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Research Article

Nematophagous Fungi Isolated from Municipal Waste-contaminated Soil in Medan City, North Sumatera: Morphological Identification, Phylogeny Analysis and Assessment as Root-knot Nematodes Biocontrol

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Abstract: Root-knot nematodes (RKNs) are groups of nematodes that cause significant diseases in horticultural and field crops. Chemical pesticides used to control RKNs could pollute environmental resources and ultimately affect human health. Therefore, eco-friendly efforts are needed. Previous research revealed that nematode-trapping fungi (NTFs) as the biological enemies of nematodes has been observed suppressing the nematode population. This study aimed to isolate NTF species from municipal waste-contaminated soil in Medan City, Indonesia, and identified them using morphological and molecular analysis. Furthermore, their biocontrol potential against *Meloidogyne hapla* Chitwood (Nematoda: Meloidogynidae) was assessed. Soil sample covered seven districts with seven repeats for isolation and *in vitro* assessment against *M. hapla* was done on CMA and observed after 12-72 hours. Three isolates were successfully obtained and proven effective in suppressing *M. hapla* by 97.7% (isolate sH51 and sH52) and 89.27% (isolate sH53). Morphological identification on PDA and genetic analysis of ITS concluded that sH51 is *Drechslerella brochopaga* Drechsler (Ascomycota: Orbiliaceae) and sH53 is *Arthrobotrys thaumasius* Drechsler (Ascomycota: Orbiliaceae). Morphological analysis for isolate sH52 reveals it as *Arthrobotrys sinensis* but is limited to *Arthrobotrys* sp. based on phylogeny analysis thus additional gen needs to be sequenced for confirmation.

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1. Introduction

Root-knot nematodes (RKNs) are a group of several parasitic nematodes infecting various horticultural crops. Their infection leads to the formation of knots in plant roots which subsequently intervene in water and nutrient transportation systems and trigger more extensive breakdown such as canopy leaves yellowing, stunting, withering, hypertrophy, and decreased yield (Istiqomah and Pradana, 2017; Asyiah et al., 2021). The most well-known and significant of them is *Meloidogyne* spp. (Nematoda: Meloidogynidae). Most of the efforts to control RKNs especially in Asian countries are the

utilization of chemical pesticide such as carbofuran (Sim et al., 2019) which involves soil microbiomes intervention (Güven and Koç, 2020) and further promoting bioaccumulation and biomagnification (Mishra et al., 2020). Sustainable alternatives are employing various biological agents to inhibit the development of some stages in their life cycle (Mendoza-de Gives, 2022) without intervening with other non-target organisms in ecological systems such as natural metabolites (Göze Özdemir et al., 2021), bacteria (Migunova and Sasanelli, 2021), and fungi (Zhang et al., 2020).

Several biological agents possessing antagonistic interaction towards RKNs have been used to control their population such as fungal nematode-trapper (Sharma et al., 2021; Youssef and El-Nagdi, 2021). Nematode-trapping fungi (NTFs) are important species of fungi known for evolving a wide variety of traps to enamor and predate nematodes as a food source. Also, trap forms are various including constricting rings, adhesive knobs, columns, and networks (Su et al., 2017). Previously, several NTFs from Family Orbiliaceae (Phylum: Ascomycota) such as *Dactylella* sp., *Monacrosporium* sp., and *Arthrobotrys* sp. had been successfully isolated from soil (Hastuti and Faull, 2018). Furthermore, some species had been proven experimentally to be able to reduce the juvenile population of *Meloidogyne* spp. and reduce root cavities by more than 80% (Kang et al., 2019; Singh et al., 2019; Yusuf, 2019). *Arthrobotrys vermicola* Rifai was observed repressing nematodes by 99.8% and lessening root cavities by 60% (Tarigan, 2021). On the other hand, *Arthrobotrys oligospora* Fresen, *Candelabrella musiformis* Rifai and Cooke, and *Dactylaria eudermata* Drechsler showed an ability to reduce vermiform of *Meloidogyne incognita* Chitwood and root-knot in tobacco after 7, 15, 30 days (Hastuti and Faull, 2018). These results are extremely and sustainably satisfying; thus, NTF should be evaluated as a substitution for less environmental-friendly pesticides such as carbofuran. The objectives of the research were to isolate NTFs from municipal waste-contaminated soil in Medan, North Sumatera, Indonesia. Their ability to suppress *Meloidogyne hapla* Chitwood population was observed *in vitro*. Furthermore, these potential isolates would be identified morphologically and compared genetically with other previously recorded species through DNA sequencing and a phylogeny tree will be established.

2. Material and Methods

2.1. Soil sampling and isolation

Seven districts in Medan City, North Sumatera, Indonesia were selected and seven spots nearby areas contaminated by municipal waste were determined at each district. For each spot, the soil was sampled by making three plots sized 50 × 50 cm each and having a distance of minimally 10 meters between plots. A hundred grams of soil was removed from each plot at a 10-15 cm depth and the soil was mixed. Then, 100 g soil was removed from this mixture, wrapped with aluminum foil, and placed in a labeled sterile plastic container. All samples were preserved in an icebox at 4^{o}C for five days (Tarigan, 2021).

