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THREE RARE DIOECIOUS *CAENORHABDITIS* NEMATODE SPECIES (*C. tribulationis*, *C. yunquensis*, AND *C. zanzibari*) DO NOT LIVE IN THE SAME HABITATS

Le Tho Son✉, Nguyen Thi Thu Hang, Nguyen Thi Thu

College of Forestry Biotechnology and F-School, Vietnam National University of Forestry, Xuan Mai Town, Chuong My District, Hanoi, Vietnam

✉To whom correspondence should be addressed. E-mail: sonlt@vnuf.edu.vn

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SUMMARY

The *Caenorhabditis* nematodes are among the best model systems for studies verifying the molecular-to-phenomenon consequences at different magnitudes. Hundreds of strains within nearly 60 species have been found in the wild and used for studies of evolution. However, the insights of evolution could be uncovered deeper when more *Caenorhabditis* isolates are gained and accessible. Unfortunately, the effort to isolate wild-type strains is not efficient in the habitats where the *Caenorhabditis* nematodes do not naturally distribute. In contrast, we surveyed many samples in various locations and revealed the presence of *Caenorhabditis* nematodes, which had already been seen globally. This suggests that rarer nematode decedents may live in the same habitats. In this research, we report the current isolations and the identifications of three *Caenorhabditis* species (*C. tribulationis*, *C. yunquensis*, and *C. zanzibari*) with low frequencies of discovery in the wild. Molecularly, they are highly similar to a few previous relatives from other territories.

Keywords: *Caenorhabditis*, nematodes, new cheap media (NcM)

INTRODUCTION

The nematode genus *Caenorhabditis* is tiny (~1 mm) and plays a role in understanding basic concepts of biological issues concerning evolution, for example, genomic and proteomic comparison (Crombie *et al.*, 2019; Stevens *et al.*, 2019; Caro *et al.*, 2022), genome (Adams *et al.*, 2023), sexual dimorphism (Le *et al.*, 2017), and diversity, for example, adaptation (Caraballo *et al.*, 2020; Caraballo *et al.*, 2021).

Although the most appreciated contributors are *C. elegans* and *C. briggsae*, the other species do contribute to the understanding of evolutionary contexts. More than 50 species with hundreds of strains have been isolated from the wild to do deeper research of evolutionary traits and diversity in the genus (Kiontke, Sudhaus, 2006; Le *et al.*, 2017; Le *et al.*, 2021). We continue the long-term research of isolating the *Caenorhabditis* species in different habitat styles in Vietnam. In this report, we

report the ridiculous findings of bare species, which are *C. tribulationis*, *C. zanzibari*, and *C. yunquensis*.

MATERIALS AND METHODS

Media preparation

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

Nematode Growth Media (NGM) was prepared: 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 of 1M KPO_4 + 17 g of agar + 4 mL of 0.75 g/mL NaCl + 1 L of water.

Nematode selection

Vegetation samples were collected from different sites in two national parks (Cuc Phuong and Cat Tien) within two years (from 2020 to 2022).

Caenorhabditis strains were isolated as in our previous description (Le *et al.*, 2021). An amount of 5 to 10 g of each vegetation compost (leaves, fruits, and flowers) was put on the surface of one *Escherichia coli* OP50-seeded media petri plate (10 cm) and incubated at room temperature (approximately 25^{o}C) for three days. Subsequently, each of the two worms (mode) per sample plate was transferred onto and grown on an OP50-seeded media petri plate (5 cm) in a (19 ± 1)^{o}C incubator for several generations. Each worm developed its population and was defined as a strain.

Morphologically, the translucence, approximately 1-mm length, and rod shape of the body were more likely similar to many other *Caenorhabditis* species under microscopes (4X). Further determination was the pharyngeal morphologies, which had two circular bulbs under microscopic magnification (40X); again, this was recognized as the appearance of *C. elegans* (Barriere, Felix, 2006). Regarding sexes, of the strain candidates, the ones presenting two sexes (males and isofemales) were concerned in the next examinations.

Species determination

Total DNA was isolated using the "Single Worm Lysis" method for tiny nematodes (Ahringer, 2006; Le *et al.*, 2023).

