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A modified efficient protocol for DNA extraction in Silkworm (*Bombyx mori* L.)

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Abstract
For molecular analysis of any organism a superior quality of the DNA sample is required which can be obtained from a suitable DNA extraction protocol. The present study was undertaken to isolate DNA more efficiently from silkworm larvae using liquid nitrogen. Isolated DNA was first treated with Tris-saturated Phenol followed by treatment with Phenol: Chloroform: Isoamyl alcohol (25:24:1) and Chloroform: Isoamyl alcohol (24:1) for obtaining pure DNA sample. Isolated genomic DNA from Silkworm was found suitable for identifying diverse breed Susing microsatellite markers for taking up directional breeding programmes to push up bivoltine silk production.

Keywords: Genomic DNA, silkworm larvae, microsatellite marker

Introduction
Silkworm (*Bombyx mori* L.) is a domesticated insect having been cultured for a period of over 5000 years [1] which possesses excellent characteristics as an experimental organism. It has genome size of 530 Mb broken into 28 chromosomes which is utilized as a framework genome to organize information for other Lepidopterans, which represent a diverse and important group of insect pests in agriculture [2]. There are numerous characters in all stages of silkworm that are heritable and morphological characters like body color, shell weight, cocoon weight, etc. [3]. has been traditionally used to identify a strain. The isolation of intact, high-molecular-mass genomic DNA is essential for many molecular biology applications including PCR, endonuclease restriction digestion, Southern blot analysis and genomic library construction [4-7]. The cloning of important genes requires the extraction of large insert genomic DNA. Therefore, the first and foremost step in undertaking any such molecular work is the extraction of quality DNA from the desired sample [8]. In case of insects including Silkworm, the best source of DNA for molecular study is fresh/live insects. In this context, the present study was undertaken to isolate highly pure DNA from twenty silkworm strains by modifying the genomic DNA extracation protocol given by [9, 10] and determined the suitability of DNA using SSR markers.

Materials and Methods
The experimental materials of the present investigation comprised twenty parental breeds obtained from the Division of Sericulture, SKUAST- Jammu. Thebreeds used in the present investigation included PO_1, PO_3, ND_2, ND_3, ND_5, NSP, SPO, UDHEY-1, UDHEY-2, UDHEY-3, UDHEY-4, UDHEY-5, UDHEY-6, UDHEY-7, UDHEY-8, CSR_{18}, CSR_{19}, CC_1, $SH_6$and NB_4D_2.Fresh 5th instar Silkworm larvae were collected in labeled perforated plastic polythene bags(Fig.1) and were carried to the Plant Genomics Laboratory, School of Biotechnology, SKUAST-J, Chatha for extraction of DNA. DNA was extracted by using three methods namely, DNAzol reagent method [9, 11]. However, good quality of DNA was not obtained using these methods. So, Buhroo *et al.* protocol was modified for obtaining superior quality of DNA. For this the modified protocol named as a Phenol-Chloroform method of DNA extraction was followed which yielded high molecular weight DNA

Fig 1: Fresh silkworn collection in polythene bags for DNA extraction

Suitable for genomic studies. The steps of the modified protocol are given as under

1. The whole silkworm larvae were taken in pre chilled pestles motor (Each pestle contained a single silkworm larvae) and alimentary canal was removed. Remaining larvae was crushed along with sufficient quantity of liquid nitrogen by pestle motor and silkworm larvae was ground in liquid nitrogen using a fresh pre chilled mortar till it becomes powder like material-homogenate.
2. The powdered content was transferred to fresh/autoclaved centrifuge tubes containing DNA extraction buffer (100 mMTris–HCI, pH 8.0, 50 mM EDTA 1% SDS, having 100 µg/mL Proteinase- K and 4µl RNase.
3. Sufficient quantity of lyses buffer (800µl for a single silkworm larva) was added to the samples and mixed well.
4. The content in the tubes was mixed thoroughly but slowly and the samples were incubated at 55^{o}C overnight in water bath.
5. Next day the tubes were taken out from water bath and equal volume of Tris-saturated Phenol was added using a glass pipette, the tubes were mixed gently by inverting the ends of tubes and allowed to remain at room temperature for 5 to 10 minutes.
6. The samples were centrifuged at 10,000 rpm for 20 minutes in room temperatureor 4 ^{0}C. The supernatant was collected in a clean, autoclaved centrifuge tube using 1ml pipette.
7. Equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to the supernatant obtained and step 6 was repeated.
8. Equal volume of Chloroform: Isoamyl alcohol (24:1) was added and step 6 was repeated.
9. Equal volume of chloroform was added and step 6 was repeated.
10. The supernatant was collected in sterile, autoclaved centrifuge tubes and immediately kept on -20^{0}c for 10 minute.
11. 100µl of 3M Sodium acetate (pH 5.2) and 700µl of chilled absolute ethanol was added and the samples were kept at 4 ^{o}C for overnight.
12. Next day the samples were taken from the refrigerator and the DNA (which was found floating in the tube).
13. The tubes were centrifuged at 10,000 rpm for 5 minutes and the supernatant was discarded.
14. 500µl 70% alcohol was added to the tubes and centrifuged at 10,000 rpm for 10 minutes at 4^{o}C. The supernatant was discarded.
15. The pellet was dried for about 2 hours at room temperature.
16. After the pellet was dried completely, 100µl of TE (pH 8.0) buffer was added and let to dissolve completely in the buffer so that the DNA is uniformly dissolved in the buffer.

