

Metabarcoding of nematode communities associated with *Ananas comosus* L. (Pineapple)

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Abstract

Plant-parasitic nematodes are serious pests causing important crop losses worldwide. Hence, this study was conducted to determine the occurrence of plant-parasitic nematodes (PPNs) in healthy and unhealthy pineapple crops. To determine if the infestation is prevalent, a soil metabarcoding analysis was done. Six soil samples were obtained from the rhizospheres of Red Spanish pineapple farm in Mabitac, Laguna, Philippines. They were freeze-dried and brought to the laboratory for metagenomics analysis using Primers NemF and 18Sr2b. The results showed uncultured Eukaryotes (43.1%) nematodes (31.2%), Platyhelminthes (6.9%), Apicomplexa (6.5%), Annelida (5.0%), Rotifera (3.6%), Arthropoda (1.8%), Ascomycota (1%), unidentified (0.4%) and Basillariophyta, Cercozoa, Chytridiomycota, Mollusca and Mucoromycota with 0.1%. In total, 374,410 sequence reads were obtained and were clustered into 117 OTUs at 97% similarity. In assessing the nematode community structure, it yielded 26,565 nematodes; 5,315 nematodes were obtained from the rhizosphere of healthy samples, and 21,250 were from the unhealthy ones. The fungivore *Apelenchus avenae* (Bastian 1865) was the most prevalent (47.8% and 43.9%) in both locations. Other nematodes found on the plant rhizospheres were *Rhabdolaimus aquaticus* (de Man), *Acrobeloides varius*, *Rotylenchulus reniformis* (Linford and Oliveira, 1940), *Aphelenchoidinae sp.*, *Panagrolaimoidea sp.*, and two unidentified nematodes. The *Basiria sp.*, *Alaimus sp.*, and two other unidentified nematodes were the only ones found in the rhizosphere of unhealthy pineapple plants. Meanwhile, *Mesocriconema onoense*, *Aphelenchoides sp.*, *Ditylenchus gilanicus*, and an unidentified nematode were found in the rhizosphere of healthy pineapple plants. Phylogenetic analyses of the nematode communities show that only Orders *Rhabditida* and *Enoplida* are associated with the crop and the Family *Aphelenchoididae* is distant from *Rhabditida*, thus, dividing the clade into three. The first clade consists of diverse nematode communities of fungivores, bacterivores, predators, and plant and insect parasites. The second clade consists of bacterivore nematodes found in moist environments, and the third clade comprises mainly *Aphelenchoididae* species, mostly plant parasites.

Keywords: Metabarcoding, *Acrobeloides varius*, *Aphelenchus avenae*, *Ditylenchus gilanicus*, *Mesocriconema onoense*, *Rhabdolaimus aquaticus*, *Rotylenchulus reniformis*

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Introduction

Pineapple is one of the major fruit crops in the world, including the Philippines. The country ranks third in production worldwide, with an estimated production of 2.86 million metric tons for the past five years (Shahbandeh, 2023). The pineapple plantation area is more than 40,000 hectares, and the concentration of production is in Mindanao and Luzon and is controlled by international companies. The most widely grown cultivars are Queen, Cayenne, Philippine Native or Red Spanish, and the newly developed MD2. In 2019, the country was the second largest pineapple exporter, according to the Food and Agriculture Organization (Hossain, 2016), and was considered the primary export product of the Philippines, same with bananas (Voora et al., 2020).

Pineapple production and exportation can be considered at their peak in the Philippines (Balito, 2010). However, problems and challenges might arise and need to be addressed. These include natural calamities, soil degradation, and disease and pest infestation. One of the pests that pose a significant threat to the crop is the plant-parasitic nematodes (Bugnay-Inso and Pedroche, 2021). It has been reported by Garcia et al. (2022) that these roundworms can affect root function, which could lead to crop deterioration and, eventually, death. Thus, it would lead to a substantial decline in yield (approximately 60%). Nematodes are one of the most diverse creatures on earth. Due to its enormity and complexity, the Environmental Metagenomic DNA sequencing technique was used to shed light on the biodiversity and function of the local nematode community. The technique gives details to find new taxa and genes, construct novel genomes, assess community structure and diversity, and find out which metabolic pathways are encoded in the community (Sharpton, 2014). In this study, environmental metagenomics analysis was performed to determine the diversity of nematodes associated with healthy and unhealthy pineapple plants.

Since nematodes are diverse, this study aimed to identify the nematode communities associated with healthy and unhealthy pineapple plants. Thus, it will provide a glimpse of the nematodes that could potentially be pests in pineapple.

Material and Methods

Sampling site description

The Pineapple farm sampling site is located at

14°27'31.56588 North and 121°22'6.6 East of Mabitac, Laguna, Philippines, and with an estimated size of 10,000 m² where the Red Spanish pineapple variety is grown, a native in the Philippines (Figure 1-A and B). The area can be characterized by patches of healthy and unhealthy pineapple plants. Soil sampling was done during the pineapple's fruiting stage, around October 2021. The characterization of healthy and unhealthy plants was based on their aboveground symptoms, such as chlorosis and absence or inability to bear fruits.



Figure 1. Pineapple plantation in Mabitac, Laguna, Philippines. A) Healthy pineapple plants B) Unhealthy pineapple plants

Soil sample collection

Using the Protocol of Sapkota and Nicolaisen (2015), Soil samples were collected from the rhizospheres of healthy and unhealthy pineapple plants. Three soil samples were collected from each field and were mixed thoroughly. A kilogram of the composite samples was obtained. Subsequently, 1 g of soil for each sampling tube was taken and freeze-dried for 48 hours.

Library construction

DNAs/RNAs were extracted from the samples. Quality control was done, and the samples advanced to library construction. The sequencing library was prepared by arbitrary fragmentation of the DNA or cDNA sample, followed by 5' and 3' adapter ligation. PCR amplification and gel purification followed for the adapter-ligated fragments (Head et al., 2014). To efficiently determine the nematode communities accurately and quantitatively, Next-Generation Sequencing (NGS) was used, where the nucleotide sequences were determined using PCR amplification. DNA sequencing followed, and nematodes were classified based on their DNA barcode sequences (Kenmotsu et al., 2020). Phylogenetic analyses were also done to determine the

relationship of nematodes found associated with pineapple.

Primer designing

The primer design that was used was based on the method of Porazinska et al. (2009) and Sapkota and Nicolaisen (2015) and can mostly exclude bacterial, fungal, and plant DNA.

Table-1. Primers used in the study.

