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WILD-TYPE *CAENORHABDITIS SINICA*, A MODEL NEMATODE FOR SPECIATION AND EVOLUTION, MASSIVELY FOUND IN VIETNAM

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SUMMARY

Caenorhabditis sinica is a male/female species, in which the genetic diversity is possibly high. Thus, the species is advantageous for the understanding of the mechanisms of diversity, evolution, and adaptation within the nematode genus *Caenorhabditis*. Previous studies reported the geographic distribution of *C. sinica* only in China; nonetheless, this should be more convincing for its ecology. We allegedly surveyed the *Caenorhabditis* species and their diversity in the forests of Vietnam. We found 59 *C. sinica* strains, and this therefore indicates the vast variation that is 85.88% and 100% identical within isolated wild-type strains. They unequally present in habitats and are enormous in the northern forest, and rarer in the southern forest. The comparison of 18S rDNA barcode sequences from 59 *C. sinica* isolates by nucleotide sequence alignment showed the consistent diversity among the strains in or off the same ecologies, and all are comparable with the ever-first isolated strain JU727.

Keywords: *Caenorhabditis briggsae*, CFB strains, JU727, speciation

INTRODUCTION

Caenorhabditis sinica is a nematode within the genus *Caenorhabditis* and it was only reported to have a vast diversity within the territory of China (Wang *et al.,* 2010; Le *et al.,* 2023). The species and *C. briggsae* are supposed to be closest sisters and possibly descendants of a common ancestor. *Caenorhabditis sinica* is a male/female species while *C. briggsae* is an androdioecious species. However, the mechanism of their speciation has remained uncovered (Howe, Denver 2008). So, the two species are fundamental to shed light on the biological evolution of the *Caenorhabditis* species. Consequently, the research of each species can accumulate the understanding of their speciation that took millions of years back.

Besides, *C. sinica,* with many wild-type

variants in the wild, inherits mutations in its genomes (Wang *et al.,* 2010), indicating they are samples for investigating the evolutionary strategies that are uncovered in the genus *Caenorhabditis*. Therefore, the wild-type isolates of *C. sinica* play a key role in an evo-devo manner.

Theoretically, *C. sinica* and *C. briggsae* would present in the same habitats. In previous studies, *C. briggsae* was found to distribute broader while *C. sinica* has been only found in the midlands of China (Howe, Denver 2008; Huang *et al.,* 2014), which shares a long border with Vietnam, suggesting that *C. sinica* is restricted in Southeast Asia. In our recent research, we reported the presence of many *Caenorhabditis* nematodes including *C. briggsae* in the forests of Vietnam (Le *et al.,* 2023). These indicate that *C. sinica* should present in the neighborhoods of China and Vietnam. In this research, we report the isolation of *C. sinica* from the vegetation samples that were collected from forests and the analyses of their molecular diversity using DNA barcodes.

MATERIAS AND METHODS

Media

NaCl; peptone; agar; nutrient agar; cooking oil; Yeast extract; cholesterol; CaCl2; MgSO4; KH2PO4; K2HPO4; pig fat and mushroom solutions. New Cheap Media No.18 (NcM18) (0.4 g of pig fat + 20 mL of mushroom solutions + 17 g of agar + 4 ml of 0.75g/L NaCl + 1 L distilled water); "Nematode Growth Media" (NGM) (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 + 17 g of agar + 4 mL of 0.75 g/mL NaCl + 1L water) (Le *et al.,* 2021).

Isolation of *Caenorhabditis sinica* strains

Sampling: Vegetation samples were collected alongside the main path from the main gate to Bong Station in Cuc Phuong National Park and alongside the main path from the main gate to Bau Sau Station in Cat Tien National Park)

Isolation: C. sinica was isolated as our previous description (Le *et al.,* 2021). Five to 10 g of each vegetation sample (decomposed leaves, rotten fruits and flowers) was placed on one *Escherichia coli* OP50-seeded media plate (10 cm) and incubated at room temperature (approximately 25ºC) for three days. Subsequently, each of two worms per sample plate was transferred onto and grown on OP50-seeded media plate (5 cm) in a (19 ± 1) ºC for generations. The single worm raised its population so-called a strain.

