Journal of Biotechnology Research
ISSN(e): 2413-3256, ISSN(p): 2413-8878
Vol. 11, Issue. 3, pp: 16-23, 2025
URL: https://arpgweb.com/journal/journal/16
DOI: https://doi.org/10.32861/jbr.113.16.23

Academic Research Publishing Group

Original Research **Open Access**

Comparative Analysis of DNA Extraction Protocols for Molecular Study of Fall Armyworm

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Article History
Received: 25 May, 2025
Revised: 22 June, 2025
Accepted: 11 July, 2025
Published: 30 July, 2025

Abstract

Fall armyworm (FAW) is a polyphagous and ravenous pest, destroying maize plants world over. It was first observed in Nigeria in the year 2016 and since has spread and engulfed the farming system in the continent of Africa. Several species have begun to spring up with very similar destructive pattern and this necessitates more objective molecular characterization and genomic research works toward integrated pest management practices. Many research works have proposed different DNA extraction methods that could provide quality DNA for molecular downstream analysis. This study objectively compared and modified four conventional protocols that are cost effective, rapid and yield good quality DNA for further studies. The CTAB (Cetyl Trimethyl Ammonium Bromide) method, CTAB-PVP (Cetyl Trimethyl Ammonium Bromide-Polyvinyl pyrolidone - PVP) method, SDS (Sodium Dodecyl Sulfate) method and Urea method. The result of the Analysis revealed significant variation in DNA purity at 1% ($p<0.01$) level with values ranging from 1.83 ± 0.2154 (Urea) to 2.05 ± 0.2123 (SDS). Also, DNA yield or concentration among the protocols at 1% ($p<0.01$) level with values ranging from 784.77 ± 388.80 (PVP) to 2854.08 ± 1274.87 (CTAB). This indicates that CTAB has the best yield while PVP has the least yield. We concentrated on the CTAB yield and beefed up the washing method for higher quality. Mitochondria Cytochrome oxidase 1 (mtCO1) region was used to amplify the DNA and the product sequenced. Hence, the CTAB protocol yielded the highest DNA suitable for downstream analysis.

Keywords: Fall armyworm; DNA extraction protocol; CTAB; Cytochrome oxidase sequences.

1. Introduction

Fall armyworm (FAW) or *Spodoptera frugiperda* (Noctuidae: Lepidoptera) is an important pest that ravages maize plants, thereby causing a significant yield loss annually across countries [1]. It was formerly discovered in the Western Hemisphere where it is a major pest of corn and many other that belong to the grass family (Poaceae) [2]. The FAW was discovered in West Africa in 2016 but rapidly spread across and beyond the continent, to eastern and southern Africa, India and southeastern Asia [3-6]. The pest was also reported by FAO where opined that from the year of emergence in 2016 it has continued to spread from one season to another, one country to another and from

continent to another continent [7, 8]. The disruption of agricultural practice in Africa has a significant effect on the people because the continent largely depends on it for food and industrial raw material production. Apart from the anthropogenic factors, occasioned by upsurge in population, there are several arrays of natural phenomena responsible for inadequacy of food production. Hence, the contribution of agricultural sectors to food security and poverty reduction is hampered by interaction of biotic and abiotic factors in the sub-region [6, 9]. The region is faced with the challenge of feeding its rapidly growing populations and the outbreak and spread of FAW is a major setback in sub-Sahara Africa (SSA) for causing huge damage to maize crops which is the major staple food for more than 300 million farmers in Africa [10, 11]. It was reported by Rwomushana, *et al.* [12] that up-to-date appraisals from about 12 African countries submitted an annual loss of 4.1 to a massive 17.7 million tons of maize due to FAW. Farm-level estimates from Ghana and Zambia suggested a yield loss of 22–67 per cent [13], 47 per cent in Kenya [14] and 9.4 per cent in Zimbabwe [15] due to FAW invasion. Apparently, if appropriate, effective and perhaps an integrated control strategies are not implemented, FAW will continue to occasion enormous destruction to maize and exacerbate the already dangerous food security and livelihood conditions of millions of smallholder farmers across the countries of SSA. The Food and Agriculture Organisation (FAO) has stated that fall armyworm is estimated to cause Africa nearly $10 billion in annual maize yield losses. The Director General of FAO, Mr. Qu Dongyu, revealed this at the 5th Steering Committee Meeting on Global Action for Fall Armyworm Control in Rome. He further stressed that over 70 countries in Africa, Asia and the Pacific, and the Near East are reporting infestations of the pest, warning that FAW knows no boundaries as it accelerates its march across the planet [16].

