Jurnal Sumberdaya HAYATI April 2023
Vol. 9 No. 1, hlm 17-23

Available online at:
https://journal.ipb.ac.id/index.php/sumberdayahayati

First Record of Free-Living Nematode *Mylonchulus hawaiiensis* from Bondowoso Regency-Indonesia

ANKARDIANSYAH PANDU PRADANA[1*], TOYOSHI YOSHIGA[2]

[1]Department of Plant Protection, Faculty of Agriculture, University of Jember, Jember 68121, Indonesia
[2]Department of Applied Biological Sciences, Saga University, Saga 840-8502, Japan

Received 12 August 2022/Accepted 18 April 2023

Monochids are predatory nematodes that can be found in various ecosystems. In this study, we analyzed monochids from the rhizosphere of Arabica coffee in Bondowoso Regency-Indonesia. The morphological analysis showed that the nematode belonged to the *Mylonchulus hawaiiensis* (Cassidy, 1931) species. The analysis results were also confirmed by a molecular analysis using DNA from a single nematode. The study also involved a polymerase chain reaction process using a forward primer SSU F07 (5'-AAA GAT TAA GCC ATG CAT G-3') and reverse primer SSU R 81 (5'-TGA TCC WKC YGC AGG TTC AC-3'), which amplifies the small subunit (SSU) region of rDNA. Phylogenetic analysis of the sequence obtained formed a cluster with those of *M. hawaiiensis* found in GenBank. The sequences obtained had homology ranging from 99.64 to 99.70% compared with *M. hawaiiensis* sequences found in GenBank. This study is the first report of *M. hawaiiensis* from Indonesia.

Key words: 18S rDNA, Monochids, Morphometric, Predator, Description

INTRODUCTION

Monochids are predatory nematodes that can be easily recognized by their clear buccal cavity and feeding apparatus (Bilgrami & Kulshreshtha 1993; Pervez *et al.* 2000; Bilgrami *et al.* 2001). They are spread in many countries and can be easily found in various types of soil, especially fertile soil in the natural environment (Ahmad & Jairajpuri 2010). One of the reported genera of the monochids group along with adequate morphometric and molecular data is *Mylonchulus*. This genus has been reported to be found in several countries in Asia, America, the Middle East, and Australia (Shokoohi *et al.* 2013).

Research on the genus *Mylonchulus* in Indonesia is still underexplored. Gafur and Ajizah (2022) reported that *Mylonchulus brachyuris* (Bütschli 1873) had been found and characterized in various locations on the island of Borneo-Indonesia. Conducted through nematode surveys in Indonesia, other reports related to the genus *Mylonchulus* only mentioned that this genus existed, but these works did not document any morphometric and molecular descriptions at the species level. The reporting of new geographical records of species holds significant importance for scientific and ecological purposes. It facilitates researchers in comprehending species distribution, including abundance, rarity, and the factors that influence it. Moreover, the reporting of new geographical records can aid in understanding the distribution and diversity of a species, assist in developing conservation strategies, and advance knowledge of nematode taxonomy and biodiversity. Hence, the reporting of new geographical records of species is crucial for scientific and ecological advancements.

A survey conducted on Arabica coffee plantations in Bondowoso Regency-Indonesia, collected specimens from monochids and preserved them in DESS solution. The specimen was later identified as Mylonchulus. The specimen was further identified as Mylonchulus hawaiiensis. This study reported the morphometric and molecular characters of the small subunit (18S) region of rDNA. The research gap addressed in this study concerns the differences in morphometric size between *M. hawaiiensis* specimens reported in this study and those in previous studies. The smaller size of the specimens reported in this study is a notable difference from those reported previously. The reporting of this discrepancy is an important contribution to the understanding of morphometric variations in *M. hawaiiensis* across different regions. By addressing this gap, this study provides new insights into the taxonomy and biodiversity of *M. hawaiiensis*, which can be used to inform further research and conservation efforts. Additionally, the reporting of this research gap in the study is a valuable addition

[*]Corresponding author:
E-mail: pandu@unej.ac.id

to the field of nematology and provides important information on the morphometric characteristics of *M. hawaiiensis*. This is also the first *M. hawaiiensis* report in Indonesia.

