ORIGINAL ARTICLE

Optimization of DNA extraction methods for genomic analysis of rice root-knot nematode (*Meloidogyne graminicola*) using PCR (polymerase chain reaction) and sanger sequencing

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Abstract

The root-knot nematode *Meloidogyne graminicola* is an economically important pest in rice production. The identification of a nematode species is an important basis in nematode management to reduce yield losses by extracting nematode DNA as an early step in molecular identification. This study aimed to investigate the optimal extraction method and number of *M. graminicola* for nematode genomic analysis based on PCR (polymerase chain reaction) and Sanger sequencing. The DNA extraction methods used in this study were the CTAB, SDS, and commercial kit (GeneAidTM Tissue/Blood DNA Mini Kit). The results revealed that the three DNA extraction methods could be used to analyze the nematode genomics based on PCR and Sanger sequencing using one nematode, both in a second-stage juvenile and a female, equipped with the process of nematode destruction by freezing. This finding was shown by the amplification of all DNA templates with Mg-F3 and Mg-R2 primers through PCR with a size of 370 bp, while Sanger sequencing obtained 372 bp.

Keywords: DNA extraction, genomic analysis, *Meloidogyne graminicola,* nematode stage, Sanger sequencing

Introduction

Plant parasitic nematodes are commonly found in rice-producing countries, including Indonesia. Some of the rice parasitic nematodes identified are *Hirschmaniella* spp., *Aphelenchoides besseyi*, and *Meloidogyne* spp. (Indarti *et al*. 2020; Khan and Ahamad 2020). Among them, *M. graminicola* is a species that poses a serious threat to the sustainability of rice production (Khan and Ahamad 2020). Since first identified in Indonesia in 1993 (Netscher and Erlan 1993), the distribution of the nematode *M. graminicola* has expanded to rice production centers in Java Island, Indonesia, such as Yogyakarta, Bogor, Cirebon, and Sukabumi (Nurjayadi *et al*. 2015), as well as South Sulawesi (Mirsam and Kurniawati 2018). According to Mirsam and Kurniawati (2018), crop loss due to the attack of *M. graminicola* is 20–80% in several regions in Southeast Asia.

The first step in controlling rice root-knot nematodes is the identification of the nematode species. Molecular nematode identification is a method to quickly identify nematode species for research and for further purposes in determining appropriate nematode control management (Oliveira *et al.* 2020). An important step in the molecular identification of plant parasitic nematodes is the extraction of nematode DNA. Each DNA extraction method has different effects on the quality and quantity of DNA produced. The extraction procedure affects the real-time PCR (polymerase chain reaction) amplification and the quality of the DNA template is also a crucial factor in conventional PCR for diagnosis (Trzewik *et al*. 2016). The DNA yield is influenced by many factors, including the organism species, the extracted tissue, the preservation method, the extraction procedure, and the deposition method. The ideal extraction method should optimize DNA yield, minimize DNA degradation, and be efficient in terms of cost, time, labor, and supplies. In addition,

a good extraction method should also be suitable for extracting many samples and producing minimal hazardous waste (Chen *et al.* 2010).

The number of nematode cells increases along with the development of nematode stages (Oliveira *et al.* 2020). Furthermore, the size of nematodes is quite small, thus limiting their genetic material to be obtained through DNA extraction for molecular identification. Accordingly, the collected samples are important in providing the basis for the construction of the nematode genome, yet currently, there is no standard method for extracting high-quality DNA from individual nematodes (Sloan *et al.* 2021). A large number of nematodes in the extraction process causes genetic variations of nematodes within a species, hence requiring efforts to use a minimal number of nematodes to prevent genetic variations. Lastly, it is necessary to study the optimization of DNA extraction methods with variations in the number of nematodes and nematode stadia.

