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Ten (10) M Ammonium acetate is an efficient molecular concentration for the extraction of genomic DNA from small insects used for rolling circle amplification

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Most of the published methods for DNA isolation from vectors (insects) are not always effective in mealy-bugs, aphids and leafhoppers because they have high amount of polyphenols in their body systems. A simple, rapid, reliable labor-effective and carcinogenic compound-free salting out method for isolation of high molecular DNA from these insects is described. The ratio of OD_{260}/OD_{280} ranged between 1.8 to 2.0. The method was successfully applied to Rolling Circle Amplification and in restriction analysis, indicating the removal of the common inhibitors.

Key words: *Pseudococcidae*, *Aphididae*, *Auchenorryncha*, DNA extraction, rolling circle amplification, restriction analysis.

INTRODUCTION

Insects species that directly feed on plants play important functional roles in agricultural ecosystems, as they contribute to nutrient recycling. Moreover, most species can be serious pests and vectors of crop diseases, having economically important consequences for the agricultural industries. Due to their ecological and economical importance, research on systematic, phylogenetics, ecological genetics and molecular methods for detection and control of vector transmitted diseases of insect species are needed. Nonetheless, the isolation of high quality DNA for molecular studies and diagnosis of the disease from the vectors has been hindered by lack of simple, efficient and cheap molecular tools for DNA isolation. A fundamental requirement for molecular biology is the rapid DNA isolation and amplification of the specific DNA sequences. Typically, many DNA isolation protocols from the class insecta have been developed but none of the protocols can be used to extract the DNA from two or more species separately (Philips and Simon, 1995; Chen and Ronald, 1999; Starks and Peters, 2002; Strauss and Zangerl, 2002; Calderon et al., 2010). These protocols differ in the extraction buffer components and the pH of the buffer. Most of them use proteinase K digestion and chelex extraction especially while dealing with fresh specimens, but give poor quality DNA extracts (Strauss and Zangerl, 2002) due to phenolics and tannins in the insects' gut.

Phenolics are the major contaminants in DNA preparations from plant pests (Couch and Fritz, 1990; Lodhi et al., 1994; Kim et al., 1997). Phenolics, as powerful oxidizing agents, can reduce the yield and purity of DNA; inhibiting enzymatic modifications of the DNA such as

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restriction endonuclease digestion and poly-merase chain reaction (PCR) (Lodhi et al., 1994; Horne et al., 2004; Friar, 2005; Padmalatha and Prasad, 2006; Arif et al., 2010). Other additives such as polyvinylpyrroli-done (PVP) (Deobagkar, 1982; Strauss and Zangerl, 2002) and citrate have been used as antioxidant in the extraction buffer to inhibit oxidases in mealy-bugs (Aljanabi and Martinez, 1997). High concentration of ammonium acetate inhibits competitively the covalent binding of the phenolic compounds to the isolated nucleic acids through the formation of ionic bond. More so, the salt concentration enhanced the cell lysis due to the crenation effects. However, salting out protocol (Wahl, 1984) has been used to isolate DNA from small insects (aphids and whiteflies), but the salting agent was not included in the extraction buffer. This makes the protocol to be long and time consuming and the nucleic acids gets degraded by nucleases. In addition, most of the des-cribed methods are laborious, costly and are not amenable to isolation of high quality DNA. Most of the known DNA extraction buffers used in insect and other animal tissues does not employ the crenation effect to lyse the cells and nuclear membranes. Most of the isola-tion procedures usually involve marceration of the insect tissue in a boiling potassium hydroxide (KOH) solution (Rose et al., 1994; Knolke et al., 2005). These methods sclerotise structures of the abdomen and genitalia while soft tissues are discarded during preparation leading to a loss of a considerable amount of DNA. In addition, the high temperatures used during the boiling process in KOH can lead to the nicking of the DNA.

DNA extraction is invasive and causes damage to the specimens; although, efforts have been made to minimize the destruction by using small portions of the insects in other species (Mitchell et al., 1997; De Verno et al., 1998; Shneider et al., 1999; Cruickshank, 2002). Nondestructive DNA extraction methods have been reported (Philips and Simon, 1995; Rung et al., 2009). However, the method described by the Rung et al. (2009), is destructtive since the specimens are cut into half, whereas that of Philips and Simon (1995) can be considered semi-destructive since the abdomen is perforated several times with insect pins and submerged in the fluid to wash them. In case of the mealy-bug, aphids and leafhoppers, these protocols may not apply since the insects are very small in size especially where instars DNA is required. Several DNA isolation commercial kits such as DNeasy tissue kit (Qiagen Inc., Valencia, CA, USA), a FastDNA kit (Qbiogene, Inc., Carlsbad, CA, USA) are being used to extract DNA from leafhoppers, aphids and mealybugs (Zidani et al., 2005). These commercial kits are expensive and tend to produce low DNA yields with short storage life from tissues rich in polyphenols (Lodhi et al., 1994; Fire and Xu, 1995; Calderon et al., 2010) imposing barriers to diagnose disease caused by insect vectors. In most cases, PCR is used to amplify defined sequences but the fidelity of the Taq DNA polymerase is low and the technique is limited to amplification of short (20 to 30 bp) DNA segments (Lodhi et al., 1994). The use of PCR to detect disease causative agents in insect vectors has been complicated by the lack of quick and easy DNA extraction protocols and by the inhibition of PCR by components in insect extracts (Lodhi et al., 1994; Fire and Xu, 1995).

Linear rolling circle amplification is the prolonged extension of the oligonucleotide primer annealed to the circular template DNA (Inoue-Nagata et al., 2004; James et al., 2011). The rolling circle amplification has the advantage of not requiring a thermal cycling instrument. The technique has been used in assaying banana streak virus (BSV) (James et al., 2011) and begomovirus in plants (Reagin et al., 2003). In this work, 'rolling circle amplification' was used to amplify the isolated DNA since the *Phi29* DNA polymerase is known to be more sensitive to the inhibitory compounds in the DNA samples compared to *Taq* DNA polymerase (Liu et al., 1996; Reagin et al., 2003; Inoue-Nagata et al., 2004; James et al., 2011).

