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Morphological identification of plant parasitic nematodes found in roots and soil of pomelo (*Citrus maxima* (J. Burm.) Merr.)

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Abstract Pomelo (*Citrus maxima*) is a tropical fruit plant originated from Southeast Asia. It is generally eaten and is known for its sour taste and richness in vitamin C and was traditionally used in medicine. Additionally, Pomelo is widely distributed throughout the country's regions and is one of the predominant citruses planted. In the Philippines, the pomelo industry has created a higher market value and provides in the farming community. However, the growth in the said industry is threatened by different factors, and one of those is plant-parasitic nematodes. Thus, the study focused on identifying and determining the presence of parasitic plant nematode in *C. maxima* roots and soil within its rhizospheres. Morphological identification was done using a light microscope to classify the nematodes based on their physical characters like the stylet, knobs, tail, and buccal cavity, lip region, location of body regions, and the size and length of the body. Dichotomous keys, photographs from the database (NemaPlex), and related literature were used for genus-level identification while morphometrics was done to verify and identify the species of the plant-parasitic nematode. Based on morphological analysis of *C. maxima*, the identified free-living nematodes are *Aphelenchus*sp. under *Order* Dorylaimida, *Mononchus* sp. under *Order* Mononchida, *Rhabditis* sp. under Order *Rhabditida* and a plant-parasitic type *Tylenchulus spp.* under *Order* Tylenchida with a relative frequency of 80%, 60%, 60%, and 100%, respectively. Moreover, Molecular identification is highly recommended to determine the species level of the collected nematode.

Keywords: *Dorylaimida*, *Mononchida*, Plant-parasitic nematodes, *Rhabditida*, *Tylenchulus*

Introduction

Life on earth relies on the plants. They are an excellent source of basic human needs in clothing, shelter, and especially food. Many citrus species of plants have medicinal value that can improve our health and have many beneficial uses (Ahmed and Azmat, 2019). *Citrus maxima*, commonly known as Pomelo is a tropical fruit originated from Southeast Asia, it is cultivated for its large and round fruit. The fruit is generally eaten and is known for its sour

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taste and richness in vitamin C, and was traditionally used in medicine to treat coughs, fevers, and gastrointestinal disorders (Sawant and Panhekar, 2017). The oil from the leaf has a fungicidal activity and is also used to make perfume and other aesthetic products. Meanwhile, the fruits can be used to treat asthma, coughs, leprosy, epilepsy, and mental aberration; the bark and roots of the plant also possess antimicrobial activity (Dubey *et al.*, 2004).

Pomelo is widely distributed throughout the regions of the Philippines and is one of the predominant citrus plants (Ochasan *et al.*, 2015). The fruits are harvested only once a year throughout countries that has cultivated Pomelo between November and April (CUESA, 2018). In the Philippines, a steady increase in fruit exports provides profit to farmers. However, this growth is threatened by different factors such as natural calamities, climate change, infestations of various organisms like insects, fungi, small mammals, and nematodes. These damages created a significant loss in profit for both the farmers and the dealers.

Nematodes are considered one of the oldest and most diverse animals on Earth (Wang *et al.*, 1999, cited in Lambert and Bekal, 2002) and possess a wide range of habitat, from marine up to terrestrial habitat (Blaxter and Koutsovoulos, 2014). They are minute and vermiform in shape and can either be free-living, in which the nematode is predatory to other organisms, or a parasite to an animal or plants (Aguinaldo *et al*., 1997; Lambert and Bekal, 2002).