Polymerase chain reaction (PCR) is a widely used tool in molecular biology to generate multiple copies of nucleic acids from initial DNA templates. The amplified nucleic acids are then expected to serve different purposes, such as detection, quantification, preparation of data for sequencing, or generating constructs for cloning (Jue *et al.* 2020). Furthermore, PCR has the main principle of DNA amplification, so it can be used for detection and identification at the species level. It also has specificity, and high sensitivity, thus it is fast for identification techniques (Farmawati *et al.* 2015). DNA sequencing aims to determine the sequence of nitrogenous bases (adenine, guanine, cytosine, and thymine) in a DNA sample (Tasma 2015). Cycle sequencing of PCR products is a fast and convenient method with a wide range of practical applications. In this study, three methods of DNA extraction were tested (CTAB – cetyltrimethylammonium bromide; SDS – Sodium dodecyl sulfate, and commercial kit), with variations in the number and stadia of nematodes (females and second-stage juveniles) to obtain the best results in terms of quality and quantity of DNA and to be used for genomic analysis using PCR and Sanger sequencing.

Materials and Methods

The research was conducted from December 2021 to April 2022 at the Sub-Laboratory of Agricultural Nematology and Sub-Laboratory of Applied Entomology, Laboratory of Plant Pest Science, Department of Plant Pests and Diseases, Faculty of Agriculture, Gadjah Mada University, Special Region of Yogyakarta, Indonesia. Samples were obtained from three regions in the Special Region of Yogyakarta: Ngemplak, Berbah, and Sewon.

Nematode extraction

Meloidogyne graminicola was sampled from infected rice plant roots in the center of rice production in the Special Region of Yogyakarta, Indonesia. Second-stage juvenile nematodes were extracted from rice roots using a modified Whitehead Tray method (Southey 1986). Female nematodes were isolated from symptomatic roots using needle preparations to separate them from the roots of their host plants. The nematodes were hooked up and put into a 1.5 ml Eppendorf tube containing 50 µl of distilled water. Variations in the number of nematodes were used: 1, 5, and 10, both second-stage juveniles and females. Then the freezing process was carried out by storing in a refrigerator at –20°C for 24 hours. After freezing, the process was continued by crushing with a micro pestle to facilitate nematode destruction. Based on research by Xin *et al.* (2021), physical treatment such as freezing increases the efficiency of the cell lysis process in DNA extraction and increases DNA concentration compared to chemical treatment. The samples were then processed according to the protocol of each method used as follows:

DNA extraction

Commercial kit

The commercial extraction kit used in this study was the GeneAid™ Tissue/Blood DNA Mini Kit (Geneaid Biotech Ltd., Taipei, Taiwan). The nematode sample that had been destroyed was then added to 600 µl of Cell Lysis Buffer (GT buffer from kit) and homogenized. Next, 20 µl of Proteinase K was added to the tube, homogenized, and then incubated at 60°C for 30 minutes. Every 10 minutes, the tube was shaken to distribute the temperature evenly. After the incubation process, the protein removal step was carried out by adding 200 µl of protein removal buffer (GBT buffer from kit), then homogenized for approximately 10 seconds, and incubated at 60°C for 20 minutes.

The DNA precipitation step was performed by adding 200 µl of absolute ethanol. The solution was then transferred to the GS column from the kit, which had been placed in the collection tube and then centrifuged at 15,000 rpm for 2 minutes. The collection tube was next removed and replaced with a new one. Then 400 µl of wash buffer (W1 buffer from the kit) was added to clean the resulting pellet. Next, it was recentrifuged at 15,000 rpm for 30 seconds. The solution in the collection tube was removed, and the GS column was placed back in the collection tube. Centrifugation was repeated at 15,000 rpm for 3 minutes to dry the column matrix. The GS column was placed in a new 1.5 ml tube. The preheated 30 µl elution buffer was inserted into the center of the column matrix,

incubated for at least 5 minutes, and then centrifuged at 15,000 rpm for 60 seconds. Lastly, the suspension in 1.5 ml tubes was ready to be used for the next step or stored at –20°C (available at: https://geneAid.com/data/files/1605685391109197921.pdf).