The management and control of FAW therefore becomes a regular event in order to rescue major part of the farm if not all, towards achieving a worthwhile harvest at the end of the season. It becomes imperative therefore for researchers to focus scientific binocular on the dynamics of FAW resurgence with effort to studying modifications of their genome towards evolution in their attempt to evade the familiar chemical previously applied [17].

Several species have begun to spring up with very similar destructive pattern and this necessitates more objective molecular characterization and genomic research works toward integrated pest management practices [18]. However, research funding on many occasions are elusive to researchers in the continent of Africa to embark on long voyage of fruitful research [16]. The researchers have continued to improvise in order to maximize the little resources available. A good example is testing of many extraction protocols for quality DNA useful for various downstream analyses. Over the years, such have been employed not only on insect pest but other living organisms [19]. Many extraction protocols have been designed for insects and reported to be good and offer similar results to those that were industrially optimized [20]. Few conventional protocols have even been optimized to give good results for next generation sequencing analysis [20-22] It's on this milieu that this work was designed to optimize few and determine the best conventional DNA extraction protocol useful for large sample number of insect collection for various molecular analysis.

2. Material and Methods

FAW larvae were collected from maize fields within and outside the National Cereal Institute (NCRI) compound Ibadan with hand gloves and/or forceps into small containers and brought alive into the molecular laboratory of NACGRAB. The FAW larvae between 3rd and 4th instars were selected and transferred into well-labelled phials containing 99% ethanol prior to laboratory analysis. Four different conventional DNA extraction protocols were used for genomic studies of the insects. Several samples were collected, preserved in ethanol but only thirty (30) samples were used for each protocol, the total larvae used was one hundred and twenty (120). Nanodrop spectrophotometer 2000 model was used to check the quality and quantity of the extracted DNA. The quality and quantity of DNA recovered were compared and analyzed with statistical method to determine the best approach and that could be recommended for future genomic research works.

2.1. CTAB (Cetyl Trimethyl Ammonium Bromide) METHOD

Larva of either 3rd or 4th instar (100 to 120) mg was homogenized in 750 µL of extraction buffer (2% CTAB, 1.4 M NaCl, 20mM EDTA, 100 mM TRIS-HC1 pH 8, and 0.2% B-mercapto-ethanol). Proteinase K (10 mg/mL) (2 µL per mL of extraction buffer) was added to the homogenate and the mixture incubated at 65 °C for 30 min and the tubes inverted several times. Another 750 µL of chloroform: isoamyl alcohol (24:1) was added when cooled to room temperature and the mixture centrifuged at 12,000 rpm for 5 min to separate the phases. The aqueous phase was transferred into a 1.5 mL tube and the chloroform: isoamyl alcohol step was repeated. The supernatant was pipetted and DNA was precipitated by adding 500 µL of ice-cold isopropanol to the aqueous phase in another 1.5 mL tube and incubated at -20 °C overnight. The precipitated DNA was centrifuged at 12,000 rpm for 5 min to collect the pellet after which the isopropanol was decanted and the DNA pellet washed with 500 µL 80% ethanol. The ethanol was decanted after centrifugation at 10,000 rpm for 5 min and the washing stage repeated three more times. The pellet was afterwards air-dried and suspended in 50 µL of nuclease free, molecular grade water [23].