Morphological selection: Determination of the *Caenorhabditis* nematodes is complicated and required experience. Each strain was classified by the clearance, the 1-mm size and the rod shape of body, which were more likely similar to *C. elegans* under microscopes (4X). Further sorting was the pharyngeal morphologies, which had two circular bulbs under microscopic magnification (40X), again this was recognized as *C. elegans* (Barriere, 2006). Regarding to sexes, among the strain candidates, the ones presented two sexes (male and females) were gated.

Species determination by molecular identification

Total DNA was isolated using "Single Worm Lysis" method for tiny nematodes (Ahringer, 2006; Le *et al.,* 2023). Part of the 18S rDNA sequence was amplified with two universal primers for nematodes (SSU18A (AAAGATTAAGCCATGCATG) and

SSU26R (CATTCTTGGCAAATGCTTT CG-3') (Barriere, 2006)). Every PCR product was purified with MEGAquick-spin™ Plus Total Fragment DNA Purification Kit (iNtRON biotechnology) and got sequenced with the Sanger method by a sequencing service (ATCG Limited Co.). Each of the DNA sequences was proceeded for BLASTnt on the National Center for Biotechnology Information (NCBI).

Phylogenetic analysis

The phylogenesis of the 59 18S rDNA sequences was aligned and compared together. Next, the phylogeny was reconstructed using the Neighbor-Joining method on MEGA11 as in our previous study (Tamura *et al.,* 2021).

RESULTS AND DISCUSSION

Two hundred nematodes from 400 vegetation samples with estimated body morphology for the *Caenorhabditid* nematodes (a "two-bulb" pharynx, dioecy, transparency, and approximately 1 mm length) were isolated from forest vegetation samples. They were able to develop well on NcM18 as NGM. Comparisons of the approximate 800 bp-18S rDNA sequences of the *Caenorhabditis* nematode candidates with the DNA sequence database on NCBI (National Center for Biotechnology Information) presented 51 *C. sinica* strains from 50 sites in Cuc Phuong and seven from 50 sites in Cat Tien (Fig. 1). Moreover, we isolated one strain in Quoc Oai district in Hanoi. Previous sampling reported the finding of species in Bac Can Province (Wang *et al.,* 2010). Thus, the species likely appears in many places throughout the country, suggesting more strains are living in the wild.

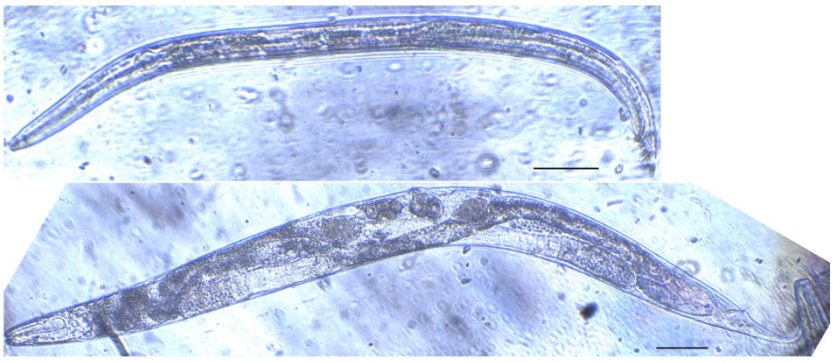

Figure 1. Two sexes presented in *C. sinica* populations, male (top) and female (bottom). Scale bar: 100 µm.

Next, we compared the 18S rDNA sequences of the 59 genomes and found that they are different, of which 57 are 99.30% identical and two are lower (CFB23: 85.88% and CFB7: 90.90%) to the sequence EU19600 of the strain JU727 in China (Tables 1 and S1). These results indicate a higher diversity of *C. sinica* in Cuc Phuong than Cat Tien by 7.28 times (a strain ratio of 51/7). The phylogenic analysis shows that likely 59 *C. sinica* have been evolved 18 times, and they are biased towards Cuc Phuong (Fig. 2). These results pursue the species discrimination of *C. sinica*, consistent with an unequal theme of the nematode distributions as in our previous reports on *C. briggsae* and parasitic *Halicephalobus* (Le *et al.,* 2023) between the long-distance forests.