The 18S rDNA sequence was amplified with two universal primers for nematodes: SSU26R (5'-CATTCTTGGCAAATGCTT TCG-3') and SSU18A (5'-AAAGATTAA GCCATGCATG-3') (Barriere, Felix, 2006). The PCR products were purified with the Intron DNA Purification Kit before they were sequenced by sequencing services using either one of the PCR primers. Each of the DNA sequences was compared with the DNA references using BLASTnt on the National Center for Biotechnology Information (NCBI) (Le *et al.*, 2021).

Phylogenetic analysis

The 18S rDNA sequences were aligned and used for the phylogenetic analysis and phylogenetic tree construction using the Neighbor-Joining method of the MEGA11 program with 100 bootstrap replicates (Tamura *et al.*, 2021). For *C. yunquensis*, a reference sequence was included in the analysis.

RESULTS

We collected 80 vegetation samples from

forests, each from one of 40 sites in Cuc Phuong National Park or 40 sites in Cat Tien National Park (Le *et al.*, 2023). The samples were used to isolate the *Caenorhabditis* nematodes. In this report, we presented three dioecious species with similar morphology (*C. tribulationis*, *C. yunquensis*, and *C. zanzibari*), and they are rarely found in the wild rather than many other species (Kiontke *et al.*, 2011).

Table 1. Identity of the 18S rDNA sequences to the references.

No.	CFB No.	Identity (%)/ reference sequence	Genbank Accession No. (NCBI)	Sample sites
***C. tribulationis* in Cuc Phuong National Park**				
1	CFB97	99.76/ MH809976.1	OQ991252	20º15'51.2"N; 105º 41'57.6"E
2	CFB85	99.88/-	OQ991253	20º15'7.6"N; 105º42'45"E
3	CFB65	99.52/-	OQ991254	20º15'23.2"N; 105º42'31.6"E
4	CFB267	99.41/-	OQ991256	20º17'41.3"N; 105º39'58.4"E
5	CFB279	99.52/-	OQ991257	-
6	CFB275	99.41/-	OQ991255	20º20'56.7"N; 105º35'46.5"E
***C. yunquensis* in Cat Tien National Park**				
7	CFB199	98.94/JN636072.1	OR863788	11º27'32.5"N; 107º20'43.9"E
8	CFB209	98.82/-	OR863789	-
9	CFB227	98.94/-	OR863790	-
***C. zanzibari* in Cuc Phuong National Park**				
10	CFB63	99.52/MH809973.1	OR863791	20º15'26.2"N; 105º42'31.6"E

"-" Repetition of the upper reference sequence or sample sites.

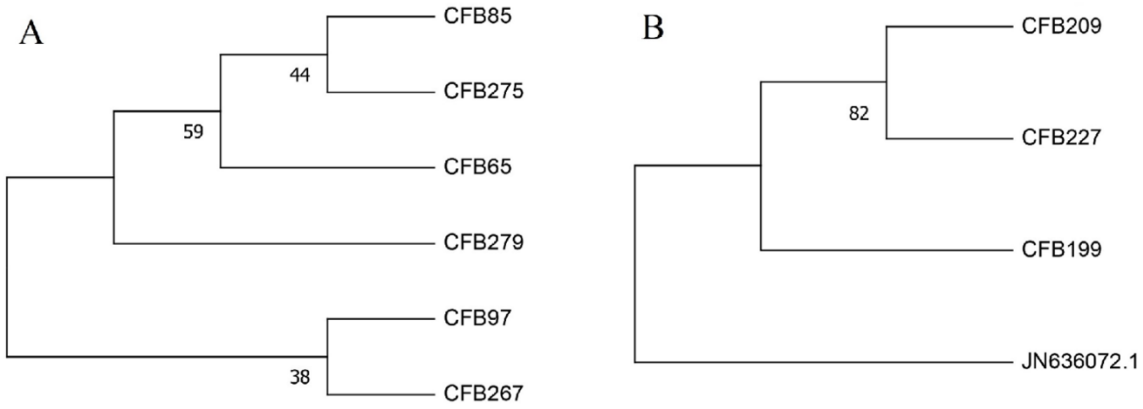

Figure 1. Phylogenetic trees of two species. (A) *C. tribulationis* from Cuc Phuong National Park. (B) *C. yunquensis* from Cat Tien National Park. The JN636072.1 sequence of the EG6142 strain is adopted from the NCBI database.