MATERIALS AND METHODS

Sampling and Nematode Collection. Sampling was carried out in an Arabica coffee plantation managed by the local farmers in Bondowoso Regency-Indonesia (114.0670193000, -8.0048203200) (Figure 1). A total of 500 g of soil from a depth of ±20 cm was extracted using the white head tray technique (Bell & Watson 2001). The extracted nematodes were then preserved in DESS solution (containing 0.25 M EDTA, 20% DMSO, at pH 8 and saturated with NaCl) and stored at room temperature until the next analysis stage (Yoder *et al.* 2006).

Morphological Identification. Morphological identification was performed by taking nematodes from the DESS solution. Nematode collection was carried out under a stereo microscope Olympus SZX9. Nematodes' morphological properties resembling *Mylonchulus* sp. were taken and transferred to a glass slide filled with water and then observed using a compound microscope Olympus BH-2 with a Toupcam M20 camera. Morphological identification was based on the main selected diagnostic characters (Table 1) (Zullini *et al.* 2002).

DNA Extraction. Total DNA was extracted from a single nematode taken from a pre-prepared DESS suspension. DNA extraction was carried out using a nematode dissolving solution (NDS) (ISOHAIR, Nippon Gene Japan) consisting of enzyme solution, lysis solution, and TE buffer at a ratio of 5:4:100 (v/v) respectively. Subsequently, the nematodes were transferred to a microtube containing 15 µl of NDS and incubated for 20 minutes at 60°C (Tanaka *et al.* 2012).

Amplification, Purification, and Sequencing of the 18S rDNA Gene. A total of 1 µl of DNA extract was added to the polymerase chain reaction (PCR) mixture containing 29.75 µl of sterile MilliQ water, 5 µl of 10× EX Taq Buffer, 4 µl of dNTP mixture (2.5 mM each), 5 µl of each primer, and 0.2 µl ExTaq. The portion of the 18S rDNA gene was amplified from two overlapping fragments, using a forward primer SSU F07 (5'-AAA GAT TAA GCC ATG CAT G-3') and a reverse primer SSU R 81 (5'-TGA TCC WKC YGC AGG TTC AC-3'). The PCR process was done using a thermal cycler, and the amplification program was set as follows: initial denaturation at 94°C (10 min); followed by 35 cycles of denaturation at 94°C (30 s), annealing at 54°C (30 s), and extension at 72°C (1 min); and the elongation process at 72°C (10 min). The PCR product was then verified using agarose gel electrophoresis to ensure the DNA was amplified properly (De Ley *et al.* 2002).