NTF isolation from soil utilized Chloramphenicol Water Agar (CHP-WA) formulated by solvating 10 grams of pure agar in 500 ml distilled water, sterilized at 120 ^{o}C, 1.02 atm for 15 minutes. The mixture was appended aseptically with 1 gram of Chloramphenicol. The previous soil mixture was inoculated one gram into CHP-WA and added with a few adult *M. hapla*. Cultures were incubated in dark storage for three days at 25 ^{o}C and examined everyday using a light microscope at magnificent 10x to see the formation of mycelium traps.

2.3. Antagonistic assessment of NTF isolates against *M. hapla In vitro*

The mycelium trapping *M. hapla* found on CHP-WA recultivated into Corn Meal Agar (CMA) for *in vitro* assessment. Approximately 1 000 adult *M. hapla* were added into the petri dish and counted using a light microscope at magnificent 10× after 12, 24, 36, 48, and 72 hours.

2.2. Macroscopic and microscopic observation of NTF isolates

The isolates were cultivated on PDA and incubated at 25 ^{o}C for 14 days (Hastuti et al., 1970). Macroscopic observations for potential isolates including characterization of colony color, morphology, conidia, and hyphae were conducted by using a light microscope with magnificent 10× and 40× (Winarto et al., 2019).

2.4. ITS alignment and phylogeny analysis

Selected isolate cultures were shipped to Macrogen, Inc. (Singapore) for internal transcribed spacer (ITS) isolation and sequencing. Cap3 Contig Assembly (Stothard, 2000) and Reverse Complement (Huang and Madan, 1999) were used for merging and reversing sequences. Subsequently, these sequences will be aligned using NCBI BLASTn (Zhang et al., 2000) and phylogeny trees were built using MEGA ver. 11 (Tamura et al., 2021).

3. Results

3.1. Antagonistic assessment of NTF isolates against *M. hapla In vitro*

Three potential NTF isolates were selected and designated as sH51, sH52, and sH53. *In vitro* assessment of the isolates against *M. hapla* on CMA are shown in Figure 1. Isolate sH51 and sH52 decreased *M. hapla* by 97.7% while isolating sH53 by 89.27% after 72 hours.

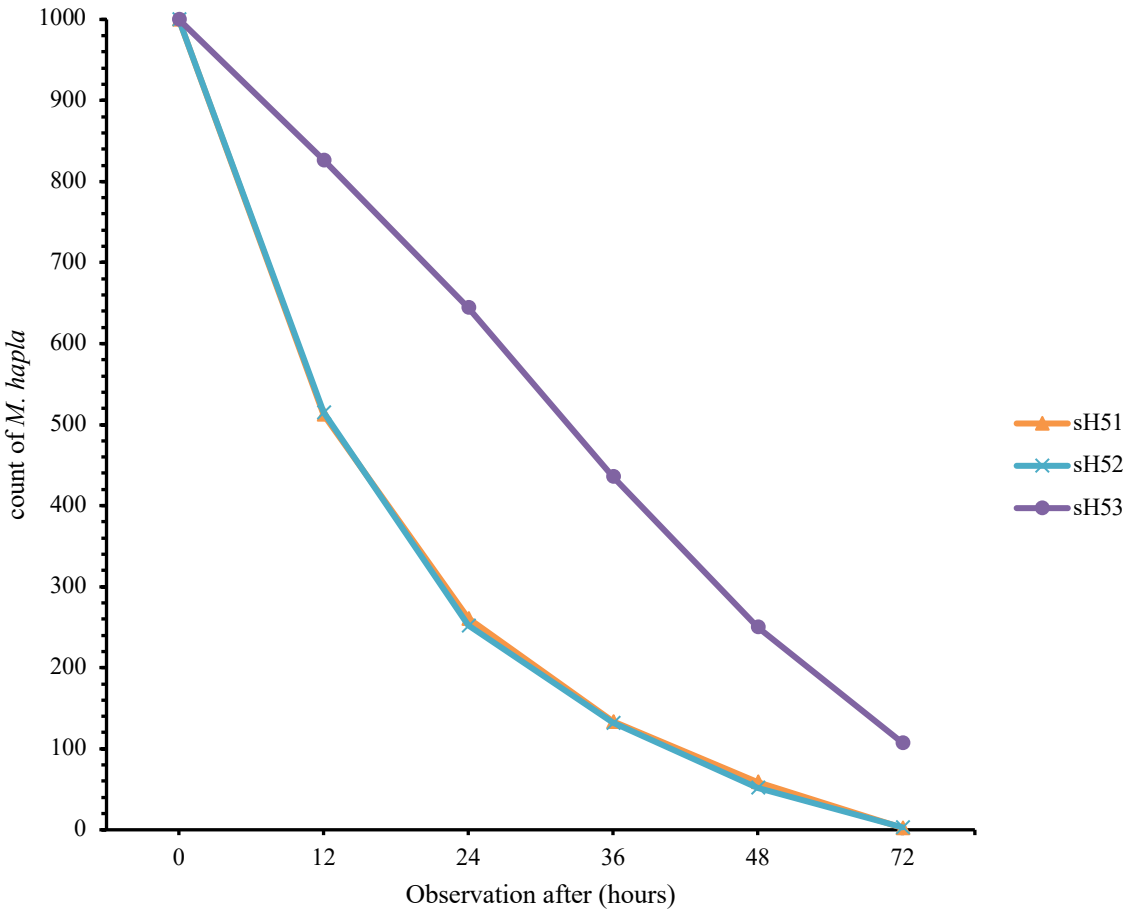


Figure 1. Observation of total count of *M. hapla* on CMA added with isolates after every 12 hours.

