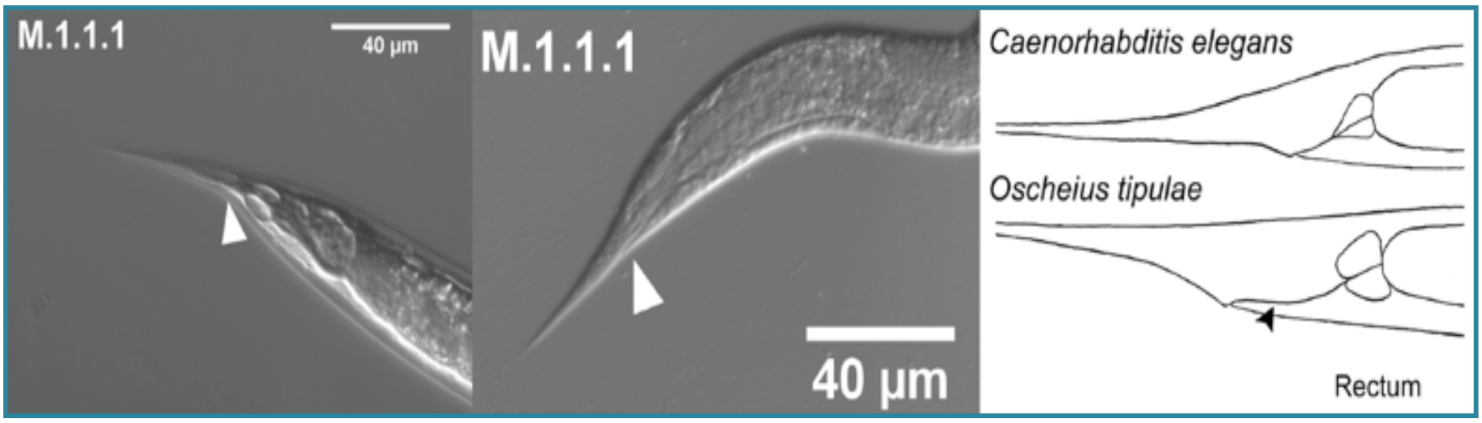

Figure 5 - Comparison of the tail of sample M.1.1.1. (two left-most images) with known morphological reproductions of C. elegans and O. tipulae. Right-most image[8].

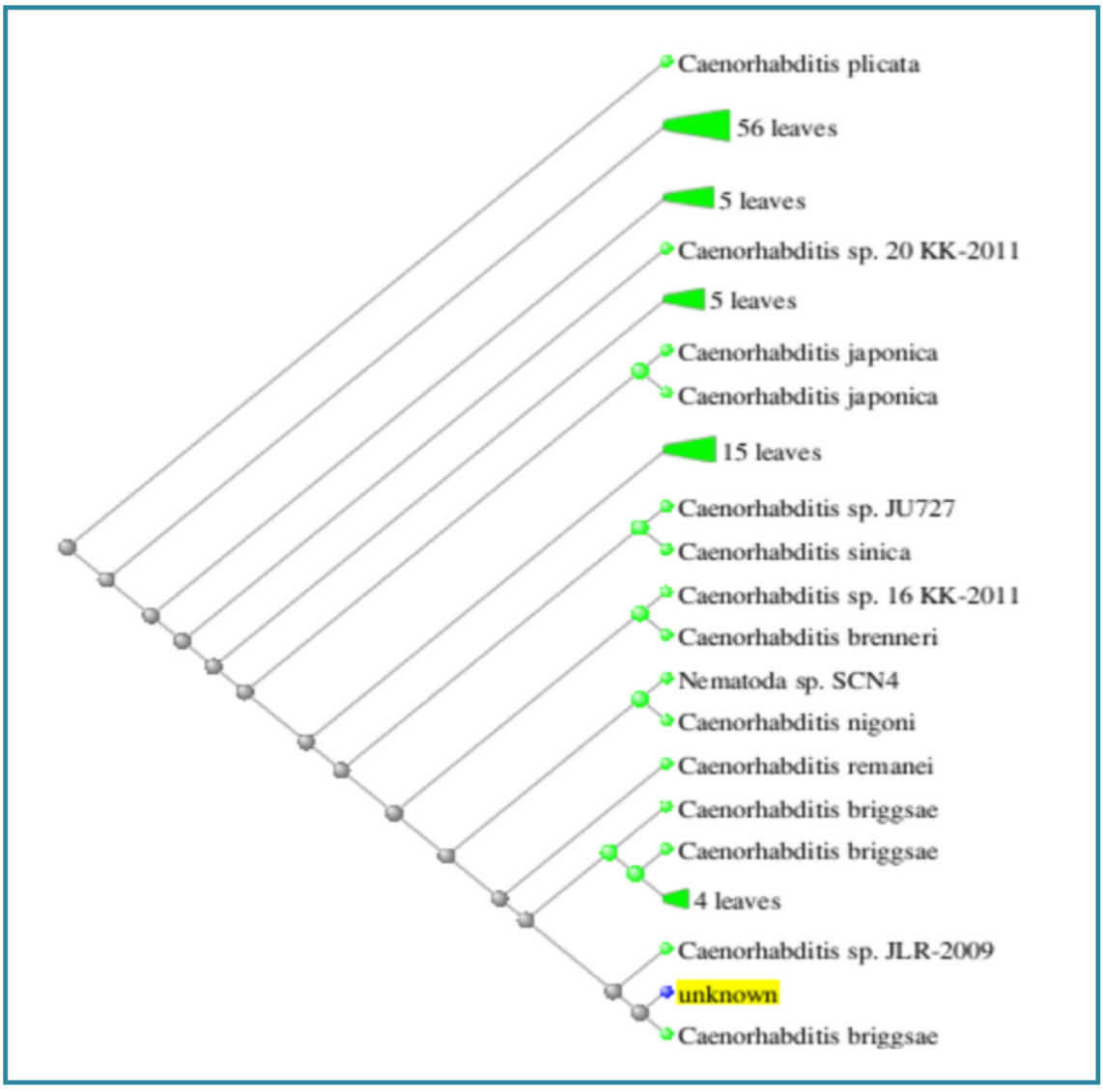

Figure 6 - A tree representing the distance of the alignment results of the 18S small ribosomal subunit rRNA gene. M.1.1.1. appears to have some genetic relation to *C. briggsae*.