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Molecular detection of *Dorylaimus stagnalis* (Durjan, 1845), in the rhizosphere of soybean plants in Jember District, Indonesia

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Abstract. *Pradana AP, Izzatika ZN, Addy HS. 2025. Molecular detection of* Dorylaimus stagnalis *(Durjan, 1845), in the rhizosphere of soybean plants in Jember District, Indonesia. Biodiversitas 26: 1913-1919.* Nematodes, members of the phylum Nematoda, are ubiquitous soil organisms whose community composition provides critical insights into soil health and ecosystem function. The genus *Dorylaimus* is of particular ecological interest due to its adaptability and role in nutrient cycling within diverse terrestrial environments. This study documents the first molecular and morphometric confirmation of *Dorylaimus stagnalis* (Dujardin, 1845), in the rhizosphere of soybean (*Glycine max*) in Jember District, Indonesia. Soil samples were systematically collected from 15 distinct sites within soybean fields using a standardized protocol, and nematodes were extracted via the Whitehead tray method. Detailed morphological observations were made using an Olympus BX50 light microscope, and morphometric measurements were obtained following De Man's ratios, which collectively confirmed diagnostic features consistent with *D. stagnalis* as reported in previous studies. Molecular characterization was performed by PCR amplification of the 28S-D2D3 region followed by Sanger sequencing. Subsequent phylogenetic analysis revealed 94% sequence similarity to established reference sequences (AY592994.1 and AY592995.1) and a low genetic distance of 0.1058, thereby substantiating the taxonomic identity of the specimens. This study's integrative approach not only verifies the presence of *D. stagnalis* in Indonesia but also indicates subtle morphometric variations potentially attributable to local environmental pressures. Its findings expand the current understanding of nematode biodiversity in Indonesian agroecosystems and provide a framework for future research on sustainable soil management practices.

Keywords: 28S-D2D3, evolution, morphology, morphometry, nematode

INTRODUCTION

Nematodes are unsegmented organisms belonging to the phylum Nematoda, a diverse group inhabiting nearly every habitat on Earth, from deep-sea environments to tropical forest soils. They play pivotal roles as decomposers, predators, and omnivores, facilitating organic matter recycling and food web dynamics (Lu et al. 2020). In agriculture, nematodes are recognized not only as crop parasites but also as key indicators of soil health, with specific species and populations reflecting soil fertility, contamination, and ecological balance (Du Preez et al. 2022; Martin et al. 2022). Among these, the genus *Dorylaimus* is frequently used as a bioindicator (Laasli et al. 2022) and has been reported across multiple regions in Indonesia, underscoring its broad distribution and adaptability to various environmental conditions.

Tarno et al. (2021) reported the presence of *Dorylaimus* in robusta coffee plantations in Malang, East Java, where the nematode interacted with other microorganisms in the plantation ecosystem. Similarly, Widowati et al. (2014) detected the genus in the rhizosphere of arabica coffee in Bondowoso District, demonstrating its capacity to inhabit various host plants and adapt to different environmental conditions. Additionally, Mutala'liah et al. (2023) reported the presence of *Dorylaimus* in the rhizosphere of potato plants, further evidencing its role in diverse agricultural systems. However, despite these frequent reports across multiple regions of Indonesia, no study has yet specifically identified species from this genus using molecular data.

During a nematode survey in soybean fields in Jember District, East Java, Indonesia, a nematode exhibiting characteristics similar to those of *Dorylaimus stagnalis* (Dujardin, 1845), was discovered. *D. stagnalis* is an omnivorous species capable of consuming various food sources, enabling it to influence soil microbial dynamics and the structure of rhizosphere communities, thereby potentially affecting plant health and productivity (Shafqat et al. 1992; Zheng et al. 2019). Notably, the presence of *D. stagnalis* has not been previously documented in Indonesia. Identifying and recording this species not only expands our understanding of nematode diversity and function in agricultural ecosystems but also paves the way for future studies on its interactions with other soil organisms and its potential role in sustainable soil management strategies.