Cetyltrimethylammonium bromide (CTAB) 2% lysis buffer

The crushed nematode samples were added to 200 µl of CTAB buffer solution and incubated at room temperature for an hour. After that, it was incubated at 65°C for 30 minutes using a water bath, and shaken every 10 minutes to even out the temperature of the suspension. The sample was then centrifuged at 2,400 rpm for 5 minutes before being homogenized for 1–3 minutes with Chloroform Isoamyl Alcohol (CIAA). The extraction process was continued by centrifugation of the sample at 9,600 rpm for 15 minutes. The supernatant was separated in a new 1.5 ml Eppendorf tube, and then cold absolute ethanol (2× the volume of the supernatant) was added. Samples were next incubated at –20°C for 24 hours. After incubation, the samples were centrifuged at 9,600 rpm for 15 minutes, and the ethanol was removed. Then, 1 ml of cold 70% alcohol was added to the Eppendorf tube and the tube was inverted, followed by centrifugation of 9,600 rpm for 15 minutes. The ethanol was removed and the pellet was dried for 3 hours. After drying, 30 µl of TE solution was added and homogenized. The extracted DNA were stored at –20°C.

Sodium dodecyl sulfate (SDS) 1% lysis buffer

The nematode sample that had been crushed was added to the 1% SDS buffer solution according to the study of Mondino *et al.* (2015) with some adjustments [SDS lysis buffer solution contains 1% sodium dodecyl sulfate (SDS), 50 mm EDTA, 100 mm NaCl, 100 µg · ml^{-1} proteinase K, 1% β-mercaptoethanol and 100 mm Tris-HCl pH 8.5]. Then, it was incubated at 60°C for 30 minutes.The supernatant was transferred to a new tube, and the CIAA solution was added. The sample was centrifuged at 10,000 rpm for 15 minutes. The supernatant was taken and separated in a new 1.5 ml Eppendorf tube, and cold absolute ethanol (2× the volume of the supernatant) was added to a new Eppendorf tube. The sample was incubated at –20°C for 24 hours. After incubation, the sample was centrifuged at 10,000 rpm for 15 minutes, then the ethanol was removed and the DNA pellet was stored. After that, 1 ml of 70% cold alcohol was added to the Eppendorf tube. The Eppendorf tube was inverted and centrifuged at 10,000 rpm for 15 minutes, then the ethanol was removed, and the pellets were air dried for 3 hours. After drying, 30 µl of TE solution was added and homogenized. The extracted DNA were stored at –20°C.

DNA quantity-quality

Analysis of the quantity and quality of nematode DNA in the extracted samples was carried out using a NanoDrop™ Spectrophotometer. The quantity of DNA from the extraction was obtained in units of ng · µl^{-1}. The purity of the DNA was calculated using a spectrophotometer with a wavelength ratio of 260 nm/280 nm (A260/280). For each treatment, DNA quality and quantity data were obtained from the spectrophotometer (Maestrogen™ MaestroNano Pro). The DNA quantity and quality data obtained from the spectrophotometer were then processed using the split plot CRD design using the STAR (statistical tool for agricultural research) application. If significant differences were present, further tests were carried out using the least significant different (LSD) test ($p = 0.05$). LSD test is an advanced procedure to determine which treatment is significantly different if the null hypothesis is rejected (Montgomery 2011).

DNA amplification

The extracted nematode DNA was then amplified using a PCR (BioRad™ T100 ThermalCycler) with a specific primer for the nematode *M. graminicola* (Mg-F3 5′-TTATCGCATCATTTATTTG-3′ and Mg-R2 5′-CGCTTTGTTAGAAAATGACCCT-3′) with an amplified target fragment of 369 bp, which was incorporated into the ITS fragment section (Htay *et al.* 2016). The PCR program was set with an initial denaturation at 94°C for 2 minutes, then followed by 35 cycles with the following steps: denaturation at 94°C for 15 seconds, annealing at 50°C for 30 seconds, and extension at 68°C for 60 seconds. The final synthesis was carried out at 68°C for 5 minutes at a final temperature of 4°C. The amplified product was then electrophoresed on 1% agarose gel and then visualized on a UV transilluminator (Reddy and Raju 2012). The final step of the PCR process was electrophoresis to visualize the result using a UV transilluminator (Bio-Rad™ UV Transiluminator 2000).