We evaluated and compared the effectiveness of the developed method and Michele et al. (2002) method for isolation of DNA from three different insect species belonging to the order Hemipteran. The protocol circumvented the deleterious carcinogenic chemicals such as phenol and chloroform compounds that may contaminate the DNA from the procedure. In this study, we developed an inexpensive and rapid DNA isolation method by incorporating 10M ammonium acetate in the extraction buffer [sodium chlorides-tris- EDTA (STE)]. We evaluated the quality of the DNA isolated using the developed extraction buffer by electrophoretic and biophotometric analysis, uncut unmethylated λ standard method, restriction endonucleases digestion and rolling circle amplification.

The isolated DNA was suitable for these molecular applications, confirming that the method can be applicable to other insect vectors.

MATERIALS AND METHODS

Sample collection

Insects were collected during a short-rains season from a banana plantation at the Kenya Agricultural Research Institute (KARI) - Kisii in Western Kenya. Direct searching method was used to collect the mealy-bugs. Leafhoppers were collected using a black cage and an aspirator was used to suck them into 50 ml vials. Banana aphids were collected using a camel hair brush and put in the vials containing 70% ethanol as described by Rung et al. (2009). The insect samples were stored at -20°C until DNA extraction.

Protocol development and nucleic acid extraction

To develop and evaluate the protocol, samples were selected to include different haplotypes of *Pseudococcidae*, *paracoccidae*, *Aphididae* and *Auchenorryncha*, and placed in an Eppendorf tube

In the laboratory, specimens were transferred to vials containing absolute ethanol and stored in a -80°C freezer until DNA extraction. The insect samples were cleaned using distilled H_2O containing 2 mM EDTA or TE by vortexing for 30 s, after which the dH_2O-EDTA solution was removed with a pippete. Four hundred micro liter (400 µL) of extraction buffer [0.5M sodium chloride, 10 mM tris (pH 8.0), 36 Mm EDTA (pH 8.0), 0.2% sodium dodecyl-sulfate (SDS), 25 µL proteinase K and 10 M ammonium acetate; the overall pH of the buffer was adjusted to 7.6] was added to the Eppendorf tube. The insects were ground with a sterile teflon Epperdorf grinder (Kontes) and the DNA was isolated as described by Liu et al. (1996). The mixture was incubated in a heating block at 55°C for 2 h (1 h is adequate for this step, but it has been shown that the more the incubation time, the better the yields). The Eppendorf tubes were centrifuged in a non-refrigerated microcentrifuge at 14000 rpm for 5 min to pellet the cell debris and precipitate proteins. This step eliminates the use of the phenols and chloroform to dissolve the proteins. Then 2 µL of RNases (10 mg/ml) were added to the supernatant in a fresh Eppendorf tube and incubated for 1 h at 37°C. This step was meant to digest all the RNA in the total nucleic acid isolated.

2 m^3 of ice-cold isopropanol were added to precipitate the DNA and the mixture was gently mixed by inverting the tubes. The tubes containing the DNA were placed at -20°C for 30 min to allow the DNA to precipitate. The tubes were centrifuged at 14000 rpm for 15 min. The supernatant was removed and the same volume of cold 70% ethanol was added to the pellet. The tubes were spun at 14000 rpm in a non-refrigerated micro centrifuge for 5 min. The ethanol from the Eppendorf tube was poured off and the tubes were air dried completely. The tubes must be air dried for at least 15 min. The DNA pellet was resuspended in 50 µL of distilled H_2O and incubated at 37°C for 30 min or at 4°C overnight. The detection of the virus was done using rolling circle amplification. In addition, the sodium tris-EDTA (STE) based DNA extraction protocols described by Michele et al. (2002) was tested on the samples and analyzed using electrophoresis and bio-photometer.

Electrophorectic analysis of the genomic DNA

Electrophoresis of DNA from mealybug, aphids and leafhopper samples isolated using the developed and the STE based (Michele et al., 2002) protocols was carried out at 100 V for 1 h in 1% agarose gels in tris-(hydroxyl-methyl)-aminomethane (tris) (40 mM)-acetic acid (20 mM)-EDTA (2 mM) at pH 8.1. Gels were stained with SYBR safe (5 µg/ml) for 30 min. DNA bands were visualized using ultra-violet illumination Gel Doc (Bio-RAD) software (USA) and photographed. Lambda DNA was used as a molecular marker (positive control).

Biophotometric analysis

DNA was quantified using OD260/280 nm methods and fully expressed as yields per gram of starting material. Fifty (50) folds dilutions (in sterilized water) were made for each sample prior to OD reading. DNA concentration was extrapolated from the OD readings. Pure preparations of DNA have OD260/280 ratio ranging from 1.8 to 2.0.

Uncut unmethylated lambda DNA standards method

DNA yield and quality (integrity) was made by visual estimation using the mini-gel method (Wachira, 1996). Two microliter (2 µL) of DNA was drawn from the test samples, 3 µL of the gel loading dye (50% of the glycerol, 250 mM EDTA pH 8.0, 0.01% bromophenol blue) was added to it, and the mixture run on a 1.5% agarose gel in 1x TBE buffer (89 mM Tris-HCl pH 8.3, 89 mM boric acid, 2.5 mM EDTA) at 50 V, alongside standards of uncut unmethylated (100, 250 and 500 ng) Lambda DNA. The mini-gel was stained with the SYBR-safe dye and visualized under UV-light (312 nm) Gel Doc (Bio-RAD) USA. The dye was intercalated between the DNA molecules which ultimately fluoresced under UV-light. Comparisons of the band size and staining intensity were made between the test samples and the standards. DNA concentration was inferred from the comparisons. The mini-gel was also used to give the indication of the intactness of the DNA samples. Photography of the gels was done on UV-light (312 nm) Gel Doc (Bio-RAD) USA.