2.2. Ctab-Pvp (Pvp) Method

DNA was extracted using CTAB-PVP (Cetyl Trimethyl Ammonium Bromide-Polyvinyl pyrolidone). Larvae that weighs 120mg was cut out (3rd instar) and grinded with pre-warmed 200µL CTAB-PVP buffer (20mM EDTA at pH 8, 100 mM Tris-HCl at pH 7.5, 1.4M NaCl, 2% w/v CTAB and 4% PVP) in a mortar and pestle that was pre-heated at 60 °C. Then, the slurry was transferred into labeled 1.5mL Eppendorf tube and 0.5mL of CTAB-PVP buffer was used to rinse the mortar and pestle into the 1.5mL tube. The mixture was incubated with occasional

mixing for 30 minutes at 60 °C. Afterwards, the mixture was allowed to cool at room temperature and 2µL RNase A was added and incubated for another 15 minutes at 37°C. After incubation, an equal volume of chloroform- isoamyl alcohol (ratio 24:1) was added. It was then mixed by inversion and centrifuged at 12,000 rpm for 8 minutes. The supernatant was pipetted into a freshly labeled 1.5 mL tube and the chloroform- isoamyl treatment was repeated. DNA was precipitated by adding 2 times volume of ice-cold absolute ethanol to the supernatant. The mixture was incubated at -40°C for 1 hour to precipitate the DNA. The precipitate was centrifuged at 12,000 rpm for 8 minutes, washed trice with 70% ethanol and centrifuged at 10,000rpm for 2 minutes each time of washing. The DNA pellet was air-dried and eluted with 50µL sterile water before storing in -40°C freezer [20].

2.3. SDS (Sodium Dodecyl Sulfate) - METHOD

The 3rd Instar larva of FAW was homogenized with 200 µL DNA extraction buffer (100 mM NaCl, 10 mM Tris-HCl, 50 mM EDTA, 0.5% SDS, 0.2% β-mercapto-ethanol, pH 8.0). The homogenate was transferred into a sterile 1.5 mL centrifuge tube. The mortar and pestle were rinsed with another 500 µL DNA extraction buffer and added to the slurry in the tube. Proteinase K (20 mg·mL-1) of 5 µL was pipetted into the tube, vortexed briefly, incubated at 65 ºC for 40 min, with occasional shaking during the incubation. The tube centrifuged for 5 min at 12,000 rpm and the supernatant pipetted to a new 1.5 mL centrifuge tube. About 400 µL of 5 M ice-cold NaCl was added and thoroughly mixed, then centrifuged for 5 min at 12,000 rpm. The supernatant pipetted to a new 1.5 mL centrifuge tube, equal volume of ice-cold isopropanol was added and mixed gently to homogenize for DNA pellet precipitation. The tubes were incubated/placed in the freezer at -40 ºC for 30 min. Tubes centrifuged for 5 min at 12,000 rpm. And the supernatant discarded. The DNA pellet was washed with ice-cold 75% ethanol, Centrifuge for 5 min at 10,000 rpm, the supernatant decanted and the washing step repeated three more times. After washing, the DNA pellet was air-dried for about three hours and 50 µL ultrapure water was added to dissolve/elute the DNA [21].

2.4. Urea Method

The 3rd Instar larva of FAW was suspended in 100 µl of TESU6 buffer (10mM Tris-HCL pH 8.0, 20 mM EDTA pH 8.0, 2% SDS, 6M Urea and 25 µg/ml proteinase K) and homogenized, mixed by vortex and then incubated at 55^0C in a shaking incubator with oscillation of 200 rpm for 15 min. Certain volume, 10 µl of 5 M NaCl was added and mixed gently by inversion after which equal volume of Phenol:Chlorophorm:Isoamyl Alcohol (25:24:1) was added and mixed by inversion. The mixture was centrifuged at 12,000 g for 5 minutes and the supernatant was pipetted out. Equal volume of ice-cold isopropanol was added and gently inverted several times and kept at -20^0C overnight for DNA precipitation. The tubes were centrifuged at 12,000 rpm for 5 minutes and the supernatant decanted. The DNA pellet collected was washed with 70% ice-cold alcohol, centrifuged at 10,000 rpm for 2 mins. The upper phase (alcohol) was decanted and the washing was repeated two more times. The DNA pellet was drained further by centrifugation and decantation and the pellet was air-dried for about three hours. The DNA pellet was re- suspended in 50 µl of nuclease free water to dissolve them and the DNA stored in the -40°C until use [19].