Phylum Nematoda consists of two major classes. First is Chromadorea that has only one subclass Chromadoria and the second is Enoplea, which is divided into two subclasses, namely, Dorylaimia and Enoplia as cited by (Blaxter and Koutsovoulos, 2014). Despite the class differences, all the subclasses possess both free-living and parasitic nematodes. Apparently, the plant-parasitic nematodes' lifestyle is considered as homoplasious between different subclasses (Blaxter and Koutsovoulos, 2014). Plant-parasitic nematodes (PPNs) infect plants, including cultivated crops worldwide. PPNs feed on most parts of the plant, i.e., roots, stems, and leaves, and possibly including the flowers and seeds. Though there are various ways in which the PPNs feed on its host, all use a specialized mouth spear called a stylet (Lambert and Bekal, 2002). Studies had suggested, depending on the level of infection, 10% to 30% loss yield due to plant-parasitic nematode (Duncan and Cohn, 1990). Mature trees can tolerate large numbers of PPNs nematodes before exhibiting a lack of vigor. However, young trees grow poorly if replanted into nematode-infested soils (Duncan and Cohn, 1990). Typical symptoms caused by PPNs in *C. maxima* are stunted and slow growth, yellowing, and reduced foliage, increased fruit drop-rate, and reduced fruit size and yield.

The objectives were to identify and determine the presence of parasitic plant nematode in *C. maxima* roots and soil within its rhizospheres; to gather information regarding its morphometrics, and to establish the MycoNema Bank of the Polytechnic University of the Philippines.

Materials and methods

Roots and soil sample collection

The soils were obtained at the lowest point of the rhizosphere, approximately 15 cm below the soil surface using a trowel. Each of the soil samples was put in a ziplock and mixed thoroughly then were processed in the laboratory of the Institute of Weed Science, Entomology, and Plant Pathology at the University of the Philippines Los Baños (UPLB).

Soil analysis

Soil analysis is a process that determines the amount of plant nutrients like the nitrogen, phosphorus, and potassium present in the soil. It also tests the physical and biological properties like texture, density, and pH value that is significant for plant nutrition, or the health of soil (Folnovic, 2019).

Soil analysis was used to test the sample collected. One kilogram of the soil sample from the two different collection dates was segregated and sent to the Bureau of Soil and Water Management (BSWM) at Visayas Ave., Quezon Memorial Circle, Quezon City, Philippines.

The researchers followed the protocol for collection, extraction, processing, and preparation of nematode slide mounts given by Mr. Romnick Latina, an Assistant Professor at the Institute of Weed Science, Entomology, and Plant Pathology at the University of the Philippines.

Nematode extraction

The samples were processed for isolation using the Baermann Tray method at the Institute of Weed Science, Entomology, and Plant Pathology at the University of the Philippines Los Baños (UPLB). A volume of two hundred ccs of soil and one hundred grams of roots per plant samples were put in a two-ply of tissue paper and then placed above the screen inside the tray. An ample amount of water was poured to cover the whole sample and was incubated. After one week, the water and the debris on the bowls were filtered using the method of wet sieving. The suspension was poured on the stack of sieves (108 µm, 45 µm, and 38 µm, from top to bottom). The sieves were rinsed one by one while tilted to an angle of 45˚. The soil nematode suspension was collected from the terminal sieve by concentrating the particulates on one side and carefully poured into a glass bottle (Latina, 2018).

Fixative and mounting medium

Double strength (2x) formalin-glycerol was used as a fixative for nematodes. It was prepared using 100 ml/ 40 % formalin, 10 ml of glycerol, and 450 ml of distilled water with a total volume of 560 ml. From the (2x) FG, (1x) FG was used as a mounting medium. It was prepared by adding an equal volume of distilled water (Latina, 2018).

Killing and fixation

For killing and heat fixation, a two-step procedure was done. In this method, fixation was done after killing. Water was heated to nearly boiling point in a pot. The glass bottles containing nematode suspension was placed in a basket with hot water or 2-3 minutes. After that, the glass bottles were removed, and an equal amount of double strength fixative was added. For 24 hours, the suspension was allowed to be fixed at room temperature (Latina, 2018).

Preparation of temporary mounts

Temporary mounts were prepared for the identification of nematodes. A nail polish ring on a glass slide was first made. A circle was engraved in the slide by letting the tip of the nail polish brush touch it while rotating the ringer device. The thickness of the ring was ensured to be even and was allowed to harden.

