Short Communication

First Record of *Caenorhabditis briggsae* (Nematoda: Rhabditidae) from the Giant African Land Snail, *Lissachatina fulica* (Gastropoda: Achatinidae) in the Southern Philippines

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Abstract | A new population of *Caenorhabditis briggsae* (Nematoda: Rhabditidae) was recovered from the giant African land snail, *Lissachatina fulica* (Gastropoda: Achatinidae), in Bukidnon province (8.0515° N, 124.9230° E) of the southern Philippines. This species was identified by morphological characterization and analysis of the D2-D3 expansion segments of the 28S rDNA sequence. The D2-D3 rDNA sequence alignment of the four isolates, B2R1 (MT459236), B2R2 (MT459237), B2R3 (MT459238), and B3R1 (MT459239), do not exhibit any nucleotide differences with each other or with previously described populations of *C. briggsae* from China (MN519141), Germany (EF417140), and the USA (AY604481, JN636061); therefore, they are conspecific. This is the first report of *C. briggsae* isolated from *L. fulica* in the southern Philippines. This study provides an additional account of *C. briggsae* to existent worldwide descriptions, thereby extending its geographic distribution and habitat range.

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Introduction

Nematodes can be found dwelling inside several species of gastropods, and their relationships can range from paratenic to parasitic and even pathogenic (Grewal *et al*., 2003). *Lissachatina fulica*, a member of the gastropod family Achatinidae, is often known as the giant African land snail (GAS). This species is a widespread terrestrial snail that is found across the Philippines and serves as a reservoir for a variety of parasites, including nematodes of different taxa (Constantino-Santos *et al*., 2014; d'Ovidio *et al*., 2019; Silva *et al*., 2019).

Caenorhabditis briggsae (Nematoda: Rhabditidae) is under the Elegans group, a monophyletic clade

consisting of seven species within the genus *Caenorhabditis* (Sudhaus and Kiontke, 1996). To date, this "Elegans" group solely contains two hermaphroditic species, namely, *C. briggsae* and *C. elegans*, whereas other are designated as gonochoristic species (Nigon and Dougherty, 1949; Sudhaus and Kiontke, 1996). *Caenorhabditis briggsae* is described as reproductively isolated and phylogenetically distinct from the model nematode, *C. elegans* (Nigon and Dougherty, 1949; Baird, 2001). This species was reported in various countries such as China, Europe, India, Japan, Kenya, Reunion Island, South Africa, Taiwan, the United States, and South America, mainly from soils, mushrooms, rotting chestnuts, cabbage leaves, fig fruits, and snails (Dougherty and Nigon, 1949; Baird, 2001; Kiontke and Sudhaus, 2006; Kiontke *et al*., 2011; Guerrero *et al*., 2018). In the Philippines, the first ever recorded *C. briggsae* isolate was also recovered from *L. fulica* in the capital city (Metro Manila) using the SSU rDNA marker via the ash digestion method (Constantino-Santos *et al*., 2014). Herein, we report for the first time an androdioecious nematode population (4 isolates) isolated from the GAS, *L. fulica,* in different areas of Bukidnon province, southern Philippines. The use of morphology and analysis of the D2-D3 expansion segments of the 28S rRNA were employed.

Materials and Methods

Nematode isolates

Four isolates of *C. briggsae* were recovered from *L. fulica* using the methods described by Diano *et al.* (2022). Specimens of *L. fulica* were gathered from Bukidnon province (8.0515° N, 124.9230° E) in Mindanao Island, southern Philippines. Snail samples were methodically washed with sterile distilled water (SDW) to remove the adhering dirt and then placed in a sterilized plastic container while carrot disks were added as food until snail death. A total of four snail cadavers were washed with SDW, the shells were taken out, and were transferred aseptically onto a nutrient agar (NA) media (in Petri plates, 60 mm x 15 mm), which acted as the seed cultures. After 24 h of incubation at 27 °C, the Petri plates were examined under the dissecting microscope to check for the presence of nematodes. Agar blocks with live nematodes were transferred to freshly plated NA medium, seeded with a 24-h *Proteus* sp. bacterial culture isolated from the cadaver of *L. fulica* , as a food source. Nematode monocultures were attained through sub-culturing by picking and transferring one hermaphrodite to a freshly plated NA medium.

