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NEW WILD-TYPE ISOLATES OF THE NEMATODE GENERA *OSCHIEUS*, *ALLOIONEMA*, AND A DIFFERENT RHABDITID GENUS (NEMATODA: RHABDITIDA) FOUND IN FORESTS OF VIETNAM

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ABSTRACT

In this report, we describe the isolation and determination of four new free-living nematode (FLN) strains of three species in different nematode genera (*Oschieus*, *Alloionema*, and another rhabditid genus) from Cat Tien and Cuc Phuong National Parks, Vietnam using the isolation protocol for *Caenorhabditis* nematodes. Of the four strains, three strains were similar to the previous determinants and one strain may be a new rhabditid species that was first found in Vietnam. They can grow well on the *E. coli* OP50 – seeded media (New Cheap Media No. 18 (NcM18) and Nematode Growth Media (NGM)). Compared with the isolation of the *Caenorhabditis* nematodes in the same scenario, the frequencies of finding these three genera were low because of the inappropriate isolation method employed in the *Caenorhabditis* nematodes. This data supports knowledge of the FLN diversity and implies that FLNs that stay uncovered in the forestry ecosystems should be uncovered using different isolation methods for the nematode order Rhabditida, i.e. entomopathogenic nematodes (EPNs), helminths, and the superfamily Rhabditidae.

Keywords: Entomopathogenic nematodes, free-living nematodes, helminths, New Cheap Media (NcM), parasitic nematodes.

INTRODUCTION

Nematodes are predicted to be widely distributed among multicellular organisms on Earth and they developed in various ecosystems in soil and water (Neher, 2010; Ridall, Ingels, 2021). Free-living nematodes (FLNs), which are mostly microscopic, play roles in the micro-habitats. Many of them eat bacteria and subsequently are consumed by other bacteria, fungi, and tiny animals. The other types of FLNs live in the soil and have the potential to interact with plants and animals, known as parasitic nematodes. The regularly encountered nematodes are parasitic to many crops and ruminants, and they are vectors for many associated viruses and bacteria.

So far, most studies reported biological descriptions for morphology, diversity, and ecology (Viney, 2017), and less for the applicable properties of helminths and EPNs (Son *et al.*, 2022; Son *et al.*, 2023). EPN and helminths become ecological and recent

bioindicators for water resources and soil health (Thieltges *et al.*, 2008; Semprucci *et al.*, 2015; Lu *et al.*, 2020). However, FLNs are tools for studying biological concepts and they become organismal models to analyze molecular genetics (Consortium, 1998; Gupta, Sternberg, 2003) and ecological evolutionary developmental biology (Eco-Evo-Devo) (Montanara *et al.*, 2022).

Every aspect of nematology benefits from the knowledge of FLN biodiversity. Nonetheless, FLNs have been found across continents worldwide, they should have been hesitated in many microhabitats due to uncountable reasons.

We pursue the research of the diversity of FLN throughout Vietnam. In previous studies, we reported the isolation, characterization, and culture of vast FLN species within three nematode genera *Caenorhabditis*, *Pristionchus*, and *Halicephalobus* in forests (Le *et al.*, 2022; Son *et al.*, 2023; Son TL *et al.*, 2023). This study aims to investigate more different FLNs that are living in the forests using a similar isolation protocol as in our previous studies. Consequently, we found four nematodes in the genera *Oschieus*, *Alloionema*, and another rhabditid genus. This result supports the knowledge of the biodiversity of the Rhabditida genera and facilitates other research such as Eco-Evo-Devo.

METHODS AND MATERIALS

Media

New Cheap Media No.18 (NcM18): 17 g of agar, 0.4 g of pig fat, 4 ml of 0.75g/L NaCl, 20 ml of mushroom solutions, and 1 L distilled water (Le *et al.*, 2021).

Nematode Growth Media (NGM): 17 g of agar, 1 mL of 5 mg/mL cholesterol, 2.5 g of peptone, 1 mL of 1M $CaCl_2$, 1 mL of 1M $MgSO_4$, 25 mL 1M KPO_4, 4 mL of 0.75 g/mL NaCl, and 1 L water (Stiernagle, 2006; Le *et al.*, 2021).

Isolation of nematodes

Vegetation samples were directly collected from 80 different sites, each 40 in either National Parks (Cat Tien or Cuc Phuong), which were described for their geography in previous studies (Le *et al.*, 2022; Son *et al.*, 2023; Son TL *et al.*, 2023). The sample collection at each site was repeated four times through the years (2020-2023). Briefly, an amount of 5 to 10 g of each vegetation sample was placed on the agar surface of an *Escherichia coli* OP50-seeded media plate (9 to 10 cm) and incubated at 25ºC for three to five days. The worms growing on the plate were selected for further cultivation.