Rolling circle amplification of circular genomes (mitochondria DNA and banana streak virus genome)

The rolling circle amplification (RCA) technique was carried out using the standard protocol of Sambrook and Russell (2001) as modified by James et al. (2011). Amplification of BSV circular DNA was performed using a TempliPhi™ Kit (GE Healthcare, UK) following the manufacturer's protocol. The templiphi kit contained sample buffer, reaction buffer (salt and dNTPs) and enzyme mix (*Phi29* DNA polymerase and random primers in 50% glycerol). Rolling circle amplification degenerate primer mix was also added as described by Wambulwa et al. (2013). Twenty nanogram (20 ng) of total nucleic acid was dissolved in sample buffer, denatured for 3 min at 95°C and cooled down in ice for 3 min. After adding 5 µL of reaction buffer and 0.2 µL of enzyme mix, the reaction was run for 18 to 20 h at isothermal temperature of 30°C. The reaction was stopped by the incubation for 10 min at 65°C to inactivate the *Phi29* DNA polymerase. Aliquots corresponding to 250 ng nucleic acids in 10.2 µL volume of rolling circle amplification product were digested using the restriction enzyme (*Stu*1) for 2 h according to the manufacturer's protocol. Restriction products were resolved on a gel SYBR-safe green stained 1% agarose gel, using TBE as running buffer, and visualized under UV-light transillumination (AlphaDigiDoc, Cambridge, UK). DNA molecular weight marker (Hyperladder™ 1, Bioline) was loaded for band size comparison. To estimate unknown fragment sizes, migration distances were compared to those of reference fragments from the molecular marker (Hyperladder™ 1, Bioline).

Restriction enzyme digestion of the rolling circle amplification and gel electrophoresis

Ten microlitre (10 µL) of the TempliPhi reaction product from each of the insects' samples were incubated separately with the restrictiton enzyme *Stu 1* (Gibco BRL, Eggenstein) for 2 h. A 20 µL aliquot of the digested TempliPhi product was mixed with 2 µL of 5x gel loading dye (Biolabs) and electrophoresed for about 20 min at 100 V on a 1% SYBR safe stained agarose gel using 1x TAE as the running buffer. The gel was visualized under ultra violet (UV) illumination with Gel Doc (Bio-RAD) software (USA). Internal standards (positive controls) for banana streak virus (BSV) isolates generated using *Stu*1 (New England BioLabs*)* were used to identify the isolates present on each sample (James et al., 2011).

Genomic DNA restriction enzymes analysis

Restriction enzymes, *Kpn*1 and *Stu*1 were obtained from New England Biolabs, USA. Digestion was carried out according to the conditions specified by the manufacturer. Two microgram (2 µg) of the mealybug, aphids and leafhopper DNAs were digested for 6 h at 37°C with four units of the enzyme. Electrophoresis of DNA was carried out at 50 V for 8 h in 1% agarose gels in tris-(hydroxyl-methyl)-aminomethane (tris) (40 mM)-acetic acid (20 mM)-EDTA (2

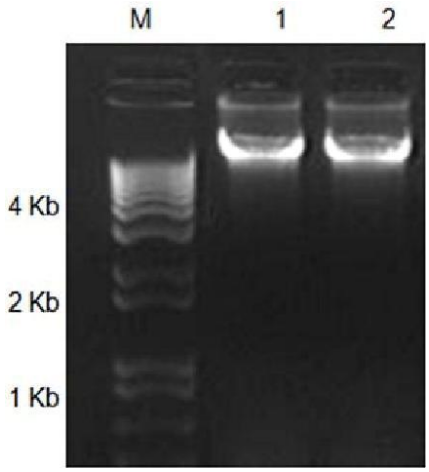

Figure 1. Agarose gel analysis of mealy-bugs' gDNA isolated using the new protocol. Lane M represent molecular marker (Invitrgen, Carlsbad, CA, USA), lane 1 represent mealy-bugs' gDNA and lane 2 aphids' gDNA.

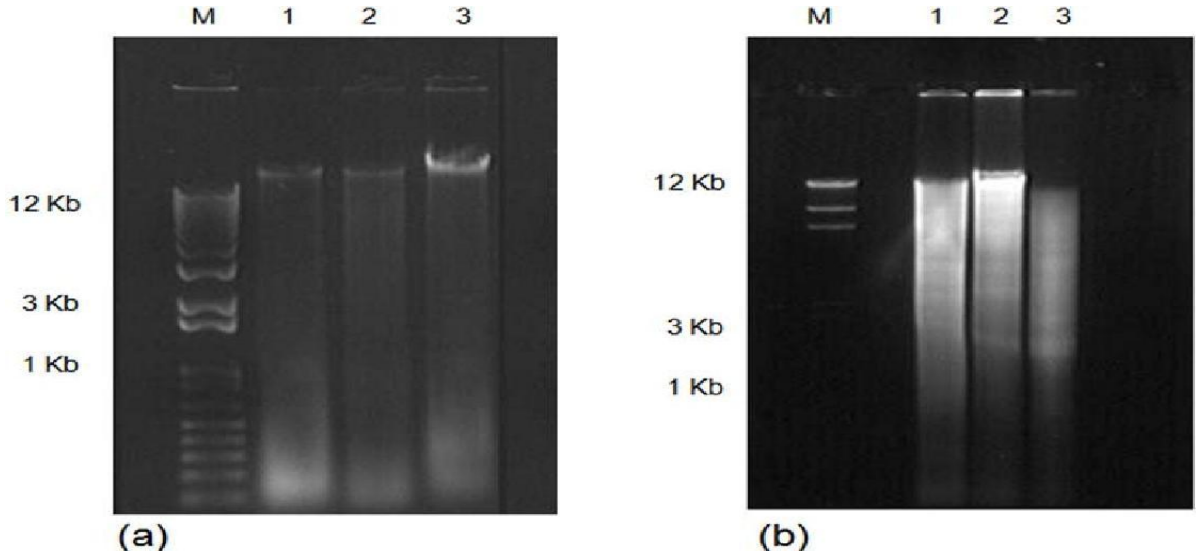

Figure 2. Agarose gel analysis of DNA prepared from mealy-bugs, aphids and leafhoppers using Michele et al. (2002) protocol. Lane M represent molecular marker (Invitrogen, Carlsbad, CA, USA), lane 1 represent mealy-bugs' gDNA, lane 2 represent aphids' gDNA and lane 3 represent leafhoppers' DNA.