Sequencing and molecular phylogenetic analysis

DNA sequencing was conducted by sending the PCR product to the Laboratory of Integrated Research and Testing, Gadjah Mada University, Yogyakarta, Indonesia. The results were analyzed using the basic local alignment search (BLAST) tool on the website of the National Center for Biotechnology Information (NCBI). The nucleotide sequences obtained were then analyzed using ClustalW multiple alignments on Bioedit software. The relationship between isolates was constructed using the Molecular Evolutionary Genetic

Analysis (MEGA11) software with a maximum likelihood approach (Rahman *et al.* 2018). Furthermore, GenBank genomic data was used to identify most species of plant parasitic nematodes (Nisa *et al.* 2022). In addition, the sequencing data results were also analyzed using electropherogram and nitrogen base sequence.

Results

DNA quantity-quality

The concentration values obtained from the extraction of DNA using three methods and variations in the number and stadium of nematodes revealed varying concentrations and purity of nematode DNA (Table 1, 2, 3). Overall, SDS DNA extraction method showed the highest DNA concentration both in juvenile and mature female stages (Table 1). DNA extraction using commercial kits tended to have a low concentration yield compared to the other two methods (CTAB and SDS) in both female and juvenile stages.

For the purity value, commercial kits, SDS, and CTAB at each stage, and the number of nematodes tested, met the criteria for good DNA quality. The absorbance values were in the range of 1.8–2.0 (Table 3). The values of purity in each extraction result were not significantly different. They also fulfilled the criteria for pure extraction results since they had passed the threshold for RNA contaminant levels (A260/A280 >2.00) and protein contaminant levels (A260/A280 <1.80).

Table 1. Pairwise mean comparison of extraction methods in extracted DNA concentration (ng · µl^{-1})

Extraction methods	Female				extraction methods	Juvenile			
	number of nematodes					number of nematodes			
	1	5	10	$\bar{x}2$		1	5	10	$\bar{x}2$
CTAB	27.96	53.87	41.30	41.04 b	CTAB	12.74	16.81	22.86	17.47 b
SDS	38.72	55.52	40.37	44.87 a	SDS	42.72	45.64	56.09	48.15 a
Kit	4.43	4.89	4.89	4.74 c	Kit	4.87	5.75	5.87	5.50 c
$\bar{x}1$	23.70 b	38.09 a	28.85 ab		$\bar{x}1$	20.11 a	22.73 a	28.27 a	

Values followed by the same letter for each row and column were not significantly different according to quantitative factorial set (p = 0.05), n = 3 replications; CTAB – cetyltrimethylammonium bromide; SDS – sodium dodecyl sulfate; $\bar{x}1$ – the average of DNA concertration of each number of namatodes with different DNA extraction methods; $\bar{x}2$ – the average of DNA concentration for each DNA extraction methods with different number of nematodes

Table 2. Comparison of extraction methods at each level of number of nematodes in concentration per individual extracted nematode (ng · µl^{-1})

Extraction methods	Juvenile			extraction methods	Female		
	number of nematodes				number of nematodes		
	1	5	10		1	5	10
CTAB	27.96 b	10.77 a	4.13 a	CTAB	27.96 b	10.77 a	4.13 a
SDS	42.72 c	9.13 b	5.609 a	SDS	38.72 c	11.10 b	4.04 a
Kit	4.87 a	1.15 a	0.587 a	Kit	4.43 a	0.98 a	0.49 a