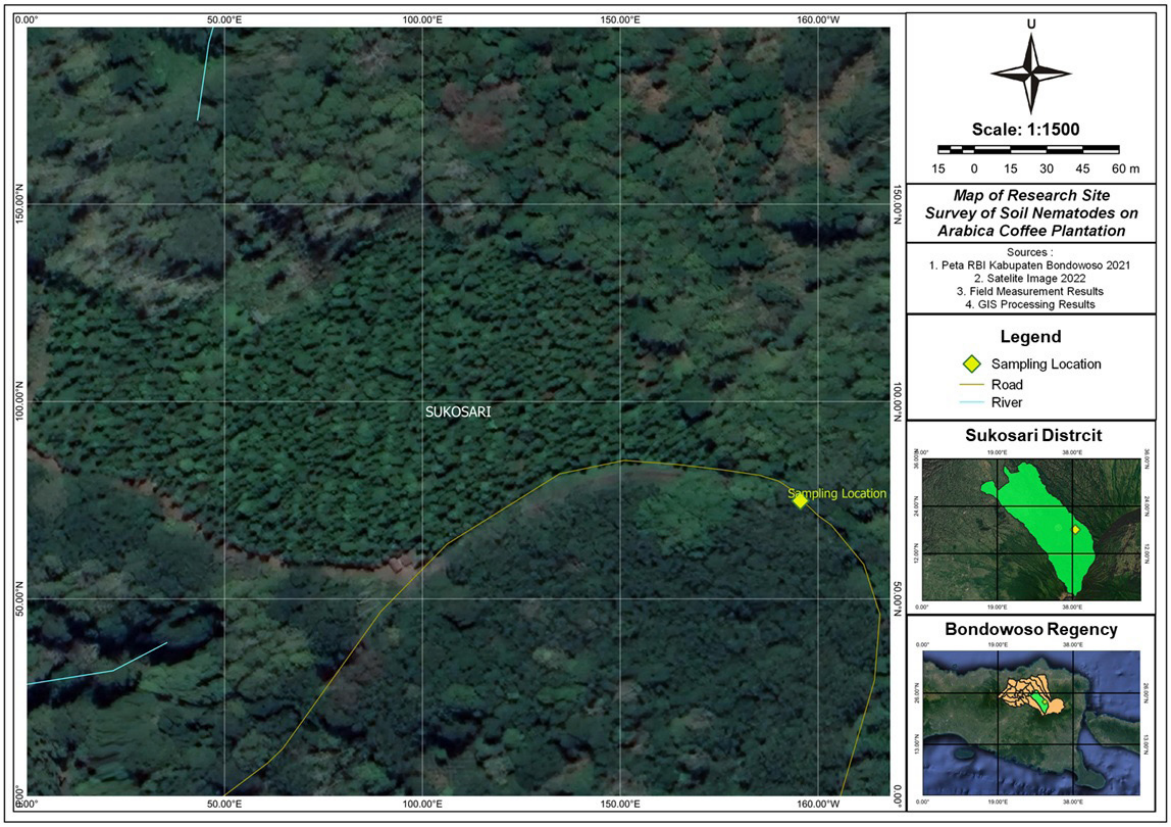

Figure 1. Sampling location in Arabica coffee plantation, Bondowoso Regency, Indonesia

Table 1. Morphometric of *M. hawaiinensis* (all absolute measurements in µm)

Properties/ratios*	*M. hawaiiensis* this study	*M. hawaiiensis* Zullini *et al.* (2002)
N	4	1.00
L	884.50±0.32 (622–1,300)	1.23
A	19.40±4.11 (15.64–23.31)	22.0
B	2.95±0.91 (2.18–4.04)	3.50
C	37.36±0.51 (36.67–37.87)	35.0
c’	1.38±0.08 (1.31–1.50)	1.30
V	56.54±4.69 (50.30–61.25)	59.0
Body diameter		
at lip region	26.79±5.05 (23.13–34.21)	25
at mid-body	44.64±7.58 (39.75–55.76)	57
at anus	27.07±1.48 (24.99–28.42)	27
Pharynx	307.62±44.44 (271.64–372.21)	352
Buccal capsule length	28.56±1.45 (27.15–30.20)	27
Buccal capsule diameter	14.70±0.50 (14.24–15.26)	14
Tail	23.65±8.55 (16.53 – 34.87)	34

*Abbreviations: L = body length; a = body length ÷ greatest body diameter; b = body length ÷ distance from anterior to esophago-intestinal valve; c = body length ÷ tail length; c’ = tail length ÷ tail diameter at anus or cloaca; V = % distance of vulva from anterior

The amplified PCR product was purified using ISOSPIN PCR Product (Nippon Gene, Japan). The Sanger sequencing process was done by Macrogen Japan Corp. using the same primers used for amplification.

Phylogenetic Analyses of 18S rDNA Gene Sequence. The newly obtained sequences of *M. hawaiiensis* (18S rDNA) and other sequences of the genus *Mylonchulus* from GenBank were used for phylogenetic analysis. The sequences were analyzed using the basic local alignment search tool (BLAST) program on the National Center for Biotechnology Information (NCBI) website. The nucleotide sequences obtained were then analyzed using Clustal W multiple alignments. The relationship between isolates was constructed using molecular evolutionary genetic analysis (MEGA) software version 11.0.10 with a bootstrap involving 1,000 replicates.