mM) at pH 8.1. Gels were stained with SYBR safe (5 µg/ml) for 30 min. DNA bands were visualized using ultra-violet illumination Gel Doc (Bio-RAD) software (USA) and photographed. Lambda DNA digested with both *Kpn* 1 and *Stu* 1 was used as a molecular marker (positive control).

RESULTS

Protocol development and nucleic acid extraction

The results from the new protocol indicated high molecular weight genomic DNA (Figure 1). However, the results from the study of Michele et al. (2002) protocol were undesirable; the DNA was degraded (Figure 2).

Biophotometric analysis

In this study, the ratio of the OD260/280 nm obtained from the isolated DNA samples ranged between 1.8 to 2.0 which was certainly due to nucleic acids. Yields obtained using the OD260 readings were variable amongst the insects' DNA samples. The quantity of the DNA ranged from 2 ng/µl for leafhopper DNA to 7.2 ng/µl for

Table 1. UV-quantification of DNA from the three types of insects using the new protocol.

Sample number	DNA concentration (ng/µl) at OD260	OD260/280	X50 dilution factor (ng/µl)
Mealy-bugs			
1	7.2	2.00	360
2	7.2	1.86	360
3	2.6	1.95	130
Aphids			
1	5.4	1.82	270
2	5.3	1.89	265
3	4.2	1.93	210
Leafhoppers			
1	2.0	1.87	100
2	2.8	1.85	140
3	3.5	1.87	165

Table 2. UV-quantification of DNA from the three insects using the Michele et al. (2002) protocol.

Sample no.	DNA concentration (ng/µl) at OD260	OD260/280	X50 dilution factor (ng/µl)
Mealy-bugs			
1	2.0	1.21	100
2	1.9	1.19	95
3	1.8	1.22	90
Aphids			
1	1.7	1.25	85
2	1.4	1.30	70
3	1.6	1.19	80
Leafhoppers			
1	2.4	1.48	120
2	2.7	1.39	135
3	3.6	1.29	180

OD260/OD280 ratio results obtained using Michele et al. (2002) protocol ranged from 1.19 to 1.48 indicating high level of proteins in the nucleic acids. The concentration of the DNA was below that of the new protocol (ranged from 1.4 ng/µl (aphids) to 3.6 ng/µl (leafhoppers) as indicated in Table 2.

Uncut unmethylated lambda DNA standards method

The results obtained by the method revealed that the DNA isolated was of good quality, relatively intact and of high molecular weight as indicated in Figure 3.

Rolling circle amplification test

The results reveal that the organellar DNA was isolated by the new protocol and were amplified by the non-specific random primers in the reaction mix. However, the results confirmed the absence of the banana streak virus (BSV) in the insects gut as shown in Figure 4.

Endonuclease digestion of the genomic DNA

The results reveal that the nucleic acids isolated were free from phenolic compounds. The DNA was restritable by *Kpn* 1 and *Stu* 1 and restriction enzymes as shown in Figures 5 and 6.

DISCUSSION

The type of contaminations arising in DNA isolated from biological material varies according to its origin such as organism, tissue and life stage (Aljanabi and Martinez, 1997). Therefore, the type and condition of specimens and tissues are key factors in selecting a DNA isolation

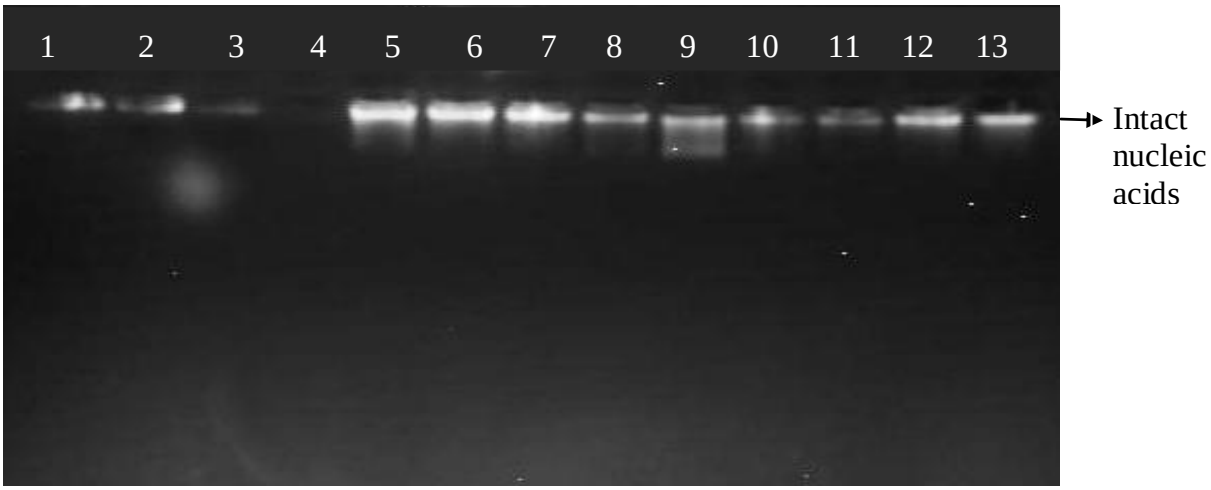

Figure 3. Quantity and quality of isolated DNA from the three insects using new protocol. Lanes 1 to 3 represent uncut unmethylated λ DNA standards (750, 500 and 250 ng, respectively), lane 4 represent negative control (SDW), lanes 5 to 7 represent aphid DNA, lanes 8 to 10 represent leafhopper DNA, Lanes 11-13 represent mealy-bugs DNA.

