AN EFFICIENT AND RELIABLE METHOD FOR EXTRACTION OF GENOMIC DNA FROM STINK BUG SPECIES (HETEROPTERA: PENTATOMIDAE)

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ABSTRACT: *The author represents a very simple, rapid, an extremely efficient and modified method of DNA extraction from different stink bug species belonging to order Heteroptera. These bugs pose huge financial problems to the farmers because of their resistance to commonly used pesticides in the agricultural fields, their tendency to form large populations within short period of time, causing remarkable damage to food crops and fruit trees, thereby, reducing the global yield and quality of food. They are considered one of the major agricultural pests of economically important crops throughout the world that includes legumes (soyabeans), cereals (rice, wheat) and tree crops (coconut, palms, citrus, cocoa, coffee). Most of the species are plant specific and thus depending on the type of plant they infest, their economic importance varies greatly from species to species. Molecular techniques have entered successfully in the field of taxonomy in order to supplement the taxonomic data of the worker. However, due to varied habitats, habits, feeding behavior, breeding behaviors, different life cycles patterns and very large populations of insects, a single method of DNA extraction cannot be used for all the insects belonging to different orders. So it becomes necessary to develop a suitable protocol for a particular group of insects in order to yield reproducible products. Thus a simple, time savior and a cost efficient method is recommended for the extraction of genomic DNA when a large number of bug samples are to be processed in a short period of time or even if a single specimen is available, this method should act as a best and convenient method to evaluate the result of that small amount of DNA in a sample.*

Key Words: : *Stink bugs, pests, Genomic DNA, DNA extraction,*

INTRODUCTION

The stink bugs falls in the category of the most important pests of economically important agricultural crops and food trees. Some of these are easily identified on the basis of their external morphologically or their external genitalic features such as spermatheca (Kaur *et al*., 2017), but sometimes extremely small size of an insect or availability of damaged specimens or partial fragments of body parts act as a barrier in identification process. The identification of cryptic species in the population sometimes poses a natural hindrance in their biological and chemical control. Such limitations can be triumphed over by supplementing the use of molecular biology techniques. These techniques are usually PCR-based. It is a very realistic point that working with the insects in laboratory for molecular analysis during the process of DNA extraction, usually some amount of DNA is always lost due to mishandling of specimens, erroneous preparation of chemicals, over or under use of lysis buffers, or harsh effect of the strong and denaturing chemicals used in the process. The result of PCR products depends mostly upon the first step of extraction i.e homogenizing of the tissue. Nowadays, taxonomists working on insect populations belonging to different groups contentedly invite molecular techniques so as to supplement their taxonomic and phylogenetic data with genetics. A good Molecular technique enables the extraction of DNA from a large number of samples in very short period of time or even extraction of small amount of DNA in case if the specimen is damaged, with faded coloration, lost parts or or less in number. The technique should be cost effective and must use easily accessible equipments. Several published methodolgy of DNA extraction on various plant and animal tissues have been published using Chelex-100, Sarcosil with Proteinase K, CTAB, Polyvinyal pyrolidone and mercaptoethanol ,Only CTAB ,CTAB with mercaptoethanol ,CTAB with SDS, DIGSOL buffer etc. (Walsh *et. al*, 1991; Lardeux *et.al,* 2008 and Lagisz *et.al,* 2010). A protocol should be such that it should be - non destructive on insect specimen and should not harm the originality of the preserved dried or fresh specimen. A basic CTAB method for genomic DNA extraction with some modification standardized specifically for stink bugs was used in the present study to perform PCR - based reactions (Aljanabi and Martinez, 1997).

MATERIALS AND METHODS

During the research work, the author did not employ the available commercial kits for DNA isolation and PCR, since they are not cost effective and also they involved use of expensive lab chemicals. So, it became

mandatory to develop an easy and efficient DNA extraction protocol in the lab which could be used for stink bug's species anywhere in simple and feasible allowed conditions.