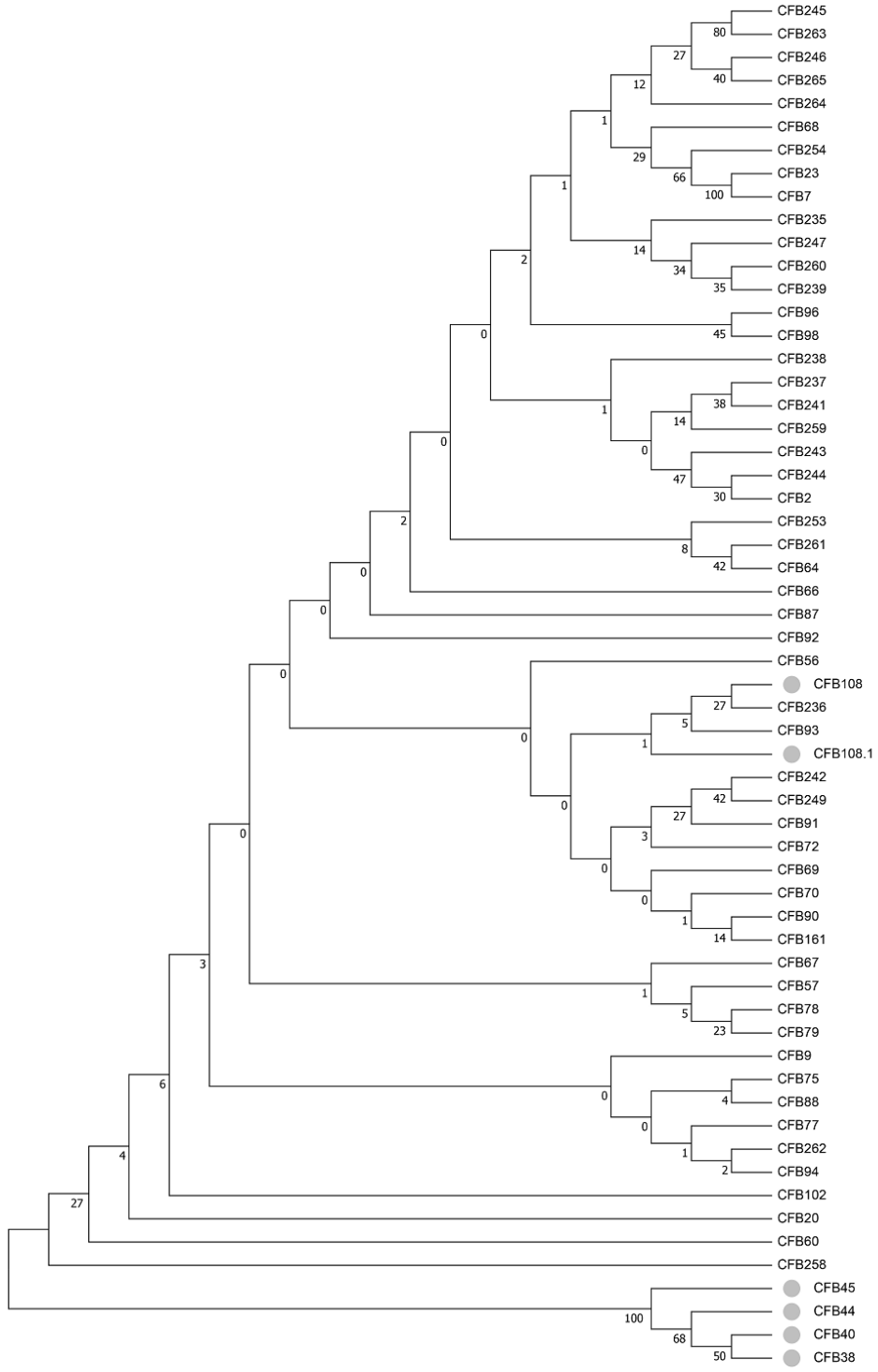

Figure 2. Phylogenetic tree of *C. sinica* strains isolated in Cat Tien (●), CFB235 in Quoc Oai, and the rest in Cuc Phuong.

Table 1. Comparison of 18S rDNA sequences of *C. sinica* strains in Vietnam with EU19600 (JU727) in China.