method. Tissues in the digestive tracts of vectors of plant diseases are rich in phenolics and tannins. These secondary metabolites must be removed to obtain DNA free from contaminants. Phenolics and other secondary metabolites cause damage to DNA and inhibit restriction endonucleases and DNA polymerases (Lodhi et al., 1994; Friar, 2005; Padmalatha and Prasad, 2006; Arifa et al., 2010). The widely used methods occasionally fail to remove all phenolics from DNA preparations. To tests the effects of inclusion of 10M ammonium acetate in our DNA isolation method, we compared the developed method with the traditionally used methods described by Michele et al. (2002). The results indicated good yields of high molecular weight DNA using the developed protocol. The ratio of the OD260/OD280 obtained for the isolated DNA samples ranged from 1.8 to 2.0 which was certainly due to nucleic acids (Table 1). A ratio less than 1.8 could indicate the presence of proteins and/or other metabolites in the DNA samples, in which case it would be necessary to re-precipitate the DNA (Honeycutt et al., 1992; Draper and Scott, 1998; Simon et al., 2003).

A DNA extract contaminated by chloroform and/or phenol normally gives a ratio higher than 2.0 in which case the extract should be re-precipitated with isopropanol (Brondmann, 2008). All the samples in this study indicated a ratio that was within the range which was attributed to relatively pure DNA. Like any other antioxidant, the ammonium acetate forms complex ionic bonds with proteins and other secondary metabolites and co-precipitate with cell debris upon cell lysis (Lodhi et al., 1994; Couch and Fritz, 1990). The acetate complexes accumulate at the interface between the organic and aqueous phases and can be eliminated from the DNA preparations (Reineke et al., 1998). High concentration of the ammonium acetate helps to reduce the browning of the DNA preparations produced by the oxidation of the secondary metabolites (Horne et al., 2004; Li et al., 2007). However, there is one complexity of the mealy-bug system and other vectors; this pertains to the presence of certain yeast and bacteria-like symbionts in both sexes of the insects. They are transmitted by the mother to the egg. The symbionts invade certain polyploid cells which form a small organ called the mycetome whose function is not known. When DNA is isolated from whole insects, the possibility of “contamination” of mealy-bug DNA by the symbiont DNA cannot be avoided (Deobagkar et al., 1982). This may become important, particularly when one is studying differences between DNA isolated from males and females, because the two sexes are vastly different in size. At least, in the stock that was used in this study, it would have been difficult to demonstrate symbionts in adult mealy-bug females, whether virgin or gravid (Aljanabi and Martinez, 1997). However, in any kind of study, specific primers are used to amplify only the sequences of interest BSV and therefore solving a problem of contamination by nucleic acids from symbionts.

Yields obtained using the OD260 readings were variable amongst the insects that the DNA was isolated. These results were attributed to the variation of the hardness of the vectors cuticle; mealy-bugs have the softest cuticle while leafhoppers have the hardest cuticle. This quantity of DNA obtained was adequate for PCR and RCA techniques which require about 0.3 ng/µl for amplification to occur. Indeed, it has long been known that polysaccharides like other contaminants are impossible to detect by non-degradative analytical techniques and usually they interfere with quantification of the nucleic acids spectrophotometrically and may even cause anomalous hybridization kinetics (Kim et al., 1997). However, the OD260/280 nm ratio for the Michele et al. (2002) protocol (1.19 to 1.48) indicated a higher level of contamination in the DNA preparations (Table 2). The

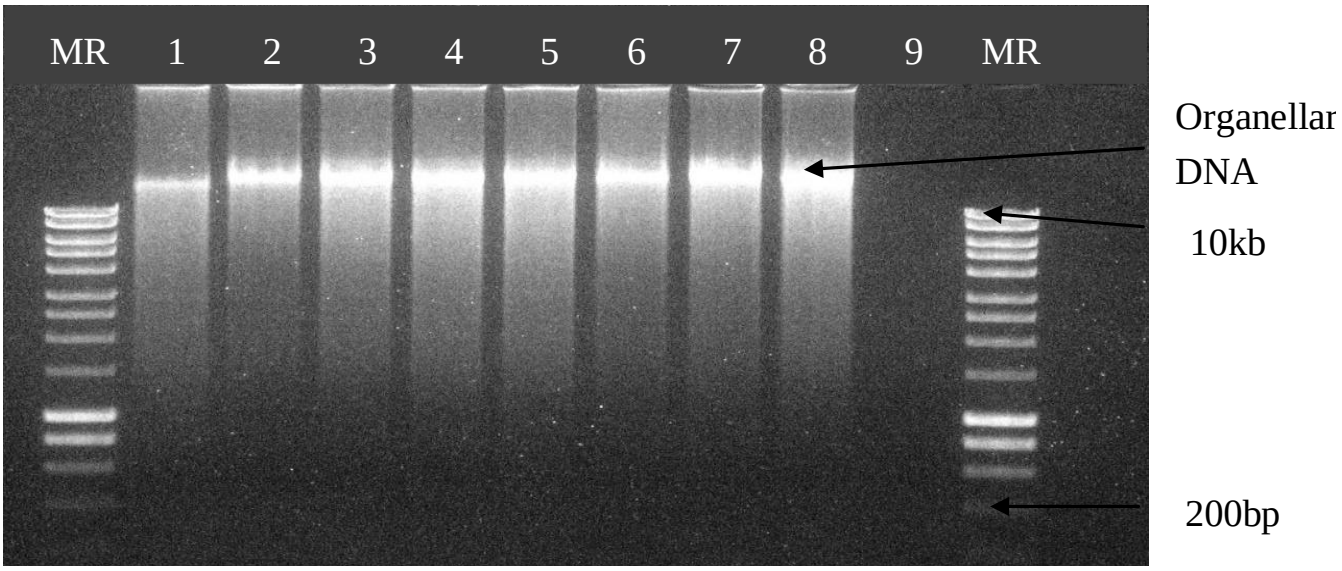

Figure 4. Rolling circle amplification of the mitochondrial circular DNA from the three insects. Lane MR represent molecular marker (Hyperladder^{TM} 1, Bioline), lanes 1 to 3 represent mealy-bugs DNA, lane 4 to 5 represent aphids DNA, lanes 6 to 8 represent leafhopper DNA and lane 9 represent negative control (SDW).