Values followed by the same letter for each row and column were not significantly different according to quantitative factorial set (p = 0.05), n = 3 replications; CTAB – cetyltrimethylammonium bromide; SDS – sodium dodecyl sulfate

Table 3. Comparison of extraction methods at each level of number of nematodes in DNA purity (A260/280)

Extraction methods	Female			extraction methods	Juvenile		
	number of nematodes				number of nematodes		
	1	5	10		1	5	10
CTAB	1.95	1.95	1.8	CTAB	1.87	1.90	1.94
SDS	1.89	1.95	1.87	SDS	1.85	1.85	1.92
Kit	1.90	1.91	1.87	Kit	1.89	1.84	1.85

CTAB – cetyltrimethylammonium bromide; SDS – sodium dodecyl sulfate

The results of the nematode DNA extraction using the SDS and the commercial kit methods did not show a significant difference in the results of the DNA concentration values at the nematode stadia tested, both female and second-stage juvenile nematodes. The CTAB method of different stadia showed a significant difference in the concentration of DNA produced. The SDS method produced the highest DNA concentration compared to the other two extraction methods (commercial kit and CTAB) (Table 2). Each DNA extraction method, commercial kit, CTAB, or SDS, resulted in the highest concentration of nematode DNA in the amount of one extracted nematode and the lowest concentration yield per individual was found when using 10 extracted nematodes (Table 3).

DNA amplification

All extraction methods at all stadia and variations in the number of nematodes used were able to amplify well at 370 bp as targeted even though there were some thin bands (SF1) and smears (CJ1, CJ5, CJ10, CF1, CF5, and CF10) (Fig. 1).

Sequencing and molecular phylogenetic analysis

The results of DNA extraction using the commercial kit method with one second-stage juvenile nematode had the lowest number of bases (Table 4) and obtained bases with poor amplification results, indicated by the presence of red bars of 46 and 36 bases, yellow bars of 27 and 23 bases, and HiSQV values of 275 and 285 on the sequencing results using forward (Mg-F3) and reverse (Mg-R2) primers. The CTAB and SDS methods had the highest number of bases compared to the commercial kit method.

Figure 2 revealed the phylogenetic tree of the *M. graminicola* sample (from Sewon, Ngemplak, and Berbah) with several *M. graminicola* in other regions as well as several species from other *Meloidogyne* genera. *M. graminicola* sampled from Sewon and Berbah had a closer relationship than *M. graminicola* from Ngemplak.

Discussion

DNA extraction is an important process in molecular biology and a fundamental step for initiating other steps such as sequencing, amplification, hybridization, ligation, cloning, and biodetection. Previous researchers have studied DNA extraction for potential applications, e.g., disease diagnosis, pathogen detection, and gene therapy (Min *et al*. 2014). The yield and quality of genomic DNA (gDNA) are the determinants before sequencing in the identification process of plant parasitic nematodes. On the other hand, extraction of nematode DNA is not easy because of the thick nematode cuticle layer (460 nm) and is resistant to chemical, enzymatic, and mild physical disturbances (Seesao *et al.* 2014).

The method of extracting the DNA of the nematode *M. graminicola* in this study was initiated by freezing the isolated nematode suspension in a 1.5 ml Eppendorf tube. The freezing process was carried out using an additional 50 µl dH2O. The function of the freezing process in this early stage was to facilitate the grinding

Table 4. Quality value (QV) analysis of sequencing results by three extraction methods with different nematode stages

Extraction methods	Stage	Primer	Number of amplified base pairs				Total base amplified
			red bar	yellow bar	blue bar	HiSQV	
CTAB	juvenile	Mg-F3	26	12	306	306	344
		Mg-R2	19	5	316	316	340
	female	Mg-F3	26	4	313	313	343
		Mg-R2	21	18	301	301	340
SDS	juvenile	Mg-F3	26	11	307	309	344
		Mg-R2	21	8	312	312	341
	female	Mg-F3	26	16	297	299	339
		Mg-R2	40	28	272	274	340
Commercial Kit	juvenile	Mg-F3	46	27	271	275	344
		Mg-R2	36	23	284	285	343
	female	Mg-F3	22	14	306	306	342
		Mg-R2	38	7	297	298	342