For molecular studies, it is important to isolate genomic DNA (gDNA) from adult specimens. Usually legs, thorax or part of head is used for isolation to prevent any kind of parasite and blood meal contaminants that could be present in the abdomen of an insect. After many laboratory repetitive experiments, legs were considered as the most suitable tissue for the extraction process with respect to the quantity and quality yield. The methodology of molecular techniques (PCR-RAPD) for the selected bugs belonging to family Pentatomidae was standardized as follows:

METHODOLOGY FOR MOLECULAR STUDY

The outline procedure for DNA extraction and PCR technique include following steps: -

a) Isolation of genomic DNA.
b) Testing the presence and integrity of intact DNA
c) Estimation of intact DNA through Agarose gel electrophoresis or spectrophotometric assay.
d) Amplification of DNA by polymerase chain reaction.
e) Agarose gel electrophoresis of amplified products.
f) Photography of amplified products in Gel-Doc system.

a) PROTOCOL FOR ISOLATION OF GENOMIC DNA

The adult individuals were first identified on the basis of morphology and then genomic DNA was extracted by modifying various extraction techniques including CTAB (Cetyltrimethylammonium Bromide) method. The quantity of DNA in samples averaged 20–30 ng/µl. Both male and female adults were used for DNA isolation (if both available).

- Insect tissue taken for DNA extraction was grinded in standardized 4:1 ratio of **homogenization buffer** (0.1M NaCl, 0.2M Sucrose, 0.01M EDTA, 0.03M Tris-Hcl, pH-8.0) and **Lysis buffer** (0.25M EDTA, 2.5% (w/v) SDS, 0.5M Tris-Hcl, pH-9.2) and final volume was made upto 500µl.
- Then 5µl Rnase, 5µl proteinase K and 10µl 2-merceptoethanol were added in the eppendorf tube containing the sample.
- The mixture was incubated at 65°C for 1 hour in the water bath.
- 0.15ml of 8M Potassium acetate (pH-5.2) was added and gently vortexed.
- The mixture was then incubated in ice for 1 hour.
- The contents of the eppendorf were centrifuged at 12,000 rpm for 15 min at 4°C.
- The supernatant was collected and to it 400µl of 95% chilled ethanol was added.
- The eppendorf tube was then incubated overnight at -20°C.
- The mixture was again centrifuged at 12,000 rpm for 10 min at 4°C.
- 1000 µL of 70% ethanol was added to the reaction mixture and vortexed for a few seconds to precipitate DNA.
- The mixture was allowed to air dry at room temperature.
- DNA pellet was resuspended in 100 µl TE buffer.
- Final solution containing the extracted DNA was stored at -20°C in deep freezer for future use.

b) CHECKING/TESTING OF INTEGRATED DNA BY AGAROSE GEL ELECTROPHORESIS

The electrophoresis works on a principle of the movement of charged molecules or ions towards appropriate electrode on application of electric field, and this movement depends upon the strength of electric field applied, size of DNA, pore size of the gel and also on the viscosity of the medium or buffer. The presence and integrity of DNA was checked on 0.8% agarose.

c) PREPARATION OF GEL SOLUTION

- 50 ml of 1X TE buffer solution was taken in a beaker.
- Added 0.8g of agarose to beaker containing the buffer and stirred properly to suspend the gel particles.
- The solution was heated in microwave until agarose was completely dissolved.
- The melted agarose was then cooled slightly before casting.
- 3µl of 10mg/ml of ethidium bromide was added to the above agarose solution and the mixture was thoroughly mixed by gentle shaking.

d) CASTING OF AGAROSE GEL

- The plastic tray was cleaned properly and its edge was sealed with broad cello tape to form a mould.
- Gel comb was placed at one end of the tray to form sample wells or cavities in the gel.

- The prepared agarose gel solution was poured into the tray and the gel was allowed to settle completely for 30 minutes.
- The comb and the tape were removed carefully and the tray was placed gently in the electrophoretic chamber containing 1X TBE buffer. The gel was overlaid in the buffer to a depth of about 1mm.
- Mixed 7µl of DNA with 3µl of Bromophenol blue dye. The samples were loaded in the gel cavities very carefully.
- The lid of electrophoretic chamber was closed and connected to electric field. As per principle, DNA being negative charged tends to move towards the positive electrode in the buffer when the field is applied.
- The samples were allowed to run till the tracking dye migrated from the wells and was visible moving towards the other side of gel in the tray.

