



Research Article

Pathogenicity Test and Molecular Identification of Entomopathogenic Nematodes from Various Sugarcane Cultivation Sites in Yogyakarta, Indonesia on White Grub Instar *Lepidiota stigma* L.

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Abstract | One of the main pests in sugarcane is white grub *Lepidiota stigma*, which can reduce production by up to 60%. Chemical pesticides, though commonly used, pose risks like pest resistance and environmental harm. Entomopathogenic nematodes (EPNs) offer a sustainable alternative, effectively controlling pests through symbiosis with toxin-producing bacteria. This study aims to identify species and the pathogenicity of entomopathogenic nematodes from various locations of sugarcane cultivation in Yogyakarta, Indonesia, against white grub consisting of young instars (instars 1 and 2) and old instars (instars 3 and 4). The pathogenicity study used a two-factor, completely randomized design (CRD) + 1 control without EPNs. The first factor was the origin of EPN isolates (Madukismo, Banyuroto, and Jangkang Ayu), and the second was white grub instar (young and old). Data were analyzed using a 5% level of variance and continued with a 5% DMRT test. Species identification was performed molecularly using PCR techniques with the D2A/D3B primers in the 28s rDNA region. The results showed that entomopathogenic nematode isolates from Madukismo, Banyuroto, and Jangkang Ayu sugarcane cultivation sites had pathogenicity that was not significantly different from *L. stigma* white grubs. EPN treatment on young instars of white grubs caused higher mortality than old instars, with a mortality value of 44.44% (15 days after application). The LT50 value in the treatment of isolate origin and white grub instar ranged from 13.33 to 16.48 days. Based on molecular identification, EPN from Madukismo was identified as *Metarhabditis amsactae*.

Received | December 24, 2024; **Accepted** | March 07, 2025; **Published** | April 26, 2025

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Citation | Solichah, C., R. Brotodjojo, S.A. Kuncoro, M.E. Poerwanto and M. Ajri. 2025. Pathogenicity test and molecular identification of entomopathogenic nematodes from various sugarcane cultivation sites in Yogyakarta, Indonesia on white grub instar *Lepidiota stigma* L. *Pakistan Journal of Nematology*, 43(1): 80-87.

DOI | <https://dx.doi.org/10.17582/journal.pjn/2025/43.1.80.87>

Keywords | Biological control, Entomopathogenic nematodes, *Lepidiota stigma*, *Metarhabditis amsactae*, Sugarcane, Sustainable agriculture



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Introduction

Sugarcane (*Saccharum officinarum* L.) is one of the Graminaeae plants used as raw materials for

making sugar. It contains sugar water with levels reaching 20%, from the base of the stem to the shoot. Processed sugar cane is used as a sweetener in the form of sugar used for household consumption in the

food industry. Apart from being used for domestic consumption, some sugar cane products are exported to increase foreign exchange so that they can become a source of income for the national economy.

The area of sugarcane land in Indonesia was around 489.338 hectares (BPS-Statistics Indonesia, 2024). The area is divided into smallholder plantations, large private plantations, and large state plantations. Large state plantations have an area of around 58.99 thousand hectares, a decrease in land area from 2022 by 7.49%. Large private plantations in 2023 experienced an increase of 3.74%, amounting to 142.15 thousand hectares. Smallholder plantations have an area of 288.20 thousand hectares, which increased by 0.41% from 2022 (BPS-Statistics Indonesia, 2024). In Indonesia, there are twelve provincial areas producing sugarcane. Java Island dominates by placing East Java, Central Java, West Java, and Yogyakarta Special Region as national sugarcane producers.

One of the factors that cause a decrease in sugarcane production is the presence of a pest that destroys the roots of sugarcane, namely *Lepidiota stigma*. The attack of white grub *L. stigma* in Java Island was reported in 2011. Sugarcane production in Bondowoso Regency experienced a decrease in yield of up to 60% due to the attack of white grub *L. stigma*, which is an average of only about 40 tonnes/ha, which is usually a normal yield of around 100 tonnes/ha. Farmers who did not control the infestation only produced 5 tonnes/ha (Alimin *et al.*, 2014). The level of white grub pest attacks on smallholder sugarcane plants in Sleman Regency, including in Kalasan, Depok, Ngemplak, Prambanan, Mlati, Ngaglik and Pakem areas, was highest in 2014, namely 35.46% (Utami *et al.*, 2021).

