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A method for isolation of DNA from Polyphagous Insects by cetyltrimethylammonium bromide (CTAB-PVPP method)

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Abstract
Insects are diverse in their distribution amongst kingdom Animalia. Order Lepidotera (butterflies & moths) stands second after coloeoptera in diversity. Noctuidae is one amongst the important families of order Lepidoptera with 36 families and 4200 genera in it. As moths exhibit great diversity besides the presence of many sub-species and cryptic species it has turned out to be tough conducting the taxonomical studies on them.
The DNA isolation of polyphagous insects by SDS based methods and commercially available kits tend to produce low DNA yields with short storage life from tissues rich in phenolics. We evaluated the efficacy of the traditionally used CTAB-PVPP method for isolation of DNA from the Polyphagous insect *Spodoptera frugipedra*.

Keywords: coloeoptera, *spodoptera frugipedra*, polyphagous insect

Introduction
Insects are most diverse in their distribution amongst kingdom Animalia (Chapman, 2006). Lepidotera stands second after coleoptera in diversity (Erwin and Terry, 1997), whereas Noctuidae is one amongst important families of this order with 36 families and 4200 genera in it.
The great diversity exhibited by moths of noctuidae family besides the presence of many sub-species and cryptic species has made it difficult for taxonomic studies. Earlier, many taxonomists used anatomical tools, the phenotypic features such as wing pattern, body spots, band hairs, spines, and eye colour for identifying and classifying these moths. With the beginning of present century, anatomical tools are quite taken over by molecular tools such as protein markers and genetic markers.
Use of phenotypic markers is time consuming, and error contained process because these are infrequent and difficult to score, whereas protein markers pose stability problem hence are mostly used to study pathogen identification and insecticide resistance. Different genetic markers like nuclear and mitochondrial markers, with DNA as the basic source, being more stable are used to study taxonomy, thus phylogeny. They are also used to study not only genetic inheritance but also to study mating behaviour and dispersal patterns (Gilbert *et al.*, 1988; Nijhout, 1991) besides studying parentage and kinship, Insect plant interaction, Insect pathogen interaction, Insecticide resistance, Prey, predator and parasites, genome and QTL mapping, Comparative genomics and cytogenetic of insect (Behura 2006) [4].
DNA markers are more important because DNA is more stable than protein and help to detect variation due to mutation on intron or in gene codon (Behura 2006 [4]; Usman *et al.*, 2015). In either of methods importance of DNA remains same. Hence, DNA isolation becomes extensively important step in these studies. Most of the published methods for DNA isolation from Insects are SDS/proteinase K based protocols (Henry *et al.*, 1990 [13, 14]; Juen and Traugott 2006) [18] and commercially available kits (Ball and Armstrong 2008 [3]; Stone *et al.*, 2007) [28]. These methods usually use adult specimens or specific tissues from thorax, head, wings or leg muscles to avoid contaminants. However, these methods are not useful for the isolation of high quality DNA due to the presence of phenolics and other plant contaminants in the digestive tract of polyphagous insects.

Isolation of DNA is difficult from the tissue in the digestive tract (Juen and Traugott 2006 [18]; Serrano et al 1999) [27], or when insects are too small to dissect them. Additionally, SDS based methods and commercially available kits tend to produce low DNA yields with short storage life from tissues rich in phenolics (Lodhi *et al.*, 1994 [22, 23]; Zidani *et al.*, 2005) [29, 30], which make them unsuitable for some molecular applications (e.g., Southern blot analysis, construction of genomic libraries, DNA fingerprinting, *etc*.). Phenolics are recognized as the major contaminants in DNA preparations from plants (Lodhi *et al.*, 1994 [22, 23]; Couch and Fritz 1990 [7, 8]; Kim *et al.*, 1997) [19, 20]. Phenolics, as powerful oxidizing agents, can reduce the yield and purity of DNA by binding covalently with the extracted DNA, thereby inhibiting further enzymatic modifications of the DNA such as restriction endonuclease digestion and polymerase chain reaction (PCR) (Lodhi et al, 1994 [22, 23]; Horne *et al.*, 2004 [16, 17]; Arif *et al.*, 2010) [2]. Higher concentrations of cetyltrimethylammonium bromide (CTAB) and the addition of antioxidants such as polyvinyl-pyrrolidone (PVP) and β-mercaptoethanol to the extraction buffer can help to remove phenolics in DNA preparations from plants (Lodhi *et al.*, 1994 [22, 23]; Kim *et al.*, 1997 [19, 20]; Horne *et al.*, 2004 [16, 17]; Chen and Ronald 1999 [6]; Li *et al.*, 2007) [21]. However, PVP usually is not used in the methods reported for DNA isolation from insects (Henry *et al.*, 1990 [13, 14]; Hill and Gutierrez 2003 [15]; Serrano et al 1999 [27]; Feeley *et al.*, 2001) [11].