Morphological characterization

The nematode samples were treated with anhydrous glycerol following the standard methods of Seinhorst (1959) as modified by De Grisse (1969). Three different solutions were used to fix nematodes specifically, Solution 1 (2 portions glycerol, 8 portions of 4% formaldehyde, and 90 portions water), Solution 2 (5 portions glycerol and 95 portions of 97 % ethanol), and Solution 3 (pure glycerol). After the fixation method, nematodes of different developmental stages were subsequently picked and transferred to glass slides for mounting. Mounted nematodes in glass slides were examined under a compound microscope to check for the key characters. Each fixed nematode from different developmental stages was measured using an Olympus BX41 (Tokyo, Japan) compound microscope equipped with a digital sight camera, Nikon DP27 and an imaging software, Cell Sens.

Molecular, sequence alignment, and phylogenetic analysis

A total of 100 monocultured hermaphroditic nematodes were independently picked and placed in a 1.5 ml Eppendorf tube with a drop of SDW, frozen, and punctured with a sterile blunted end micropipette tip. The extraction of genomic DNA (gDNA) was then performed in accordance with the manufacturer's methodology (Dongsheng Biotech, Inc., Guangdong, China.). Extracted gDNA was kept in a 1.5 ml Eppendorf tube and was sent to Macrogen, Inc. in South Korea for PCR amplification, purification, and sequencing of samples. The D2-D3 expansion fragments of 28S rDNA (LSU) primer pair used were: D2A: 5'-ACAAGTACCGTGAGGGAAAGTTG-3' and D3B: 5'-TCCTCGGAAGGAACCAGCTACTA-3' (Nunn, 1992). The PCR amplification was programmed with an initial denaturation at 95°C for 5 min, then by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45s, extension at 72°C for 1 min and a final extension at 72°C for 10 min (Ye and Giblin-Davis, 2013).

Caenorhabditis briggsae D2-D3 obtained sequences was first trimmed/edited and then matched with other available sequences in the GenBank using the basic local alignment search tool (BLASTN) of the National Center for Biotechnology Information (NCBI) (Altschul *et al*., 1990). The sequences

were finally submitted to NCBI with accession numbers MT459236, MT459237, MT459238, and MT459239. An alignment of D2-D3 expansion segments of the 28S rDNA sequence of present nematode samples along with other sequences of *Caenorhabditis* species was done using default Clustal W parameters in MEGA X (Kumar *et al.*, 2018) and manually optimized using a BioEdit software (Hall, 1999). All poorly aligned end regions were also manually trimmed, and all characters were treated as equally weighted and gaps as missing data. Phylogenetic trees were particularized using the Bayesian inference method executed in the program MrBayes 3.2.7 (Ronquist *et al.*, 2012). Bayesian phylogenetic analysis was performed using the GTR + I + G nucleotide substitution model; analyses were run under 1×10^6 generations (4 runs), and Markov chains were sampled every 100 generations, with the first 250 sampled trees discarded as "burn-in". Finally, a 50% majority rule consensus tree was created. The Bayesian tree was finally visualized using the FigTree program 1.4.3 (Rambaut, 2018).

Results and Discussion

A hermaphroditic species of the free-living nematode was isolated and classified from the corpse of *A. fulica*. The photomicrograph of the isolated nematode presented the key morphological characteristics as described by Dougherty and Nigon (1949) (Figure 1).

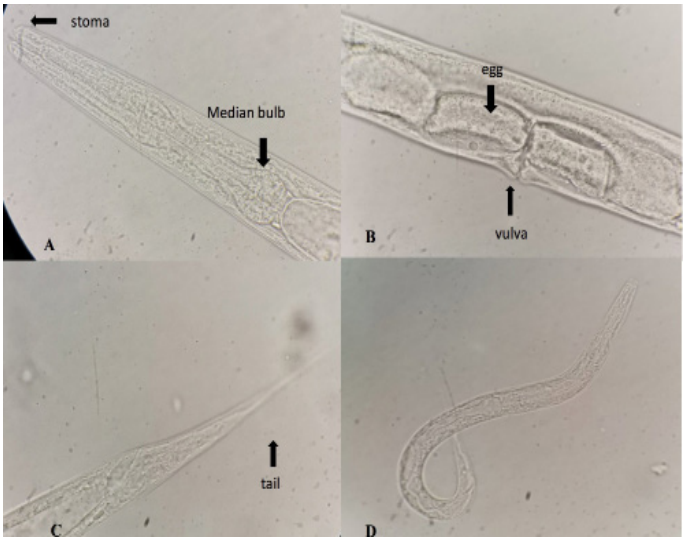

Figure 1: *Photomicrograph of Caenorhabditis briggsae hermaphrodite isolated from Lissachatina fulica from Bukidnon, Philippines. A: anterior region showing stoma and median bulb (arrows); B: lateral view showing vulva and egg (arrows); C: posterior region showing female tail and anus (arrows); and D: juvenile.*

