Morphological and molecular characteristics of
*Hemicriconemoides cocophillus* from the origin of Robusta
coffee plantation in Malang, East Java

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**Abstract.** *Hemicriconemoides cocophillus* is one of the phytonematode associated with Robusta coffee plantation. Information regarding morphology, morphometry, and molecular characters of *H. cocophillus* has not been reported in Indonesia. This study is aimed to describe characters of *H. cocophillus* that was extracted from soil samples taken from Robusta coffee plantation in Malang, East Java. Nematode extraction was conducted using the flotation-centrifuge method. Morphology and morphometric characters were observed from the permanent nematode slides. Single nematode DNA extract was amplified at the D2D3 segment of 28S and ITS1-5.8S-ITS2 with universal primers. The amplicon was sequenced and analyzed for similarity and phylogenetic tree analysis. The female morphological key character of *H. cocophillus* are sheath looks clear and thin, stylet strong with anchor-shaped knobs, and tail tapering to a finely rounded tip. Female morphometrics key characters are n=5, L=446.5±22.5 µm, a=15.8±0.9, b=5.3±0.2, c=13.9±1.5, stylet length=51.6±2 µm, tail length=32.3±3.2 µm. Based on the D2D3 and ITS1-5.8S-ITS2 regions, *H. cocophillus* have similarity level of 99% and 98%, respectively with *H. cocophillus* from NCBI. Phylogenetic tree result using Maximum Likelihood at the D2D3 segment of 28S and ITS1-5.8S-ITS2 regions show that *H. cocophillus* in this study was included in one clade with *H. cocophillus* from Mozambique.

1. Introduction

Low population of ring nematode (*Hemicriconemoides*) was found on Robusta coffee plantation in the Sumber Asin Experimental Station, Malang, East Java. Previously, *Hemicriconemoides* has not been reported to be associated with Robusta coffee plants in Indonesia. *Hemicriconemoides* spp. is a phytonematode which has a special characteristic in females covered by an outer accessory layer or sheath with smooth annuli. The sheath does not exist in juveniles, which only have a single cuticle ornamented by rows of scales and spines [1].

*Hemicriconemoides* spp. is a migratory ectoparasite phytonematode. These phytonematodes are found in soils [1]. *Hemicriconemoides* spp. has been reported infecting coffee plants in India. In Arabica coffee seedling that are artificially infected with *H. cocophillus*, *H. coffeae*, and *H. gaddi* can cause "crinkle leaf disorder" under greenhouse conditions [2]. Artificial inoculation by *H. cocophillus* on Arabica and Robusta coffee seedling significantly reduced the stems height and roots weight both...
of coffee plants [3]. Therefore, it is essential to characterize *Hemicriconemoides* species that infect Robusta coffee in this study.

Characterization of *Hemicriconemoides* spp. only based on morphology is still a difficulty to differentiate between species if done by non-taxonomists [1]. The best way in characterizing *Hemicriconemoides* species is combination of all existing methods. This research will use morphology, morphometry and molecular characters to describe *Hemicriconemoides* species in this study.

In recent years, the sequence data from the ribosomal rRNA genes has been increasingly used to provide a valuable tool in the identification of nematodes and reconstruction of phylogenetic relationships. The ribosomal RNA (rRNA) region consisting of 18S, ITS1-5.8S-ITS2, and 28S is widely used for the molecular identification of nematodes. It is fast in detecting the evolutionary process for diversity between genera, between species and between sub-species [4]. Molecular identification of *Hemicriconemoides* spp. with amplification target D2D3 segments of 28S rRNA and ITS1-5.8S-ITS2 regions has been previously reported [5, 6]. This study aims to characterize *Hemicriconemoides* sp. based on morphology, morphometry and molecular characters.

2. Methods

2.1. Sampling

*Hemicriconemoides* species was obtained during a survey of phytonematode communities in Robusta coffee plants. *Hemicriconemoides* species was extracted from soil samples of Robusta coffee roots. Soil samples were taken at the Sumber Asin Experimental Station, Malang, East Java in July 2018 with a coordinate point of 8° 16'199' S 112° 42.608' E and an altitude of 607 m asl. Soil samples taken from the main stems of coffee plants are 30-60 cm apart. Shade trees, that are white lead (*Leucaena leucocephala*) and coconut are growing among the sampled plants.