Primer	5' – 3' sequence	Reference
NemF	GGGGAAGTATGGTTGCAA	Sapkota and Nicolaisen (2015)
18Sr2b	TACAAAGGGCAGGGACGTAAT	Porazinska et al. (2009)

DNA extraction and PCR amplification

The starting genomic material was quantified using Picogreen (Invitrogen, cat. #P7589) using Victor 3 fluorometry. Subsequently, gel electrophoresis was done to evaluate the state of the DNA. For the library QC method, a library size check was done, and the size of PCR-enriched fragments was verified. The template size distribution was checked by running on Agilent Technologies 2100 Bioanalyzer using a DNA 1000 chip. For Library Quantity Check, the Illumina library was used to achieve the highest quality data on Illumina sequencing platforms. The prepared libraries were quantified using qPCR according to the Illumina qPCR Quantification Protocol Guide. To generate a standard curve of fluorescence readings and calculate the library sample concentration, Qubit standard Quantification solution and calculator were used (Ahn et al., 1996).

Metabarcoding of nematode populations

Using the NGS method in a metagenomic amplicon, samples underwent three stages: the preliminary processing and clustering stage using CD-HIT-OUT (MiSeq FLX), pre-processing and clustering stage using rDNA tools (PacBio), and lastly, the taxonomic assignment and diversity statistics stage (QIIME). In the first stage, sequences were assembled through MiSeqPE using FLASH, producing assembled reads. Filtered reads were clustered at 100% identity using CD-HIT-DUP. Assembled reads were pre-processed and clustered using CID-HIT-OUT to identify chimeras and denoising clusters of size, filtering non-chimeric clusters at a user-specified 97% ID at species level OTU cut-off (Li et al., 2012). Short, ambiguous

reads and extra-long tails were removed, including identity chimeras using rDNA tools PacBio (Schloss et al., 2009). The stage generated a distance matrix and clustered using the average neighbor method.

Taxonomic assignment and diversity statistics were computed using QIIME using representative sequences from each OTU to assign taxonomy. Phylogenetic trees were constructed from aligned and filtered representative sequences of OTUs. In constructing diversity analysis, Alpha diversity was computed using alpha_diversity.py. Taxonomic compositions were identified using UCLUST/ RDP (16s or UNITE (ITS) program application. Alpha-rarefaction.py program was used to compute Alpha rarefaction and produced the rarefaction curve graph. PCoA graphs -2D were made using make_2D_plots.py from PCoA. In making the UPGMA tree, upgma_cluster.py was used in the UPGMA tree graph. The heat map was made using the make_otu_heatmap.html.py to produce an OUT-heat map. Finally, the publication of quality graphic results was made through statistical analysis and visualization. For a taxonomic assignment, QIIME-UCLUST was used for picking OTUs at 99% similarity since this has been found to be suitable in defining Nematodes OTUs (Porazinska et al., 2012). The RDP 16S rDNA, UNITE-ITS, NCBI-18S, and CO1 were used as references for taxonomic assignments of OTUs. While for diversity analysis and visualizations, QIIME alone was used (Caporaso et al., 2010).

Evolutionary analysis by Maximum Likelihood method

The Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980) were used to create inferences for evolutionary history. The evolutionary tree with the highest log likelihood (-3778.56) was presented, and the percentage of the trees showing the associated taxa clustered together next to the branches was shown. Neighbor-Join and BioNJ algorithms were used and projected by the Maximum Composite Likelihood (MCL) approach for the empirical search of the initial tree(s). A distinct Gamma dispersal was used to determine the evolutionary differences among sites (5 categories (+G, parameter = 0.4215). The analysis involved 37 nucleotide sequences and 538 positions in the final dataset. Evolutionary analyses were then conducted in MEGA 11 (Tamura et al., 2021).



Results

Nematodes found associated with pineapple

A higher proportion of uncultured Eukaryotes were obtained (43.1%) based on the Phylum rank from the soil samples. It was followed by nematodes (31.2%), *Platyhelminthes* (6.9%), *Apicomplexa* (6.5%), *Annelida* (5.0%), *Rotifera* (3.6%), *Arthropoda* (1.8%), *Ascomycota* (1%), unidentified (0.4%) and *Basillariophyta*, *Cercozoa*, *Chytridiomycota*, *Mollusca* and *Mucoromycota* with 0.1%. In total, 374,410 sequence reads were obtained and clustered into 117 OTUs at 97% similarity. Of the total clustered sequences, 16% were classified as belonging to Phylum *Nematoda* (Figure 2).

A total of 21,250 (Table 1) nematodes were found in the rhizospheres of unhealthy pineapple plants, while only 5,350 were found in the rhizospheres of healthy pineapple plants. *Aphelenchus avenae*, *Rhabdolaimus aquaticus*, *Acrobeloides varius*, *Rotylenchulus reniformis*, *Aphelenchoidinae sp.*, *Panagrolaimoidea sp.*, and two unidentified nematodes were found in the rhizospheres. At the same time, only *Basiria sp.*, *Alaimus sp.*, and two unidentified nematodes (Accession num. EU880141.1 and EU880042.1) were found in soil with unhealthy pineapple plants. *Mesocriconema onoense*, *Aphelenchoides sp.*, *Ditylenchus gilanicus*, and an unidentified nematode (Accession No. EU880147.1) were found in the soil with healthy pineapple plants.

Among the nematode species found in the rhizospheres of healthy and unhealthy pineapple plants, *Aphelenchus avenae* was the most numerous (47.83% - healthy, 43.93% -unhealthy (Figure 2). At the same time, the unidentified nematode (EU880054.1) (9.84%) and *Acrobeloides varius* (7.30%) were much higher in the rhizospheres of unhealthy pineapple plants than those found in healthy ones. The rest, such as *Rhabdolaimus aquaticus* (31.33%), *Rotylenchulus reniformis* (13.72%), *Aphelenchoidinae sp.* (1.83%), *Panagrolaimoidea sp.* (0.41%) and one unidentified nematode (Accession num. EU880051.1) (1.57%) were higher in the rhizospheres of healthy pineapple plants. In the case of nematodes found only in the

rhizospheres of unhealthy pineapple plants, *Basiria sp.* was found next to *Aphelenchus avenae*, constituting 19.06%. It was followed by unidentified nematodes (Accession number. EU880141.1 and EU880042.1), constituting 10.46% and 0.09%, respectively. While nematodes exclusively found in the rhizospheres of healthy pineapple plants were *Mesocriconema onoense*, *Aphelenchoides sp.*, *Ditylenchus gilanicus* and an unidentified nematode (Accession No. EU880147.1) constitute 0.34%, 0.22%, 0.28%, and 0.06% respectively.

Phylogenetic analysis of the nematodes associated with pineapple

Not all nematode lineages were represented on the dataset. The phylogenetic tree (Figure 3) shows three clade groups, the first clade of nematodes belongs to the group under Order *Rhabditida*, the second under Order *Enoplida*, and the third clade of nematodes also belongs to Order *Rhabditida* under the family *Aphelenchidae* (Ruiz-Cuenca and Abolafia, 2022).