Picking out and mounting of nematodes was next done. A (1x) FG mounting medium was dropped at the center of the nail polish ring then the fixed nematode suspension was poured in a petri dish and was examined under a dissecting microscope. The target nematode was located and transferred in a mounting medium at the center of the nail polish ring using a picker (fine needle or bamboo splinter). The slide was examined thoroughly under the microscope to confirm if the nematode was successfully transferred. The coverslip was carefully placed in the slide, and labels were written at the end of the glass slide (Latina, 2018).

Population, sample size, and sampling technique

Sarian Farm, located in Juan Sumulong Road, Teresa, Rizal, wasselected as a sampling site due to the abundance of citrus plants in the location. Four seedlings were randomly collected base on the symptoms of the presence of nematodes. The first two seedlings were collected in the month of November, and the last two seedlings were collected in the month of January. Two hundred cc of soil and one hundred grams of roots per plant samples were processed.

Nematode count

For occurrence rate, the petri dish was divided into four quadrants then the nematode suspension was poured and counted.

Morphological identification

Mounted nematodes were observed under a compound light microscope (Euromex) for identification. Morphological identification was the method used to classify the base of the nematode on their physical characters like the stylet, knobs, tail, and buccal cavity, lip region, location of body regions, and the size and length of the body. Photographs were taken after each observation.

Dichotomous keys (Mekete *et al.,* 2012) and pictures from the database (NemaPlex) and related literature (Inserra *et al.,* 1988) were used for genus-level identification while morphometrics was done to verify the species of the plant-parasitic nematode. The measurements and ratios obtained were then compared to other related studies for verification.

All the observed slides and photographs were examined by Asst. Prof. Romnick A. Latina of the University of the Philippines Los Baños.

Results

Collection of nematodes

The soil samples were collected at Sarian Farm, Juan Sumulong Road, about 30 meters on the right side before the Teresa-Morong boundary, Teresa, Rizal. The coordinates of sampling site are 14°34'11.92"N and 121°13'21.26"E with an elevation of 53.79 Meters (176.48 Feet) above sea level and a temperature of 25˚C/32 ˚C. Sarian Farm is a 2-hectare farm that has a variety of different fruit-bearing plants. It has a variety of makopa, mangoes, and exotic plants like cacao, marang, and pomegranate, and most especially types of citrus fruits were available in the farm like lime, calamansi, orange, and pomelo.

Table 1. Qualitative soil analysis

LAB NO.	Description	Test Result pH Nitrogen (N) Phosphorus				
		pH	Nitrogen (N)	Phosphorus (P)	Potassium (K)	Texture Feel Method
S-307	11/13/2018	5.0	Low	Moderately High	Sufficient +++	Light
S-308	1/29/2019	6.5	High	Medium	Sufficient +++	Heavy
S-65	Bumanglag and Soliman, 2019	6.9	Moderately low	High	Sufficient +	Heavy

Two types of soil were tested by the researchers in the Bureau of Soils and Water Management in Diliman, Quezon City to describe the soil type, the presence of Nitrogen, Phosphorus, and Potassium and the potential of Hydrogen of the soil. The soil was differentiating based on the time it was acquired by the researchers. Another Soil Analysis result is presented, which soil is also obtained from Sarian Farm.

Sample S-307 is a light soil with a pH level of 5.0. Its Nitrogen level is low, the Phosphorus level is moderately high, and the Potassium level is Sufficient+++. On the other hand, sample S-308 is a heavy soil with a pH level of 6.5. Its Nitrogen level is high, the Phosphorus level is medium, and the Potassium level is also sufficient+++. Sample 65 is a heavy soil with a pH level of 6.9. Its Nitrogen level is moderately low, Phosphorus level is High, and the Potassium level is sufficient+.