In general, *L. stigma* control uses chemical pesticides. The percentage of sugarcane pest control chemically carried out by sugarcane plantation business households is 55.64% (Statistics of Jawa Tengah Province, 2015). Chemical pesticides have several disadvantages, among others, and in the long term, they can make pests resistant to these pesticides. Pest resistance to chemical pesticides can lead to the use of higher doses, thus increasing the amount of pesticide residue remaining. In addition, the use of chemical pesticides can trigger an increase in pest populations. As an alternative to controlling *L. stigma*, it can be done biologically by utilizing biological agents, including entomopathogenic nematodes (Wagiyana

et al., 2021). Entomopathogenic nematodes (EPNs) can infect and kill the host quickly because they can symbiosis with EPNs symbiont bacteria that produce toxins (Gaugler and Kaya, 1990). EPNs can cause mortality in their hosts within 24-48 hours because they are assisted by their respective symbiont bacteria, namely *Xenorhabdus* sp. on *Steinernema* sp. and *Photorhabdus* sp. on *Heterorhabditis* sp. (Dubey *et al.*, 2013).

Insect larvae of each instar have different morphology and size. It is possible that older larval instars will also be more difficult to penetrate by entomopathogenic nematodes, so control will be more effective when the young instars are more vulnerable. Entomopathogenic nematodes are recognized as potential biological control agents and have been used to control various soil-dwelling insects due to their superior host-seeking ability. Several EPNs species have been shown to have potential in controlling various species of white beetle larvae such as *Steinernema scarabaei*, *S. longicaudum*, *S. glaseri*, *Heterorhabditis bacteriophora*, and *H. zealandica* (Li *et al.*, 2023). *S. feltiae* was more effective against larvae, achieving 63.30% mortality, while *H. bacteriophora* was more effective against pupae, causing 48.30% mortality (Meşepinar and Kepenekci, 2022).

The inability to accurately identify nematodes using morphological approaches has led to the development of alternative identification techniques, such as molecular identification. Molecular identification of entomopathogenic nematodes (EPNs) using the D2A/D3B region of the *28S rRNA* gene is a robust method widely adopted due to its effectiveness in distinguishing between species. A study successfully identified EPNs, including *Heterorhabditis amazonensis* and *Metarhabditis rainai*, using this molecular technique (de Brida *et al.*, 2017). Another study identified the entomopathogenic nematode species *H. zealandica* Bartow by sequencing the D2-D3 region of the *28S rRNA* gene (Lulamba and Serepa-Dlamini, 2020).

Efforts to develop entomopathogenic nematodes require the utilization of entomopathogenic nematodes with a conservation strategy that refers to measures to maintain the presence of entomopathogenic nematodes in certain agricultural areas. The initial stage in applying biological control techniques is exploration. Therefore, it is necessary

to explore and characterize entomopathogenic nematodes in various regions and soil types in the Yogyakarta sugarcane plantation areas as part of the effort to develop biological agents. This study aims to explore, identify species, and assess the pathogenicity of entomopathogenic nematodes from various locations of sugarcane cultivation in Yogyakarta, Indonesia, against young and old white grub instar.

Materials and Methods

The research was conducted at the Plant Protection Laboratory, Faculty of Agriculture, UPN 'Veteran' Yogyakarta, Indonesia, from April to October 2024 using a two-factor completely randomized design (CRD) + 1 control without EPNs. The first factor was the origin of EPN isolates (Madukismo, Banyuroto, and Jangkang Ayu), and the second factor was white grub instar (young instars 1-2 and old instars 3-4). Each treatment combination was repeated three times. Data were analyzed using 5% level of variance and continued with 5% DMRT test.

Exploration and extraction of entomopathogenic nematodes

Soil samples were taken from three sugarcane cultivation areas in Yogyakarta, namely Madukismo, Banyuroto, and Jangkang Ayu. In one plot of land, 5 soil samples were taken diagonally (1 center point and 4 diagonal points), each with 200 grams. The soil samples were then placed in plastic cups. Each glass was filled with 20 *T. larvae* and kept in a damp and dark place for 7 days until the larva died (Saputra *et al.*, 2017). Then, the dead larvae were extracted using the Whitehead tray method. *T. molitor* larvae were placed on the filter paper and observed until the nematodes in the *T. molitor* larvae moved into the water. The nematode suspension was then harvested and cultured in dog food media.