2.5. PCR Amplification and Sequencing of Cytochrome Oxidase gene on extracted DNA

The DNA extracted was further tested with gene amplification to determine their suitability for downstream analysis. The gene used for polymerase chain reaction (PCR) was Cytochrome oxidase 1 (CO1), from mitochondrial region. The primer pairs include: JM76 F GAGCTGAATTAGGAACTCCAGG JM77R ATCACCTCCACCTGCAGGATC [22] with molecular weight of 569 bp. The amplification condition includes the following: initial denaturation for 3 minutes at 95°C; 40 cycles of denaturation for 15 seconds at 95°C; annealing for 20 seconds at 56 °C and elongation for 25 seconds at 72 °C; followed by a final extension step of 10 minutes at 72°C using Eppendorf Mastercycler nexus gradient. The amplicons were separated on 1% w/v agarose gel using 1 x TBE (Tris- Borate- EDTA) and Safeview™ Classic nucleic acid stain (Canada). The product of amplification was sequenced and the sequences analyzed. The bioinformatics software employed includes; Bioedit, MEGA and NCBI database.

3. Results

The data result generated from Nanodrop spectrophotometer were subjected to statistical analysis and the following results were recovered.

Table-1. The ANOVA result showing significant difference in the concentration and purity of the extracted DNA among the extraction protocols used

		Sum of Squares	df	Mean Square	F	Sig.
DNA Purity	Between Groups	.993	3	.331	7.182	.000**
	Within Groups	5.346	116	.046		
	Total	6.339	119			
DNA Conc.	Between Groups	66996759.620	3	22332253.207	22.151	.000**
	Within Groups	116948474.747	116	1008176.506		
	Total	183945234.367	119			

**: Significant at 1% (p<0.01) level

The purity or quality of DNA extracted by the four extraction methods used were significantly different from each other as seen in Table 1.

Table-2. Mean separations result comparing the concentration and purity of the extracted DNA among the 4 extraction protocols

Variables	CTAB	PVP	SDS	Urea
DNA purity	1.99 ± 0.2907^{a}	1.87 ± 0.0915^{b}	2.05 ± 0.2123^{a}	1.83 ± 0.2154^{b}
DNA yield	2854.08 ± 1274.87^{a}	784.77 ± 388.80^{c}	1797.57 ± 948.49^{b}	2162.14 ± 1164.74^{b}

Values represent mean ± standard deviation. Values along the same row with different superscripts are significantly different at 1% (P<0.01) level.

Table 2. is the result of the Analysis of Variance (ANOVA) showing variation in DNA purity and DNA yield among the four different protocols. The result reveals significant variation in DNA purity among the protocols at 1% ($p<0.01$) level with values ranging from 1.83 ± 0.2154 (Urea) to 2.05 ± 0.2123 (SDS). This shows that urea protocol has the best purity while SDS has the least purity. Similarly, the result reveals significant variation in DNA yield among the protocols at 1% ($p<0.01$) level with values ranging from 784.77 ± 388.80 (PVP) to 2854.08 ± 1274.87 (CTAB). This indicates that CTAB has the best yield while PVP has the least yield.