3.2. Observation and morphological identification of NTF isolates

Photomicrographs of the isolates were shown consecutively in Figure 2, Figure 3, and Figure 4. Isolate sH51 (Figure 2) at 25 ºC on PDA after seven days had a diameter of 4 cm, and upper and lower medium surfaces were whitish. Mycelium was sticky, branched, and septate. Conidiophores single, lined-vertically, colorless, septate. Conidia 5-9 (6) × 2.5-3.8 (3) µm, elongate ellipsoidal or cylindrical, hyaline, thin-walled, 3-septate. The trap formed a constricting ring. These data suggest that sH51 is identified as *Drechslerella brochopaga* Scholler, Hagedorn and Rubner (Ascomycota: Orbiliaceae) based on the same species described by Yu et al. (2014).

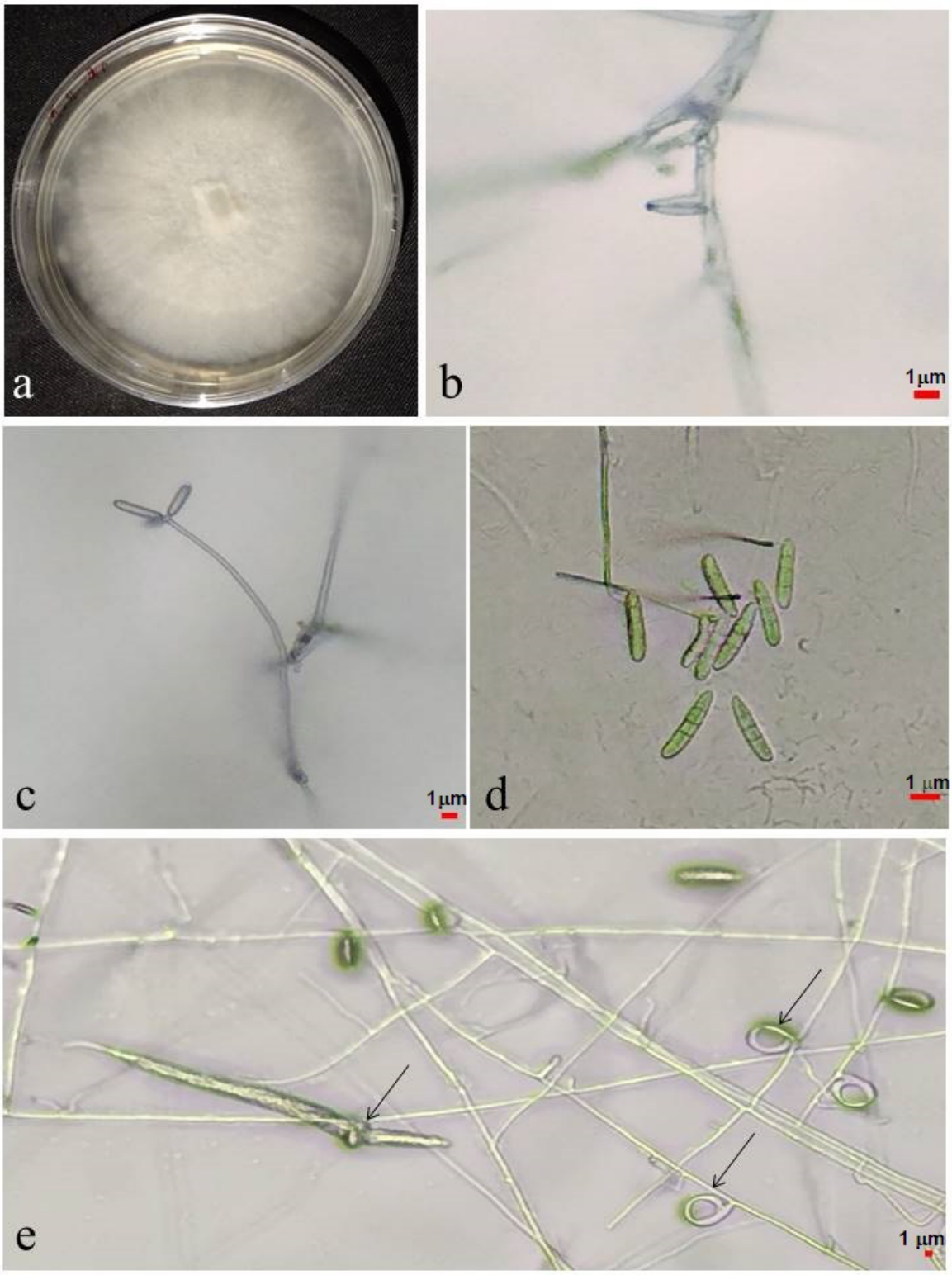


Figure 2. Observation of isolate sH51 on PDA: (a) colony, (b) early conidia, (c) elderly conidia, (d) conidiophore, (e) traps (arrow). Bars: 1 µm.

