**A Simple, Rapid and Cost-effective Protocol for Species Identification of Plant Parasitic Nematodes** by **PCR based Downstream Application**

**Abstract**

Plant parasitic nematodes are tiny, aquatic, obligate biotrophic worms that primarily impact below-ground parts of plants. Accurate identification of nematode species is crucial for assessing the global distribution of economically significant nematode pests and devising effective management strategies. Traditional methods of species-level identification rely on morphometric data, but this approach is becoming outdated due to a shortage of nematode taxonomists and the advent of molecular tools. In recent years, molecular techniques have gained popularity for nematode species identification. However, obtaining sufficient high-quality genomic DNA from nematodes is challenging due to their microscopic size, the need for a large number of nematodes, and the presence of multi-species nematode communities in the plant rhizosphere. Therefore, there is a pressing need to develop a rapid, cost-effective, and efficient protocol for identifying species using single or few nematode through molecular approaches. In this study, we have successfully developed and standardized a protocol that involves the direct use of one or a few nematodes for genomic DNA amplification through PCR. This innovative approach eliminates the need for isolating, storing, and maintaining nematode DNA samples, streamlining the identification process and making it more accessible and practical.

## Keywords: Plant parasitic nematodes, Simple extraction protocol, molecular techniques, PCR, identification

**Running title:** Simple extraction protocol for molecular diagnosis of nematodes

1. **Introduction**

Plant parasitic nematodes are basically aquatic microbes mostly reside either in the soil around the rhizosphere or within tissues of belowground plant parts *viz*., roots, rhizomes, corm *etc*. They occur as polyspecific communities and often interact with each other. Traditionally, identification of plant nematodes at species level involves morphometric database involving measurement of various body parts and their ratio. With the advance of molecular tools and paucity of traditional taxonomists in recent times, plant nematode identification by sequencing and annotation using NCBI database is getting popular and pragmatic.

Identifying nematode species through molecular methods entails the isolation of DNA, amplification of the DNA fragment of interest using oligonucleotide primers through Polymerase Chain Reaction (PCR), and sequencing the amplified DNA fragment with subsequent annotation using the NCBI portal to maximize similarity. However, extracting DNA from microscopic macrofauna like nematodes poses a significant challenge. To obtain a sufficient quantity of DNA from nematodes, a considerable number of individual nematodes (approximately 1000 nematodes) must be meticulously picked under a stereo-binocular microscope and this process is intricate, time-consuming, and tedious. Compounding the difficulty is the presence of mixed populations of different nematode species within the same genus in the rhizosphere. Furthermore, obtaining an adequate number of nematodes during sampling and extraction is not always guaranteed (Sakai, 2010; Swain et al., 1995). There is also the possibility of resorting to multiplying nematodes through carrot disc culture or pot culture before DNA extraction. However, this necessitates specific equipment, facilities, and trained professionals to conduct the culturing process (Kaplan and Davis, 1990).

Earlier attempts at molecular taxonomy using single or few nematodes were made, and protocols were established by Hominick et al. (1997), Swain et al. (1995), and Sakai (2010). These methods relied on conventional DNA isolation protocols, which included a series of chemical processes to prepare nematodes for PCR analysis. However, these processes are becoming outdated with the progress in molecular techniques and the availability of ready-to-use kits for DNA extraction. Ready-to-use kits have gained popularity among molecular biologists and are widely adopted due to their simplified procedures and high efficiency in DNA extraction.

Addressing the aforementioned challenges, a study was undertaken to establish a straightforward, cost-effective, and dependable technique for identifying nematode species from a limited number of specimens. The underlying principle of this technique is to bypass the DNA extraction process and conduct PCR using the simple extraction protocol for molecular diagnosis of nematodes.

**2. Materials and Methodology**

**2.1. Isolation of Nematodes**

 The experiment focused on two economically significant nematode genera, namely the root lesion nematode (*Pratylenchus* sp.) and the spiral nematode (*Helicotylenchus* sp.). These nematodes were isolated from a banana field affected by nematode infestation and were cultured using potted tissue culture banana plants of the Nendran cultivar. The cultured nematodes were extracted by cutting infected banana roots in to 1cm length pieces and incubating them in tap water overnight. The resulting suspension was then passed through sieves, first through a BSS 300 sieve, followed by a 500 sieve.

**2.2. PCR amplification**

A single nematode was picked from the nematode suspension and placed in a 500 µl tube with 10 µl of TE buffer. 1X TE buffer was prepared by combining 1.0 ml of 1 M Tris-Cl (pH 8.0) and 0.2 ml of 0.5 M EDTA (pH 8.0). The volume was adjusted to 100 ml by adding 98.8 ml of water. The samples were incubated in a water bath at 65°C for 2 minutes. Subsequently, the samples were mashed with an autoclaved micropestle, vortexed for 30 seconds, and then centrifuged at 12,000 g for 30 seconds. The resulting supernatant was transferred to a new 500 µl tube and directly used for PCR. PCR was conducted in 10 µl reactions, comprising 2 µl of supernatant, 5 µl of Taq 2x master mix RED (Ampliqon, Denmark), and 10 pmol each of forward (D2A - 5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and reverse primers (D3B - 5′-TCCTCGGAAGGAACCAGCTACTA-3′); the remaining volume was made up with water. The PCR amplification was carried out in a thermal cycler (Biorad C1000 touch thermal cycler, USA) with an initial denaturation at 94°C for 7 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 56°C (primer-specific temperatures) for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The same PCR protocol was applied using genomic DNA of nematodes extracted through the DNeasy for blood and tissue kit (Qiagen, USA), following the manufacturer's protocol, for comparison and result validation