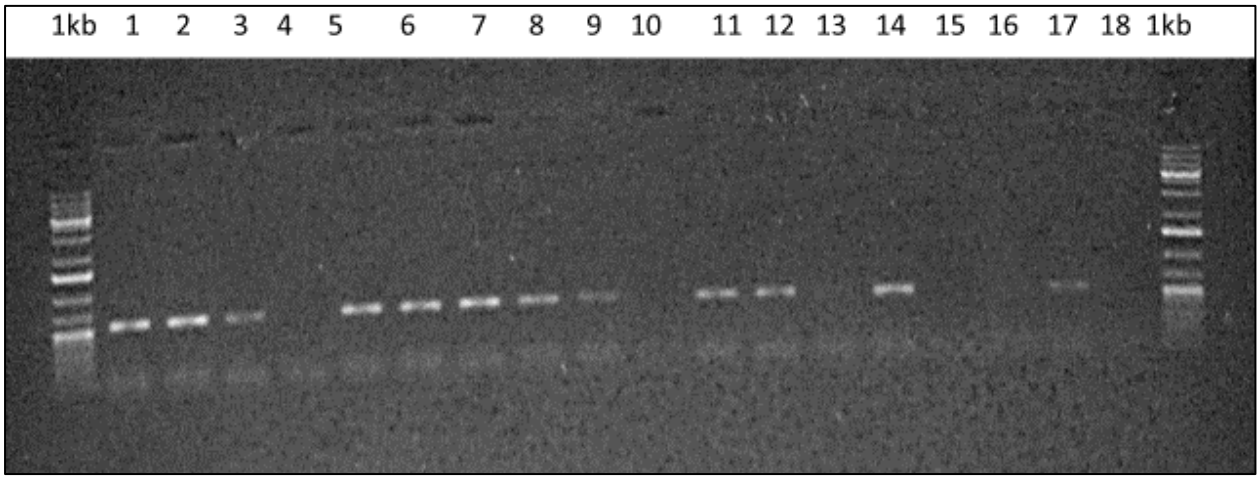

Figure-1. Gel electrophoresis image of CO1 gene amplification for FAW strains in with 18 samples

The sequences analyzed were able to fully identify *Spodoptera frugiperda* from the genebank. They were consequently submitted with the accession numbers: MW807332_Strain NGOY5, MW807337_Strain NGOY3, MW807341_Strain NGOY2 and MW807342_Strain NGOY9.

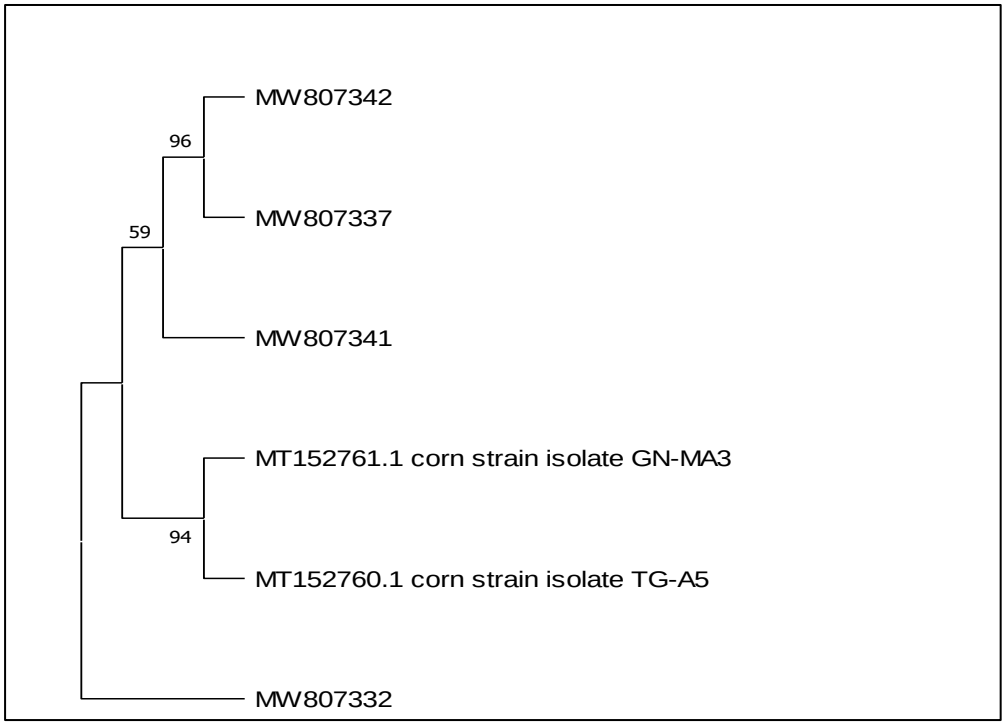

Figure-2. Phylogenetic tree of four strains of Fall Armyworm sequenced with Cytochrome Oxidase 1 gene using the DNA recovered from CTAB extraction protocol