The colony of isolate sH52 (Figure 3) at 25 ºC on PDA after seven days had a diameter of 8 cm, whitish. Mycelium hyaline, branched and septate. Conidiophore erect, hyaline. Conidia 4-8 (8) x 7-13 (11) µm, hyaline, ellipsoidal or obovoidal, 1-3-septate. Nematode trap formed adhesive three-dimensional trap. These suggest that sH52 is identified as *Arthrobotrys sinensis* Scholler, Hagedorn and Rubner (Ascomycota: Orbiliaceae) based on the same species described by Yu et al. (2014).

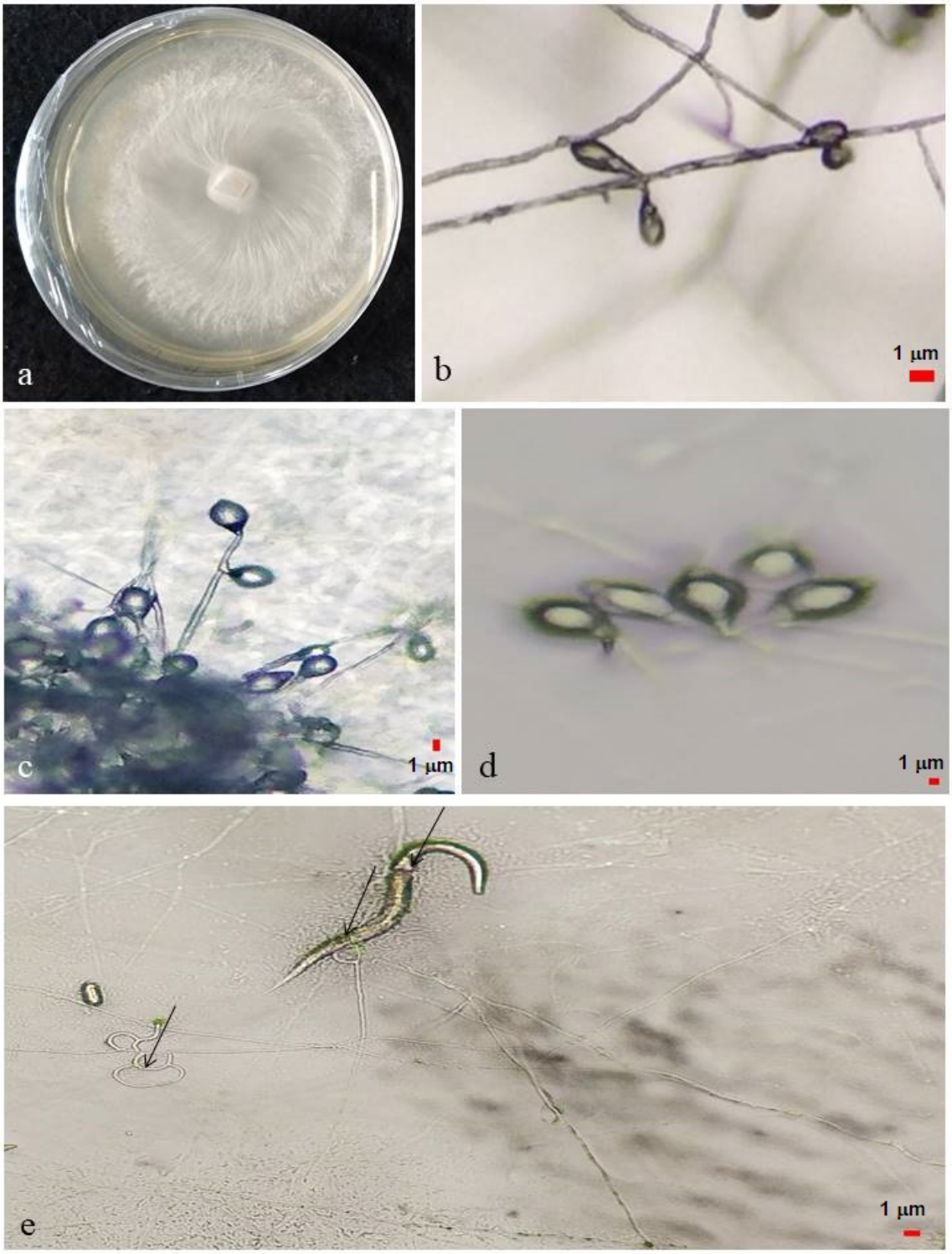


Figure 3. Observation of isolate sH52 on PDA: (a) colony, (b) early conidia, (c) elderly conidia, (d) conidiophore, (e) traps (arrow). Bars: 1 µm.