**2.3. Agarose gel electrophoresis**

The amplified products underwent electrophoresis on a 1.5% agarose gel using 1X TAE buffer, stained with ethidium bromide, and run alongside GeneRuler 1 Kb Plus DNA Ladder (Thermo Scientific, USA). Gel visualization was achieved under a UV transilluminator, and documentation was performed using a gel documentation system (Medicare GELSTAN). The PCR products were then purified from agarose gel, quantified and sequenced. The sequence data obtained in this study have been deposited into GenBank, and the details are as follows.

|  |  |  |
| --- | --- | --- |
| **Nematode** | **Method** | **GenBank ID** |
| *Pratylenchus coffeae* | PCR using genomic DNA of nematode isolated from DNeasy for blood and tissue kit (Qiagen, USA) | MH087065 |
| *Helicotylenchus multicinctus* | MH087063 |
| *Pratylenchus coffeae* | Simple extraction protocol | OR649325 |
| *Helicotylenchus multicinctus* | OR649327 |

**3. Results and Discussion**

This study successfully established a simple, fast, and reliable protocol for nematode characterization through a simple extraction protocol from a single or few nematodes. While other available DNA extraction protocols, such as the DNeasy for blood and tissue kit, provided high-quality DNA (**Fig. 1**), they were either characterized by lengthy procedures or were relatively expensive. Moreover, these protocols were not suitable for extracting DNA from single or a small number of nematode samples (5-10 nematodes). In the Simple extraction protocol described here, the nematode cell and nuclei are disrupted using mechanical force, releasing DNA into the TE buffer. Subsequent centrifugation co-precipitates cell debris with polysaccharides and protein complexes, which can interfere with the quality of the DNA (Sakai, 2010; Sika et al., 2015).

To assess the quality of genomic DNA extracted using the simple extraction protocol, PCR amplifications were performed on the supernatant obtained from this method, along with genomic DNA isolated from the kit method, for *P. coffeae* and *H. multicinctus*. Successful amplification of a single target fragment of 760 bp was achieved for both *P. coffeae* and *H. multicinctus* (**Fig. 2**). After gel extraction, the amplicon was quantified, and the simple extraction protocol yielded a sufficient quantity of amplicon for sequencing (**Table 1**). This suggests that the DNA extracted using this method is suitable for nematode characterization and is free from secondary metabolites that could interfere with DNA yield and quality (Porebski et al., 1997) and additionally extracting DNA in TE buffer helps prevent degradation of DNA.

The developed protocol proves to be user-friendly, with a simple buffer composition that eliminates the need for special kits in PCR amplifications. The procedure is labor-saving, and TE preparation is simplified when a manufactured solution is utilized. Notably, successful amplifications were achieved from crude DNA supernatant, including TE buffer. It is worth mentioning that higher concentrations of TE buffer can inhibit PCR reactions and other molecular analyses of the extracted DNA due to the presence of the chelating agent EDTA (Aboul-Maaty et al., 2019). However, in the described method, the EDTA concentration in the supernatant was significantly reduced in the PCR reaction mixture, being less than 0.001%. At this concentration, TE buffer has no inhibitory effect on PCR (Khosravinia and Ramesha, 2007). Therefore, the DNA extraction method outlined here is suitable for diagnostic purposes, particularly because mitochondrial DNA and rDNA are prevalent genetic regions used for discriminating nematode species.

**4. Conclusion**

In summary, this study introduces a rapid and cost-effective protocol for genomic analysis through direct PCR, facilitating the characterization of nematode species using only one or a few nematodes. This innovative approach eliminates the cumbersome steps of isolating, storing, and maintaining nematode DNA samples, streamlining the identification process for increased accessibility and practicality. This protocol holds promise for applications in various fields, offering a user-friendly and efficient solution for nematode characterization with potential implications for diagnostics and research.

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**Ethical Approval**

No ethical issues involved

**Consent for publication**

All the authors agreed to publish the content.

**Authorship contribution statement**

CA: Conceptualization, data curation, investigation, work design, methodology developed, validation, visualization, analyzed the data, Writing – Original draft, review and editing, Formatting; PG: Project administration, nematode isolation, Writing – Original draft, review and editing

**Conflicts of interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Table 1. Concentration of the amplified fragment of different nematode samples after PCR and gel purification.**

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| --- | --- | --- | --- |
| **S. No.** | **Method** | **Nematode Samples** | **Concentration (ng/µl)** |
| 1.  | DNeasy for blood and tissue kit (Qiagen, USA) | *P. coffeae* | 72 |
| 2.  | *H. multicinctus* | 75 |
| 3.  | Direct PCR method | *P. coffeae* | 51  |
| 4.  | *H. multicinctus* | 58  |



**Fig. 1.** DNA isolation using DNeasy blood and tissue kit (Qiagen, USA). Lane M - 1 kb plus DNA ladder (SM1331); Lane 1 - DNA from *P. coffeae*; Lane 2 - DNA from *H. multicinctus*.



**Fig. 2.** **PCR products amplified with D2A and D3B primers from nematode DNA samples extracted by** **(a) DNeasy blood and tissue kit (Qiagen, USA).** Lane 1 – Amplified fragment using *P. coffeae* DNA; Lane 2 - Amplified fragment using *H. multicinctus* DNA; Lane M - 1 kb plus DNA ladder (SM1331). **(b) Simple extraction protocol**. Lane 1 – Amplified fragment using *P. coffeae* supernatant; Lane 2 - Amplified fragment using *H. multicinctus* supernatant; Lane M - 1 kb plus DNA ladder (Cat. No. SM1331).