5. Discussion

The results obtained from the four (4) DNA extraction protocol were good and produced a range of results acceptable for further analysis. It is good to note that majority of these extraction reagents are actually required to disrupt the cellular structure, coagulate or precipitate them and wash the exposed nuclear materials in the innermost part of the cells, but the concentration varied across each protocol. Essentially, high concentrations of inert salt removes polysaccharides; Polyvinyl-pyrrolidone (PVP) and β-mercaptoethanol takes care of phenolic compounds while the chloroform-isoamyl alcohol treatment with RNase treatment ensures DNA is free of protein and RNA contaminants [24, 25]. Oftentimes, the component at very high concentration carries the name of the protocol. However, these have significant effects on the purification of cellular inclusions based on the variation in their locations, densities and nature among others. According to the result recovered, the DNA concentration/quantity was least in PVP (388.80 ng/µL) lowest limit and highest in CTAB (2,854.08 ng/µL). The purity or quality of the extracted DNA is equally good with values ranging from 1.83 ± 0.2154 in Urea, to 2.05 ± 0.2123 in SDS. We recall that the DNA standard purity value is 1.80 [19], Urea protocol produced the closet to the standard value. Having this information, we proceeded with CTAB protocol for downstream analysis with little modification/improvement of further cleaning to achieve higher purity. Other researchers have also reported the preference they had for the CTAB protocol especially for polyphagous (such as the FAW), xylophagous insects and subsequently employed them in their insect pest molecular studies [26-28]. In another study, Ogunkanmi and others reported the superiority of CTAB protocol over other four protocols with respect to DNA yield, purity, availability and cost of reagents, time required for extraction among others [29]. Apparently, the product of amplification was good as we have in Figure 1. Furthermore, mitochondrial cytochrome oxidase I (mtCOI) region has been reported by various researchers to be a standard barcode to identify the diverse array of insect groups [25]. As long as the DNA purity and quantity which is fundamental is achieved, insect identification and their DNA barcoding is a mission-accomplished task. The amplified region of the insects was sequenced with the barcode gene and their sequences submitted into the genebank with their accession numbers as earlier reported (MW807332_Strain NGOY5, MW807337_Strain NGOY3, MW807341_Strain NGOY2 and MW807342_Strain NGOY9). Figure 2.shows the phylogenetic tree constructed with other two strains from the genebank. The two genebank samples formed a monophyletic group, flanked by Nigeria samples. This shows that they are closely related strains within the same ecosystem or continent of Africa [30].

In addition to these, the collection of larvae at their early years of development; between 1st and 3rd instars is prone to error of admixture and misidentification [31]. Singh and others have reported that the cytochrome oxidase gene are invaluable molecular markers, for the identification of ambiguous, cryptic and forensically relevant species. Not only that, there are convincing similarities and congruence between the phylogenetic relationship constructed by the morphological data of the insects and those of their cytochrome oxidase gene [32]. This corroborative evidences gave cytochrome oxidase a higher popularity among other useful genes [33, 34]. There were larvae of stem borers attacking the same stand of corn as well as the larvae of another species of the same genus of armyworm known as African Armyworm (AAW. *Spodoptera exempta*) and possibility of a newly reported variety of Southern armyworm (SAW), *Spodoptera eridania* found in many Asian countries [18] but the beauty of mtCO1 gene to unravel and demystify the ambiguity is second to none [1, 22, 35]. In this study, several look-alike larvae were encountered on the field but following a designed field collection protocol and authentication via molecular/DNA barcoding method, mtCO1 gene is an effective and reliable molecular procedure for authentication of field study towards complete identification [36].

6. Conclusion

In conclusion, there are many methods that researchers have employed and will still employ but the CTAB DNA extraction protocol as described in this study is efficient for quality FAW DNA isolation, suitable for downstream analysis.

Declarations:

Ethical Approval

Not applicable: No approval is required on right to control insect pests, especially if its negative value to humans is sufficiently high in relation to its ecological value as we have in the case of Fall Armyworm.

Authors' Contributions

A. - designed the project, carried out PCR analysis, sequencing, analyzed the results and wrote up the manuscript

A. B.- made the insect collections on the field

E. F. - Carried out PCR analysis and took part in writing of the manuscript

A. B.C. G - carried out the Literature review, DNA Isolation and took part in the write-up

D. H. I. - Carried out the gel electrophoresis

Data Availability Statement No Data associated in the manuscript

Financial interests

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Competing Interests

I declare that the authors have no competing interests or other interests that might be perceived to influence the results and/or discussion reported in this paper There is not conflict of interest among the authors before, during and after the preparation of the manuscript.

Dual Publication

The results/data/figures in this manuscript have not been published elsewhere, nor are they under consideration (from you or one of your Contributing Authors) by another publisher.

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