Note: juvenile – second-stage juvenile

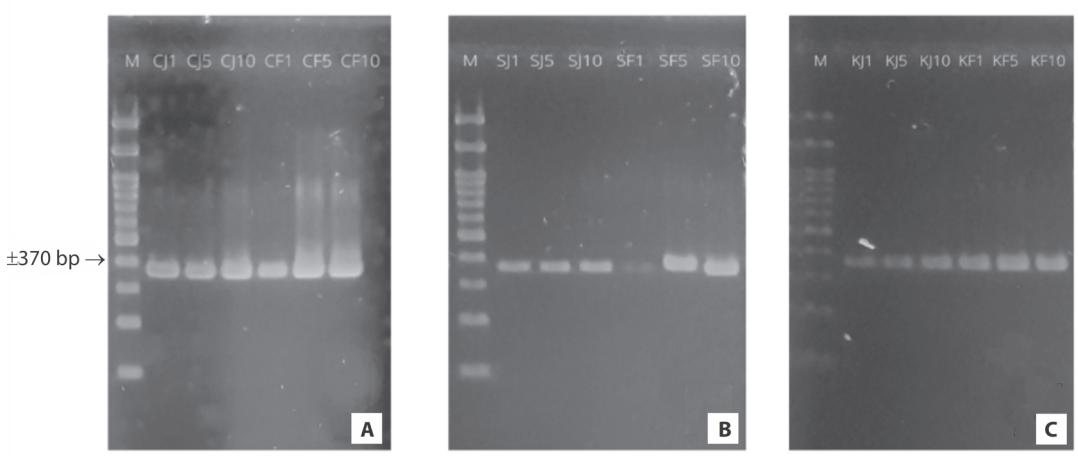

Fig. 1. Visualization of PCR results using specific primers Mg-F3 and Mg-R2 on the treatments of three extraction methods: CTAB – A, SDS – B, and commercial kit – C, the nematode stage, and the number of Meloidogyne graminicola. M – marker 100 kb; C – CTAB; S – SDS; K – commercial kit; J – second-stage juvenile; F – female; 1, 5, and 10 – the number of nematodes

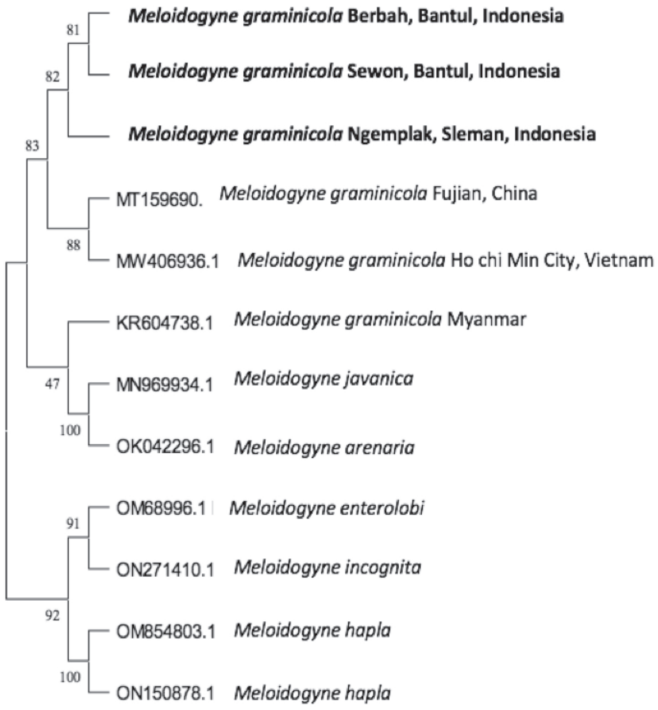

Fig. 2. Maximum likelihood phylogenetic tree of *Meloidogyne graminicola* from Sewon, Ngemplak, and Berbah (in bold) with other *M. graminicola* and other species

process of nematodes since grinding the frozen suspension more easily destroys the nematode body and can facilitate the process of removing DNA from the nematode body cells. Based on the research that has been done by Xin *et al.* (2021) it has been stated that physical treatment such as freezing can increase efficiency in the cell lysis process in DNA extraction and can increase DNA concentration compared to chemical treatment.