Nematodes found in Citrus maxima

A total of 2,275 individual species of isolated nematodes were recorded in *C. maxima*, including three free-living orders: *Dorylaimida*, *Mononchida*, and *Rhabditida*; and one genus of plant-parasitic nematodes namely, *Tylenchulus*. There are also unidentified free-living and unidentified plant-parasitic nematodes recorded.

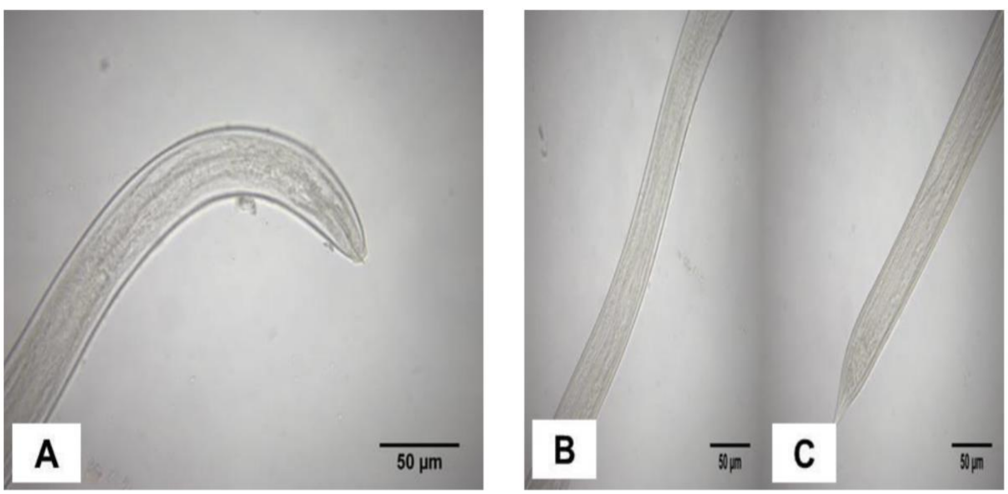

Figure 1. *Dorylaimidan Aphelenchus* spp. in HPO: (A) Head; (B) Middle region; (C) Tail (Scale bars = 50 µm)

Figure 2. *Mononchid* (*Mononchus* spp.**)** in LPO: (A) Whole body, (Scale bars = 200 µm)

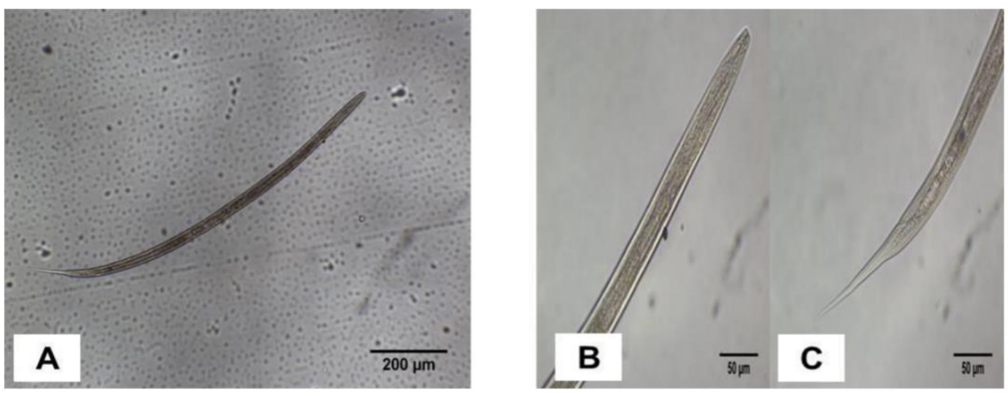

Figure 3. *Rhabditis* spp. under *Order* Rhabditida (A) Whole body in LPO (Scale bars = 200 µm); (B) Head in HPO (Scale bars = 50 µm); (C) Tail in HPO (Scale bars = 50 µm)