results also indicated low amounts of DNA than the new developed protocol. Running the isolated DNA against different concentrations of uncut unmethylated lambda (λ) phage DNA (Figure 3), was a realistic approach used in this study to determine the intactness of the isolated DNA. Although, the results obtained by this method revealed that the DNA isolated was of good quality, relatively intact and of high molecular weight, degradation of part of the isolated DNA is always inevitable. For adequate resolution of PCR, or RCA, and restriction fragment length polymorphism (RFLPs), it is known that native DNA should migrate as a tight band of high molecular weight (Brondmann, 2008).

The isolated DNA using high concentrated amount of ammonium acetate was tested for amplification reactions (Figure 4). The rolling circle amplification technique was used to amplify a DNA sample to confirm whether the sample of DNA contained inhibiting compounds or not. The technique is more sensitive to the inhibitory compounds in the isolated DNA (CIMMYT, 2005) than the PCR. The high sensitivity of the technique is due to the nature of the enzymes (*Phi29* DNA polymerase in RCA) used. *Phi29* DNA polymerase is known to recognize and amplify as little as 1 picogram of the template DNA (Reagin et al., 2003). In this study, the amplification products of the organellar DNA were obtained using RCA technique which was proof that DNA were free from inhibitory substances such as phenolic compounds that are known to form complexes with the DNA. Most common contaminants in DNA preparations are polysaccharides, RNA and phenolics compounds (Lodhi et al., 1994; Fire and Xu, 1995; Kim et al., 1997; Reineke et al., 1998; Michiels et al., 2003; Rung et al., 2009; Arif et al., 2010). Polysaccharides and phenolics produce highly viscous and brown coloured solutions (Henry et al., 1990; Couch and Fritz, 1990; Puchooa and Venkatasamy, 2005). They also reduce the storage lifespan of the DNA preparation (Lodhi et al., 1994). Given that RNA contamination is normally removed by treatment with RNase (Puchooa and Venkatasamy, 2005), and the isolated DNA was not viscous, it is likely that phenolics are the contaminants present in the Michele et al. (2002) isolated DNA. In addition, the inclusion of 10M ammonium acetate cleared the DNA solutions. This suggests DNA isolated by the developed protocol had lower concentrations of phenolics compared with the Michele et al. (2002) method.

The isolated DNA sample was further validated for its quality by determining its digestability by restriction enzymes. Indeed, the restrictability of the DNA is essential often before setting up large-scale digestion experiments. The *Stu*1 and *Kpn*1digest results obtained in this study with the DNA of the developed isolation protocol suggested that the DNA had no phenolic compounds that are known to inhibit the restriction enzymes (Figures 5 and 6) and DNA polymerase even without addition of the antioxidant (citrate) as described in other protocols (Aljanabi and Martinez, 1997). Phenolic compounds usually are formed during the isolation procedure and these bind firmly to DNA. Since many of these phenolic compounds contain methyl groups (Aljanabi and Martinez, 1997), they can end up hindering the restrictability of the isolated DNA. The production of these phenolic compounds is particularly catalyzed by the polyphenolic oxidases (Aljanabi and Martinez, 1997) in insects. Since many factors can cause a restriction digestion to fail or succeed, a single digestion should not be the decisive factor to immediately make conclusions. Other factors such as

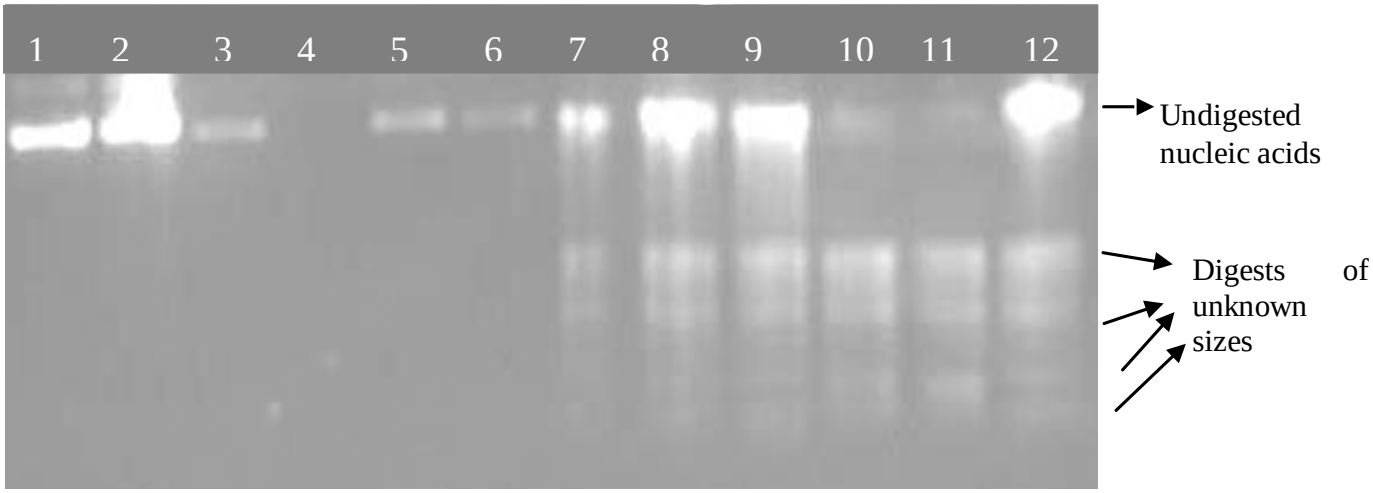

Figure 5. Analysis of non-digested and digested DNA from the three insects and lambda phage DNA using Kpn1. Lanes 1 to 6 represent undigested DNA while lanes 7 to 12 represent digested DNA. Lanes 1 and 2 represent undigested λ DNA, lanes 3, 5, 6 represent undigested mealy-bugs, leafhoppers and aphids DNA, lane 4 is sterilized distilled water, lanes 7 and 8 represent mealy-bug DNA, lanes 9 and 10 represent leafhopper DNA, lane 11 represent aphids DNA and lane 12 represent λ DNA.