Conventional nematode identification techniques, which primarily rely on morphological characteristics, often encounter significant challenges, particularly concerning time efficiency and the accuracy of results. Morphology-based identification demands specialized expertise and can be highly time-consuming, especially when distinguishing between species with highly similar morphological traits,

which is often a difficult task (Seesao et al. 2017). Misidentifications may arise when relying solely on limited morphological features, particularly in species with considerable morphological variation or those characterized by very small body sizes (Bogale et al. 2020).

The advancement of molecular technologies, such as DNA barcoding, has significantly improved the speed and accuracy of nematode species identification. DNA barcoding is a molecular technique for species identification and phylogenetic analysis based on sequence variation in this highly informative ribosomal expansion segment. DNA barcoding allows for the identification of species using unique, species-specific genetic sequences. This method accelerates the identification process and enhances accuracy, particularly for species that are difficult to differentiate based solely on morphological traits (Nega 2014). In addition, progress in these molecular techniques enables more efficient mapping of nematode diversity, offering a clearer picture of nematode biodiversity and their ecological roles across ecosystems (Floyd et al. 2002).

This study utilized molecular detection methods and DNA barcoding to confirm the presence of *D. stagnalis* in the rhizosphere of soybean plants in Jember District, Indonesia. By integrating molecular analysis with morphological and morphometric data, the study enriches the current nematode biodiversity data in Indonesia and provides precise insights into the occurrence of *D. stagnalis* within local agricultural ecosystems. The findings support the development of sustainable agricultural management strategies by underscoring the importance of soil biodiversity in promoting productivity and maintaining ecological balance. Furthermore, this research establishes a foundation for future studies on the interactions between *D. stagnalis* and other soil organisms.

MATERIALS AND METHODS

Study time and location

This study was conducted from January to July 2023. Morphometric analyses were performed in the Zoology Laboratory of the Biology Education Study Program, Faculty of Teacher Training and Education, Universitas Jember. Molecular analyses were conducted at the Center for Development of Advanced Sciences and Technology, Universitas Jember, Indonesia.

Soil sampling and nematode extraction

Soil samples were collected from soybean fields located in Bangsalsari Sub-district, Jember District, East Java, Indonesia. Sampling was conducted from 15 different points at a depth of 25 cm, collecting approximately 500 g of soil from each point. All samples collected were composited into a single homogeneous sample. From this composited soil sample, a 1-L soil subsample was measured volumetrically to standardize the extraction process and minimize bias associated with variations in soil moisture content. Nematodes were extracted using the Whitehead tray method, incubated for 48 hours, and subsequently filtered through a 400-mesh sieve (Perry et al. 2020).

Nematode preservation

The extracted nematodes were first observed under a stereomicroscope to identify individuals morphologically similar to *D. stagnalis*. A total of five nematodes showing morphological characteristics consistent with *D. stagnalis* were selected and preserved individually in DESS solution. The DESS solution consisted of 0.25 M disodium EDTA and 20% Dimethyl Sulfoxide (DMSO), adjusted to pH 8 and saturated with NaCl. The nematodes preserved in DESS solution were stored at room temperature until further molecular analysis. The DESS preservation solution was selected because of its ability to maintain both the morphological integrity of nematode specimens and the quality of their DNA for subsequent analyses (Yoder et al. 2006).

Nematode morphometric analysis

Nematode specimens were examined using an Olympus BX50 light microscope equipped with Olympus cell software. Observations were performed at multiple magnifications and focal adjustments to capture specific morphological characteristics. Morphological identification followed standard taxonomic keys, and morphometric measurements were performed using De Man's formula (Lee 2002; Perry et al. 2020).

Nematode DNA extraction

Nematodes preserved in DESS solution were soaked for 1 hour in a Petri dish containing distilled water. DNA extraction was performed following a protocol from Holterman et al. (2006) with modifications. A single nematode with confirmed morphology was placed in a 0.2 mL PCR collection tube containing 25 µL of nuclease-free water. An additional 25 µL of Holterman Worm Lysis Buffer, consisting of 200 mM NaCl, 200 mM Tris-HCl (pH 8), 1% β-mercaptoethanol, and 800 µg/mL proteinase-K, was added to the tube. The mixture was vortexed for 1 minute and then incubated in a PCR machine at 65°C for 90 minutes, followed by 99°C for 5 minutes. These incubation conditions differ slightly from the original protocol to enhance lysis efficiency. The resulting nematode DNA was ready for amplification or could be stored at -20°C for later use.