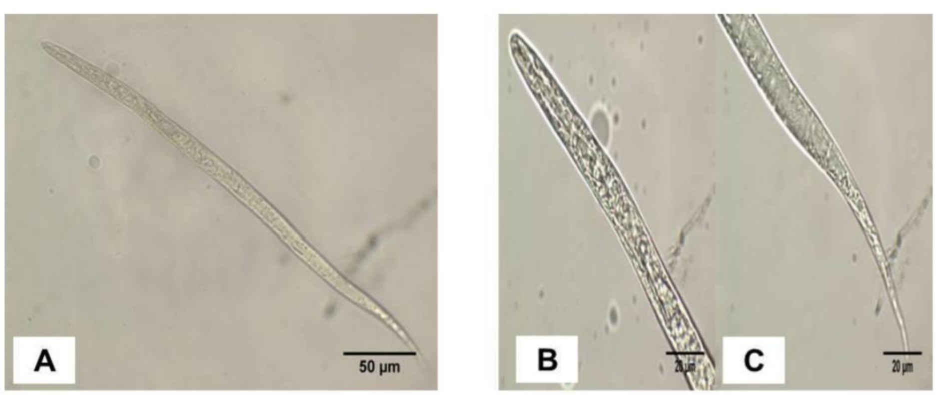

Figure 4. *Tylenchulus* sp.: (A) Whole body in HPO (Scale bars = 50 µm), (B) Head in OIO (Scale bars = 20 µm), (C) Tail in HPO (Scale bars = 20 µm)

Table 2. Occurrence rate of isolated nematodes

Nematodes	**Count**	**per 100 cc**	**Count**	**Frequency**	**Relative Frequency (%)**	**Remarks**
			Free-living			
Aphelenchus spp.	52	2.6	<5	4	80	Very Low
Rhabditis spp.	48	2.4	<5	3	60	Very Low
Mononchus spp.	6	0.3	<5	3	60	Very Low
Unidentified FLN	137	6.85	7	2	40	Very Low
			Plant parasitic			
Unidentified PPN	2	0.1	<5	1	20	Very Low
Tylenchulus sp.	2030	101.5	101	5	100	Low

The highest in count per individual was the *Tylenchulus* sp., with more than 2000 individuals. Though the count suggested the presence of infestation, the intensity was low, with 101 juveniles/100 g compared to the damage threshold of 850 juveniles/100 g (Duncan, 2005). Generally, the biotic factors affect the population of plant-parasitic nematode; however, the density of Mononchids was very low to affect the *Tylenchulus* (Yadav, 2017; Bernard *et al.*, 2017).

The count of *Aphelenchus* spp. (52 individuals) and *Rhabditis* spp. (48 individuals) nematodes are significantly higher than *Mononchus* spp. (6 individuals).

Discussion

Symptoms on the presence of nematodes on C. maxima

The seedlings collected exhibits signs of the presence of nematodes like chlorosis, reduced vigor, leaf fall, and stunted growth. The symptoms observed are typical if there is a nematode. Still, they are not diagnostic because these damages are also influenced by several factors, including aggressiveness of the nematode population, soil characteristics, susceptibility of the rootstock, presence of other pathogens like fungi, and grove management practices (Duncan and Cohn, 1990).

Aboveground symptoms of nematode damage are lack of vigor, twig dieback, a decline in growth, and reduced fruit size and yield. Nematode infestations can occur without inducing any aboveground symptoms. Below ground symptoms of a citrus nematode infestation are weak growth of feeder roots, soil adhering to roots giving them a dirty appearance, and causes galling of root tips.

Soil analysis

According to Ardakani *et al.* (2014), a soil pH of seven shows a maximum number of nematodes, and an increase or decrease from seven would decrease the nematode population. The S-65 has the highest pH level of the three samples, next to S-307 and S-308. Thus, findings suggest that S-65 has a higher number of nematodes than that of S308 and S-307, based on pH levels.