Those belonging to Order *Rhabditida* were divided into six families: *Anguinidae*, *Aphelenchidae*, *Cephalobidae*, *Criconematidae*, *Hoplolaimidae*, and *Tylenchidae*. The unidentified nematode (132) obtained was identical to that of unidentified species found in Costa Rica (EU880054.1) and 98% related to *Acrobeloides varius*. At the same time, *Aphelenchus avenae* (3733, 28156) is 75% like that of *A. avenae* found in Belgium (EU306347.1). *Ditylenchus gilanicus* (7712) is 100% identical to that of *D. gilanicus* found in Iran (MG742324.1). Same with the unidentified nematode (2813), *Basiria sp.* (14772), *Rotylenchulus reniformis* (45371, 2468), *Mesocriconema onoense* (8610), and *Panagrolaimoidea sp.*, (18920), which were 100% similar to that of the unidentified nematode found in Costa Rica (EU880054.1), *Basiria sp.* from the Netherlands (KJ869354.1), *R. reniformis* (KR153027.1, KR152997.1), *M. onoense* (MF094909.1) and *Panagrolaimoidea sp.*, (AB663162.1) from the USA.



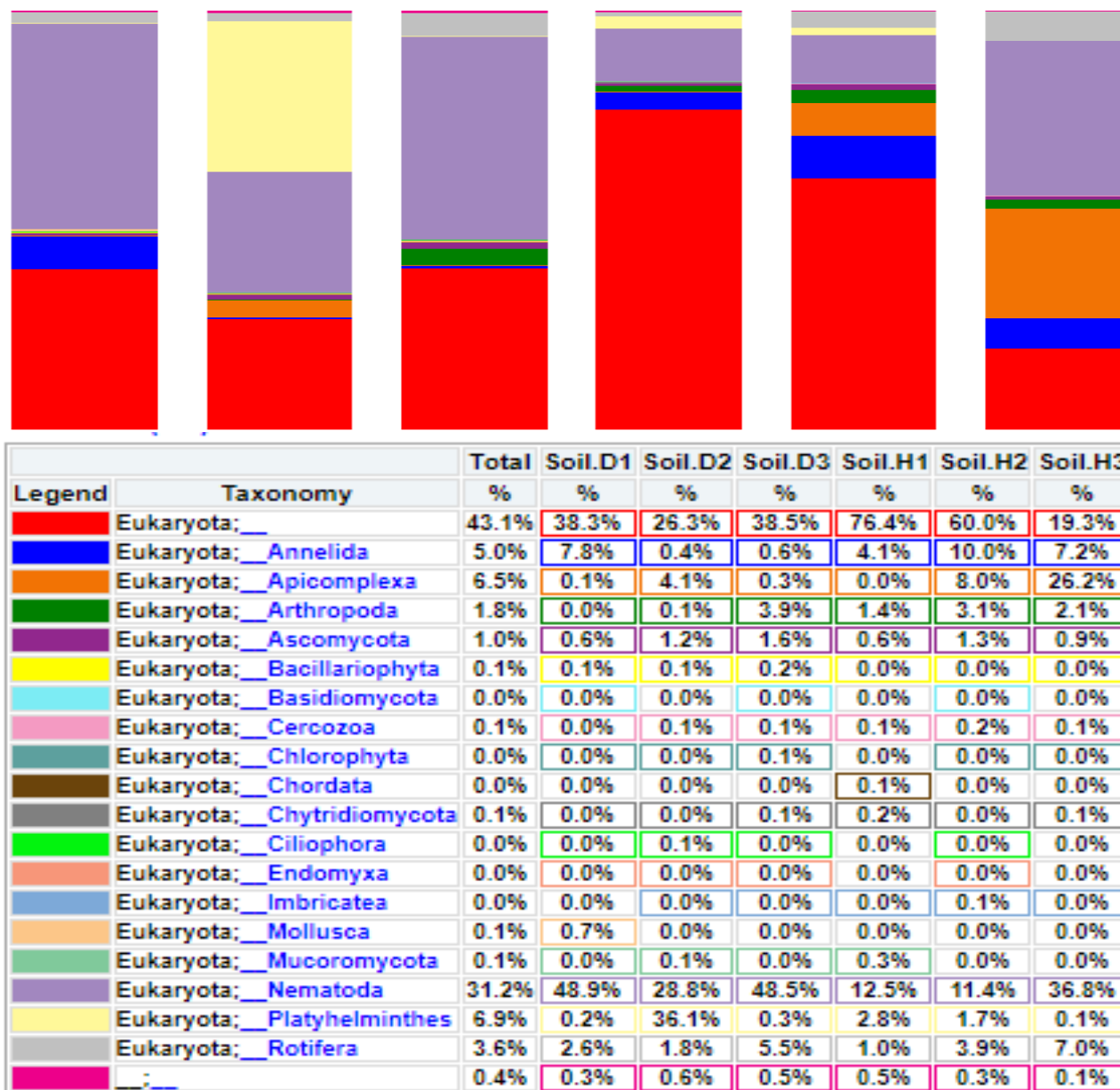


Figure-2. The comparative distribution of sequences in the total dataset at phylum rank for all soil samples. (Soil D1, D2, D3 – Unhealthy pineapple plants; Soil H1, H2, H3 – Healthy pineapple plants)

Table-2. Nematode species found in the rhizospheres of healthy and unhealthy pineapple plants.

Species	Rhizospheres Occurrence in Healthy Pineapple	Percentage of Occurrence	Rhizospheres Occurrence in Unhealthy Pineapple	Percentage of Occurrence
<i>Aphelenchusavenae</i>	2559	47.83	9335	43.93
<i>Basiria sp.</i>	0	0	4050	19.06
<i>unidentified nematode</i>	0	0	2222	10.46
<i>Rhabdolaimus aquaticus</i>	1676	31.33	485	2.282
<i>unidentified nematode</i>	70	1.308	2090	9.835
<i>Acrobeloidesvarius</i>	55	1.028	1552	7.304