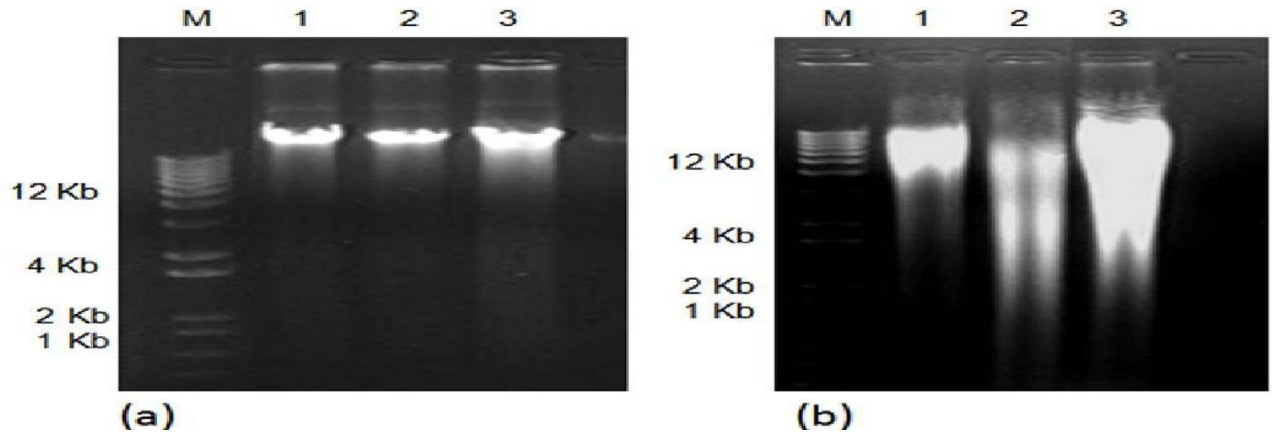

Figure 6. Digestability of DNA from the three insects using *Stu*1 (biolabs): Lane M represent molecular marker, lane 1 represent mealy-bugs' DNA, lane 2 represent aphid DNA and lane 3 represent leafhopper DNA.

poor reaction conditions could account for these results.

Conclusions

Ten molar (10M) ammonium acetate is an efficient molecular concentration for extraction of genomic DNA from small insects used for rolling circle amplification. The developed protocol from the modified sodium chloride-ris-EDTA based buffer isolated suitable DNA for rolling circle amplification (RCA) and restriction analysis. The method is simple, rapid and labor effective for DNA isolation from the three mentioned insects. Additionally, the method circumvents the use of the potentially carcinogenic compounds that may be deleterious to the users.

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REFERENCES

Aljanabi SM, Martinez I (1997). Universal and rapid salt extraction of high quality genomic DNA for PCR-based techniques. Nucleic Acids Res. 25: 4692-4693.

Arif IA, Bakir MA, Khan HA, Ahamed A, Al Farhan AH, Al Homaidan AA, Al Sadoon M Bahkali AH, Shobrak M (2010). A simple method for DNA extraction from mature date palm leaves: impact of sand grinding and composition of lysis buffer. Int. J. Mol. Sci. 11: 3149–3157.