Concentration results of this research were consistent with the research by Chen *et al.* (2010) who showed that the SDS and CTAB methods, which use buffers prepared in the laboratory, result in a higher level of DNA yield than commercial kits. Trzewik *et al.* (2016) also obtained higher quality and quantity of DNA that was suitable for conventional PCR and real-time PCR amplification with the CTAB based extraction method than the commercial kit method. However, in terms of the time needed for the extraction of nematode DNA, the commercial kit requires less time (approximately 1.5–2 hours per extraction process) than CTAB and SDS methods.

The low level of DNA purity might be caused by the high level of measurable impurities such as proteins that are not completely degraded. Contamination caused by protein might come from cell components that were not lysed during the isolation process or from phenol, as an ingredient added to the isolation process to precipitate DNA. Additionally, the low level of purity might also be caused by the presence of other impurity components, e.g., RNA, lipids, and polysaccharides (Alaey *et al*. 2005).

The yield of DNA extraction is influenced by many factors such as species, tissue, preservation method, extraction procedure, and deposition method (Chen *et al*. 2010). The highest DNA concentration is produced by the SDS method which could be because the SDS method employs Proteinase K and β-mercaptoethanol in a 1% SDS lysis buffer. Proteinase K alone is commonly used for DNA extraction research with different methods. Smaha *et al*. (2019) also used Proteinase K for their DNA extraction process. According to Braun-Kiewnick and Kiewnick (2018), lysis buffer containing Proteinase K and/or β-mercaptoethanol improves DNA extraction because these two materials allow protein degradation and cell wall building. In the DNA extraction process, SDS is an anionic detergent to lyse cells and nuclei to release RNA and DNA. In addition, enzymes such as nuclease, ribonuclease (RNase) and deoxyribonuclease (DNase) activity will also be inhibited by SDS (Farrell 2010). In the DNA extraction process, the commercial kit method uses only Proteinase K and the CTAB method uses only β-mercaptoethanol. High concentrations of CTAB were also used to disrupt cell and nuclear membranes to expose genetic components (Amani *et al*. 2011). In this method, the CTAB buffer also contained 2-β-mercaptoethanol which successfully removed the polyphenols (Aboul-Ftooh *et al*. 2019).

The more nematodes extracted, the lower the concentration of DNA produced (Table 2). Because of the large number of nematodes required in the extraction of nematode DNA it might fail the extraction process. The more nematodes which are extracted, could mean that the nematodes have not been extracted completely so that the concentration of DNA produced is not optimal. Whereas using one nematode, the cell lysis process and DNA extraction can run optimally. According to Natarajan *et al*. (2016), the high concentration of DNA obtained from the CTAB and SDS DNA extraction methods might be caused by the presence of an extraction buffer containing more anionic and cationic surfactants, which allowed the isolation of the adsorbed DNA. Although the quantity of DNA produced from the extraction method using a commercial kit was less than the other two DNA extraction methods (CTAB and SDS), the commercial kit method can be carried out in a short amount of time as it only takes 1.5–2 hours to complete one extraction process.