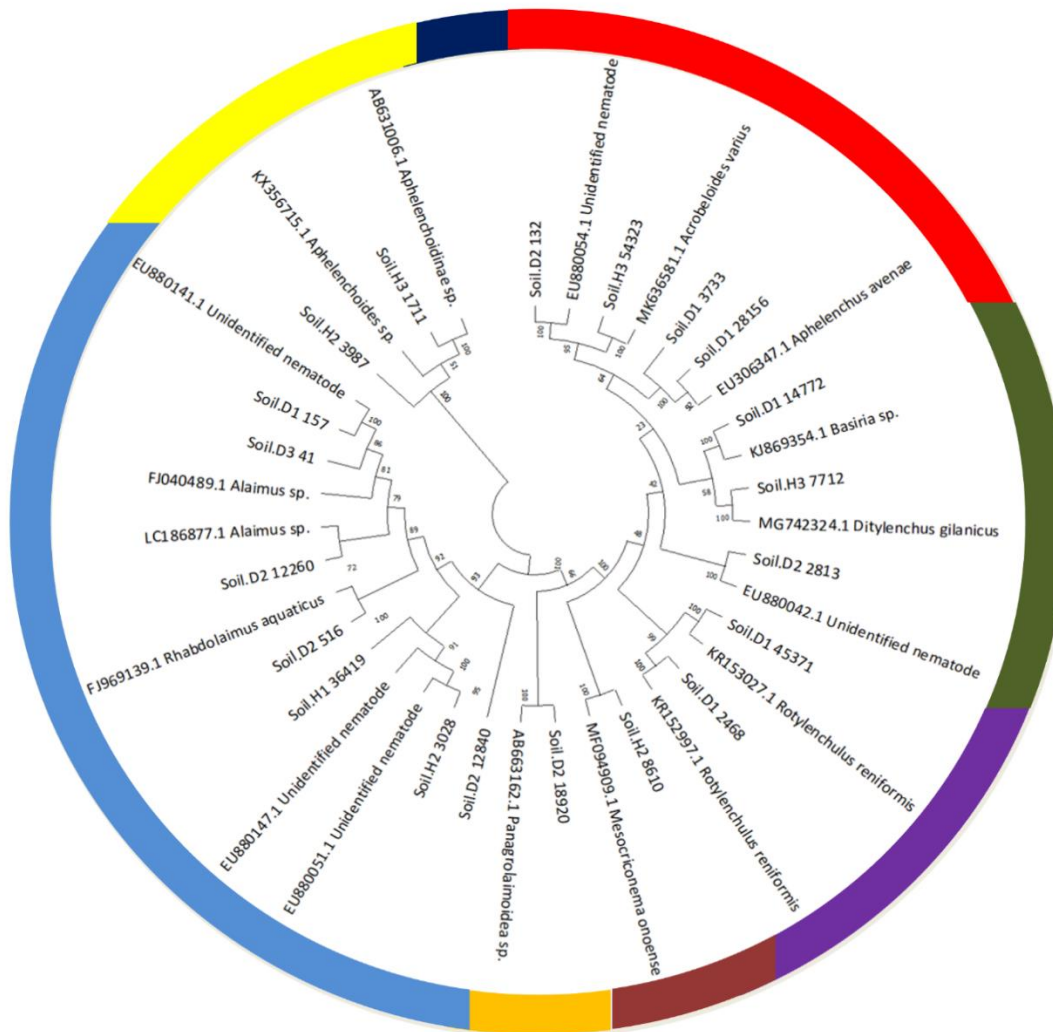


<i>Alaimus sp.</i>	0	0	1357	6.386
<i>Rotylenchulusreniformis</i>	406	7.589	23	0.108
<i>Rotylenchulusreniformis</i>	328	6.131	20	0.094
<i>Aphelenchoidinae sp.</i>	98	1.832	15	0.071
<i>Panagrolaimoidea sp.</i>	22	0.411	14	0.066
<i>Aphelenchusavenae</i>	3	0.056	23	0.108
<i>Mesocriconemaonoense</i>	18	0.336	0	0
<i>Aphelenchoides sp.</i>	12	0.224	0	0
<i>Ditylenchusgilanicus</i>	15	0.28	0	0
<i>unidentified nematode</i>	84	1.57	42	0.198
<i>unidentified nematode</i>	0	0	19	0.089
<i>Alaimus sp.</i>	1	0.019	3	0.014
<i>unidentified nematode</i>	3	0.056	0	0
TOTAL	5,350	100	21,250	100

Table-3. List of nematode species identified and their GenBank Accession Number

Species Name	Strain Isolate Number	Substrate of Isolation	Origin	GenBank Accession Number of Reference sequences
				SSU
<i>Aphelenchusavenae</i>	Soil.D1 3733	Plant Rhizosphere	Mabitac, Laguna, Philippines	EU306347.1
<i>Basiria sp.</i>	Soil.D1 14772	Plant Rhizosphere	Mabitac, Laguna, Philippines	KJ869354.1
<i>unidentified nematode</i>	Soil.D2 132	Plant Rhizosphere	Mabitac, Laguna, Philippines	EU880141.1
<i>Rhabdolaimusaquaticus</i>	Soil.D2 516	Plant Rhizosphere	Mabitac, Laguna, Philippines	FJ969139.1
<i>unidentified nematode</i>	Soil.D2 132	Plant Rhizosphere	Mabitac, Laguna, Philippines	EU880054.1
<i>Acrobelloidesvarius</i>	Soil.H3 54323	Plant Rhizosphere	Mabitac, Laguna, Philippines	MK636581.1
<i>Alaimus sp.</i>	Soil.D3 41	Plant Rhizosphere	Mabitac, Laguna, Philippines	FJ040489.1
<i>Rotylenchulusreniformis</i>	Soil.D1 45371	Plant Rhizosphere	Mabitac, Laguna, Philippines	KR153027.1
<i>Rotylenchulusreniformis</i>	Soil.D1 2468	Plant Rhizosphere	Mabitac, Laguna, Philippines	KR152997.1
<i>Aphelenchoidinae sp.</i>	Soil.H3 1711	Plant Rhizosphere	Mabitac, Laguna, Philippines	AB631006.1
<i>Panagrolaimoidea sp.</i>	Soil.D2 18920	Plant Rhizosphere	Mabitac, Laguna, Philippines	AB663162.1
<i>Aphelenchusavenae</i>	Soil.D1 28156	Plant Rhizosphere	Mabitac, Laguna, Philippines	EU306347.1
<i>Mesocriconemaonoense</i>	Soil.H2 8610	Plant Rhizosphere	Mabitac, Laguna, Philippines	MF094909.1
<i>Aphelenchoides sp.</i>	Soil.H2 3987	Plant Rhizosphere	Mabitac, Laguna, Philippines	KX356715.1
<i>Ditylenchusgilanicus</i>	Soil.H3 7712	Plant Rhizosphere	Mabitac, Laguna, Philippines	MG742324.1
<i>unidentified nematode</i>	Soil.H2 3028	Plant Rhizosphere	Mabitac, Laguna, Philippines	EU880051.1
<i>unidentified nematode</i>	Soil.D2 2813	Plant Rhizosphere	Mabitac, Laguna, Philippines	EU880042.1
<i>Alaimus sp.</i>	Soil.D2 12260	Plant Rhizosphere	Mabitac, Laguna, Philippines	LC186877.1
<i>unidentified nematode</i>	Soil.H1 36419	Plant Rhizosphere	Mabitac, Laguna, Philippines	EU880147.1





- Aphelenchoides sp.***
- Unidentified nematodes, *Alaimus sp.*, *Rhabdolaimus aquaticus***
- Panagrolaimoidea sp.***
- Mesocriconema onoense***
- Rotylenchulus reniformis***
- Unidentified nematode, *Ditylenchus gilanicus*, *Basiria sp.***
- Aphelenchus sp.*, *Acrobeloides varius*, unidentified nematode**

The unidentified nematode (3028) is 94% related to that unidentified nematode found in Costa Rica (EU880051.1). In contrast, the other unidentified nematode (36419) found was 88% related to that of another unidentified nematode found in Costa Rica

(EU880042.1), both belonging to the second clade under Order *Enoplida*. *Rhabdolaimus aquaticus*, on the other hand, was discovered to be 100% like those discovered in Germany (FJ969139.1). On the other hand, the *Alaimus sp.* (12260) collected is 72%



identical to that found in Japan (FJ040489.1) as opposed to another *Alaimus* sp. (41), sharing 73% similarity of the one found in the Netherlands (LC186877.1) and 66% parallel to the previously mentioned *Alaimus* species. Another unidentified nematode (1711) found in the rhizospheres of unhealthy pineapples is 100% identical to that found in the Costa Rican soil (EU880147.1) and 86% related to *Alaimus* sp. found in the Philippines.