Supply and maintenance white grub L. stigma

White grubs were obtained from the Experimental Garden of the Faculty of Agriculture, UPN Veteran Yogyakarta, Indonesia. The white grubs used were young instars of *L. stigma* (instars 1-2), which weighed <9 g, and old instars (instars 3-4), which weighed >10 g. The white grubs were placed in plastic cups with 300 g/glass of soil. White grubs were put into plastic cups with 300 g/glass of soil. In each glass, 1 white grub was reared and filled with fresh carrot pieces as food.

Pathogenicity test of entomopathogenic nematodes

The pathogenicity study used a two-factor, completely randomized design (CRD) + 1 control without EPNs. The first factor was the origin of EPN isolates (Madukismo, Banyuroto, and Jangkang Ayu), and the second was white grub instar (young and old). Each treatment was repeated seven times, with each experimental unit comprising 10 white grubs. The white grubs were placed in plastic cups containing 300 grams of soil. Both young and old instars were inoculated with entomopathogenic nematodes from various isolate sources at a concentration of 200 infective juveniles (IJ)/mL and incubated at room temperature (25°C). The inoculation was performed by introducing the nematode suspension into the soil. The parameters observed were:

Mortality of white grub L. stigma

Observations were made 3 days after application (DAA), 6 DAA, 9 DAA, 12 DAA, and 15 DAA. The *L. stigma* larvae mortality percentage was calculated using the following formula: $M = (n/N) \times 100\%$. Description: M: Mortality (%), n: Number of white grubs that died of infection (tail), N: Number of white grubs tested (tails).

Mortality rate of white grub L. stigma

The mortality rate was calculated using the formula: $V = (T_1N_1 + T_2N_2 + T_3N_3 + \dots + T_nN_n) / n$. Description: V: death rate, T: observation time, N: number of insects that die, n: number of test insects.

Feeding ability

Feeding ability was carried out by weighing the weight of feed before and after being eaten at observations of 3 days after application (DAA), 6 DAA, 9 DAA, 12 DAA, and 15 DAA.

Lethal time 50% (LT50)

LT50 data was calculated using Probit analysis.

Molecular identification

The molecular identification was performed using the polymerase chain reaction (PCR) method. The nematode identified through molecular techniques was collected from Madukismo. Nematode DNA was extracted with the GeneAid™ Tissue DNA Mini Kit. The universal primers D2A (5'-CAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') were employed to amplify the D2/D3 expansion

segment of the 28S rDNA (Ajri *et al.*, 2021). The PCR protocol included an initial denaturation at 95°C for 1 minute, followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 57°C for 15 seconds, and extension at 72°C for 10 seconds. This was followed by a final synthesis step at 72°C for 5 minutes, and the reaction was completed with a final hold at 4°C. Each PCR reaction consisted of a 25 µl reaction volume containing 2.5 µl of DNA sample, 12.5 µl of PCR Kit (MyTaq HS Red Mix Boline), 2.5 µl of forward primer, 2.5 µl of reverse primer, and 5 µl of distilled water.

The PCR products were analyzed using 2 % agarose gel electrophoresis and visualized using a UV transilluminator. The PCR products were sent to a sequencing service company (Apical Scientific Sequencing, 1st BASE DNA Sequencing). The sequencing results were then processed to construct a phylogenetic tree. The sequence data were input into the BLAST (Basic Local Alignment Search Tool) program via the NCBI (National Center for Biotechnology Information) website. Several isolates related to the test sample were analyzed using the Mega 11.0 program.

Results and Discussion

Pathogenicity test of entomopathogenic nematodes

Mortality observations were made every 3 days after the application (DAA) of entomopathogenic nematodes, with the observation period starting from day 3 to day 15 after application (3, 6, 9, 12, 15 DAA). There was no interaction between the treatment of isolate origin and white grub instar (Table 1). The average treatment showed higher mortality than the control. Treating the origin of entomopathogenic nematode isolate did not significantly affect the mean mortality parameter of *L. stigma* white grub. These results can be obtained because the three treatments of entomopathogenic nematode isolates originated from the same province with a distance of no more than 25 miles. This proximity causes the three isolates to mostly come from the same genus, commonly found in agricultural fields, thus increasing the chance that the entomopathogenic nematodes tested have similar characteristics and responses (Majić *et al.*, 2018). Previous studies have shown that closely related geographical distributions can influence the similarity of entomopathogenic nematode species and their effectiveness in infecting the same insect

host (Koppenhöfer and Fuzy, 2006).