We evaluated the efficacy of the traditionally used CTAB-PVPP method for isolation of DNA from the Polyphagous insect *Spodoptera frugipedra* (Lepidoptera: Noctuidae). In this study, we developed a rapid DNA isolation method by modifying several existing methods (Lodhi *et al.*, 1994 [22, 23]; Kim *et al.*, 1997 [19, 20]; Horne *et al.*, 2004 [16, 17]; Chen and Ronald 1999 [6]; Li *et al.*, 2007 [21]; Doyle and Doyle 1990) [9, 10] for Insect DNA isolation. We evaluated the quality of the DNA isolated by using Gel electrophoresis, quantifying the amount of genomic DNA by Nano Drop and also by subjecting the isolated DNA for PCR amplification using some of the mitochondrial markers.

Materials and Methods

Specimens and its collection

Larvae of *spodoptera frugipedra* were procured from NBAIR and also collected from wild population i.e. from their host plant *Zea mays* (Maize) were brought to lab conditions and were reared using artificial medium formulated by Shobha *et al.*, (2009) with slight modifications in plastic cups covered with a thin cloth for providing aeration. The development of larvae was studied in detail on daily basis till larvae pupates and emerges into adults. The body parts like thorax, legs and abdomen were separated and stored in 70% ethanol for molecular work and remaining parts were stored as voucher specimens for morphological studies.

DNA Isolation

Genomic DNA was extracted using CTAB method (Doyle *et al.*, 1990) [9, 10] used for insect material and CTAB-PVPP method (Calderon *et al.*, 2010) used for plant material with slight modification. Few samples were processed with the Samples (Larvae/Adult body parts like legs, thorax and abdomen) were homogenised using 250µl Extraction buffer (1% w/v CTAB; 1M NaC1; 100 mM Tris; 20 mM EDTA; 1% w/v polyvinyl polypyrolidone, PVPP). The buffer was prewarmed before addition to avoid CTAB precipitation. PVPP was added to the buffer just before use. The tubes were mixed by inverting them several times and then heated in a waterbath for 30 min at 70 °C before adding one volume of chloroform: isoamyl alcohol (24:1 v/v). The resulting complex was mixed by inverting the tube, and centrifuged for 5 min at 10000 g at room temperature. The upper aqueous phase was collected in a new tube and the slurry and lower layers were discarded. Two volumes of precipitation buffer (1% w/v CTAB; 50 mM Tris-HC1; 10 mM EDTA; 40 mM NaC1) were added to the supernatant and mixed well by inversion for 2 min. The mixture was centrifuged for 15 min at 13000 g at room temperature and the pellet was collected. The pellet was resuspended in 350µl of 1.2M NaC1, to which one volume of chloroform: isoamylalcohol (24:1) was added. This was mixed vigorously and centrifuged for 5 min at 10000 g at room temperature. The upper phase was removed to a new tube and 0.6 volume of isopropanol was added. This was mixed by inversion and the tube was placed at -20 °C for 15 min. The final pellet was collected by centrifugation for 20 min at 13000 g at 4 °C. To get RNA-free DNA 2µl of 10mg/m1 RNAase was added to the sample and incubated at 37 °C for 30 min before the second chloroform treatment. The final pellet was washed with 1 ml of 70% ethanol and recollected by centrifugation for 3 min at 13000g at 4°C. The pellet was drained and dried at 50°C prior to resuspension in TE buffer (10 mM Tris pH 7.4, 1 mM EDTA). Thus obtained genomic DNA was stored in -20°c until next use.