2.2. Nematode extraction

Extraction of *Hemicriconemoides* species from the soil was carried out using the centrifugation flotation method. Soil samples were mixed until homogeneous. A 100 mL soil sample was mixed with 900 mL of water in a container. The soil suspension was stirred until homogeneous and deposited for ± 40 sec. The soil suspension was poured into the stack of nematode sieves with pore size of 50, 100 and 400 mesh, respectively. The suspension was poured into a 15 mL capacity centrifugation tube. The suspension was centrifuged for 5 min at a speed of 1500 rpm, then the water was removed, and the precipitate was added with a 40% sugar solution and then homogenized. The suspension was centrifuged for 1 min at 1700 rpm. The supernatant was poured through 400 mesh nematode sieve and rinsed with distilled water. The nematode suspension was stored in a collection bottle containing 25 mL of a nematode suspension to be immediately observed or stored in a refrigerator at a temperature of 10 °C [7].

2.3. Phytonematode permanent slides

A total of 1 mL phytonematode suspension was poured in 1.5 mL collection tube then let stand for 20 min. The volume of the suspension was reduced slowly until the remaining 60 µL. FA 4:1 solution (4% formalin and 1% acetic acid) at 90 °C was poured into a phytonematode suspension to a volume of 1000 µL. The suspension was incubated at a water bath of 85 °C for 1 hr for fixation then let stand for 24 hr at room temperature. FA solution in phytonematode suspension was diluted with distilled water 3 times. The phytonematode suspension was diluted 3 times. Each dilution was taken 800 mL nematode suspension carefully, then added 800 mL distilled water. Phytonematode suspension volume was reduced to 150 µL carefully so that the phytonematode were not wasted. Two drops of glycerol were dripped on a microscope slide which has a 1 cm diameter paraffin ring followed by 3 drops of distilled water on it. The phytonematode suspension was dripped as much as 150 µl slowly over the glycerol and distilled layers to form a mound of water and then wait for 12 hr. Phytonematodes that
already contain glycerol were transferred to a microscope slide containing 20 μL 100% glycerol. Phytonematodes were covered with a cover glass and sealed with transparent nail polish. Permanent phytonematode slides were labelled and ready to be observed [8].

2.4. Phytonematode morphology and morphometry characters

Permanent nematode slides were observed using an Olympus BX 51 binocular light microscope with a 2 MP CCD camera. Morphometric measurements and photographs using ToupView® software. Morphometric characters are analyzed based on De Man's formula [9, 10].

2.5. Molecular character and phylogenetic tree analysis

DNA extraction of Hemicriconemoides followed the Holterman’s method [11]. A single Hemicriconemoides morphologically confirmed was cut into 2 parts using the tip of the syringe and then inserted into a 0.2 mL PCR collection tube containing 25 μL of Nuclear Free Water. A total of 25 μL of Holterman Worm Lysis Buffer solution containing 200 mM NaCl, 200 mM Tris-HCL pH 8, 1% 2-mercaptoethanol, and 800 μg/mL Proteinase K were added into a 0.2 mL PCR collection tube that already contained Hemicriconemoides. The mixture was vortexed for 1 min followed by incubation using the GeneAmp® PCR System 9700 PCR machine from Applied Biosystems at a temperature of 65 °C for 90 min and followed by 99 °C for 5 min. Extracted DNA is ready to be amplified.