The colony of isolate sH53 (Figure 4) at 25 ºC on PDA after seven days had a 6 cm diameter, whitish. Vegetative hyphae hyaline, branched, septate. Conidiophore erect, hyaline. Conidia ellipsoidal or musiformis, hyaline, 1-4-septate, 3-5 (4) x 4-7 (6) µm, forming a three-dimensional adhesive trap. These data are similar to the *Arthrobotrys thaumasius* Schenck, Kendr and Pramer (Ascomycota: Orbiliaceae) described by Yu et al. (2014).

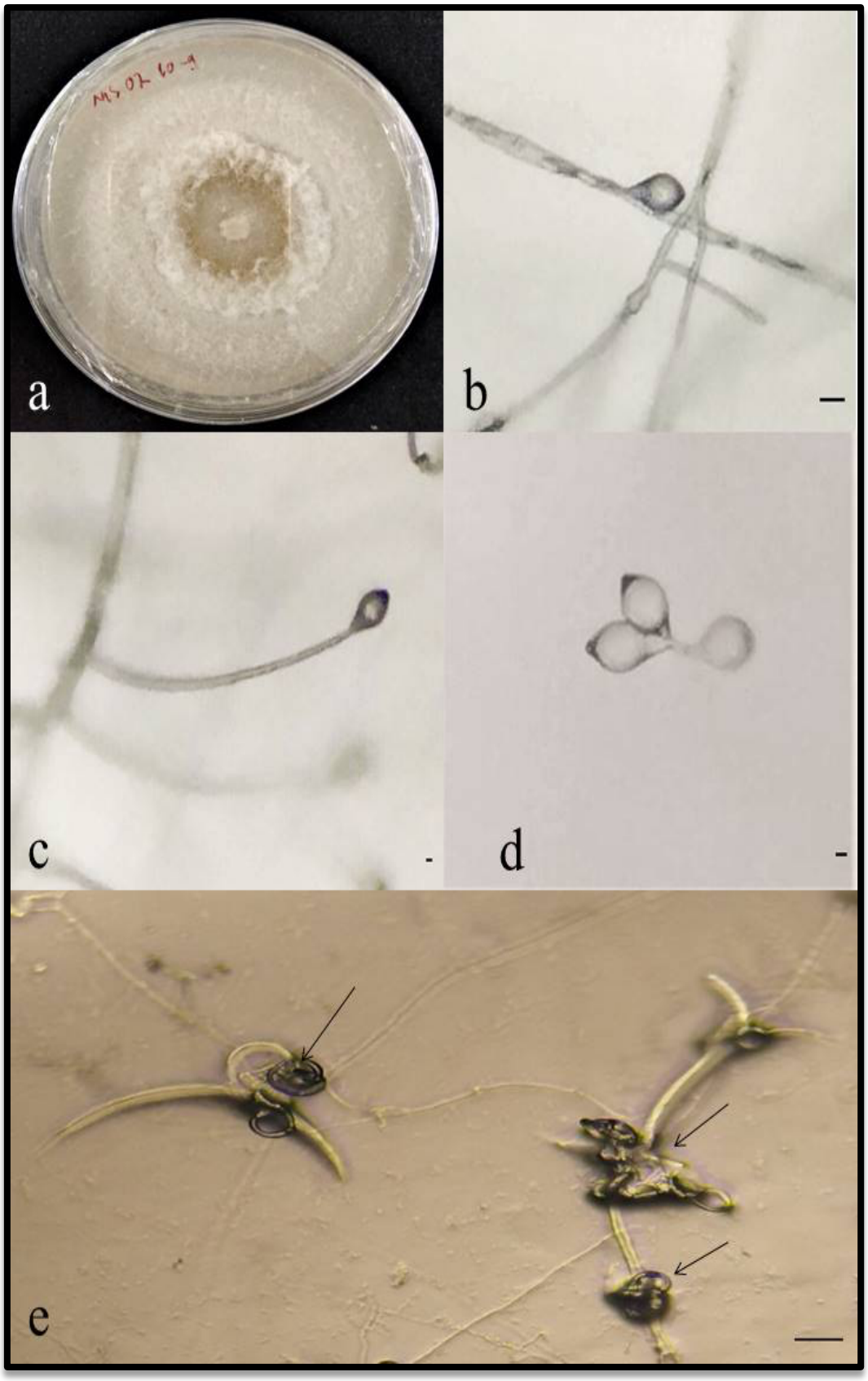


Figure 4. Observation of isolate sH53 on PDA: (a) colony, (b) early conidia, (c) elderly conidia, (d) conidiophore, (e) traps (black arrow). Bars: 1 µm.

3.3. DNA Sequence and Phylogeny Analysis

Isolates sequences obtained from Macrogen were aligned through BLASTn using the standard database (nucleotide collection), mega blast optimized and excluding models and uncultured genome. Ten species of the result for each isolate with the highest percent identity among others were collected and shown in Table 1.