Table 1: Mean mortality of *Lepidiota stigma* observation 3–15 days after application (%).

Treatment	Observations days after application (DAA)				
	3	6	9	12	15
Origin of isolate					
Banyuroto	03,33 a	11,67 b	23,33 b	33,33 b	38,33 b
Jangkang Ayu	00,00 a	11,67 b	23,33 b	36,67 b	36,67 b
Madukismo	00,00 a	03,33 b	21,67 b	30,00 b	35,00 b
White grub instar					
1-2 (young)	00,00 p	06,67 p	27,78 p	42,22 q	44,44 q
3-4 (old)	02,22 p	11,11 p	17,78 p	24,44 p	28,89 p
Mean	1,11 x	8,89 x	22,78 x	33,33 x	36,67 x
Control	00,00 x	00,00 y	00,00 y	00,00 y	00,00 y
Interaction	(-)	(-)	(-)	(-)	(-)

Description: Treatment mean followed by the same letter in one column shows no significant difference based on 5% DMRT test. The sign (-) indicates no interaction between treatments. Data were transformed with square root.

Mortality of young white grub instars was higher than older instars on the 12th and 15th days after application (Table 1). This occurs because older instars have thicker cuticles that are more difficult to penetrate by entomopathogenic nematodes. These results also align with the statement of (Muliani and Srimurni, 2022) that in younger instars, insects are more vulnerable to biotic factors such as pathogenic microorganisms and natural enemies and abiotic factors such as temperature and humidity. Late instars or old instars are the least parasitized insect instars. Late instars of holometabola insects have much stronger physiological defenses than early instars or young instars (Gols *et al.*, 2019).

Observations of *L. stigma* white grub mortality rate in the treatment of entomopathogenic nematode isolate origin and white grub instar had no interaction and no significant difference between treatments (Table 2). The LT50 value in the treatment of isolate origin and white grub instar ranged from 13.33 to 16.48 days (Table 3). The insect mortality rate is influenced by the ability of entomopathogenic nematodes to infect insects, so host instar has no effect on this parameter. The three isolates originated from the same genus, so their ability to infect is not much different. The ability of nematodes to infect insects is influenced by the ability of entomopathogenic nematodes to find hosts, which is influenced by the sensitivity of sensor neurons in capturing signals from CO₂ compounds

released by insects to find the location of host insects. The ability to find hosts is also influenced by the sensitivity of entomopathogenic nematode sensors in sensing insect-specific cues in the form of chemicals released by insects. These chemicals can be pheromones, exuviae, or feces. This sensitivity comes from the age and experience of entomopathogenic nematodes in exposure to chemicals released by the host (Zhang *et al.*, 2021). Pathogenicity is also influenced by the type of bacteria symbiotic with the nematode (Cahyono *et al.*, 2020).

Table 2: Average mortality rate of white grub *Lepidiotia stigma* (days).

Instar white grub	Origin of isolate			Mean
	Banyuroto	Jangkang Ayu	Madukismo	
1-2 (young)	09,53	09,15	09,45	9,37 p
3-4 (old)	09,66	08,66	10,67	9,67 p
Mean	09,59 a	08,90 a	10,06 a	
Interaction				(-)

Description: Treatment means followed by the same letter in a row or column show no significant difference based on 5% DMRT test. The sign (-) indicates no interaction between treatments.

Table 3: Value of lethal time 50 (days).

White grub instar	Origin of isolate		
	Banyuroto	Jangkang Ayu	Madukismo
1-2 (young)	13,33	13,37	14,79
3-4 (old)	13,51	13,51	16,48

Table 4: Mean of feeding ability of *Lepidiotia stigma* larvae at several observation times (g).

Treatment	Observations days after application (DAA)				
	3	6	9	12	15
Origin of isolate					
Banyuroto	06,28	06,05	07,13	07,36	07,50
Jangkang Ayu	06,79	06,13	05,99	07,56	07,83
Madukismo	05,56	07,48	06,68	07,64	07,36
White grub instar					
1-2 (young)	05,33 p	08,36 p	07,63 p	09,83 p	08,41 p
3-4 (old)	7,09 q	11,31 q	12,17 q	12,74 q	14,29 q
Interaction	(-)	(-)	(-)	(-)	(-)

Description: Treatment means followed by the same letter in a row or column show no significant difference based on 5% DMRT test. The sign (-) indicates no interaction between treatments.