After electrophoresis, the DNA was visualized through UV transilluminator. The presence of fluorescent dye Ethidium bromide in the gel, having an ability to intercalate between DNA bonds, enabled to visualize DNA in the form of shining fluorescent bands wherever the dye binds with DNA in the genome. Another way to analyse DNA in the gel is to perform the spectrophotometric assay to calculate DNA concentration in the form of optical density.

THE REAGENT USED FOR ASSAY IS TE BUFFER (PH – 8.0)

- The ratio of 1: 10 was used for DNA sample and TE buffer.
- 1µl of DNA sample was taken in cuvette.
- Added 10 µl of TE buffer and mixed the contents.
- The absorbance was noted at 260 nm and 280 nm on UV Spectrophotometer and DNA concentration was calculated accordingly.

OPTIMIZATION OF THE HEXADECYLTRIMETHYLAMMONIUM BROMIDE (CTAB) METHOD (MODIFIED FROM DOYLE AND DOYLE 1987 & 1990)

Various problems are encountered during the isolation of DNA from the stink bugs due to the following reasons:

1. Firstly, the small size of the insect imposes a difficult task for extracting out the small amount of genomic DNA.
2. Secondly, in Arthropoda group, the class insecta contains a very thick chitinous exoskeletion protective layer which is a stringent mucopolysaccharide composed of N-acetyl glucosamine linkages. This plays a huge hindrance in initial lysis of the tissue.
3. Thirdly, since the stink bugs are exclusive sap suckers, so they usually contain a large amount of contaminants in the form of polyphenolics from plants sap. The polyphenols are tannins which are produced by the plants naturally or when there is some tissue damage to plant. These compounds have a phenol ring that efficiently binds to DNA. The insects which feed on them tend to have these compounds in their guts which may interfere in Insects own DNA extraction and may result in mixing with contaminants.
4. Also, a large amount of protein in the sample hampers DNA extraction process.

So, an ideal protocol is needed which should optimize DNA yield and quality for a particular group of insects. Apart from the above mentioned protocol for DNA extraction, some of the following modifications were specifically standardized for the group of these stink bugs so as to increase the efficacy and viability of this method.

- The basics of DNA extraction remain the same in all protocols. One can modify the composition and molarity of various lysis buffers, lytic enzymes, detergents used in the experiment.
- Legs was chosen as a preferred tissue for DNA extraction.
- In addition to lysis buffer, liquid nitrogen was also used in case of fresh specimens which allowed easy breaking of cell wall of an insect to access the DNA.
- The volume of all reagents used was also reduced as compared to normal CTAB method.
- The incubation period was standardized accordingly to ensure a more thorough lysis of cells to increase quantity of DNA obtained from the tissue.
- Rnase, 5µl proteinase K and 10µl 2-merceptoethanol, 100 Mm Tris buffer, 100 mM NaCl were added to it with an overnight incubation to improve the lysis step.

- Incubation time in 95% ethyl alcohol was also standardized so as to stop further lysis of specimen. After this the pellet was completely dried to avoid any physical damage to the extracted tissue.
- Effectual quantity of Potassium acetate was standardized which is known to remove any kind of cellular and histone proteins bound to the DNA. This step was done prior to the DNA-precipitation.

CONCLUSION:

This standardized protocol was found highly suitable to amplify very small amount of genomic DNA of stink bugs to carry out polymerase chain reactions (PCR). This further helped in performing other molecular biology techniques such as such as RFLP (Restriction Fragment Length Polymorphism), AFLP (Arbitrary Fragment Length Polymorphism) and RAPD (Random Amplified Polymorphic DNA) that reveal higher level of polymorphism as compared to other protein based markers. The proposed extraction protocol from these bugs yielded good quantity and quality DNA, and exceptionally better than commercially available kits .The protocol was evaluated by polymerase chain reaction (PCR) and RAPD was also performed on some selected specimens to study polymorphism. The protocol of DNA extraction is a time saver so that other parameters can be carried out by the workers. The chemicals, consumables and other material required in this protocol are easily available in majority of laboratories, and thereby can be readily adoptable by the preliminary workers to work on large numbers of pests samples to study polymorphism or barcoding DNA in a stipulated period of time.