Standard critical levels of Nitrogen, Phosphorus and Potassium in the soil of the citrus is habitable for nematodes with a directly proportional relationship with the increase to this level, except for the Nitrogen levels, wherein it has an inversely proportional relationship with the nematode population (Ardakani *et al.*, 2014). The Nitrogen and Phosphorus level of S-308 is high and medium, making it have a less dense population than S-307 with Low N level and Moderately High P level. S-65 has more or less the same population density of S-308 with moderately low N level and High P level.

The potassium content of the soil was positively related to densities of J2 and males in soil, a relationship known to occur in other crops (Sharma *et al.*, 2005). Both samples have the result of Sufficient+++, making them equal in terms of population density for J2 and male nematodes. S-65 has the result of Sufficient+, which is lesser than the two samples.

Dorylaimida

Dorylaimids are known to have an odontostyle, a movable mural tooth, or spear, originating from a migrated stomal cell. Their cheilostome, a lip cavity of the stoma, includes a vestibulum and a guiding apparatus that can be seen as a single or double ring around the odontostyle. Odontostyle extension originates from Esophostome, an esophageal cell. The cuticle is smooth or striated, in some species. It has a two-part esophagus that is slender anteriorly and has a swollen posterior glandular and muscular region. Cardia is present at the esophageal-intestinal junction. Nerve ring surrounds the thin part of the esophagus. The female reproductive system varies from monovarial, prodelphic, or opisthodelphic, or diovarial, amphidelphic. Male with paired spicules and a long vas deferens leading to paired and opposed testes. Gubernaculum can be seen sometimes. Tail shape is varied, often diverging between sexes. The species of nematodes in most families of the *Dorylaimida* do not feed on higher plants; they feed on algae, fungi, or predator of other nematodes (Ferris, 2018a).

The nematode was identified to be an *Aphelenchus* spp. because of the tapering to narrow tip at the posterior part of the body. Female *Aphelenchus* spp. have a medium sized body, ranging (733.30 ± 28.85) µm long. Body is linear to ventrally arcuate upon fixation. Cephalic region is low, flattened and slightly offset. They possess 5 annuli in the cephalic region. Body cuticle coarsely annulated. With lateral fields, each has a single ridge (2 incisures) running throughout the length of the body. the odontostylet is slender without basal swellings, used for piercing the fungi. the procorpus is cylindrical, from the anterior end of the body, and somewhat narrowing just before joining the large muscular, ovoid median bulb and with centrally placed, crescentic valve plates. The Oesophageal glands are on a dorsal lobe, oesophago–intestinal junction from the anterior cephalic region. the nerve ring behind the median bulb from the anterior end of the cephalic region. Excretory pore above the nerve ring from the cephalic end. Vulva posterior at about 70–75 % of the body length. The vulval lips in females is slightly protuberant and the body often narrowing sharply just behind the vulva. Vagina is sloping anteriorly. Genital tract is monoprodelphic, outstretched, spermatheca not fully segmented, filled with sperms, oocytes arranged in single row, tip of the oocytes sometimes reaching the base of the end bulb. Post uterine sac narrow elongated. Tail long, cylindrical, sometimes slightly ventrally concave, ending in a broadly rounded terminus.

Male Aphelenchus nematodes have spicules paired, slender, and ventrally arcuate. Gubernaculum is linear; Bursa not clearly seen. Tailed elongated a caudal papilli with a large mucro-like structure. Tails are long, cylindrical, sometimes slightly ventrally concave, ending in a broadly rounded terminus (Bina Chanu *et al*., 2017).

Mononchus species

Species under Order *Mononchida* are distinguished by a strong, cuticularized stoma with one or more large teeth. Stoma may also have rows of denticles. They have long, muscular cylindrical esophagus. They are known to be predaceous and cannibalistic, with juveniles may be bacterivore. Mononchids inhabits only soil and freshwater, and has no marine species (Ferris, 2018b). *Mononchus* spp. have a distinct large dorsal tooth in a barrel-shaped buccal cavity. the buccal cavity has a transverse subventral rib and the tail has a distinct terminal spinneret.