Caenorhabditis tribulationis

We isolated and raised six *C. tribulationis* strains from five of 40 sample sites in Cuc Phuong (12.5%) but none were from 40 sites in Cat Tien National Park (0.0%) (Table 1). Previous studies reported that the species (strain: JU2774) is in Australia (Stevens *et al.*, 2019; Liu *et al.*, 2022). These suggest that the species are not globally redundant.

Comparison of the 18S rDNA sequences showed that six strains have a high identity to the reference MH809976.1 of JU2774 (Table 1), suggesting they were quite conserved over time with probably four times of evolution (Fig. 1A).

Caenorhabditis yunquensis

We isolated and raised three *C. yunquensis* strains from one site in Cat Tien (2.5%) (Table 1) but none were from Cuc Phuong (0.0%). Previously, this species was reported to live on two islands in America (Puerto Rico and French Guiana) (Felix *et al.*, 2014). These indicate that the species is not globally redundant.

Comparison of the 18S rDNA sequences showed that three strains have a "mild" identity to the reference JN636072.1 of EG6142 (or *Caenorhabditis* sp. 19 from Puerto Rico) (Kiontke *et al.*, 2011) (Table 1), suggesting they are not well conserved with possibly two times of evolution over time (Fig. 1B).

Caenorhabditis zanzibari

We isolated and raised one *C. zanzibari* from one site in Cuc Phuong National Park (2.5%) (Fig. 2 and Table 1), and none were from Cat Tien National Park. This species was previously found as JU2161 in Tanzania (Stevens *et al.*, 2019). These suggest the species is not redundant. The 18S rDNA of *C. zanzibari* CFB63 has a high identity with the reference MH809973.1 (Table 1).

We isolated three rare newest descendants of the *Caenorhabditis* ancestors (Felix *et al.*, 2014; Stevens *et al.*, 2019). Previous studies and our current research reveal lower frequencies of isolating the three species than many other nematodes such as *C. briggsae* and *C. brenneri* in our previous reports (Le, Nguyen, 2021; Son TL *et al.*, 2023). This result can be the nature of the species. Thus it is critical to investigate factors regulating the quantity of isolates such as ecology, the nature of species, and the laboratory. However, we do not rule out the possibility of technical problems during the forestry sampling because most work was manually handled.

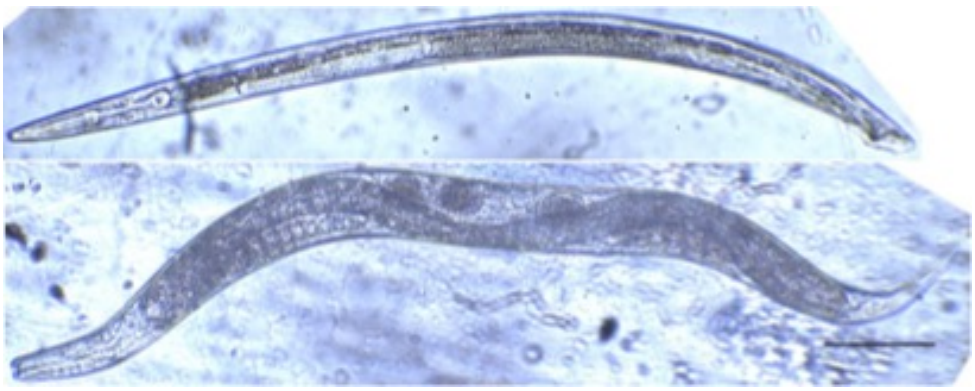

Figure 2. *C. zazibari* CFB63. A male (top) and female (bottom). Scale bar-100 µm.

CONCLUSION

Three rare species of *Caenorhabditis* nematodes were isolated from the forests and cultured. They inhabit different forests, in which *C. tribulationis* and *C. zanzibari* live in Cuc Phuong National Park while *C. yunquensis* present in Cat Tien National Park. It is worth uncovering the ecological adaptation of the species as part of the evolution within the *Caenorhabditis* nematodes.

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