The feeding ability of *L. stigma* larvae was observed every 3 days after the application of entomopathogenic nematodes, with the observation period from day 3 to day 15. There was no interaction between the

treatment of isolate origin and white grub instar (Table 4). The treatment of the origin of entomopathogenic nematode isolates Banyuroto, Jangkang Ayu, and Madukismo did not show significant differences in the feeding ability of white grub *L. stigma*. This result suggests that the effectiveness of entomopathogenic nematodes in suppressing larval feeding activity may be more dependent on nematode species and virulence rather than their geographic origin. Previous studies have shown that variations in entomopathogenic nematode effectiveness are influenced by factors such as host susceptibility, nematode strain aggressiveness, and environmental conditions rather than solely by the geographic source of the isolates (Kaya and Gaugler, 1993). Additionally, the mode of action of entomopathogenic nematodes, primarily through bacterial symbionts that release toxins to kill the host, may contribute to similar feeding suppression across different isolates (Dillman *et al.*, 2012).

EPN treatment on young instars showed lower feeding ability than old instars. This occurs because older instars with larger bodies require more energy during eating. These results align with the research of (Manullang *et al.*, 2017). which found that late-instar larvae consume more feed than early instars because they require a lot of energy reserves during the process.

Molecular identification of entomopathogenic nematodes PCR electrophoresis result showed that the EPN from Madukismo, Yogyakarta was successfully amplified at ~650 bp (Figure 1). The expansion region of D2 D3 28S rDNA was sequenced for molecular analysis. The BLAST-N results for the EPN sample showed high identity to *Metarhabditis amsactae* from India (MT872508) with 99.8 % identity and *Metarhabditis amsactae* (OR185554) with 98.2% identity (Table 5). Sequence results for the *Metarhabditis amsactae* sample from Madukismo, Yogyakarta, Indonesia, were submitted to GenBank with accession number PQ578621. *Metarhabditis amsactae* demonstrates significant potential as a biological control agent against various insect pests due to its facultative entomopathogenic nature. The nematodes can be mass-cultured both in vivo and in vitro, making them suitable for agricultural applications (Kumari *et al.*, 2023).

The *M. amsactae* isolate from Yogyakarta clusters closely with other *M. amsactae* isolates from the Philippines and India (Figure 2). *Steinernema feltiae*

Table 5: The percentage of nucleotide base similarity between the *Metarhabditis amsactae* from Yogyakarta-Indonesia and nematodes published in the NCBI database.

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13
PQ578621 <i>Metarhabditis amsactae</i> (Indonesia)	ID												
MT872508 <i>Metarhabditis amsactae</i> (India)	99,8	ID											
OR185554 <i>Metarhabditis amsactae</i> (Philippines)	98,2	98,4	ID										
OR335536 <i>Metarhabditis amsactae</i> (India)	98,3	98,5	98,5	ID									
EU195965 <i>Rhabditis blumi</i>	84,2	84,4	82,5	82,1	ID								
OP646462 <i>Metarhabditis blumi</i> (Colombia)	84,1	84,3	82,4	82,0	100,0	ID							
PQ362854 <i>Metarhabditis blumi</i> (Brazil)	83,9	83,9	82,0	81,5	100,0	100,0	ID						
OQ954517 <i>Metarhabditis rainai</i> (Mexico)	83,3	83,5	81,8	81,7	80,0	80,0	79,6	ID					
PQ106986 <i>Metarhabditis rainai</i> (India)	83,1	83,3	81,6	81,5	79,8	79,9	79,4	99,8	ID				
AM399058 <i>Pellioditis marina</i>	80,8	81,0	79,2	79,4	78,7	78,8	78,3	87,0	86,9	ID			
MK418537 <i>Oscheius myriophilus</i> (Mexico)	81,1	81,3	79,5	79,7	78,4	78,4	77,9	82,7	82,5	83,1	ID		
MN389639 <i>Oscheius myriophilus</i>	81,1	81,3	79,5	79,7	78,4	78,4	77,9	82,7	82,5	83,1	100,0	ID	
BQ705086 <i>Steinernema feltiae</i>	38,2	38,5	38,5	38,6	39,7	39,6	39,1	37,8	38,0	40,1	38,0	38,0	ID