One to two gravid worms (P_0) with a similar appearance of the *Caenorhabditis* nematodes, i.e. the transparency, the 1-mm size, and the rod shape of the body under microscopes (4X) were transferred onto an OP50-seeded media plate (either NcM18 or NGM) to reproduce next generations. The incubation was followed up at room temperature (approximately 25ºC) for three to seven days

Species determination by molecular identification

The total genomic DNA of each strain was extracted from a few worm bodies using the “Single Worm Lysis” method suitable for tiny nematodes (Ahringer, 2006; Le *et al.*, 2023). Next, the 18S rDNA sequence of the nucleus DNA was amplified with two universal primers, (SSU18A (5'-

AAAGATTAAGCCATGCATG-3 ') and SSU26R (5' -CATTCTTGGCAAATGCTT TCG-3 ') (Barriere, Felix, 2006)). The PCR products were purified with MEGAquick-spin™ Plus Total Fragment DNA Purification Kit (iNtRON Biotechnology, South Korea) before getting sequenced with the Sanger method by a sequencing service (ATCG Limited Co., Viet Nam). Each of the DNA sequences was compared with the DNA database of the National Center for Biotechnology Information (NCBI).

Phylogenetic analysis

Phylogeny of the 18S rDNA sequences of the isolates was analyzed using the Neighbor-Joining method on MEGA11(Tamura *et al.*, 2021).

RESULTS

We picked a total number of 320 vegetation samples from Cat Tien and Cuc Phuong National Parks. The samples were incubated to grow nematodes within. In the nematode isolation, over 300 nematode individuals developed and survived well on either NcM18 or NGM over multiple generations and each population was defined as a strain. However, nearly 200 nematodes died due to unknown reasons. In the species determination, among 300 survival nematode strains that were sequenced for the 18rDNA barcode, many were FLNs within the genus *Caenorhabditis*, and fewer were different nematodes. In this research, we report four different FLNs, which are new wild-type isolates.

Following the cultivation, the four nematodes were examined with PCR. The PCR products are detected as single bands that range from 800 bp to 900 bp in size, resembling other nematodes using the same molecular method in our previous reports (Le *et al.*, 2022; Son *et al.*, 2023; Son TL *et al.*, 2023) (Figure 1a). Next, we determined the taxa for the nematodes by comparing their unassembled 18S rDNA sequences with the nucleotide database of NCBI. We found that they were grouped into three different genera in the order Rhabditida.

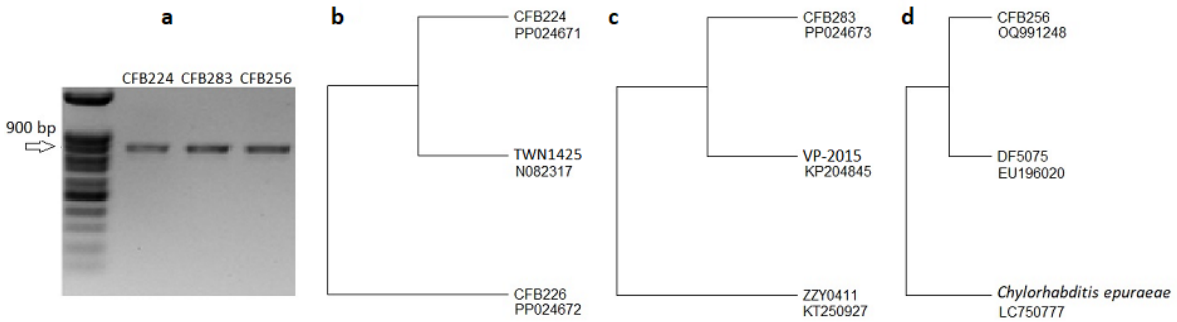

Figure 1. Taxonomy determination of isolated nematodes. **(a)** Electrophoresis gel image of PCR products. **(b)** Phylogeny of *Oscheius* sp. CFB224 and CFB226, **(c)** *Alloionema* sp. CFB283, and **(d)** *Cephaloboides* sp. CFB256. In the phylogenic trees, the Genbank sequence ID is below the strain number. Sequence N082317 of *Oscheius* sp. TW1425, KP204845 of *Alloionema* sp. VP2015, ZZY0411 of *Alloionema* sp. KT250927, and EU196020 of *Cephaloboides* sp. DF5071, and LC750777 of *Chylorhabditis epuraeae* were achieved from the database of NCBI.