Nematode DNA amplification

DNA amplification was carried out using a PCR machine targeting the D2D3 region of the 28S rDNA. The primers used were forward primer D2Ab (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and reverse primer D3B (5'-TCGGAAGGAACCAGCTACTA-3'), yielding an expected PCR product size of approximately 766 bp (De Ley et al. 1999). The D2D3 region was chosen because it provides sufficient variation for accurate species identification and phylogenetic analysis in nematodes. The PCR reaction mixture consisted of 25 µL of 2× MyTaq HS Red Mix, 1 µL of forward primer D2Ab, 1 µL of reverse primer D3B, 20 µL of nuclease-free water, and 3 µL of DNA template, with a final reaction volume of 50 µL in each 0.2 mL PCR tube.

The amplification process began with an initial pre-denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds, with a final extension at 72°C for 10 minutes.

The amplification products were visualized by electrophoresis on a 1% agarose gel stained with ethidium bromide (EtBr). A 5 µL aliquot of the PCR product and 2 µL of a 1 kb DNA ladder marker were loaded into separate gel wells. Electrophoresis was performed at a constant voltage of 100 volts for approximately 30 minutes in 1× TBE buffer. DNA bands were visualized and documented using a UV transilluminator.

Purification of DNA amplification products and sequencing

The amplified DNA was purified using Phenol-Chloroform-Isoamyl alcohol (PCI), a mixture of three organic solvents employed for cell lysis, cellular component separation, and DNA purification (Gupta 2019). The purification process began with measuring the volume of the PCR product, followed by mixing it with Tris-EDTA (TE) buffer to achieve a final volume of 200 µL. The mixture was then combined with an equal volume of PCI (v/v) and centrifuged at 4°C at 10,000 rpm for 15 minutes. The upper aqueous phase (200 µL) was carefully transferred to a new tube containing 10% sodium acetate (v/v). Subsequently, 500 µL of 99% absolute ethanol was added for overnight precipitation at -20°C. After centrifugation at 4°C at 15,000 rpm for 20 minutes, the supernatant was discarded, leaving a pellet. The pellet was washed with 200 µL of 70% ethanol and centrifuged again at 4°C for 10 minutes. The supernatant was discarded, and the pellet was air-dried before resuspension in 50 µL of TE buffer as an elution buffer. TE buffer serves to maintain a stable pH, dissolve DNA, prevent enzymatic degradation, and dilute the DNA for further applications or storage (Nakayama et al. 2016).

Phylogenetic tree analysis and DNA barcoding

The PCR products were sequenced for nucleotide analysis using the Sanger dideoxy sequencing method at First BASE Laboratory, Malaysia. The obtained nucleotide sequences were aligned using MEGA version 11 software and subjected to BLAST analysis through the National Center for Biotechnology Information (NCBI) database. The sequences were compared with reference sequences retrieved from NCBI and aligned using the ClustalW multiple sequence alignment algorithm. Subsequently, a phylogenetic tree was constructed using the maximum likelihood method in MEGA 11, with 1000 bootstrap replicates to assess branch reliability (Pradana and Yoshiga 2024).

RESULTS AND DISCUSSION

The morphology and morphometry of *D. stagnalis*

In this study, nematodes exhibiting morphological characteristics consistent with those of *D. stagnalis* were identified. The species displayed distinct features, such as a wrinkled cuticle. The lip region appeared slightly narrower than the adjacent body section, indicated by a subtle depression (Figure 1).

The nematodes identified in this study exhibited morphological characteristics consistent with those of *D. stagnalis* as described by Shafqat et al. (1992). According to Shafqat et al. (1992), *D. stagnalis* has six fused lips when viewed en face: two ventrosubdorsal, two lateral, and two ventrosublateral. Each lip features papillae, which are small protrusions, on both the inner and outer rings. The amphid, a sensory organ, is stirrup-shaped, with a slit-like opening at the junction of the lip and body regions. The odontostyle, a tooth-like structure, is hollow and cylindrical, with a smooth tip, while the odontophore, which supports the odontostyle, is a simple rod-like structure slightly longer than the odontostyle and embedded within the esophageal tissue. These observations align with the findings of this study, wherein the nematodes possessed an odontostyle-type stylet.