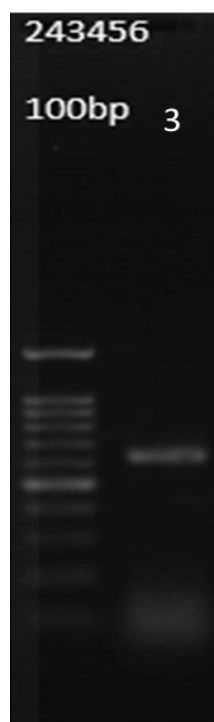


Figure 1: Visualization PCR electrophoresis: Marker DNA ladder (100bp); *Metarhabditis amsactae* from Yogyakarta, Indonesia amplified at ~650 bp (3).

was chosen as the outgroup because it has a much lower percentage of nucleotide similarity compared to species in the *Metarhabditis* and *Rhabditis* groups. This close grouping, supported by extremely low branch length values (near zero), indicates high genetic similarity among these isolates. This suggests a shared evolutionary history or recent divergence, likely influenced by ecological or geographical factors in Southeast Asia. The presence of *M. amsactae* in Yogyakarta, Indonesia, and its close relationship

with isolates from other tropical regions suggests that this species thrives in warm, humid climates. Its evolutionary proximity to Indian and Philippine isolates may also point to historical biogeographical connections, such as shared agricultural practices or natural dispersal routes involving host insects.

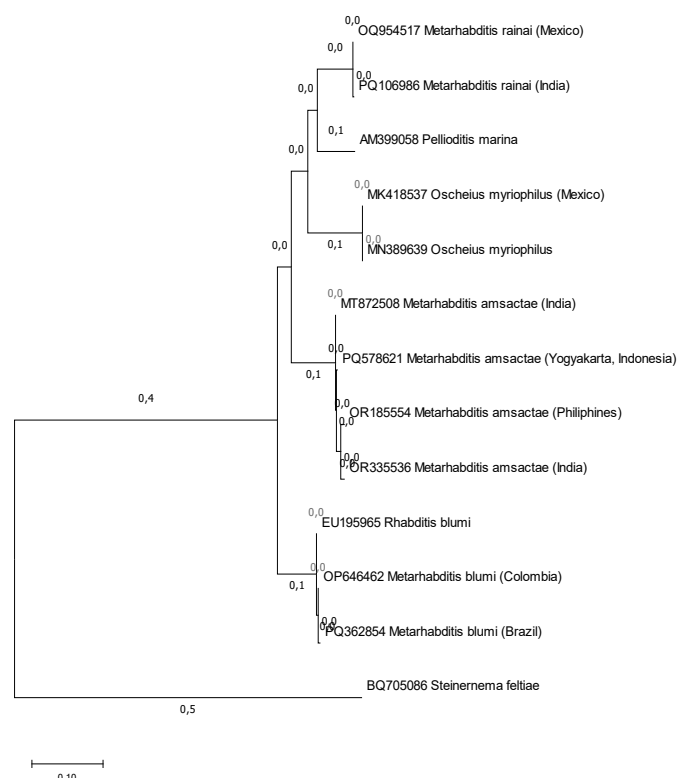


Figure 2: Maximum-likelihood phylogenetic tree with Neighbor-joining (NJ) Kimura 2 model of *Metarhabditis amsactae* and other represented species based on 28S rDNA gene sequences.

Conclusion

The entomopathogenic nematode isolates from Madukismo, Banyuroto, and Jangkang Ayu sugarcane cultivation sites have pathogenicity that is not significantly different from the white grub of *L. stigma*. EPN treatment on young instar white grub caused higher mortality than on old instar with a mortality value of 44.44% at 15 days after application. The LT50 value in the treatment of isolate origin and white grub instar ranged from 13.33 to 16.48 days. Based on molecular identification, EPN from Madukismo was identified as *Metarhabditis amsactae*.

Acknowledgments

We would like to express our sincere gratitude to the Research and Community Service Institute (*Lembaga Penelitian dan Pengabdian Masyarakat-LPPM*) of UPN "Veteran" Yogyakarta for the financial support provided through the 2024 Internal Research Grant under the Applied Research Scheme.

Novelty Statement

This study is the first to report the effectiveness of *Metarhabditis amsactae* as an entomopathogenic nematode in Indonesia, highlighting its significant virulence against specific host targets and its potential for sustainable biological control. These findings contribute to the development of locally adapted bio-control agents suited to tropical ecosystems.

Author's Contribution

All authors contributed equally to the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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