The third clade belongs to the Family *Aphelenchoididae*, where two genera were identified: *Aphelenchoidinae* sp. and *Aphelenchoides* sp. The genus *Apelenchoinidae* sp. (1711) obtained from the healthy pineapple rhizospheres was 100% identical to that found in Japan (AB631006.1). At the same time, *Aphelenchoides* sp. (3987) was 45% like that found in Belgium (KX356715.1).

Discussion

Nematodes associated with both healthy and unhealthy pineapple

Among the nematodes associated with healthy and unhealthy pineapple plants, *A. avenae* was the most numerous (91.96%). It is a fungivore nematode known to control fungi infestation (Haraguchi and Yoshiga, 2020). They are considered highly active nematodes (Khan et al., 2020), very diverse, and live in all soil habitats. However, they can also be found in leaf covers, root cortex, and crowns, such as in pineapple (Kumari, 2012). They can also be considered free living and found to be associated with plant debris, lichens, cork, and wood tunnels bore by insects (Khan et al., 2020). Its damaging effects on crops are insignificant, but it could be a carrier of potential pathogens such as bacteria and fungi (Kumari, 2012). The result of the study was coherent with the study of (Khan et al., 2020), where *A. avenae* was the most abundant species (40%). This nematode was also found to be associated with different vegetable crops (Anwar et al., 2013), such as cucumber, tomato, chili, and bell pepper. They stated that *A. avenae* is an ectoparasite of epidermal root tissue and not considered a dangerous pest of vegetables.

Nematodes associated with unhealthy pineapple plant

In addition, *Basiria* sp. is one of the plant-parasitic nematodes found in the rhizospheres of unhealthy pineapple plants. It belongs to Family *Tylenchidae* and is highly diverse in soil habitat. It has been said that

the feeding habit of *Tylenchidae* sp. is indefinite, thus, classifying them as epidermal feeders, mycophagous, or herbivores (Munawar et al., 2021). While in the study of Kanwar et al. (2021), *Basiria* sp. served as one of the preys for predatory nematodes such as *Mylonchulus dentatus* and *Aporcelaimillus nivalis* and was regarded as plant parasitic. Some of these *Basiria* sp. were also found to be associated with pomegranate, fig, grapevine, berry, date palm, lemon, and orange (Azimi, 2019). In Belgium, some species of *Basiria* were also recorded for the first time on its nematofauna (Bert and Geraert, 2000). This species was also found to be associated with *Solanum melongena* in Egypt (Ibrahim et al., 2010). In contrast, this species was absent in the rhizospheres of healthy pineapple plants, as results had concluded. Further investigation is recommended to determine the factors resulting in such behavior of the organism.

The other species found in the rhizospheres of unhealthy pineapples were classified as unidentified nematodes (EU880054.1). Based on the phylogenetic tree, it has the same lineage as *Alaimus* sp. (Figure 3) and *Rhabdolaimus* sp., both free-living nematodes. This species was first reported by Powers et al. (2009) in the Costa Rican Humid Rainforest. They stated that limited nematode diversity was observed in tropical environments rather than temperate ecosystems.

Nematodes found to be associated with healthy pineapple plants

In the case of nematodes found in the rhizospheres of healthy pineapple plants, next to *A. avenae* is *Rotylenchus reniformis* (406 and 308/100 g of soil). It is a known-plant parasitic nematode of pineapple (Wang, 2007), particularly an ectoparasitic herbivore (Benzonan et al., 2021; Daramola et al., 2013). Symptoms associated with the infestation done by this nematode would be chlorosis, stunting, plant decline, stunted development, and reduced fruit production and usually associated with a dense population (Soler et al., 2021). In a study by Sipes and Schmitt (2000), above 1000/g soil of this species would lead to economic yield loss. Evidence of the association with pineapple was reported by Kiriga et al., 2018; Soler et al., 2021. However, it can be noted that the damage could have been more evident since the crop still bore fruits during the time of sampling. Other nematodes found in the rhizospheres of healthy pineapples were *Mesocriconema onoense*, *Aphelenchoides* sp., and *Ditylenchus gilanicus* and



considered to be plant parasitic. They were found to be also associated with pineapple (Kiriga et al., 2018; Soler et al., 2021). However, a small percentage was recorded on the soil samples collected. The unidentified nematode (Accession No. EU880147.1) found associated with healthy pineapple plants was first reported again by Powers et al. (2009).

Phylogenetic analysis of the nematodes found to be associated with pineapple

Generally, there were only two classes in Phylum *Nematoda*, Class *Chromadorea* and *Enoplea*. There were 16 to 20 orders, and among those, only two orders appeared in the nematode communities associated with pineapple, *Rhabditida*, and *Enoplida*. However, the family *Aphelenchoididae* from the Order *Rhabditida* is distant from the lineage because of its polyphyletic nature. Order *Rhabditida* comprises many bacterivores, fungivores, Phyto-feeders, and parasites of vertebrate and invertebrate animals. It thrives in soil or freshwater niches (Abolafia and Peña-Santiago, 2007). As mentioned, (Ruiz-Cuenca and Abolafia, 2022), there are around 450 genera under this Order. However, numerous phylogenetic studies have been published and challenged the classification (Ahmed and Holovachov, 2021) since nematodes are very diverse and evolving. In the family *Anguinidae*, only the species *Ditylenchus gilanicus* was found. It is a new species discovered in Iran and belongs to the *D. trififormis* group (Yaghoubi et al., 2018). This species is characterized by a rounded tail tip and lateral field of six lines and is considered a fungivore (Qiao et al., 2016). In *Apelenchidae* family, only the species *Apelenchus avenae* was found to be associated with pineapple. It is also a fungivore characterized by a white body, tapered but rounded on both ends, with a short style. It has a short stylet, and metacarpus fills the whole-body diameter, which is the distinguishing characteristic of this species (Kumari, 2012). As discussed by Karim et al., 2009, this nematode evolved to have a set of genes encoding a range of enzymes that could degrade cell walls. These findings suggest that it could also be a plant parasite. In the family of *Cephalobidae*, two species were found, *Acrobeloides varius* and the unidentified nematode (132). The species are known to share other characteristics as that of other *Acrobeloides* sp., including an elongated swelling corpus and lateral incisures ranging from 1-5 μ that extend up to the pointed tail (Kim et al., 2017). This species is also known to be a bacterial feeder

(Trap et al., 2021).