Table 1 presents a comparison of the morphometric data of *D. stagnalis* from three different studies. These studies demonstrate variations in the physical characteristics of the nematode, which are likely attributable to geographical factors. In this study, the body length of *D. stagnalis* ranged from 2173 to 2344 µm, which is shorter than that of samples from India (2630 to 4410 µm). The body length of samples from Japan (3000 µm) was also greater than that of the Indonesian samples. On average, *D. stagnalis* from Indonesia was approximately 11% shorter than the Indian samples and 22-27% shorter than the Japanese samples.

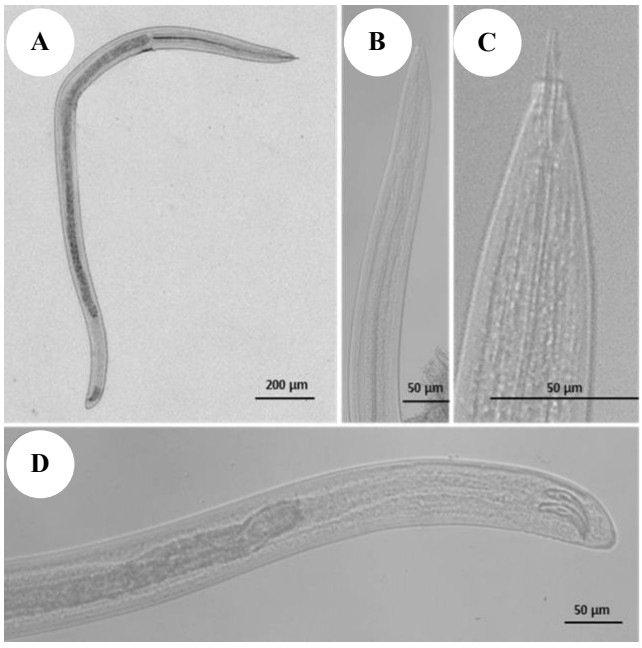

Figure 1. Morphological features of *Dorylaimus stagnalis* identified in this study: A. Entire body; B. Anterior region; C. Anterior region with a focus on the stylet; D. Posterior region

Table 1. Morphometric comparison of *D. stagnalis* in this study and previous reports

Characteristics *D. stagnalis* (Current study) (Indonesia) ♂	*D. stagnalis* (Shafqat, 1992) (India) ♂	*D. stagnalis* (nies.go.jp) (Japan) ♂
n	5	50	NA
L (µm)	2173-2344	2630-4410	3000
b	4.13 ± 0.4	3.97	4.4
s	33.60 ± 1.8	-	41
a	26.52 ± 0.25	27.38	36
c	15.83 ± 0.13	12.3	14

Note: n: Number of samples observed; L: Body length; b: Body length/distance from the anterior valve to the esophagus; s: Odontostyle length; a. Body length/maximum body width ratio; c. Body length/tail length ratio

The body length to maximum body width ratio (a) for *D. stagnalis* in Indonesia was 26.52 ± 0.25, which is slightly lower than that of the India samples (27.38) and significantly lower than that of the Japanese samples (36). The 3% difference in the ratio compared to the Indian sample is not biologically significant; however, the 26% lower ratio compared to the Japanese sample indicates a more pronounced morphological divergence. This difference may reflect local adaptations of *D. stagnalis* in Indonesia to distinct environmental conditions, such as variations in resource availability or predation pressures (Liu et al. 2015; Luan et al. 2020).

In contrast, the body length to tail length ratio (c) in Indonesia was recorded as higher (15.83 ± 0.13) compared to that in India (12.3) but lower than that observed in Japan (14). This variation in the c ratio may reflect differences in body proportions potentially linked to varying behaviors or survival strategies across different environments (Grzelak et al. 2016). The 29% increase in the c ratio compared to that in India suggests that *D. stagnalis* in Indonesia possesses a relatively shorter tail in relation to its body length, which could represent an adaptation to the specific ecological conditions of this region (Majdi et al. 2019).