A total of 2 DNA regions, D2D3 segment of 28S rRNA and ITS1-5.8S-ITS2 were amplified separately. Each DNA region was made into a mixture of PCR solution with a composition of 2 μL of Hemicriconemoides DNA template, 12.5 μL 2x Go Taq® Green Master mix (Promega), 1 μL of 10 μM forward primer, 1 μL of reverse primer 10 μM, and 8.5 μL Nuclease Free Water is put into a 200 μL microtube. The primers used in the D2D3 segment of 28S rRNA consists of the forward D2A ‘5- ACAAGTACCCTGAGGGAAGTGTG -3’ and reverse D3B ‘5- TCAGAGGAACCGCTACTA -3’ [12], while the ITS1-5.8S-ITS2 consists of the forward TW81 ‘5- GTCAGCTAGCTGACCTGCTGC -3’ and reverse AB28 ‘5- ATATGTTAAGTTCAGCGGT -3’ [13]. The PCR machine used was GeneAmp® PCR System 9700 from Applied Biosystems. Each DNA region had the same PCR cycle and annealing temperature and was amplified separately. The PCR cycle for each DNA target was 40 cycles. Each cycle consisted of denaturation (94 °C, 60 sec), annealing (55 °C, 60 sec), extension (72 °C, 120 sec). The cycle begins with initial denaturation (94 °C, 4 min) and ends with 72 °C of the final extension for 10 min. The amplicon was electrophoresed with 1% agarose gel and visualized with UV transilluminator.

PCR products were analyzed for their nucleotide sequences by the sanger dideoxy sequencing method. The DNA Sequence Assembler v4.7 software trial version is used for contiq. In the contiq process, the ambiguous sequence was cut. The results of the nucleotide sequence were aligned with BLAST® from the National Center for Biotechnology Information (NCBI). Bioedit version 7.2.6.1. used for the alignment of nucleotides with reference sequences from NCBI. MEGA 10.05 was used for phylogenetic tree analysis with the Maximum Likelihood method, bootstrapping 1000 times, and using the model with the lowest BIC (Bayesian Information Criterion) value on the model test results.

3. Results and discussion

3.1. Morphology and morphometry characters

Only female nematodes were found. The body was slightly curved in ventral. The sheath (extra cuticles) was clearly visible and thin. The 1st annulation was smaller than the 2nd annulation in the anterior part. Stylet short and strong with anchor shaped. Dorsal pharyngeal gland opening was very close to the stylet base. The anus was right or one posterior to the vulva. The spermatheca was small, round to oval and has no sperm. The tail was tapered to a finely rounded tip. Based on this morphology character, the nematode was identified as Hemicriconemoides cocophillus (Fig. 1).

The female character of genus Hemicriconemoides is having a sheath [14]. The description of H. cocophillus in this study is consistent with previous study [10, 15]. H. cocophillus was 1st described...
Figure 1. Light microscopic photographs of female *Hemicriconemoides cocophillus* population from Malang. Female, A: entire body, B-D: anterior region, E: female, F: posterior region. Scale bar: A=100 µm, B-F= 30µm.

by Loos in 1049 [16] from the grass and coconut soil in Sri Lanka. *H. cocophillus* has never been reported in the rhizosphere of the Robusta coffee plants in Indonesia.

Morphometry characters of *H. cocophillus* was measured based on 5 females from the population of Malang, East Java (Table 1). Measurement of female morphometry are (µm): body length (L)= 446.5±22.5 (407.2-464.1) µm, DGO= 5±0.5 (4.3-5.5), V= 91.6±1% (90.2-92.5), Genital tract length = 199.2±7 (191.4-209.4), OV= 44.7±1.7 (43.1-47), Stylet= 51.6±2 (49.8-55), Stylet knob height = 3.6±0.5 (3-4.2), Stylet knob width = 7.3±0.5 (6.7-7.8), Diameter at mid-body 28.3±1.6 (26.3-29.9), Diameter at anus =18.1±1.4 (16.2-19.7), Diameter at vulva = 21.4±0.9 (20.2-22.2), Tail length = 32.3±3.2 (28.6-37.4), Pharynx length = 84.5±3.1 (80.7-87.9), V-anus distance = 5.2±2.1 (2.8-7.4), First lip annulus diameter = 18.4±0.8 (17.5-19.1), Sec lip annulus diameter = 20.2±1.1 (19-22.1), Third body annulus diameter = 21.5±1.3 (20.1-23.4). Body length ratio, i.e. a= 15.8±0.9 (15.2-17.3), b= 5.3±0.2 (5-5.5), c= 13.9±1.5 (12.2-15.8), c'= 1.7±0.2 (1.6-2), dan o= 9.7±1 (8.5-11.1). Morphometric characters of *H. cocophillus* have similarities with the previous study [10].
Table 1. Morphometry of *Hemicriconemoides cocophillus* from Malang, Indonesia compared to the Mozambique.