RESULTS

Environmental Conditions. The sample was obtained from an area at an altitude of ±1,260 m above sea level with humidity around 30 to 85%. The Arabica coffee grown in this area was about 7 years old, and pine trees were grown as a canopy. The soil was mostly covered by coffee and pine leaf litter. The average soil pH was 4.5 to 5.7. Some portraits of the area where the sample was obtained are presented in Figure 2.

Morphology and Morphometry of *Mylonchulus hawaiiensis*. In this study, a predatory nematode was found with various body lengths, ranging from 622 to 1,300 µm. The diameter of the nematode body ranged from 39.75 to 55.76 µm. Based on these measurements, the ratio between the total body length and width ranged from 15.64 to 23.31. At various magnifications under the microscope, the nematode showed a relatively thick but smooth cuticle. Several teeth were found on the anterior, indicating the characteristics of predatory nematodes. The morphometric measurements showed that the buccal capsule length ranged from 27.15 to 30.20 µm. Furthermore, the diameter of the buccal capsule ranged from 14.24 to 15.26 µm. In the lip region, the body width ranged from 23.13 to 34.21 µm. *M. hawaiiensis* had a slightly bent tail with a length ranging from 16.53 to 34.87 µm. The morphometric data of *M. hawaiiensis* are presented in Table 1 and Figure 3.

Molecular Characterization and Phylogenetic Position of *M. hawaiiensis.* The morphometric data of the predatory nematode were obtained through molecular analysis so that the identification results at the species level had higher confidence. The results of agarose gel electrophoresis showed that the 18S rDNA gene was successfully amplified. The analysis results showed only 1 band with values around 1600 bp (Figure 4).

The sequences obtained in this study were submitted to GenBank with accession number OP077090. The sequences were then compared with several other sequences found in GenBank. The compilation of the phylogenetic tree showed that the discovered nematode had high homology with other *M. hawaiiensis* with accession numbers AB361442.1; AB361440.1; AB361439.1; AB361438.1; and AB361441.1. In the first 4 most hit accession numbers, the sequence has a percent identity of up to 99.76%, while in the fifth accession code the percent identity reaches 99.58%. The results of the phylogenetic analysis are presented in Figure 5.

Figure 2. Coffee plantation where soil samples were taken (a) coffee plants with pine trees as a canopy and soil conditions covered with leaf litter; (b) post-excavation soil conditions during sampling