These morphometric variations suggest significant differences among *D. stagnalis* populations across different regions. The differences in body length, body length to width ratio, and body length to tail length ratio indicate that environmental factors play a critical role in shaping the morphology and size of these nematodes (Liu et al. 2015; Majdi et al. 2019).

Molecular characterization and phylogenetic position of *D. stagnalis*

The phylogenetic analysis based on the D2D3 region of the 28S rDNA gene revealed clear evolutionary relationships between the analyzed *D. stagnalis* specimens and closely related species within the nematode group (Figure 2). The D2D3 expansion region of 28S rDNA is frequently used as a molecular marker in nematode taxonomy and phylogenetics due to its relatively high sequence variability between closely related species and its conserved regions within species. The *D. stagnalis* specimens from this study formed a robust clade with previously identified reference sequences available in public genetic databases, supported by a high bootstrap value of 94%. This strong clustering confirms the reliability of the D2D3 region for differentiating *D. stagnalis* from closely related species and validates the taxonomic identification of these specimens.

The clade containing *Clavicaudoides* sp. and *Paractinolaimus decraemerae* displayed a lower bootstrap value (59%) that nevertheless suggests that these species share a relatively recent common ancestor in evolutionary terms. The phylogenetic tree indicates that despite some morphological or ecological differences, these species remain genetically closely related. Several studies have noted that phylogenetic proximity is often associated with similarities in ecological habitats or survival strategies (Kern et al. 2020).

D. stagnalis is a cosmopolitan species found across a range of freshwater and terrestrial environments. As outlined in previous studies, this species exhibits adaptations that enable it to thrive in diverse conditions, with its broad distribution across ecosystems reflecting high ecological flexibility (Zheng et al. 2019; Zhylina and Shevchenko 2019). The phylogenetic tree not only deepens our understanding of the evolutionary relationships within the *Dorylaimus* genus but also provides a basis for further research on the ecological adaptations and evolutionary trajectories of this species across various ecosystems.

A heatmap generated from the genetic distance data depicts the phylogenetic relationships between *D. stagnalis* specimens from different locations and other nematode species (Figure 3). In this heatmap, genetic distances are represented by a color gradient, with lighter shades indicating closer genetic proximity and darker shades reflecting greater genetic divergence.

The *D. stagnalis* specimens from this study exhibited a very close genetic distance to AY592995.1 *D. stagnalis*, as indicated by the low genetic distance value (0.1058). This suggests that the specimens in this study are genetically identical to previously identified *D. stagnalis* specimens. These findings align with the earlier phylogenetic analysis, which placed the specimens within the same clade as *D. stagnalis*.

However, the specimens from this study exhibited greater genetic distances from other species, such as MG921257.1 *Mesodorylaimus* sp. and JN242245.1 *Nevadanema nevadense*, with genetic distance values of 0.1816 and 0.1760, respectively. This suggests that although these species belong to the same phylum, they have evolved along distinct evolutionary paths and display significant genetic divergence from *D. stagnalis*. The larger genetic distances also indicate that these species have undergone different adaptations and speciation processes, likely reflecting differences in habitat and environmental selection pressures (Kikuchi et al. 2017).