Rhabditis species

The body length of species under Order *Rhabditida r*anges from 11- 59 µm. Cuticle surface is smooth or finely annulated and is longitudinally striated, often bearing 7–11 lines (ridges or incisures) with three prominent ridges or bands in the middle. Lip region flattened anteriorly, slightly higher in females than males. Six lips bear two circles of sensilla arranged in three pairs and each lip with one apically inserted, papilliform sensillum. The stoma capsule is relatively broad and is short isomorphic/isotropic. Metacorpus is muscular that forms a swollen median bulb, and the Isthmus is distinct. Excretory pore behind basal base is about 106 µm from the anterior end (Huang *et al.*, 2015). Due to the diversity and arising of new species under Order Rhabditidae, we will confine the identity of the specimen as *Rhabditis* spp. (Sudhaus, 2011).

Tylenchulus

The body length of *Tylenchulus* ranges from 328-477 µm. Its tail is cylindrical and thick, that ends in a bluntly rounded terminus. After fixation, its body is vermiform, straight, or slightly arcuate, slender, and with faint transverse striae. A stylet is delicate that has small, rounded knobs. Procorpus and metacorpus are not interfused, the isthmus is distinct, post corpus in the bulb is also present. Excretory pore is situated at half of the body from the head. The Lip region is hemispherical, and the annuli are not distinct (Maafi *et al.*, 2012).

Tylenchulus is a nematode that occurs worldwide in citrus growing areas and causes a severe disease known as a slow decline. *Tylenchulus* is the primary cause of the decrease in the production of citrus. It does not kill the trees, but the growth and yield of infected trees slowly decrease. The severity of slow decline can vary from minor to severe reduction of infected trees. Symptom expression is influenced by several physical and chemical factors, as shown in the collected samples.

The nematodes found were divided into three-function group: Order *Dorylaimida* and *Rhabditida* comprised the bacterivorous and fungivorous group; the *Mononchida* comprised the predatory group, and *Tylenchulus* sp. composed the plant-parasitic group. Other nematodes were classified under free-living nematode primarily due to the absence of stylet. Identification did not proceed due to obscure and/or damaged characters of these individuals. Two individuals were labeled plant-parasitic due to the presence of stylet but were not identified due to damage to key characteristics. The free-living species of the former two are commonly associated with the presence of soil bacteria and fungi (Ferris, 2018a,). Hence, the effect of the organic matter in the soil dramatically affects their population (Ferris, 2018a; Bernard *et al.*, 2017).

1	Styletpresent…………………………………………	2
	Stylet absent…………………………………………	3
2	Knobs prominent, lip region round, elongated tail, tapering with round tips……………………………..	*Tylenchulus*
	Knobs absent, lip region slightly pointed, stubby tail, tapering with pointed tip……………………………..	*Dorylaimida*
3	Buccal cavity tubular, lip region pointed, amphids visible, tail elongated………………………………..	*Rhabditida*
	Buccal cavity U-shaped, lip region flat, tail either stubby or elongated…………………………………..	*Mononchida*
1	Buccal cavity U shaped …………………………...	*Mononchida*
	Buccal cavity cylindrical…………………………….	2
2	Stylet present…………………………………………	3
	Stylet absent…………………………………………	*Rhabditida*
3	Knobs present………………………………………..	*Tylenchulus* sp.
	Knobs absent…………………………………………	*Dorylaimida*
1	Esophagus1or 2 parts………………………………..	2
	Esophagus1or 2 parts………………………………..	3
2	Buccalcavity cylindrical……………………………..	*Dorylaimida*
	Buccalcavity cylindrical……………………………..	*Mononchida*
3	Stylet present…………………………………………	*Tylenchulus* sp.
	Stylet absent…………………………………………	*Rhabditida*

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