DNA Quantification

The quantity and purity of the extracted DNAs were analysed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, 2000 UK). DNA purity is the ratio of spectrophotometric absorbance of DNA at λ=260 nm and protein at λ=280 nm.

Results and Discussion

The different contaminants present in DNA isolated from biological material vary according to its origin (e.g., organism, tissue, life stage) (Aijanabi and Martinez 1997; Hill and Gutierrez 2003 [15]; Friar 2005) [12]. Therefore, the type of organism chosen and condition of specimens and tissues are the key factors in selecting for DNA isolation method. Tissues in the digestive tracts of most insects are rich in phenolics and tannins. These secondary compounds must be removed to obtain DNA free from contaminants. Phenolics and other secondary compounds cause damage to DNA and/or inhibit various activities like restriction endonucleases and Taq polymerases (Lodhi *et al.*, 1994 [22, 23]; Friar 2005 [12]; Arif *et al.*, 2010 [2]; Li et al 2007) [21]. The most conventionally used is CTAB method; it occasionally fails to remove all phenolics from DNA preparations (Lodhi *et al.*, 1994) [22, 23]. Antioxidants are commonly used to address problems related to phenolics; examples include 2-mercaptoethanol (β-Mercaptoethanol, BME, 2BME, 2-ME, orβ-met) (PVP (Polyvinylpyrrolidone), bovine serum albumin (BSA), among others (Zidani *et al.*, 2005) [29, 30]. PVP forms complex hydrogen bonds with phenolics and co-precipitates with cell debris upon cell lysis (Lodhi *et al.*, 1994 [22, 23]; Kim *et al.*, 1997 [19, 20]; Michiels *et al.*, 2003) [24]. These PVP-phenolic complexes accumulate at the interface between the organic and aqueous phases and can be eliminated from DNA preparations. On the other hand, high concentrations of β mercaptoethanol, helps to reduce the browning in DNA preparations produced by the oxidation of phenolics (Horne *et*

al., 2004 [16, 17]; Li *et al.*, 2007) [21]. To test the effect of the inclusion of PVPP and an increased concentration of β-mercaptoethanol in our DNA isolation method, we compared this method with the traditionally used CTAB method (Doyle and Doyle 1990) [9, 10]. The results indicated similar yields (~50 µg/100 mg fresh tissue) of high molecular weight DNA using both methods (Figure 1). Nevertheless, the A260/280 ratio for the CTAB method (1.21–1.32) and for the CTAB-PVP modified method (1.69–1.76) indicated a higher level of contamination in the DNA isolated by the traditional CTAB method.

DNA preparations containing contaminants have a shorter storage lifespan (Lodhi *et al.*, 1994) [22, 23]. The most common contaminants are polysaccharides, RNA and phenolics (Henry *et al.*, 1990 [13, 14]; Reineke 1998 [26]; Lodhi et al 1994 [22, 23]; Horne *et al.*, 2004 [16, 17]; Arif *et al.*, 2010 [2]; Puchooa and Venkatswamy 2005 [25]; Michiels *et al.*, 2003) [24]. Polysaccharides and phenolics usually produce highly viscous and brown-colored solutions, respectively (Henry *et al.*, 1990 [13, 14]; Couch and Fritz 1990 [7, 8]; Puchooa and Venkatasamy 2005) [25]. Given that RNA contamination is normally removed by treatment with RNase (Puchooa and Venkatswamy 2005) [25], and the isolated DNA was not viscous, it is likely that phenolics are the contaminants present in the CTAB isolated-DNA. In addition, the inclusion of PVPP and β-mercaptoethanol cleared the DNA solutions. This suggests that DNA isolated by the CTAB-PVP method had lower concentrations of phenolics compared with the traditionally used CTAB method.