The DNA extraction process using the CTAB and SDS methods takes a long time since these methods require a longer time for the DNA precipitation process (8–24 hours) than the commercial kit. The advantages of the SDS and CTAB methods from this study are that they are cheaper and can produce high DNA concentrations. Regardless, the CTAB and SDS methods require more time to carry out a long extraction procedure, particularly in the DNA precipitation time. Besides, the CTAB and SDS methods require more effort because the reagent solutions used in this extraction method must be prepared first. The commercial kit method is faster and simpler than the other extraction methods since all the ingredients used for extraction are already available in a set of DNA extraction kits. Yet, because of this convenience, the commercial kit costs more. The extraction method used depends on the objective use of the DNA results and also on the target nematode species (Braun-Kiewnick and Kiewnick 2018).

PCR primers can be quite specific and sensitive (one female or juvenile provides a sufficient amount of DNA for analysis). The only important prerequisite is to have a sufficient number of PCR cycles. This could be due to the small amount of target DNA in each sample. All tested source materials contained target DNA (isolated female, roots, soil) and different DNA extraction methods, including simplified protocols, which could be used for detection (Tesarova *et al*. 2003). Suparningtyas *et al*. (2018) stated that the smear formed in the isolation process indicated that there had been degradation or fragmentation of genomic DNA during the isolation process. Although the value of the DNA concentration produced by the commercial kit extraction method for both second-stage juveniles and females had the lowest value, its results could be used for PCR purposes as indicated by the band formed on the electrophoresis results.

To investigate the quality of the data obtained from the electropherogram the quality value (QV) is observed. QV is a basic estimate of base caller accuracy. Obtaining the QV of the sequencing data can be done by observing the bars formed from the sequencing data (Travers *et al.* 2010). The colored bars resulting from the visualization of the electropherogram indicate the level of confidence in the base call. The height of the bar is relatively proportional to the score. The blue bar indicates the QV prediction error rate of 20 or 1% for the base call at that position. The yellow and red bars represent QV 20 (SenGupta and Cookson 2010). Accordingly, blue bars indicate a good result. The blue color has a high-quality pure base that is formed at least 99% pure, thus the nitrogen base is read correctly. Yellow and red bars (worse than yellow) mean the invalidation of the base calling algorithm.

Bases with these values can still be read correctly. Good quality sequencing data is characterized by well-defined peak resolution and poor resolution of the first 10–25 bases, yet still acceptable (Eurofins Genomics, 2019). Poor resolution of bases can be caused by primer excess during the PCR cycle. Currently there are many methods to reduce noise due to primer carry over. This method assumes that the excess primer is present in free single-stranded form and can be easily purified or digested away from double-stranded PCR products and substantially higher in molecular weight. However, primers have the potential to form intermolecular and intramolecular structures, depending on the primer sequence and salt concentration, which can significantly affect the ability to separate primers from PCR products using this method (SenGupta and Cookson 2010). Additionally, most sequencing methods require adding an adapter sequence to the end of the DNA fragment, and there are many different strategies for adding it. For double-stranded DNA sequencing, adapters can be added either by DNA fragmentation followed by ligation or by the introduction of transposon-based adapters (Enroth *et al*. 2019).

In summary, the three DNA extraction methods, namely, CTAB, SDS, and commercial kits at each stage (second stage juvenile and female) could be used to determine nematode genomics based on PCR and Sanger sequencing, using only one nematode. The disadvantage of both SDS and CTAB methods is that they take a long time (for DNA precipitation), but both methods have the advantage of producing high concentrations and requiring more affordable costs. While the advantages of the extraction method using the commercial kit are that it requires a relatively short time for extraction, it has the disadvantage of low DNA concentration. In this regard, further research can be carried out considering that there are still many DNA extraction methods that have not been carried out in this study.

Conclusions

The commercial kit, CTAB, and SDS methods can be used for the extraction of root-knot nematode DNA. All of the DNA extraction methods from *M. graminicola* for PCR and Sanger sequencing based genomic analysis can be started with freezing treatment. If it takes a short time to get the extraction results, it can be done using DNA extraction using a commercial kit, but if there are limited costs and available time, DNA extraction using the CTAB and SDS methods can also be done.

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