Anticipated results:

This protocol was used in the PH.D work of the author on selected species of the stink bugs. The quality and quantity of DNA is generally fine. The purity value of DNA samples ranged from >1.8 and found appropriate for evaluation by polymerase chain reaction. The protocol was proved successful for various pentatomid bug species like *Eocanthecona furcellata* (Kaur *et al*., 2018), *Nezara viridula, Nezara antennata, Perillus bioculatus* etc. using OPA series primers 1. OPA-02 TGCCGAGCTG 2. OPA-05 AGGGGTCTTG 3. OPA-06 GGTCCCTGAC 4. OPA-08 GTGACGTAGG 5. OPA-10GAGATCGCAG. (Kaur *et al*., 2018).

PROTOCOL FOLLOWED FOR RAPD-PCR

A reaction mixture of 25 µl was prepared in a properly sterilized PCR amplification tube.

REAGENTS AND THEIR VOLUME USED FOR PREPARING 25ML OF REACTION MIXTURE:

REAGENTS	VOLUME USED
10X ASSAY buffer	2.5 µl
$MgCl_2$ (25Mm)	1.0 µl
dNTPs (2mM)	1.0 µl
Taq Polymerase (1U / µl)	1.0 µl
Primer (10Pm/ µl)	1.0 µl
Template DNA (50ng)	2.0 µl
Distilled water	16.5 µl
Total	**25.0µl**

- The reaction mixture containing target DNA was amplified in a preset programmed PCR Thermal cycler. Various steps of programme in Thermal cycler for 44 cycles are as follows:

Cycle 1
Denaturation at 94 ˚C - 5 minutes
Primer annealing at 37 ˚C - 1 minute
Primer extension at 72 ˚C - 2 min's
Cycle 2 - 43
Denaturation at 92 ˚C - 1 minute
Primer annealing at 37 ˚C - 1 minute
Primer extension at 72 ˚C - 2 minutes
Cycle - 44
Denaturation at 92 ˚C - 1 minute
Primer annealing at 37 ˚C - 1 minute
Primer extension at 72 ˚C - 7 minute

- Finally, the amplified product was stored at -20 ˚C in a deep freezer.
- The presence and integrity of amplified product was seen through agarose gel electrophoresis. In one of the wells DNA ladder was loaded, while in others samples were loaded.

- The amplified products containing fluorescent dye Ethidium bromide were photographed in Gel-Doc system.

For every species, the results obtained for RAPD-PCR were represented in the form of a gel plate that included three parameters for analysis - agarose gel photograph representing DNA bands (presence or absence), distance matrix constructed using Jaccard Similarity Coefficient and cladogram using NJ method. The gel plate result of one species i.e. *Eocanthecona furcellata* is shown below: (Kaur *et al*., 2018)

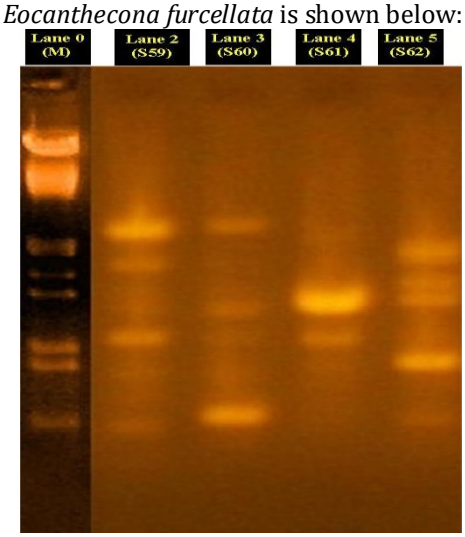

Fig. 13: RAPD-PCR pattern of genomic DNA using primer OPA 8.
Lane 0: MW marker (2000-100 size in bp);
Lane 2: *Eocanthecona furcellata* (S 59) Uttarakhand
Lane 3: *Eocanthecona furcellata* (S 60) Himachal Pradesh
Lane 4: *Eocanthecona furcellata* (S 61) Punjab
Lane 5: *Eocanthecona furcellata* (S 62) Jammu & Kashmir

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