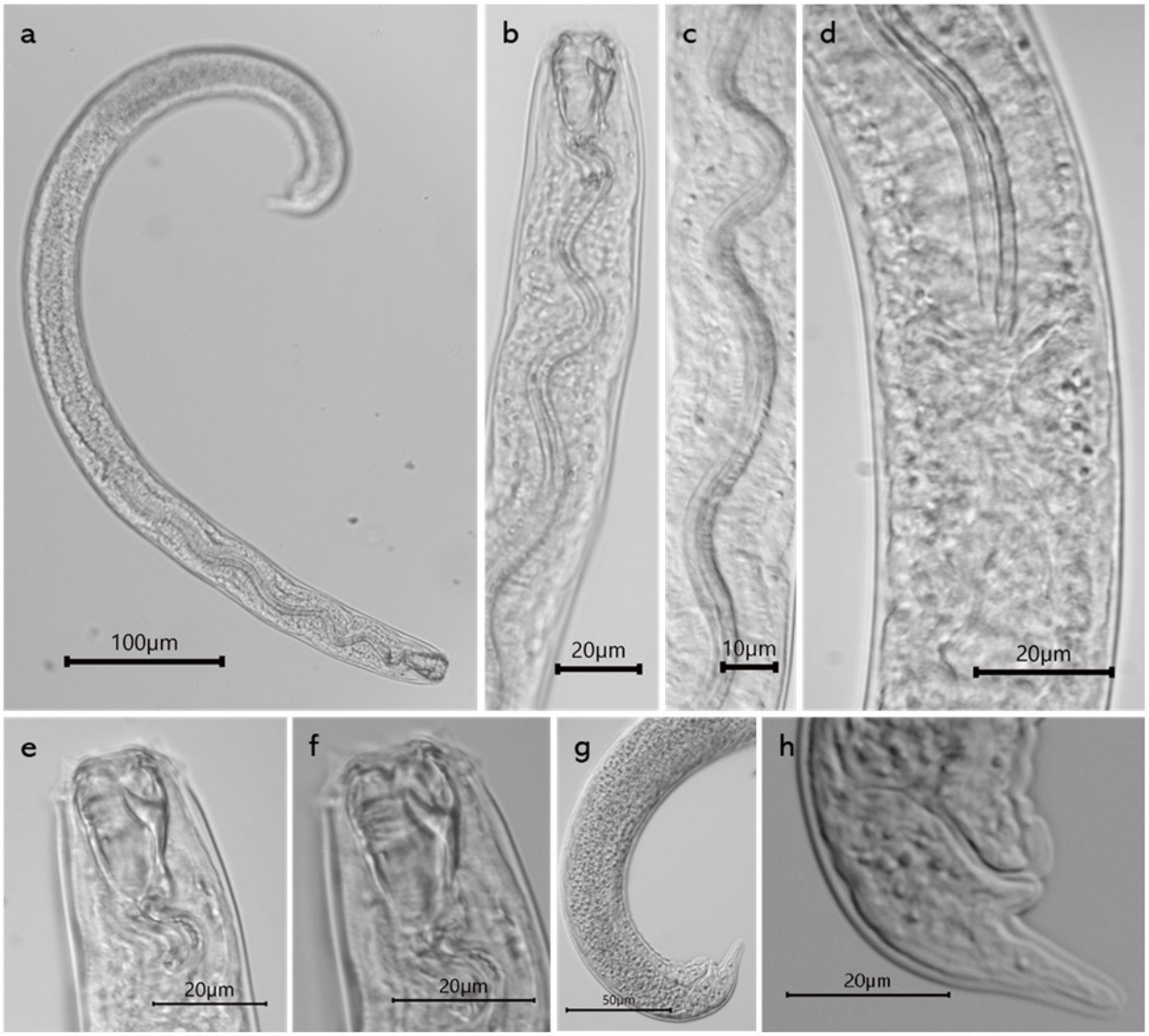

Figure 3. *Mylonchulus hawaiiensis* (Cassidy, 1931) Andrássy, 1958, from the rhizosphere of Arabica Coffee plants in Bondowoso–Indonesia. (a) whole nematodes' body, (b) anterior region, (c, d) pharyngo-intestinal junction, (e, f) anterior end with the buccal armature, (g) posterior region, (h) tail

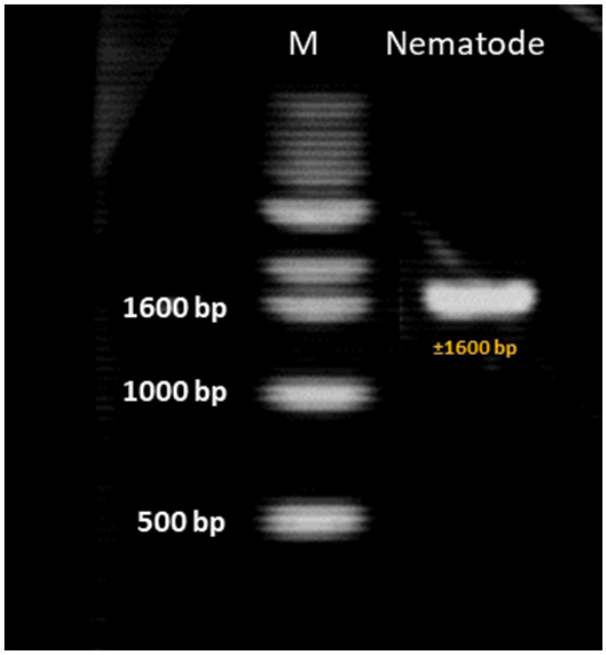

Figure 4. Analysis on 1% agarose gel electrophoresis to confirm nematode DNA successfully amplified using primers amplifying 18S rDNA gene. M = 1 kb DNA ladder band, Nematode: 18s rDNA nematode band

Based on the analysis of sequence homology, the predatory nematode obtained had the highest homology with *M. hawaiiensis* (99.64 to 99.70%). This value indicates that the isolates belong to the same species as the compared isolates.