No.	CFB catalogs	Identity (%) with EU19600†	Genbank Access (NCBI)	Sample sites‡
1	CFB254	99,42	OR130938	cP37
2	CFB253	99,88	OR130939	cP20
3	CFB260	99,53	OR130940	cP34
4	CFB259	99,65	OR130941	cP34
5	CFB258	99,64	OR130942	cP32
6	CFB239	99,76	OR130943	cP9
7	CFB238	99,88	OR130944	cP9
8	CFB237	99,76	OR130945	cP35
9	CFB236	99,88	OR130946	cP37
10	CFB245	99,76	OR130947	cP17
11	CFB244	99,65	OR130948	cP19
12	CFB243	99,65	OR130949	cP20
13	CFB242	99,65	OR130950	cP13
14	CFB241	99,64	OR130951	cP37
15	CFB249	99,30	OR130952	cP21
16	CFB247	99,76	OR130953	cP13
17	CFB246	99,76	OR130954	cP25
18	CFB265	99,76	OR130955	cP29
19	CFB264	99,88	OR130956	cP28
20	CFB263	99,65	OR130957	cP21
21	CFB262	99,76	OR130958	cP9
22	CFB261	100	OR130959	cP28
23	CFB87	99,64	OR130960	cP37
24	CFB68	99,64	OR130961	cP30
25	CFB78	99,88	OR130962	cP30
26	CFB66	99,76	OR130963	cP30
27	CFB91	99,65	OR130964	cP28
28	CFB96	99,65	OR130965	cP32
29	CFB75	99,53	OR130966	cP27
30	CFB93	99,53	OR130967	cP24
31	CFB79	99,76	OR130968	cP37
32	CFB108.1	99,64	OR130969	cT48
33	CFB77	99,76	OR130970	cP32
34	CFB94	99,76	OR130971	cP28
35	CFB88	99,76	OR130972	cP30
36	CFB98	99,76	OR130973	cP30
37	CFB60	99,64	OR130974	cP36
38	CFB56	99,64	OR130975	cP9
39	CFB70	99,76	OR130976	cP36
40	CFB69	99,76	OR130977	cP8
41	CFB64	99,88	OR130978	cP21
42	CFB57	99,64	OR130979	cP3
43	CFB92	99,64	OR130980	cP22
44	CFB90	99,53	OR130981	cP17
45	CFB72	99,65	OR130982	cP33
46	CFB67	99,64	OR130984	cP27
47	CFB20	99,64	OR130985	cP1
48	CFB9	99,64	OR130986	cP40
49	CFB23	85,88	OR130987	cP32
50	CFB7	90,90	OR130988	cP6
51	CFB2	99,53;-	OR130989	cP15
52	CFB40	99,76;-	OR130990	cT31
53	CFB44	99,88;-	OR130991	cT31
54	CFB38	100;-	OR130992	cT34
55	CFB45	99,76;-	OR130993	cT31
56	CFB161	99,53;-	OR430994	cP12
57	CFB102	99,76	OR130983	cT1
58	CFB108	99,76	OR130936	cT48
59	CFB235	99,76	OR130937	Hoa Thach, Quoc Oai, Hanoi 20°56'39" N; 105°33'38"E

† EU19600 is the first 18S rDNA sequence of *C. sinica* JU727 that was isolated from China. To see the detailed comparison of sequence alignments, access in the GenBank on NCBI (https://www.ncbi.nlm.nih.gov) and Table S1. †cT – Cat Tien National Park; cP – Cuc Phuong National Park. The 18S rDNAs were compared by BLAST in NCBI on May 27, 2023.

In addition, the isolates from Cuc Phuong are typically more diverse than those from Cat Tien (Fig. 2). According to the 18S rDNA sequences in this research, two strains (CFB108 and CFB108.1) changed at least 12 times and are more closely related to the strains from Cuc Phuong. We assume different possibilities, for example, the similarity of random changes during life histories and the mutual migration of strains within the two forests. In contrast, the four (CFB45, 44, 40, and 38) had four times and are apart from the strains in Cuc Phuong. Thus, the four of six isolates from Cat Tien likely evolved independently of the majority.

In conclusion, fifty-nine *C. sinica* were isolated from two national parks (Cuc Phuong and Cat Tien) and raised in the laboratory. The number of isolates and the comparison of 18S rDNA sequences revealed the divergence is higher in Cuc Phuong. This species will facilitate the study of evolution within the *Canorhabditis* nematodes.

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