Thus, the unidentified nematode, which is 98% identical to *A. varius*, both found in the rhizospheres of the soil samples, were categorically considered a bacterial feeder. Another family in Order *Rhabditida* is *Criconematidae*. They are also known as ring nematodes and are considered plant parasitic. They are also considered monophyletic because of their distinguishing characteristics, such as the moprodelphic ovary without a post-vulva sac (Powers et al., 2017). *Mesocriconema onoense* was one of the plant-parasitic nematodes found in the soil samples. It is one of the 90 species in the genus *Mesocriconema* and is classified as an ectoparasitic root nematode (Nguyen et al., 2021). The same nematode species was also observed in Vietnam (Nguyen et al., 2021) and reported by (Powers et al., 2017). *Hoplolaimidae* is the family to which *Rotylenchulus reniformis* belongs. The species is also known as reniform nematodes and is semi-endoparasitic. It is also one of the ten species under the genus *Rotylenchulus*. The species is 100% identical to that found in the farm soil in Alabama (Nyaku et al., 2016). *Basiria* sp., on the other hand, is generally considered plant parasitic. It belongs to the family *Tylenchidae*, one of the most diverse and numerous nematodes habitating in soils. It includes 42 genera, including *Basiria* sp. Each species under this genus has different characteristics and is monophyletic or belongs to a single common ancestor (Eisvand et al., 2019). It was described by Bert et al. (2003) as “small to medium size nematodes, with an oral opening surrounded by six papillae with a second circle of four papillae on the edges of the anterior surface.” Mostly, the tails are elongated with a rounded end, seldom spicate. Another nematode under the Order *Rhabditida* is *Panagrolaimoidea* sp., which is 100% identical to that found in the dissected body of *Coatitermes clevelandi*, a termite (Kanzaki et al., 2012), thus an insect-associated nematode. This genus belongs to the Family *Cephalobina*. Andrassy (2005) reported that this predominantly includes bacterivores that show greater phylogenetic kinships to Tylenchids, which is also the same Order as the latter (Khan et al., 2012).

Rhabditid nematodes can be found abundantly in different soil types and sediments, whether on land or freshwater. They can be free-living bacterivores of saprovores or entomopathogenic parasites. Generally, nematodes belonging to the Order *Rhabditida* can be considered as a challenging nematode group where diversity is highly far from being prominent (Abolafia



and Peña-Santiago, 2007).

Enoplida is one of the two nematode orders associated with pineapple. Only two of the 21 families under this order were associated with the crop: Family *Alaimidae* and *Rhabdolaimidae*. Most of the nematodes in this order can be found in marine or moist land environments. They are commonly classified as predators (Andrassy, 2005). Specifically, two species of nematodes (*Alaimus sp.* and *Rhabdolaimus aquaticus*) and three unidentified nematodes were found in the rhizospheres of pineapples. *Alaimus sp.* is a bacterivore nematode often associated with mosses (Andrassy, 2005).

Rhabdolaimus aquaticus is also a bacterivore and generally thrives in riverbanks or moist locations (Botha A and Heyns J, 1993). The three other unidentified nematodes were with the same lineage (94-100% similarity) as those found in the humid lowland of Costa Rica and were most likely a bacterivore.

Two nematode species of the Order *Rhabditida* belonging to the family *Aphelenchoididae* were also found in the pineapple rhizospheres. These were separated from *Rhabditida* since *Aphelenchoididae* is polyphyletic (Esmaeili et al., 2017), coming from different ancestral lineages. *Aphelenchoinidae sp.* is often associated with monocots, gymnosperms, and ferns (Aliramaji et al., 2018). *Aphelenchoides sp.*, on the other hand, is composed of fungivores and plant parasites. Sometimes, the parasitic species are referred to as foliar nematodes since they feed primarily on leaves and buds (Duncan and Moens, 2013). The species found in the soil sample was 46% like that found in Belgian soil, which was found by Sánchez-Monge et al. (2017), a plant parasite. However, according to Sánchez-Monge et al. (2017), there was a significant number of undescribed species in the Family *Aphelenchoididae* and increasing the number of taxonomic studies and evolution will shed light on the biodiversity of this family.

Conclusions

Metabarcoding of nematode communities associated with pineapple provided insights on its diversity. Fungivores, bacterivores, predators, and plant-parasitic and insect-associated nematodes were present. The presence of plant-parasitic nematodes in the rhizospheres of the crop did not yet pose a significant threat since their density is still below the level of infestation. Nevertheless, it showed a glimpse

of the plant-parasitic nematode community, which could be detrimental to pineapples.

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References

- Abolafia J and Peña-Santiago R, 2007. Nematodes of the order Rhabditida from Andalucía Oriental, Spain. The genera *Protorhabditis* (Osche, 1952) Dougherty, 1953 and *Diploscapter* Cobb, 1913, with description of *P. spiculocrestata sp. n.* and a species *protorhabditis* key. *J Nematol.* 39(3): 263-274. PMID: 19259497; PMCID: PMC2586499.
- Ahmed M and Holovachov O, 2021. Twenty years after de ley and blaxter-how far did we progress in understanding the phylogeny of the phylum nematoda? *Animals.* 11(12). <https://doi.org/10.3390/ani11123479>
- Ahn SJ, Costa J and Emanuel JR, 1996. PicoGreen quantitation of DNA: Effective evaluation of samples pre- or post-PCR. *Nucleic Acids Res.* 24(13): 2623–2625. <https://doi.org/10.1093/nar/24.13.2623>
- Aliramaji F, Pourjam E, Alvarez-Ortega S, Afshar FJ and Pedram M, 2018. Description of *Aphelenchoides giblindavisi n. sp.* (Nematoda: Aphelenchoididae), and Proposal for a New Combination. *J. Nematol.* 50: 437-452. <https://doi.org/DOI: 10.21307/jofnem-2018-035>.
- Andrassy I, 2005. Free Living Nematodes from Hungary (*Nematodaerrantia*). Hungarian Natural History Museum and Systematic Zoology Research Group of the Hungarian Academy of Science.
- Anwar SA, Mahdi MM, McKenry MV and Qadir A, 2013. Survey of plant-parasitic nematodes