Description

Adult: Characterized by having three ridges in their lateral fields, a stegostomum forming a pharyngeal sleeve half of the total stomal length, one small tooth projecting in each metarhabdion, a well-developed median bulb, and protandrous hermaphrodites with a female: male ratio of 15:1 (Guerrero *et al.*, 2018).

Female: The stoma is long and tubular. Pharynx length occupies 18.6% of the body. The vulva is located centrally, with slightly protruding lips and a slightly thicker vulval-uterine connection. They lay very early-stage eggs, which are oval. Tail is conical, about 62 µm long. *Juveniles:* Unsheathed cuticle. Body short, about 36% shorter than the female adult. Pharynx length occupies 18% of the body length. Median and basal are not well developed. The tail is elongated and conical.

In the present study, we were not able to observe male nematodes for all four isolates (B2R1, B2R2, B2R3, and B3R1) despite culturing them several times in NA medium with varying bacterial concentrations as a food source. This can likely be attributed to *C. briggsae* being a sexually reproducing nematode exhibiting androdioecy, wherein a majority of the population exists as self-fertile hermaphrodites and very uncommon males. This is similar to the model organism, *C. elegans,* and another rhabditid nematode, *Oscheius myriophila* (Kiontke *et al.*, 2004). While males in all four isolates in this study were not observed, earlier studies have reported the presence and importance of males in the morphological characterization of *C. briggsae* (Nigon and Dougherty, 1949; Fodor *et al.*, 1983; Baird, 2001). For now, we cannot pinpoint the explicit factors of male nonexistence in our samples, and further investigations are recommended. Nevertheless, from the four isolates of *Caenorhabditis briggsae* B2R1 (MT459236), B2R2 (MT459237), B2R3 (MT459238), and B3R1 (MT459239), molecular characterization was done to confirm species identification, and sequences of the D2-D3 fragment of 28S rDNA of our four isolates yielded 608, 606, 598 and 632 base pairs (bp) respectively. The alignment file of D2-D3 rDNA sequences of the present four strains did not show any nucleotide differences with each other or with the already described populations of *C. briggsae* from China (MN519141), Germany (EF417140), and the USA (AY604481, JN636061), hence being considered the same. Moreover, a comparison between the newly

isolated strains of *C. briggsae* from Bukidnon, southern Philippines, and the first recorded *C. briggsae* from Metro Manila, northern Philippines (Constantino-Santos *et al.*, 2014) is not feasible due to the lack of morphological data for the latter and the different informative markers used. For instance, SSU rDNA was used to sequence *C. briggsae* from Metro Manila, which showed 98.1% similarity to *C. briggsae* (GB JX512216). In contrast, the new isolates of *C. briggsae* from Bukidnon were sequenced using the D2-D3 fragment of 28S rDNA.

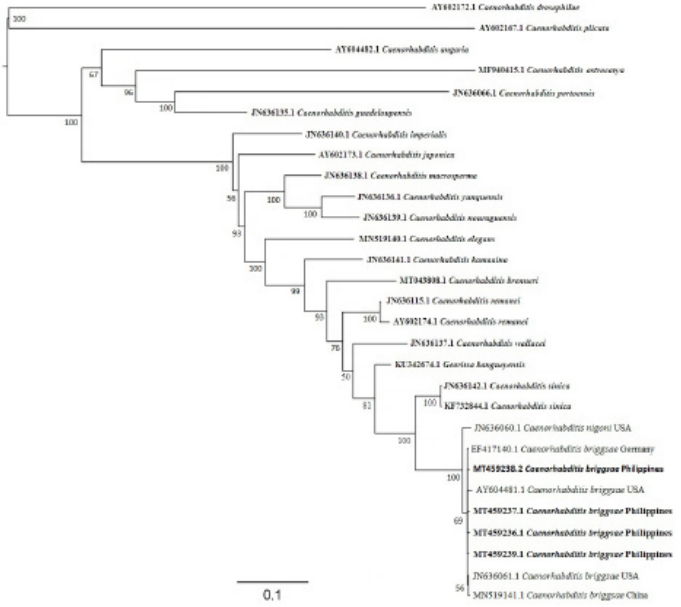

Figure 2: *Phylogenetic tree as inferred from the Bayesian inference analysis between four Caenorhabditis briggsae strains and other closely related strains are provided based on the D2-D3 fragment of 28S rDNA region. Bayesian phylogenetic analysis was carried out using the GTR + I + G nucleotide substitution model; analyses were run under 1 × 10⁶ generations (4 runs).*