Table 1. Top 10 of the highest percent identity of BLASTn match sequences

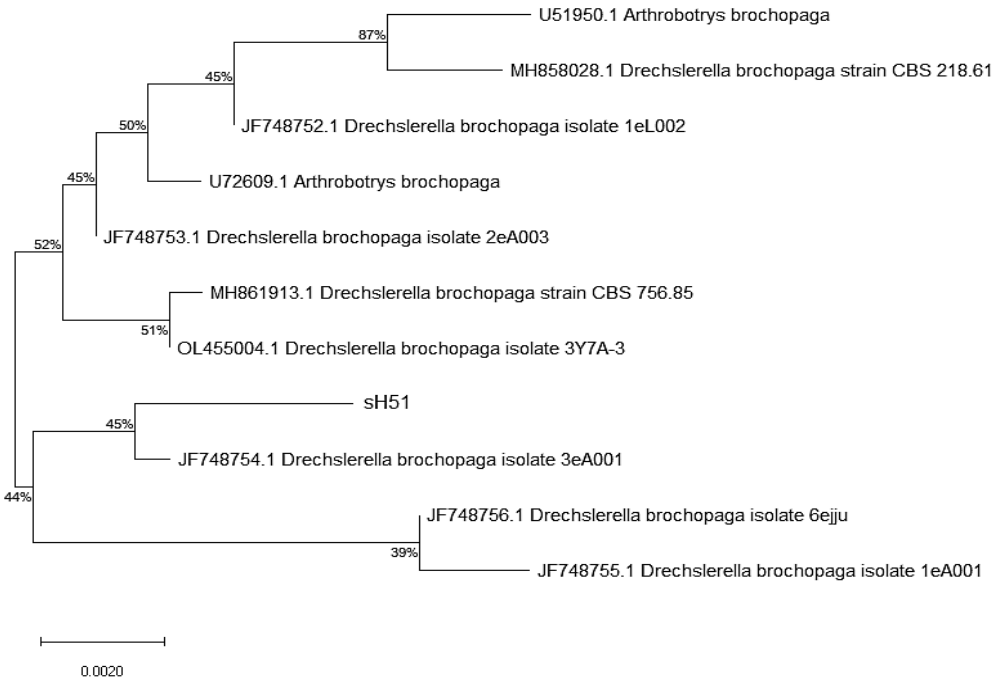
Scientific Name	GenBank Accession No.	Strain	Total Score	Query Cover	Percent Identity
Isolate sH51					
Drechslerella brochopaga	U72609.1		996	74%	100.00
Drechslerella brochopaga	JF748753.1	2eA003	909	68%	100.00
Drechslerella brochopaga	JF748752.1	1eL002	909	68%	100.00
Drechslerella brochopaga	U51950.1		1 022	77%	99.64
Drechslerella brochopaga	JF748754.1	3eA001	893	67%	99.59
Drechslerella brochopaga	JF748756.1	6ejju	797	61%	99.32
Drechslerella brochopaga	MH858028.1	CBS 218.61	1 024	78%	99.30
Drechslerella brochopaga	JF748755.1	1eA001	852	65%	99.16
Drechslerella brochopaga	MH861913.1	CBS 756.85	1 031	79%	99.13
Drechslerella brochopaga	OL455004.1	3Y7A-3	1016	78%	99.12
Isolate sH52					
Arthrobotrys sp.	MN014032.1	TWF898	1 000	79%	99.28
Arthrobotrys sp.	MN014031.1	TWF889	1 000	79%	99.28
Arthrobotrys sp.	MN014033.1	TWF1010	992	78%	99.10
Arthrobotrys sp.	MN014026.1	TWF761	998	79%	98.93
Orbiliaceae sp.	KX953548.1	SA228	1 046	83%	98.81
Orbiliaceae sp.	KX953601.1	SA323	837	67%	98.73
Arthrobotrys sp.	MN014024.1	TWF756	989	79%	98.58
Arthrobotrys sp.	MW131532.1	HK10	1 014	85%	97.34
Arthrobotrys thaumasia	MN717431.1	DS01	941	90%	94.03
Orbilia oligospora	MZ427476.1	JL1	937	90%	94.02
Isolate sH53					
Arthrobotrys thaumasia	MN014043.1	TWF1005	994	32%	99.82
Arthrobotrys thaumasia	MN014037.1	TWF588	981	32%	99.63
Arthrobotrys thaumasia	MN014039.1	TWF566	968	32%	99.62
Arthrobotrys thaumasia	EU977529.1	110	1 086	36%	99.50
Arthrobotrys thaumasia	EU977532.1	111	1 059	35%	99.49
Arthrobotrys thaumasia	MN014036.1	TWF579	963	32%	99.44
Arthrobotrys thaumasia	MN014035.1	TWF585	963	32%	99.44
Arthrobotrys thaumasia	AF106526.1	CBS 322.94	1 110	37%	99.19
Arthrobotrys thaumasia	MN947291.1	NPS014	1 026	35%	98.62
Arthrobotrys thaumasia	KX640093.1	NBS005	1 026	35%	98.62

Table 1 shows that based on the BLAST result for isolate sH51 (720 bp), all match sequences are *Drechslerella brochopaga*, which substantiates previous morphological identification (Figure 2). Isolate sH51 is closely related to the same species with accession no. U72609.1.