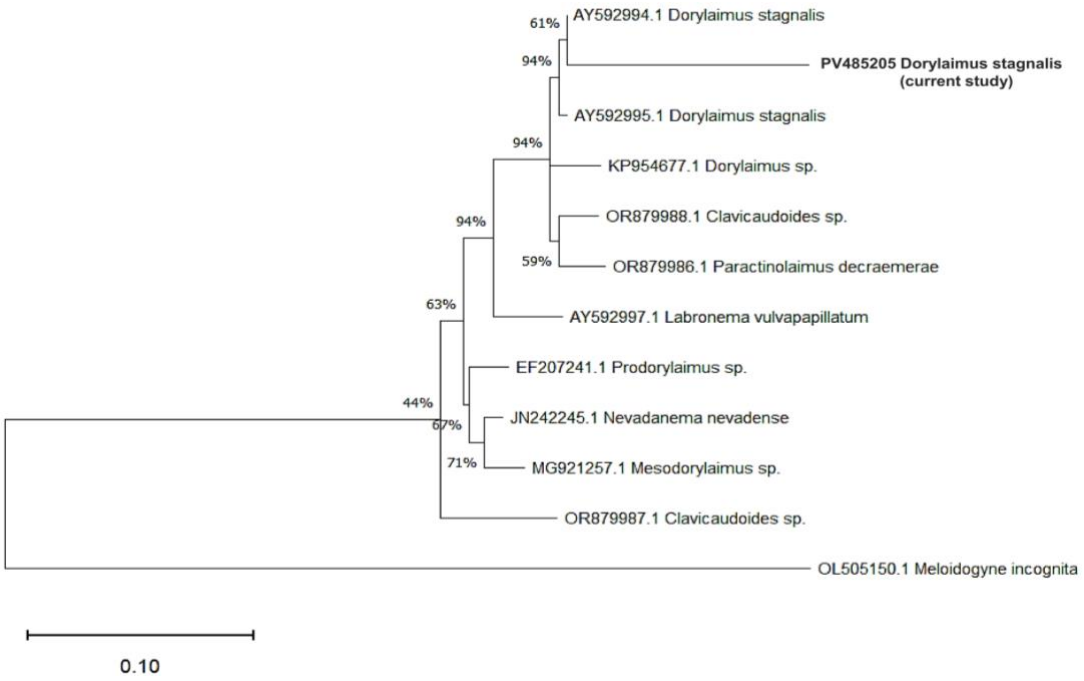

Figure 2. Phylogenetic tree of *D. stagnalis* in this study based on the D2D3 region. Tree reconstruction was performed using the Neighbor-Joining Method with the Kimura 2-parameter + gamma distribution model and 1000 bootstrap iterations