DISCUSSION

Predatory nematodes are more commonly found in natural ecosystems than in agroecosystems (Parmelee & Alston 1986; Ratnadass *et al.* 2012). In the area where the samples were taken, the coffee farmers applied a silvicultural cultivation system. The coffee plant as the main product and the pine tree as a forest plant functioning as a canopy was also taken. Leaf litter on the ground caused the soil to have a substantial amount of organic matter. Under these conditions, the soil could generally become loose and dark (Wood *et al.* 2006). These conditions are similar to soil conditions in natural ecosystems. The presence of high organic matter will provide more opportunities for bacteria and fungi to grow (Pettit 2004; Liang *et al.*

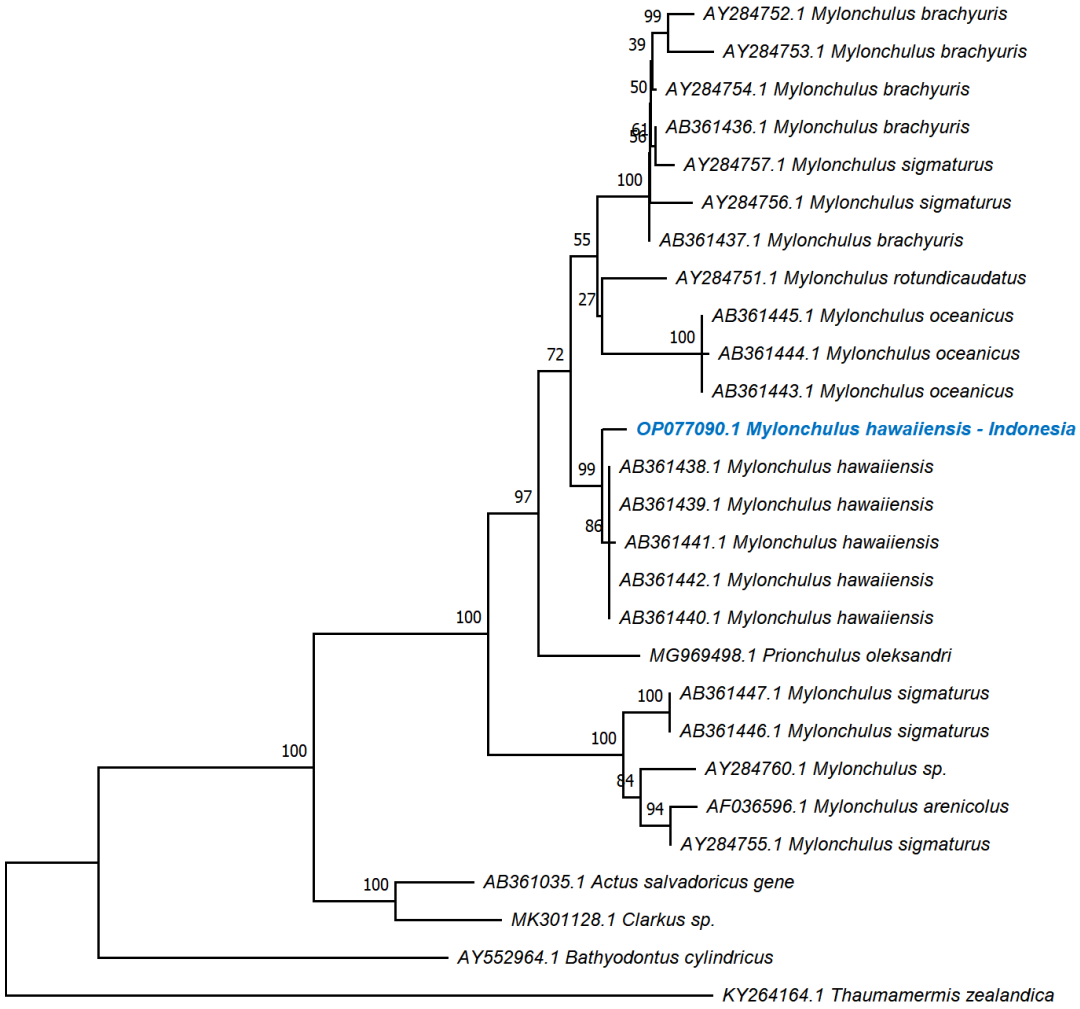

Figure 5. Phylogenetic analysis of *M. hawaiiensis* from the rhizosphere of Arabica coffee plantations in Bondowoso–Indonesia with the genus *Mylonchuulus* found in GenBank based on nucleotide sequences using the MEGA v 11.0.10 program with neighbor-joining tree method coupled with a bootstrap of 1,000 replicates

2019). The abundance and diversity of soil microbes imply the development of bacteria-feeding nematodes and fungal-feeding nematodes. In such conditions, predatory nematodes are more likely to have ample prey (Kostenko *et al.* 2015).

Compared with the *M. hawaiiensis* reported by Zullini *et al.* (2002), the present study only documented an insignificant difference. The previous study reported one nematode, so the present study aimed to complete the previously reported data. *M. hawaiiensis* usually have a ventrally curved body after being fixed in the fixation solution like DESS. The nematode body length found in this study was shorter than the nematode body length in references. Likewise, with several other characters measured, the average has a smaller value. However, in this study, some characteristics such as body diameter in the lip region, buccal capsule length, and buccal capsule diameter of nematodes had higher mean values (Ahmad & Jairajpuri 2010).