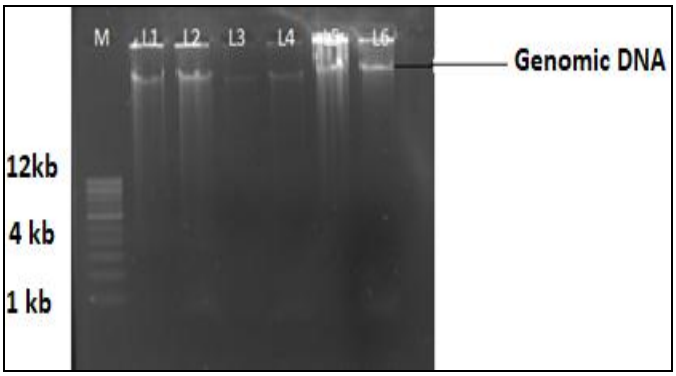

Fig 1: Agarose gel analysis of DNA prepared from *Spodoptera frugipedra* Larvae using CTAB-PVPP method of DNA isolation. M-DNA marker of 1 kb size (Invitrogen, Carlsbad, CA, USA); Lane 1 to Lane 6 genomic DNA isolated with the CTAB-PVPP method

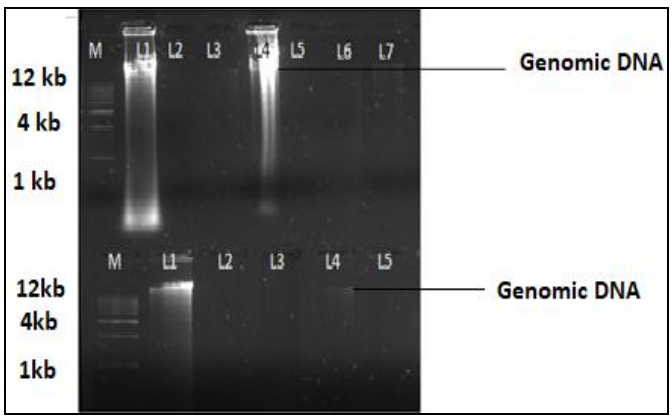

Fig 2: Agarose gel analysis of DNA prepared from *Spodoptera frugipedra* Larvae using CTAB method of DNA isolation. M-DNA marker of 1 kb size (Invitrogen, Carlsbad, CA, USA); Lane 1, genomic DNA isolated with the modified CTAB method

The isolated DNA using both methods was tested for PCR amplification. Amplifications of a mitochondrial cytochrome oxidase I (COI) gene fragment using fresh DNA obtained with both methods were successfully achieved. However, amplification of the COI gene fragment was observed only for the CTAB-PVPP isolated-DNA after the DNA samples had been stored for three months.

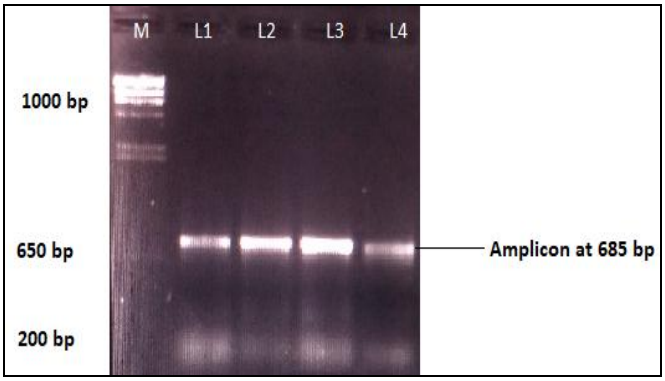

Fig 3: Amplification of a mitochondrial cytochrome oxidase I (COI) gene fragment using DNA (isolated from *Spodoptera frugipedra* larvae) that had been stored for three months. M, DNA size marker (1 Kb plus DNA ladder, Invitrogen, Carlsbad, CA, USA); Lane 1, genomic DNA isolated with the CTAB-PVPP method

These results indicate that DNA isolated by the traditional CTAB method is not suitable for longer storage periods. Similar results have been previously reported (Lodhi *et al.*, 1994 [22, 23]; Doyle and Doyle 1990) [9, 10].

Conclusions

The modified CTAB-PVP method for DNA isolation seems to be suitable for PCR analyses. This method is rapid, simple and efficient for the isolation of DNA from polyphagous insects which possess high concentrations of phenolic compounds that can interfere with DNA extraction and analysis.

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