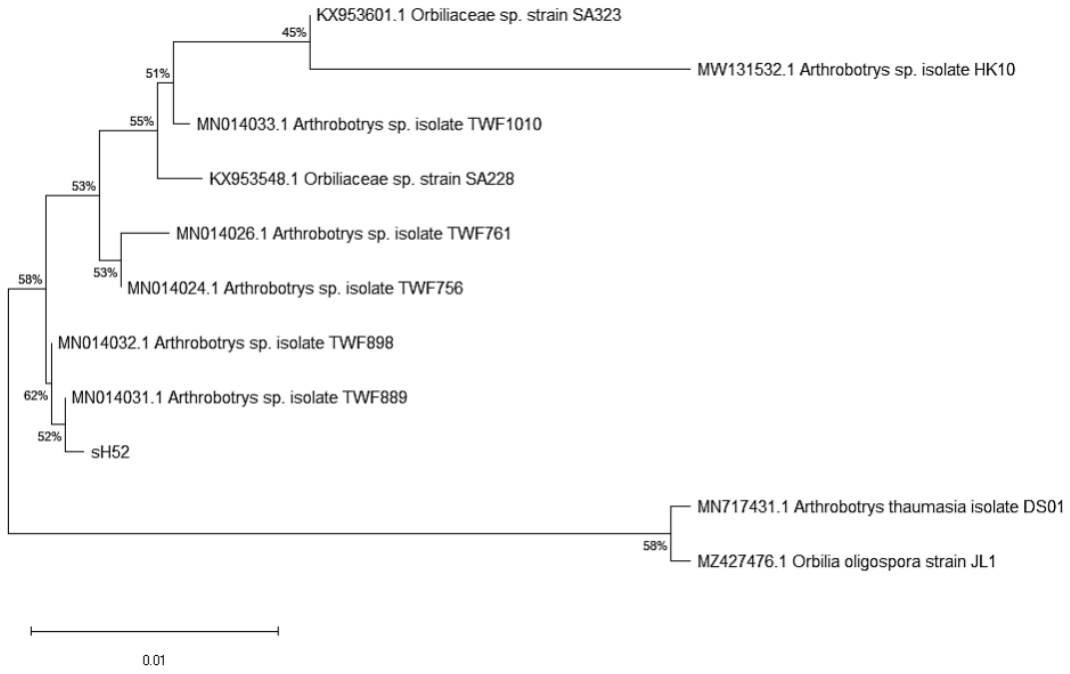
BLAST result definitely identifies isolate sH53 (1650 bp) as *Arthrobotrys thaumasia* which is in accordance with previous morphological identification (Figure 4).

Meanwhile, all compared sequences for isolate sH52 (701 bp) are diverse and its closest similarity is *Arthrobotrys* sp. var. TWF898 (accession no: MN014032.1) isolated in Taiwan. This result is also indefinite and insufficient to substantiate previous morphological identification (Figure 3) despite confirming the same genus.

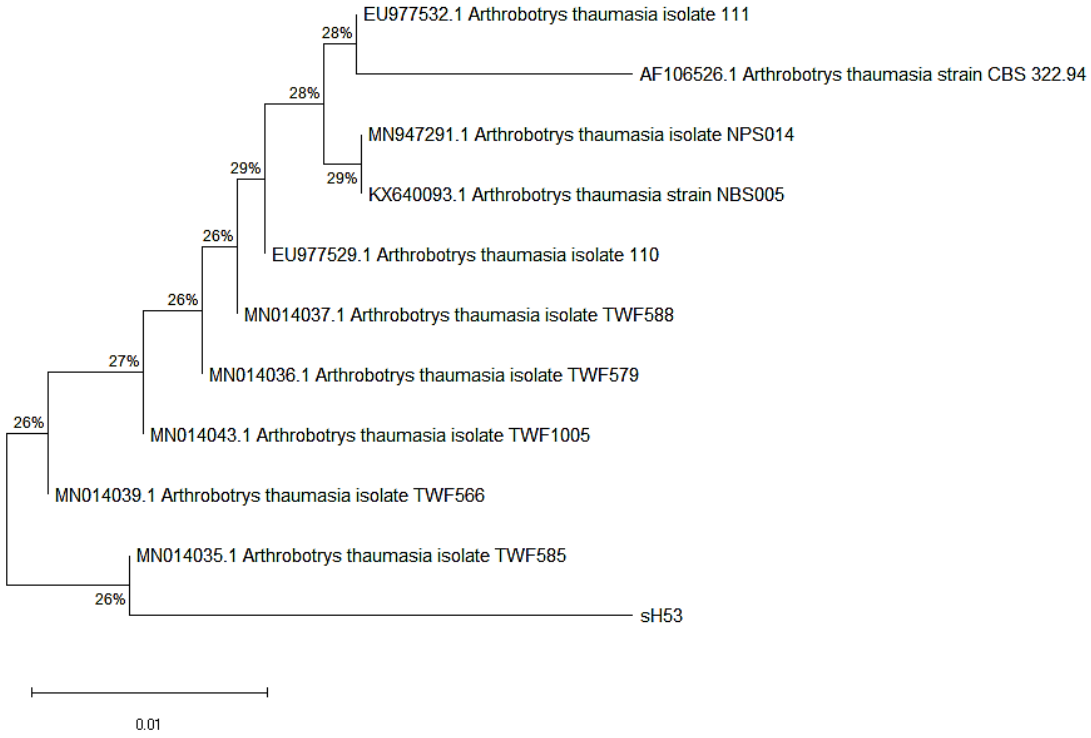
Subsequently, data in Table 1 was used to establish species-level neighbor-joining trees by using MEGA software with bootstrap replication 1000x for testing the reliability of BLAST results. Phylogeny tree results are shown in Figure 5.