- associated with four vegetable crops cultivated within tunnels. Pak. J. Zool. 45(3): 595-603.
- Azimi S, 2019. Three species of genus *Basiria* (Nematoda: Tylenchidae) from fruit orchards in Khuzestan province, Southwestern Iran. J. Biol. Stud. 2(3): 64–73.
- Balito LP, 2010. The Philippine pineapple industry. Acta Horticulturae. 902:53-62.
- Benzonan NC, Dalisay LCS, Reponte KCC, Mapanao CP, Alvarez LV, Rendon AO and Zurbano LY, 2021. Plant-Parasitic Nematodes Associated with Pineapple (*Ananas comosus*) in Selected Provinces in Luzon, Philippines. Eur. J. Mol. Clin. Med. 8(2): 945-957.
- Bert W and Geraert E, 2000. Nematode species of the order Tylenchida, new to the Belgian Nematofauna with additional morphological data. Belg. J. Zool. 130(1): 47–57.
- Bert W, Coomans A, Claerbout F, Geraert E and Borgonie G, 2003. Tylenchomorpha (Nematoda: Tylenchina) in Belgium, an updated list. Nematol. 5(3): 435–440. <https://doi.org/https://doi.org/10.1163/156854103769224412>
- Botha A and Heyns J, 1993. Species of the genera *Oxydirus*, *Dorylaimellus* (*Axodorylaimellus*), *Laimydorus* and *Rhabdolaimus* from rivers in the Kruger National Park (Nematoda: Dorylaimida and Araeolaimida). Koedoe. 36: 49–60. <https://doi.org/doi:10.4102/koedoe.v36i1.361>
- Bugnay-Inso LJC and Pedroche NB, 2021. Survey and Identification of Plant-Parasitic Nematodes Infecting Planting Materials of Garlic (*Allium sativum*) in Major Growing Areas of Ilocos Norte, Philippines. Mountain J. Sci. Interdiscipl. Res. 81(2): 33-44.
- Caporaso G, Kuczynski J, Stombaugh J, Bittinger K, Bushman F, Costello E, Fierer N, Peña A, Goodrich J, Gordon J, Huttley G, Kelley S, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung MR, Jens S, Turnbaugh JR, Walters PJ, William A, Widmann J, Yatsunenko T, Zaneveld J and Knight R, 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 7(5): 335–336.
- Daramola FY, Afolami SO, Idowu AA and Nwanguma EI, 2013. Studies on the occurrence and distribution of plant-parasitic nematodes in some pineapple-producing states in Nigeria. Asian J. Crop Sci. 5(2): 190–199. <https://doi.org/10.3923/ajcs.2013.190.199>
- Duncan LW and Moens M, 2013. Migratory endoparasitic nematodes. In M. Perry, R.N. & Moens (Ed.), Plant Nematology. 144–178. CABI Publishing.
- Eisvand P, Nejad RF and Azimi S, 2019. Description of *Basiria khuzestanensis* n. sp. (Nematoda: Tylenchidae) from Iran and its phylogenetic relationships with other species in the family. Zootaxa. 4563(3). <https://doi.org/10.11646/zootaxa.4563.3.4>
- Esmaili M, Heydari R, Fang Y and Li H, 2017. Molecular and morphological characterisation of *Aphelenchoides paraxui* n. sp. (Nematoda: Aphelenchoididae) isolated from *Quercusbrantii* in western Iran. Eur. J. Plant Pathol. 149(3): 625 – 637. <https://doi.org/10.1007/s10658-017-1212-y>
- Garcia N, Grenier E, Buisson A and Folcher L, 2022. Diversity of plant parasitic nematodes characterized from fields of the French national monitoring programme for the Columbia root-knot nematode. PLoS One. 17(3). <https://doi.org/10.1371/journal.pone.0265070>
- Haraguchi S and Yoshiga T, 2020. Potential of the fungal feeding nematode *Aphelenchus avenae* to control fungi and the plant parasitic nematode *Ditylenchus destructor* associated with garlic. Biol. Control. 143. <https://doi.org/10.1016/j.biocontrol.2020.104203>
- Head SR, Komori HK, LaMere SA, Whisenant T, Van Nieuwerburgh F, Salomon DR and Ordoukhanian P, 2014. Library construction for next-generation sequencing: overviews and challenges. BioTechniques. 56(2): 61-77 61-passim. <https://doi.org/https://doi.org/10.2144/000114133>
- Hossain MF, 2016. World pineapple production: An overview. Afr. J. Food Agric. Nutr. Dev. 16(4): 11443–11456. <https://doi.org/10.18697/ajfand.76.15620>
- Ibrahim IKA, Mokbel AA and Handoo ZA, 2010. Current status of phytoparasitic nematodes and their host plants in Egypt. Nematropica. 40(2): 239–262.
- Kanwar RS, Patil JA and Yadav S, 2021. Prospects of using predatory nematodes in biological control for plant parasitic nematodes – A review. Biol. Control. 160: 104668. <https://doi.org/10.1016/j.biocontrol.2021.104668>
- Kanzaki N, Giblin-Davis RM, Scheffrahn RH, Taki H, Esquivel A, Davies KA and Herre EA, 2012. Reverse Taxonomy for Elucidating Diversity of