| Characters a | Populations b | Mozambique [10] | Malang, Indonesia |
|-------------|---------------|----------------|------------------|
| N           | 33 females    | 5 females      |
| L*          | 512±30.1      | 446.5±22.5     |
|             | (440-570)     | (407.2-464.1)  |
| A           | 14.9±1.9      | 15.8±0.9       |
|             | (10.7-18.4)   | (15.2-17.3)    |
| B           | 5.0±0.3       | 5.3±0.2        |
|             | (4.5-5.9)     | (5-5.5)        |
| C           | 14.0±1.4      | 13.9±1.5       |
|             | (11.8-17.7)   | (12.2-15.8)    |
| c'          | 1.7±0.2       | 1.7±0.2        |
|             | (1.3-2.2)     | (1.6-2)        |
| O           | 9.4±1.2       | 9.7±1          |
|             | (6.8-17.7)    | (8.5-11.1)     |
| DGO*        | 5.5±0.6       | 5±0.5          |
|             | (4.0-6.5)     | (4.3-5.5)      |
| V           | 92.0±0.7      | 91.6±1         |
|             | (90.5-93.5)   | (90.2-92.5)    |
| Genital tract length* | 226±45.9         | 199.2±7         |
|             | (201-319)     | (191.4-209.4)  |
| OV          | 45.5±4.3      | 44.7±1.7       |
|             | (38.0-57.0)   | (43.1-47)      |
| Stylet*     | 57±1.3        | 51.6±2         |
|             | (54.0-59.5)   | (49.8-55)      |
| Stylet knob height* | 3.5±0.4       | 3.6±0.5        |
|             | (3.0-4.5)     | (3-4.2)        |
| Stylet knob width* | 9.0±0.8        | 7.3±0.5        |
|             | (6.5-10.5)    | (6.7-7.8)      |
| Diameter at mid-body* | 34.5±3.3       | 28.3±1.6       |
|             | (27.5-43)     | (26.3-29.9)    |
| Diameter at anus* | 22.0±1.9       | 18.1±1.4       |
|             | (19.0-26.5)   | (16.2-19.7)    |
| Diameter at vulva* | 25.0±1.7       | 21.4±0.9       |
|             | (20.5-28.5)   | (20.2-22.2)    |
| Tail length* | 36.8±3.5         | 32.3±3.2       |
|             | (31.5-43.5)   | (28.6-37.4)    |
Table 1. (continued).

|                         | Unit Mean ± SD | Unit Mean ± SD |
|-------------------------|----------------|----------------|
| Pharynx length*         | 103±4.2        | 84.5±3.1       |
|                         | (97-110)       | (80.7-87.9)    |
| V-anus distance*        | 5.5±1.2        | 5.2±2.1        |
|                         | (3.5-8.0)      | (2.8-7.4)      |
| First lip annulus diameter* | 11.5±0.8   | 13.4±1.7       |
|                         | (10.0-13.0)    | (11.3-16)      |
| Sec lip annulus diameter* | 15.5±0.6     | 16.6±1         |
|                         | (14.0-17.0)    | (15.5-18.1)    |
| First body annulus diameter* | 19.0±1.4     | 18.4±0.8       |
|                         | (17.0-21.5)    | (17.5-19.1)    |
| Sec body annulus diameter* | 21±1.4        | 20.2±1.1       |
|                         | (19.0-23.5)    | (19-22.1)      |
| Third body annulus diameter* | 22.5±1.5     | 21.5±1.3       |
|                         | (20.0-25.0)    | (20.1-23.4)    |

a * = unit μm. L = body length; a = maximum body length / body width; b = body length / esophageal length; c = body length / tail length; c’ = tail length / body width in the anus (female); DGO = distance from the base knob stylet to the dorsal pharyngeal gland orifice; o = DGO x 100 / stylet length; V = distance of vulva from anterior end x 100 / total body length (%); OV = length of anterior female gonad x 100 / total body length (%).

b Data presented on mean ± standard deviation (minimum - maximum).