(a)



(b)



(c)

Figure 5. Genetic relationship of (a) sH51; (b) sH52 and (c) sH53 with other most similar NTF species acquired from BLASTn results (percentages show site coverage of the sequences).

Figure 5a shows that the closest relationship for isolate sH51 is *D. brochopaga* strain 3eA001 (no. accession: JF748754.1) by 45%. Isolate sH53 in Figure 5c is closely related to *A. thaumasia* isolate TWF585 (accession no: MN014035.1) by 26%. Meanwhile, isolate sH52 is closely related to *Arthrobotrys* sp. strain TWF889 (MN014031.1) by 52%.

4. Discussion

Literature exploration revealed that this is initial research on the existence of *D. brochopaga* (isolate sH51) examined from municipal waste-contaminated soil in Medan City, North Sumatera, Indonesia. Previous studies identified *D. brochopaga* isolated from the soil of the oriental melon field (Cho et al., 2008; Singh et al., 2019) and leaf litter (Elshafie et al., 2006). Elshafie et al., (2006) and Xie et al., (2010) separately isolated *D. brochopaga* from soil and observed fungal constricting ring development which is a similar type of trap described in section 3.2 Figure 2.

Isolate sH53 can be identified assuredly as *Arthrobotrys thaumasia*. Previous reports also revealed isolating *A. thaumasia* from the soil sample in the same place (Hastuti et al., 2021) and neighboring regions in North Sumatera, Indonesia (Purba et al., 2022). However, Hastuti's isolate *A. thaumasia* DS01 (accession no: MN717431.1.) does not exist in the sH53 phylogeny tree (Figure 5c), which means they are distantly related. Other research isolating *A. thaumasia* from pasture soils, barn soils, and woodland soils in China revealed similar morphological characteristics (Wang et al., 2017).

Results for isolate sH52 are still uncertain. ITS genome sequenced does not adequately determinate its morphological and genetic identification to the species level consistently. Even though ITS is reliable for fungi identification in most cases, other regions such as large subunit (LSU) and small subunit (SSU), are also highly recommended to be sequenced to compensate for rampant cryptic speciation (Raja et al., 2017; Hastuti et al., 2021; Purba et al., 2022).

All isolates significantly suppressed *M. hapla in vitro* (Figure 1). Isolate sH52 and sH51 (*D. brochopaga*) suppressed *M. hapla* by 97.7% while isolate sH53 (*A. thaumasia*) showed suppression of 89.27%. Previous studies have also revealed that *D. brochopaga* and *A. thaumasia* isolated from soil in Korea reduce more than 70% of *M. incognita in vitro* (Kang et al., 2019). *D. brochopaga* was found to

be effective in controlling nematode, significantly increasing total chlorophyll content in leaves and activating root and shoot defense-related metabolic pathways (Singh et al., 2019). A study showed that *A. thaumasia* also suppressed the nematode population by 93% and supported plant growth when applied as a fungal suspension to tomato plants (Purba et al., 2022).

Most of the preceding studies discuss isolating NTF from farmland. This research provides basic information about novel reservoirs for acquiring NTF samples since farmlands are hardly available in urban areas. However, municipal waste-contaminated soil is very common, thus making it more accessible for following researches and supporting future sustainable urban agriculture.

Conclusion

Isolation of NTF from municipal waste-contaminated soil samples in Medan City, North Sumatera, Indonesia, successfully obtained three potential isolates that have an efficacious nematicidal impact against *M. hapla in vitro* by 97.7% (isolate sH51 and sH52) and 89.27% (isolate sH53) thus promising them as environmentally friendly bionematicide for crops. Morphological identification and ITS sequencing analysis determine that isolate sH51 is *Drechslerella brochopaga* and isolate sH53 is *Arthrobotrys thaumasia*. While morphological analysis for isolate sH52 reveals it as *Arthrobotrys sinensis* but is limited to *Arthrobotrys* sp. based on ITS sequencing and phylogeny analysis, thus additional gen regions need to be sequenced for confirmation.

Acknowledgment

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