The phylogenetic relationships as inferred from the Bayesian inference analysis between four *C. briggsae* strains and other closely related strains are provided based on the D2-D3 fragment of the 28S rDNA region (Figure 2). Phylogenetic analyses showed a clear monophyly of the group formed by the present 4 isolates (*C. briggsae* MT459236, *C. briggsae* MT459237, *C. briggsae* MT459238, and *C. briggsae* MT459239) and other described *C. briggsae* from China (MN519141), Germany (EF417140), and the USA (AY604481, JN636061), probably conspecific isolates (Figure 2), thus confirming their identification. Sequences of *C. briggsae* formed a monophyletic group with *Caenorhabditis nigoni* described by Kiontke *et al.* (2011) from the USA, and together they formed a sister clade with species of *Caenorhabditis sinica* described by Kiontke *et al.* (2011) from the USA and Huang *et al.* (2014) from China. The D2-D3 fragment of the 28S rDNA region is conservative in resolving phylogenetic relations among closely related species.

Caenorhabditis briggsae is found in different geographic regions worldwide, isolated in various habitats such as snails, soils, mushrooms, slugs, rotting fruits, and vegetable substrates (Kiontke and Sudhaus, 2006; Cutter *et al.*, 2006; Ross *et al.*, 2010; Kiontke *et al.*, 2011; Félix *et al.*, 2013; Petersen *et al.*, 2015; Guerrero *et al.*, 2018; Constantino-Santos *et al.*, 2014). The recent recovery of four isolates of *C. briggsae* from the Philippines has built up the number of tropical *C. briggsae* isolates, as noted by Cutter *et al.* (2006). Among species in the genus *Caenorhabditis*, the association between *C. briggsae* and terrestrial gastropods is classified as necromenic, wherein nematodes wait for their host to die, feed on the bacteria that proliferate on the cadaver, and resume their development (Kiontke and Sudhaus, 2006). For example, reports demonstrated that live *C. briggsae* were isolated from slug intestines (Petersen *et al.*, 2015); free from the cut tissues of GAS by the ash digestion fluid method (Constantino-Santos *et al.*, 2014); and from the cadaver of GAS (this study), suggesting an association leaning towards necromeny. Another form of association previously reported in *C. briggsae* is entomopathogenic, wherein an evolution from necromeny likely occurred through the association with an entomopathogenic bacterium (Abebe *et al.*, 2010, 2011). Abebe *et al.* (2010) found that the *C. briggsae*-*Serratia* sp. SCB1 complex formed entomopathogenic relationships in the wild, and laboratory experiments confirmed that this nematode and its natural bacterial associate can penetrate, kill, and reproduce in an insect host.

To the best of our knowledge, this is the first report of *C. briggsae* isolated from *L. fulica* in the southern Philippines. This study provides an additional account of *C. briggsae* to existing descriptions, thereby extending geographic distribution and habitat range. In addition, it is interesting to further explore the diversity of *Caenorhabditis* species in the Philippine archipelago, especially considering how *C. elegans* and the most closely related species likely diverged in this very region.

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Novelty Satement

By employing morphological and molecular diagnostic tools, this study is the first to report the nematode, Caenorhabditis briggsae from one of the worst invasive species in the world, the giant African land snail, Lissachatina fulica, thereby expanding its known distribution and habitat range.

Author's Contribution

MAD: Conceptualization, Methodology, Investigation, Data Analysis, Writing-Original draft; LD: Conceptualization, Methodology, Investigation, Writing, Editing, NPDS: Conceptualization, Methodology, Writing, Editing, NHS: Conceptualization, Methodology, Supervision, Resources, Data Curation, Validation, Writing, Editing

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Availability of data and material
All available data are included in this manuscript and sequences are deposited in NCBI GenBank.

Ethics approval
This research does not contain human participants. This research used *A. fulica* which is considered to be invasive and pest, and there was no live dissection conducted.

Conflicts of interest
The authors have declared no conflict of interest.

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