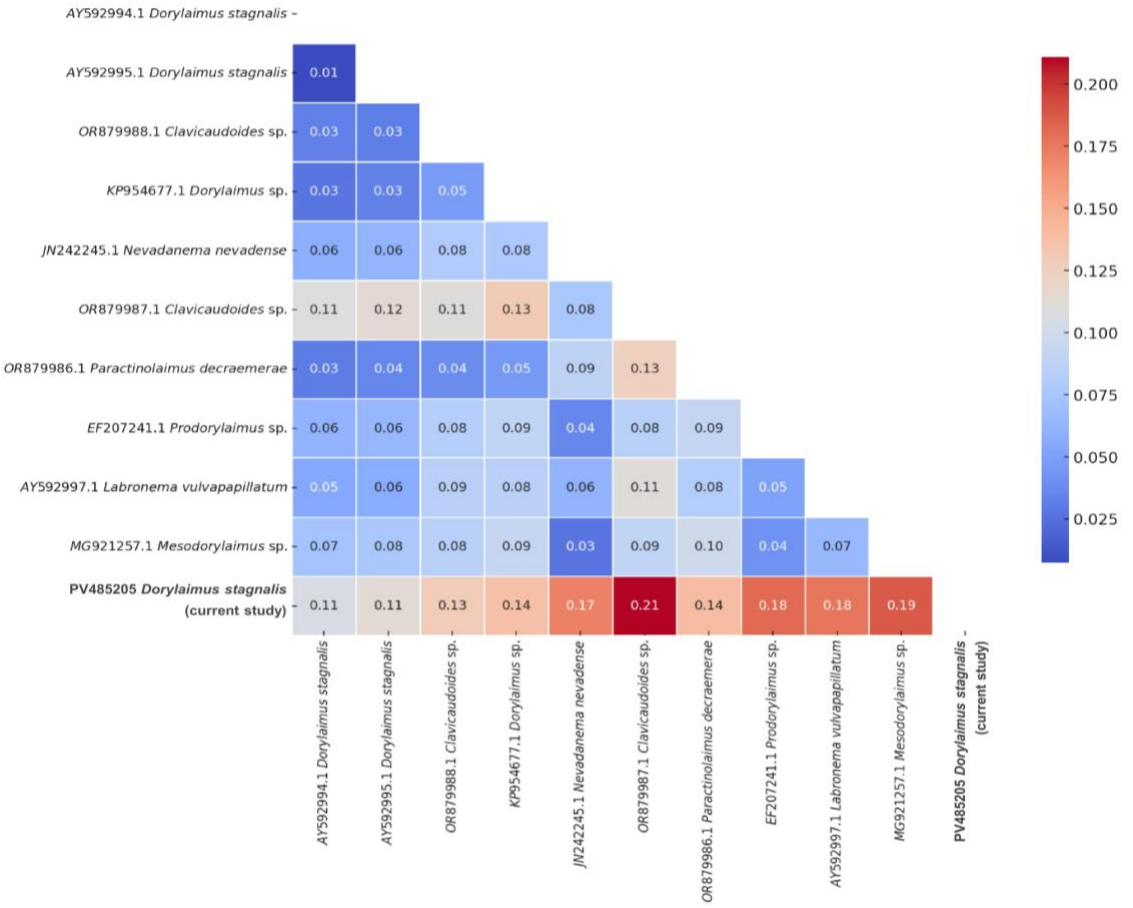

Figure 3. Genetic distance heatmap of *Dorylaimus stagnalis* and related nematode species

Figure 4. Visual representation of the DNA sequence (5' → 3') of the D2D3 region (766 bp) with color-coded nucleotides

The phylogenetic and genetic distance analyses provide valuable insights into the genetic diversity and evolutionary relationships among species within the phylum Nematoda, particularly those in the genus *Dorylaimus*. This study demonstrates that the analyzed *D. stagnalis* specimens exhibit a high degree of genetic similarity to *D. stagnalis* specimens identified in other locations, confirming their classification as the same species. This close genetic relationship further suggests that these specimens share a relatively recent common ancestor with other *D. stagnalis* populations, indicating that genetic variation within this species may be limited despite its broad geographical distribution.

The analyzed DNA sequence revealed a variable nucleotide composition (Figure 4). Guanine (G) was the most abundant nucleotide, totaling 232 nucleotides and constituting approximately 31.61% of the sequence. This was followed by Adenine (A), which accounted for 180 nucleotides or 24.52% of the total. Thymine (T) was the third most frequent, with 164 nucleotides (22.34%), while Cytosine (C) was the least represented, with 158 nucleotides (21.53% of the total sequence).

The high proportion of Guanine (G) suggests increased thermodynamic stability within the sequence as G-C base pairs form three hydrogen bonds compared to two bonds in A-T pairs, thereby contributing to greater stability under specific conditions. Conversely, the relatively balanced proportions of Adenine (A) and Thymine (T) indicate the possible presence of many A-T-rich segments, which may reflect regions of DNA that are more susceptible to melting or separation under particular biological conditions (Li et al. 2018).

Furthermore, the relatively balanced proportions of purine nucleotides (A and G) and pyrimidines (C and T) are consistent with the general structural requirements of DNA, with such balance being necessary to maintain the stability of the double helix. Small variations in these proportions could reflect specific biological roles or functions of this sequence, potentially indicating its involvement in the regulation of certain genes.

Given that *D. stagnalis* is an omnivorous nematode rather than a plant pathogen, its ecological role in soybean fields may differ considerably from that of parasitic species. Indeed, the presence of non-pathogenic, free-living nematodes, is sometimes used as an indicator of soil health. Their feeding habits, which can include bacteria, fungi, and small soil organisms, may help regulate microbial communities and support nutrient cycling. Comparisons with similar ecological assessments in soybean fields outside Indonesia suggest that the abundance and diversity of omnivorous nematodes can reflect a soil's functional capacity and overall sustainability, underscoring the importance of continued research on *D. stagnalis*' ecological contributions in diverse agricultural landscapes.

In conclusion, integrative morphological, morphometric, and molecular analyses conclusively confirm that the nematode specimen is *D. stagnalis*. Although morphometry revealed minor size variations compared to previously reported populations, the values remain within acceptable ranges, and the high sequence homology demonstrates a close genetic relatedness to other isolates, thereby both establishing the species identity and marking the first documented occurrence of *D. stagnalis* in Indonesia, which provides a valuable foundation for future research into its ecological significance within agricultural ecosystems.

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