Oscheious species

We isolated two wild-type strains CFB224 and CFB226 from Cat Tien National Park. Their 18S rDNA sequences have high identities (99.76% and 99.88%) to *Oscheious* sp. 24 TWN1425 (Schoch *et al.*, 2020) (Figure 1b and Table 1), indicating that they are the same species as *Oschieus* sp. 24 TWN1425.

Oscheius sp. CFB224 and CFB226 were infrequently found in this research (ratios of strains: 2/300, and local sites: 2/40 in Cat Tien National Park, Dong Nai Province), possibly due to the isolation techniques in the laboratory, which was specific for the *Caenorhabditis* nematodes. In our previous research, we found a vast majority of the *Oscheius tipulae* (ratios of strains: 19/90, local sites: 19/90 in Quang Nam Province) using an EPN isolation protocol (Son *et al.*, 2022); Notably, Quang Nam Province is geometrically situated between Cat Tien and Cuc Phuong National Parks. These quite opposites highly suggest that the isolation of the nematodes, at least within the territory of Vietnam, using the EPN isolation protocol is more productive than the *Caenorhabditis* protocol. However, the low frequency of finding the nematodes would be dependent on other unknown reasons since the two strains in this research may be different from *O. tipulae*.

Alloionema species

We isolated one wild-type strain, CFB283, which is 97.75% identical to *Alloionema* sp. VP-2015 (Schoch *et al.*, 2020) (Figure 1c and Table 1), indicating that they are the same species.

Alloionema sp. CFB283 was rarely found in this research (ratios of strains: 1/300 and local sites: 1/40 in Cat Tien National Park, Dong Nai Province). Many *Alloionema* nematodes are parasitic to pests, slugs, and snails and they develop to full stages in the host animals (Urek *et al.*, 2003; Laznik *et al.*, 2009; Nermut *et al.*, 2015; Holovachov *et al.*, 2016; Jaffuel *et al.*, 2019) rather than free juveniles which were not regularly selected in this research. This would be the main reason why the *Alloionema* nematodes were rare to be seen.

Intermediate conclusion: The two parks (forests) have a high diversity of animals including insects, gastropods, and ruminants, which are the hosts for many parasitic nematodes, theoretically including *Ocheius* and *Alloionema*. Thus, low frequencies of found parasitic nematodes are possibly due to the inappropriate isolation protocol. The three helminths in this research (CFB224, CFB226, and CFB283) developed well on the *E. coli* OP50-seeded media, suggesting that they own the potential of free-living.

Rhabditid species

We isolated and determined another new strain CFB256 that has a quite low identity (90.69%) to the first hit of *Cephaloboides nidrosiensis* DF5075 (Kiontke *et al.*, 2007; Carta *et al.*, 2018) and a little lower identity (90.36%) to the first-second hit that was *Chylorhabditis epuraeae* (Kanzaki *et al.*, 2020) (Figure 1d and Table 1). Thus, it is potentially a new *Cephaloboides*, *Chylorhabditis* species, or a different species within the superfamily Rhabditidae of the order Rhabditida.

Nematode strain CFB256 was rarely found in this research (ratios of strains: 2/300 and local sites: 1/40 in Cuc Phuong National Park, Ninh Binh Province). This nematode developed well on *E. coli*-OP50-seeded media, suggesting that it is an FLN.

Table 1. Molecular determination of new free-living nematodes.

Wild-type isolate	Sample sites	GenBank Sequence ID	Comparable species	Identical percentage; Sequence ID
CFB224	Cat Tien	PP024671	*Oscheious* sp. 24 TWN1425	99.76%; N082317.1
CFB226	Cat Tien	PP024672	-	99.88%; -
CFB283	Cat Tien	PP024673	*Alloionema* sp. VP-2015	97.75%; KP204845.1
CFB256	Cuc Phuong	OQ991248	*Cephaloboides nidrosiensis* DF5075 *Chylorhabditis epuraeae*	90.69%; EU196020.1 90.36%; LC750777

CONCLUSION

In this research, we found that two FLNs are similar to *Oscheious* sp. 24 TWN1425 and one FLN is similar to *Alloionema* sp. VP-2015. The frequencies of finding them were low, partly because we used an inappropriate isolation method which was designed for the *Caenorhabditis* nematodes. Last, another nematode isolate was found to be a new rhabditid species within the superfamily Rhabditida from forests. However, the four nematodes grew well on the *E. coli* OP50-seeded media. These findings indicate that the diversity of FLNs in the forests would be higher. To assess the potential FLNs in forests, it is necessary to try different isolation methods for FLNs rather than the methodology for the *Caenorhabditis* nematodes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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