A nematode species has a unique character. Principally, although each individual of the same species has a slightly different size, the ratio between these sizes is in the same range of values. In the De Man formula, several ratios are used to determine the species *M. hawaiiensis*, including body length ÷ greatest body diameter, body length ÷ distance from anterior to the esophago-intestinal valve, body length ÷ tail length, tail length ÷ tail diameter at anus or cloaca, and % distance of vulva from anterior. The ratios between species are generally different; therefore, the use of ratios in determining nematode species is essential (Zullini *et al.* 2002). In this study, although there were differences in the size of the nematodes, the ratios between variables were in the same range.

The analysis using a molecular approach corroborates the identification of the nematode as *M. hawaiiensis*. In previous studies, several researchers used molecular techniques and the 18S rDNA region for identification purposes because this region is quite common, and the database in GenBank regarding its sequence results has been sufficient. Each species has a unique DNA sequence. Identification using molecular techniques has the advantage of high accuracy. In addition, identification with this technique is free from the influence of the external environment (Bhadury & Austen 2010).

In conclusion, the results of the morphometric and molecular analysis have shown that the species discovered in this study belongs to *Mylonculus hawaiiensis*. Morphometric analysis shows that the nematodes vary in size, but these variances fall in the same range as documented in the previous studies. The homology of the nematode sequence reaches 99.70%, indicating identical molecular characteristics.

CONFLICT OF INTEREST

All reagents, chemicals, and sequencing costs during the research process were funded by the Nematology Laboratory-Saga University, Japan. The authors do not receive funding for publication from any party.

ACKNOWLEDGEMENTS

The authors would like to thank the PIU IsDB Project-the University of Jember for providing funding to A.P.P. in order to take part in the non-degree training program at Laboratory of Nematology -Saga University, Japan through contract no 26924/UN25/KP/2021. We are grateful to Ayman K. Elsayed, Ph.D. from the Faculty of Agriculture, Saga University-Japan for his valuable help with molecular data analysis.

REFERENCES

Ahmad W, Jairajpuri MS. 2010. Mononchida: The Predatory Soil Nematodes. Netherlands: Brill.