3.2. Molecular character and phylogenetic tree

The nucleotide of D2D3 segment of 28S rRNA region *H. cocophilus* in this study was 765 bp while the ITS1-5.8S-ITS2 region was 694 bp. Homology results in the D2D3 segment of 28S rRNA region for *H. cocophilus* has 99% similarity with 84% cover queries with *H. cocophilus* from NCBI (KM516171.1). Homology results in the ITS1-5.8S-ITS2 region for *H. cocophilus* has 98% similarity with 100% query cover with *H. cocophilus* from NCBI (KM516183.1) (Table 2). The Maximum Likelihood phylogenetic tree in the D2D3 segment of 28S rRNA with TN93 + G model showed that *H. cocophilus* was included in 1 clade with *H. cocophilus* from several countries with a bootstrap value of 100 and could be separated from *H. ortonwilliamsi*, *H. wessoni*, *H. macrodorus*, and *H. chitwoodi*. The Maximum Likelihood phylogenetic tree in the ITS1-5.8S-ITS2 area showed that *H. cocophilus* was included in a group with *H. cocophilus* from Mozambique with a bootstrap value of 97 and *H. cocophilus* from the USA slightly out of the clade. Based on ITS1-5.8S-ITS2, *H. cocophilus* is close to *H. ortonwilliamsi* and is strictly separated from *H. chitwoodi*, *H. wessoni* and *H. macrodorus* (Fig. 2).

Identification of *H. cocophilus* using universal primers in previous studies have been done. Molecular characterization of *H. cocophilus* should use at least 2 universal primers with different amplification DNA targets, and the results must all be identified as *H. cocophilus*. D2D3 segment of 28S rRNA and ITS1-5.8S-ITS2 region have been widely used to characterize *H. cocophilus* species [5, 6, 10]. This study used D2D3 segment of 28S rRNA and ITS1-5.8S-ITS2 regions with the results all similar to *H. cocophilus* from NCBI sequences.
Table 2. Homology of D2D3 segments of 28S rRNA and ITS1-5.8S-ITS2 sequences of *Hemicriconemoides cocophillus* from Robusta coffee with sequences from NCBI.

| Regions         | Nc (bp) | Accession     | Species                | Isolate | Qc % | E-value | Identit y % |
|-----------------|---------|---------------|------------------------|---------|------|---------|-------------|
| LSU D2D3 (28S rRNA) | 756     | KM516171.1    | *H. cocophillus*       | CD1374  | 84   | 0       | 99          |
|                 |         | KM516170.1    | *H. cocophillus*       | CD1496  | 87   | 0       | 96          |
|                 |         | MF438042.1    | *Hemicriconemoides* sp. 1 | VSS-    | 100  | 0       | 91          |
|                 |         | KF856514.1    | *H. ortonwilliamsi*    | Rociana | 87   | 0       | 94          |
|                 |         | KF856515.1    | *H. cocophillus*       | Venezeu  | 71   | 0       | 99          |
| ITS1-5.8S-ITS2  | 694     | KM516183.1    | *H. cocophillus*       | CD1374  | 100  | 0       | 98          |
|                 |         | KM516182.1    | *H. cocophillus*       | CD1496  | 99   | 0       | 95          |
|                 |         | KF856552.1    | *H. ortonwilliamsi*    | Rociana | 100  | 0       | 93          |
|                 |         | KF856553.1    | *H. brachyurus*        | P1      | 99   | 0       | 89          |
|                 |         | KF856545.1    | *H. minutus*           | CD1181  | 99   | 0       | 89          |

Figure 2. Phylogenetic relationship within populations and species of the genus *Hemicriconemoides* as inferred from Maximum Likelihood analysis using (A) the D2D3 of 28S rRNA gene sequence dataset with the TN93+G model and (B) the ITS1-5.8S-ITS2 gene sequence dataset with the K2+G model. Sequence in this study is indicated in SA1 Indonesia.
4. Conclusion
Based on morphology, morphometry, and molecular characters, *Hemicriconemoides cocophillus* in this study identically to *H. cocophillus* from Mozambique. To our knowledge, this is the 1st record of *H. cocophillus* in Robusta coffee plantation in Malang, Indonesia.

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