- Insect-Associated Nematodes: A Case Study with Termites. *PLoS One*. 7(8): e43865. <https://doi.org/10.1371/journal.pone.0043865>
- Karim N, Jones JT, Okada H and Kikuchi T, 2009. Analysis of expressed sequence tags and identification of genes encoding cell-wall-degrading enzymes from the fungivorous nematode *Aphelenchus avenae*. *BMC Genomics*. 10: 525. <https://doi.org/10.1186/1471-2164-10-525>.
- Kenmotsu H, Uchida K, Hirose Y and Eki T, 2020. Taxonomic profiling of individual nematodes isolated from copse soils using deep amplicon sequencing of four distinct regions of the 18S ribosomal RNA gene. *PLoS One*. *PLoS One* 15(10): e0240336. <https://doi.org/10.1371/journal.pone.0240336>
- Khan R, Hussain A and Tahseen Q, 2012. Descriptions of one new and two known species of Panagrolaimoidea (Nematoda) from India. *J. Nat. Hist.* 46(13–14): 867–884. <https://doi.org/10.1080/00222933.2011.653589>
- Khan S, Javed S and Khanum TA, 2020. Plant parasitic nematode of genera *Aphelenchus* and *Aphelenchoides* (Nematoda: Aphelenchoidea) from District LakkiMarwat, Khyber Pakhtunkhwa, Pakistan. *Pak. J. Phytopathol.* 32(2): 169–178. <https://doi.org/10.33866/phytopathol.030.02.0586>
- Kim T, Kim J and Park JK, 2017. *Acrobeloides varius* sp. n. (Rhabditida: Cephalobidae) from South Korea. *Nematology*. 19(4): 489–496. <https://doi.org/10.1163/15685411-00003064>.
- Kimura M, 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol.* 16(2):111–120. doi: 10.1007/BF01731581
- Kiriga AW, Haukeland S, Kariuki GM, Coyne DL and Beek NV, 2018. Effect of *Trichoderma* spp. and *Purpureocillium lilacinum* on *Meloidogyne javanica* in commercial pineapple production in Kenya. *Biol. Control.* 119: 27–32. <https://doi.org/10.1016/j.biocontrol.2018.01.005>
- Kumari S, 2012. *Aphelenchus avenae* (Nematoda: Aphelenchidae) under the rhizosphere of *Brassica napus*. *Helminthologia*. 49(1): <https://doi.org/10.2478/s11687-012-0009-y>
- Li W, Fu L, Niu B, Wu S and Wooley J, 2012. Ultrafast clustering algorithms for metagenomic sequence analysis. *Briefings Bioinf.* 13(6): 656 – 668. <https://doi.org/10.1093/bib/bbs035>
- Munawar M, Yevtushenko DP and Castillo P, 2021. First report of three tylenchidae taxa from southern alberta, Canada. *Horticulturae*. 7(11): 449. <https://doi.org/10.3390/horticulturae7110449>
- Nguyen HT, Nguyen TD, Le TML and Trinh QP, 2021. Integrative taxonomy of *Mesocriconema onoense* (Tylenchida: Criconematidae) from Vietnam highly suggests the synonymization of *Mesocriconema brevistylus* and related species. *Infect. Genet. Evol.* 95:105090. <https://doi.org/10.1016/J.MEEGID.2021.105090>
- Nyaku ST, Kantety RV, Cebert EL, Kathy S, Honger JO and Sharma GC, 2016. Principal component analysis and molecular characterization of reniform nematode populations in Alabama. *Plant Pathol. J.* 32(2): 123–135.
- Porazinska DL, Giblin-Davis RM, Faller L and Thomas WK, 2009. Evaluating high-throughput sequencing as a method for metagenomic analysis of nematode diversity. *Mol. Ecol. Resour.* 9(6): 1439–1450. DOI: 10.1111/j.1755-0998.2009.02611.x
- Porazinska DL, Giblin-Davis RM, Powers TO and Thomas WK, 2012. Nematode Spatial and Ecological Patterns from Tropical and Temperate Rainforests. *PLoS ONE*. 7(9): 1–11. <https://doi.org/10.1371/journal.pone.0044641>
- Powers DA, Nehe, P, Mullin A, Esquivel RM, Giblin-Davis N, Kanzaki SP, Stock MM and Mora LUL, 2009. Tropical nematode diversity: vertical stratification of nematode communities in a Costa Rican humid lowland rainforest. *Mol. Ecol.* 18(5): 1985–1996. doi: 10.1111/j.1365-294X.2008.04075.x. PMID: 19207247.
- Powers T, Harris T, Higgins R, Mullin P and Powers K, 2017. An 18S rDNA perspective on the classification of criconematoidea. *J. Nematol.* 49(3): 236–244. <https://doi.org/10.21307/jofnem-2017-069>
- Qiao Y, Yu Q, Badiss A, Zaidi MA, Ponomareva E, Hu Y and Ye W, 2016. Paraphyletic genus *Ditylenchus Filipjev* (Nematoda, Tylenchida), corresponding to the *D. triformis* group and the *D. dipsaci* group scheme. *Zookeys*. 2016(568): 1–12. <https://doi.org/10.3897/zookeys.568.5965>
- Ruiz-Cuenca AN and Abolafia J, 2022. Characterization of three plant or fungal feeding nematodes (Nematoda, Rhabditida,



- Tylenchomorpha) from coastal dunes in Spain including the SEM study of these species. *J. Plant Dis. Prot.* 129: 895–910. <https://doi.org/10.1007/s41348-022-00596-6>
- Sánchez-Monge A, Janssen T, Fang Y, Couvreur M, Karssen G and Bert W, 2017. mtCOI successfully diagnoses the four main plant-parasitic Aphelenchoides species (Nematoda: Aphelenchoididae) and supports a multiple origin of plant-parasitism in this paraphyletic genus. *Eur. J. Plant Pathol.* 148(4): 853–866. <https://doi.org/10.1007/s10658-016-1141-1>
- Sapkota R and Nicolaisen M, 2015. High-throughput sequencing of nematode communities from total soil DNA extractions. *BMC Ecol.* 15(1): 1–8. <https://doi.org/10.1186/s12898-014-0034-4>
- Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ and Weber CF, 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75(23): 7537–7541. <https://doi.org/10.1128/AEM.01541-09>
- Shahbandeh M, 2023. Global pineapple production by leading countries 2021. <https://www.statista.com/statistics/298517/global-pineapple-production-by-leading-countries/>
- Sharpton TJ, 2014. An introduction to the analysis of shotgun metagenomic data. *Front. Plant Sci.* 5: 209.
- Sipes B and Schmitt D, 2000. *Rotylenchulus reniformis* damage thresholds on pineapple. *Acta Hort.* 529(29): 239–246.
- Soler A, Marie-Alphonsine P, Quénehervé P, Prin Y, Sanguin H, Tisseyre P, Daumur R, Pochat C, Dorey E, González Rodríguez RG, Portal N and Smith-ravin J, 2021. Field management of *Rotylenchulus reniformis* on pineapple combining crop rotation, chemical-mediated induced resistance and endophytic bacterial inoculation. *Crop Prot.* 141: 105446. <https://doi.org/10.1016/j.cpro.2020.105446>
- Tamura K, Stecher G and Kumar S, 2021. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* 38(7): 3022–3027. <https://doi.org/10.1093/molbev/msab120>
- Trap J, Ranoarisoa MP, Raharijaona S, Rabeharisoa L, Plassard C, Mayad EH, Bernard L, Becquer T and Blanchart E, 2021. Agricultural practices modulate the beneficial activity of bacterial-feeding nematodes for plant growth and nutrition: Evidence from an original intact soil core technique. *Sustainability (Switzerland)*. 13(13): 7181. <https://doi.org/10.3390/su13137181>
- Voora V, Larrea C and Bermudez S, 2020. Global market report: bananas. International Institute for Sustainable Development. <https://www.iisd.org/publications/report/global-market-report-bananas>
- Wang KH, 2007. *Rotylenchulus reniformis* Linford & Oliveira (Nematoda: Tylenchida: Tylenchoidea: Hoplolaimidae: Rotylenchulinae). University of Florida, EENY-210. http://entnemdept.ufl.edu/creatures/nematode/r_r_eniformis.htm
- Yaghoubi A, Pourjam E, Ye W, Castillo P and Pedram M, 2018. Description and molecular phylogeny of *Ditylenchus gilanicus* n. sp. (Nematoda: Anguinidae) from northern forests of Iran. *Eur. J. Plant Pathol.* 152(3): 735–746. <https://doi.org/10.1007/s10658-018-1516-6>

Contribution of Authors

Zurbano LY: Oversaw the experiment, performed data analysis and wrote the manuscript.
Deocarís CC: Planned research and edited the manuscript.
Mapanao CP: Literature review and edited the manuscript.
Rendon AO: Collected data and wrote the methods for metabarcoding.
Alvarez LV: Oversaw the experiment, wrote the methods for phylogenetic analyses and constructed the phylogenetic tree.