Bell N, Watson R. 2001. Optimising the whitehead and hemming tray method to extract plant parasitic and other nematodes from two soils under pasture. *Nematol* 3:179-185. https://doi.org/10.1163/156854101750236312

Bhadury P, Austen MC. 2010. Barcoding marine nematodes: an improved set of nematode 18S rRNA primers to overcome eukaryotic co-interference. *Hydrobiol* 641:245-251. https://doi.org/10.1007/s10750-009-0088-z

Bilgrami AL, Kulshreshtha R. 1993. Evaluation of predation abilities of *Mylonchulus dentatus. Indian J Nematol* 23:191-198.

Bilgrami AL, Pervez R, Yoshiga T, Kondo E. 2001. Attraction and aggregation behaviour of predatory nematodes, *Mesodorylaimus bastiani* and *Aquatides thornei* (Nematoda: Dorylaimida). *Appl Entomol Zool* 36:243-249. https://doi.org/10.1303/aez.2001.243

De Ley IT, De Ley P, Vierstraete A, Karssen G, Moens M, Vanfleteren J. 2002. Phylogenetic analyses of *Meloidogyne* small subunit rDNA. *J Nematol* 34:319-327.

Gafur A, Ajizah A. 2022. First records of *Mylonchulus brachyuris* (Bütschli, 1873) (Nematoda, Mononchida, Mylonchulidae) from South Kalimantan, Indonesia. *Check List* 18:425-430. https://doi.org/10.15560/18.2.425

Kostenko O, Duyts H, Grootemaat S, De Deyn GB, Bezemer TM. 2015. Plant diversity and identity effects on predatory nematodes and their prey. *Ecol Evol* 5:836-847. https://doi.org/10.1002/ece3.1337

Liang C, Amelung W, Lehmann J, Kästner M. 2019. Quantitative assessment of microbial necromass contribution to soil organic matter. *Global Change Biol* 25:3578-3590. https://doi.org/10.1111/gcb.14781

Parmelee RW, Alston DG. 1986. Nematode trophic structure in conventional and no-tillage agroecosystems. *J Nematol* 18:403-407.

Pervez R, Bilgrami A, Yoshiga T, Kondo E. 2000. Feeding, attraction and aggregation behaviour of *Mesodorylaimus bastiani* and *Aquatides thornei* at the feeding site using *Hirschamnniella oryzae* as prey. *Int J Nematol* 10:207-214.

Pettit RE. 2004. Organic matter, humus, humate, humic acid, fulvic acid and humin: their importance in soil fertility and plant health. *CTI Res* 10:1-7.

Ratnadass A, Fernandes P, Avelino J, Habib R. 2012. Plant species diversity for sustainable management of crop pests and diseases in agroecosystems: a review. *Agron Sustain Dev* 32:273-303. https://doi.org/10.1007/s13593-011-0022-4

Shokoohi E, Mehrabi-Nasab A, Mirzaei M, Peneva V. 2013. Study of mononchids from Iran, with description of *Mylonchulus kermaniensis* sp. n.(Nematoda: Mononchida). *Zootaxa*, 3599:519-534. https://doi.org/10.11646/zootaxa.3599.6.2

Tanaka R, Kikuchi T, Aikawa T, Kanzaki N. 2012. Simple and quick methods for nematode DNA preparation. *Appl Entomol Zool* 47:291-294. https://doi.org/10.1007/s13355-012-0115-9

Wood TE, Lawrence D, Clark DA. 2006. Determinants of leaf litter nutrient cycling in a tropical rain forest: soil fertility versus topography. *Ecosystems*, 9:700-710. https://doi.org/10.1007/s10021-005-0016-7

Yoder M, De Ley IT, King IW, Mundo-Ocampo M, Mann J, Blaxter M, Poiras L, De Ley P. 2006. DESS: a versatile solution for preserving morphology and extractable DNA of nematodes. *Nematol* 8:367-376. https://doi.org/10.1163/156854106778493448

Zullini A, Loof PA, Bongers T. 2002. Free-living nematodes from nature reserves in Costa Rica. 2. Mononchina. *Nematol* 4:1-23. https://doi.org/10.1163/156854102760082168