Brondmann P (2008). DNA extraction from different matrices. Mol. Biol. Meth. Traceabil. Purp. BFR, Berlin, Germany.
Calderon CN, Quesanda M, Cano CH, Zavula PG (2010). A simple and rapid method for DNA isolation from from xylophagous insects. Int. J. Mol. Sci. 11: 5056-5064.
Chen DH, Ronald PC (1999). A rapid DNA minipreparation method suitable for AFLP and other PCR applications. Plant Mol. Biol. Rep. 17:53–57.
CIMMYT (2005). Laboratory protocols: CIMMYT Applied Molecular Genetics Laboratory. Third Edition. Mexico, D.F.: CIMMYT.
Couch JA, Fritz PJ (1990). Isolation of DNA from plants high in polyphenolics. Plant Mol. Biol. Rep. 8: 8–12.
Cruickshank R (2002) http://taxonomy.zoology.gla.ac.uk/%7Ercruicks/rcruickshark.html.
De Verno LL, Smith CA, Harrison KJ (1998). Randomly amplified polymorphic DNA evidence of introgression in two closely related sympatric species of coniferophagous *choristoneura* (Lepidoptera: *tortricidae*) in atlantic Canada. Annals Entomol. Soc. Amer. 91: 248-259.
Deobagkar DN, Muralidharan K, Devare SG, Kalghatgi KK, Chandra HS (1982). The mealy-bug chromosome system I: Unusual methylated bases and dinucleotides in DNA of a *Planococcus* species. J. Biosci. 4: 513-526.
Draper J, Scott R (1998).The isolation of the plant nucleic acids. In:
Draper J, Scott R, Armitage P, Walden R (eds) Plant Genetic transformation and gene expression. A laboratory manual. Blackwell Scientific Publications, Oxford, UK. pp. 201-226.
Fire A, Xu SQ (1995). Rolling replication of short DNA circles. Proc. Natl. Acad. Sci. USA 92: 46-4645.
Friar EA (2005). Isolation of DNA from plants with large amounts of secondary metabolites. Method Enzymol. 395: 3–14.
Honeycutt RJ, Sobral BW, Keim P, Irvine JE (1992). A rapid DNA extraction method for sugarcane and its relatives. Plant Mol. Biol. Rep. 10: 66-72.
Horne EC, Kumpatla SP, Patterson MG, Thompson SA (2004). Improved high-throughput sunflower and cotton genomic DNA extraction and PCR fidelity. Plant Mol. Biol. Rep. 22: 83a–83i.
Inoue-Nagata AK, Albuquerque LC, Rocha WB, Nagata T (2004). A simple method for cloning the complete begomovirus genome using bacteriophage Φ29 DNA Polymerase. J. Virol. Method. 116: 209-211.
James AP, Geijskes RJ, Dale JL, Hardling RM (2011). Development of a novel Rolling Circle Amplification technique to detect *Banana Streak Virus* that also discriminate between integrated and episomal virus sequences. Plant Dis. 95: 57-62.
Kim CS, Lee CH, Shin JS, Chung YS, Hyung NI (1997). A simple and rapid method for isolation of high quality genomic DNA from fruit trees and conifers using PVP. Nucleic Acids Res. 25: 1085-1086.
Knolke S, Erlachier S, Hausmann A, Miller MA, Segerer AH (2005). A procedure for combined genetalia dissection and DNA extraction in Lepidoptera. Insect System. Evol. 35: 401-409.
Li JT, Yang J, Chen DC, Zhang XL, Tang Z (2007). An optimized mini-preparation method to obtain high quality genomic DNA from mature leaves of sunflower. Gen. Mol. Res. 6: 1064-1071.
Liu D, Daubendiek SL, Zillman MA, Ryan K, Kool ET (1996). Rolling circle DNA synthesis: Small circular oligonucleotides as efficient templates for DNA polymerase. J. Am. Chem. Soc. 118: 1587-1994.
Lodhi MA, Ye GN, Weeden NF, Reisch BI (1994). A simple and efficient method for DNA extraction from gravepine cultivars, *Vitis* species and *Ampelopsis.* Plant Mol. Biol. Rep. 12: 6-13.
Michele K, Nishigushi PD, Egan M, David Kiziran D, Aloysius P, Lorenzo P, Howard C, Rosenbaum E, Yael W, Desalle R, Gonzalo Giribet G (2002). DNA isolation procedures. In:Techn Mol. System. and Evol. ed. Rob DeSelle et al.@ 2002 Birkhauser Verlag Basel/Switzerland.
Mitchell A, Cho S, Regier JC, Miller C, Poole RW, Mathews M (1997). Phylogenetic utility of elongation factor -1α in *Noctuoidea* (insecta: *Lepidoptera*): the limits synonymous substitution. Mol. Biol. Evol. 14: 381-390.
Padmalatha K, Prasad MNV (2006). Optimization of DNA isolation and PCR protocol for RAPD analysis of selected medicinal and aromatic plants of conservation concern from Peninsular India. Afr. J. Biotechnol. 5: 230–234.
Philips AJ, Simon C (1995). Simple, efficient, and non-destructive DNA extraction protocol for arthrophods. Annals. Entomol. Soc. Am. 88: 281-283.
Reagin MJ, Giesler TL, Merla AL, Resetar-Gerke JM, Kapolka KM, Mamome AJ (2003).TempliPhi: A Sequencing Template Preparation Procedure That Eliminates Overnight Cultures And DNA Purification. J. Biomol. Tech. 14: 143-148.
Reineke A, Karlovsky P, Zebitz P (1998). Preparation and purification of DNA from insects for AFLP analysis. Insect Mol. Biol. 7: 95-99.
Rose OC, Brookes MI, Mallet JLB (1994). A quick and simple nonlethal method for extracting DNA from bitterfly wings. Mol. Ecol. 3: 275.
Rung A, Miller DR, Sceffer SJ (2009). Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Method to Distinguish Three Mealybug Groups Within the *Planococcus citri–P. minor* Species Complex (Hemiptera: Coccoidea: *Pseudococcidae*). J. Gen. Entomol. 102(1): 8-12.
Shneider D, Legal L, Dierl W, Wink M, (1999). Androconial hairbrushes of the Syntomis (Amata) Phegea (L.) group (Lepidoptra, *Ctenuchidae*): A Synapomorphic Character Supported by sequence data of the mitochondria 16S rRNA gene. Zeitschrift fur Naturfarschung. 54C: 1119-1139.
Simon JC, Carre S, Bartin M, Prunier-L N, Sabate-M, Latorre A, Baurnoville R (2003). Host-based divergence in population of the pea aphids: Insights from nuclear markers and the prevalence of facultative symbionts. Proc. Biol. Sci.
Starks PT, Peters JM (2002). Semi-nondestructive genetic sampling from live eusocial wasps, *Polistes dominulus* and *Polistes fuscatus.* Insect Soc. 49: 20-22.
Strauss SY, Zangerl AR (2002). Plant-insect interactions in terrestrial ecosystems. In *Plant-Animal Interactions: An Evolutionary Approach*; Herrera, C.M., Pellmyr, O., Eds.; Blackwell Publishing: Oxford, UK, 2002.
Wachira FN (1996). Development of molecular markers in Camellia. PhD Thesis Dundee University.
Wahl DB (1984). An improved method for preparing exuviae of parasitic Hymenoptera. Entomol. New. 95: 227-228.
Wambulwa MC, Wachira FN, Karanja LS, Kiarie SM, Muturi SM (2013). The influence of host and pathogen genotype on symptom severity in banana streak disease. Afr. J. Biotechnol. 12: 27-31
Zidani S, Ferchichi A, Chaleb M (2005). Genomic DNA extraction method from pearl millet (*Pennisetum glaucum*) leaves. Afr. J. Biotechnol. 4: 862-866.