Results and Discussion

Qualitative analysis of DNA

The isolated DNA samples were subjected to 0.8% electrophoresis through agarose gel to assess the quality of DNA isolated from the silkworm breeds under evaluation in the present study. The location of DNA within the gel was determined directly by staining with low concentration of ethidium bromide (10mg/ml) which acts as a fluorescent intercalating dye. The gel was viewed under gel documentation system (UV light) to detect the quality of DNA.

Presence of highly resolved high molecular weight sharp bands following the modified protocol indicated good quality DNA (Fig. 2). These samples which were selected for amplification, while the DNA samples showing smears and bands of low intensity following other protocols were discarded.

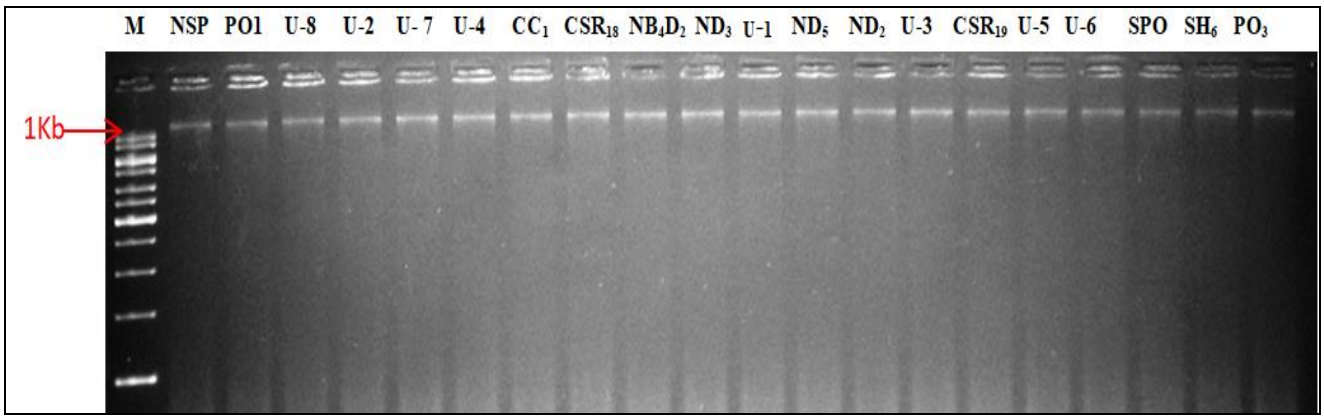


Fig 2: Genomic DNA extracted from the twenty silkworm breeds used in the study.

Quantitative Estimation of DNA

The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nmusing a UV-Spectrophotometer (Thermoscientific Inc.). Pure preparation of DNA had OD260/OD280 a value of 1.8-2.0 (Table 1).

Table 1: Quantitative analysis of silkworm breeds

S. No.	Genotype	Concentration (ng/µl)	Absorbance ($A_{260/280}$)
1	NSP	103.8	1.8
2	PO_1	345.5	1.9
3	UDHEY-8	330.4	1.6
4	UDHEY-2	639.8	1.8
5	UDHEY-7	364.5	1.8
6	UDHEY-4	307.8	1.8
7	CC_1	583.5	2.0
8	CSR_{18}	511.3	1.7
9	NB_4D_2	878.3	2.0
10	ND_3	878.2	1.9
11	UDHEY-1	438.0	1.7
12	ND_5	155.4	1.7
13	ND_2	223.7	1.8
14	UDHEY-3	387.4	2.0
15	CSR_{19}	770.7	1.8
16	UDHEY-5	73.8	1.8
17	UDHEY-6	964.6	2.0
18	SPO	412.8	1.8
19	PO_3	859.3	1.9
20	SH_6	488.2	1.8

Primer directed amplification

The amplification of DNA was carried out using standard protocol of polymerase chain reaction (PCR) with the help of microsatellite sited based 4 pairs of forward and reverse microsatellite primers namely, S0102, S0203, S0314, S0409[12,13]. The amplified products were electrophoretic ally resolved on a 3.0% agarose gel in 10X TBE buffer at 110V for 2.5 hrs. 100bp ladder was used for estimation of size of fragments. The amplified products were visualized with the help of a gel documentation system Biodoc- imaging system (Minilumi), and the size of fragments was estimated with the help of 100bp ladder (Thermoscientific).

A good quality of DNA was not obtained from given 3 methods namely, DNAzol reagent method, Sethuraman *et al.* method, and Buhroo *et al.* method. So, protocol with modification has been used for obtaining superior quality of DNA was obtained from 5thstage silkworm larva of twenty breeds (Fig.2) and amplified using microsatellite markers namely, S0203, S1019, S1904, S2019. The amplification was observed in all samples tested with little variation in intensity of the amplified bands. The $\lambda_{260}/\ \lambda_{280}$ ratio ranged from 1.6 to 2.0 indicating good quality of DNA by the method applied. After amplification S0203 produced 266-306bp, S1019 produced 218- 259bp, and S1904produced 244-295bp, S2019 produced 227-290bp, polymorphic product (Fig. 3).

The present study is concerned with the modification of the phenol chloroform method of genomic DNA extraction with by using liquid nitrogen. The crushing of silkworm under liquid nitrogen to fine powdered content which yielded high resolution DNA bands using phenol chloroform extraction method. The concentration of proteinase-K was high which extracted a high quantity of DNA. The main action of proteinase-K in extraction buffer is the removal of proteins which are attached to the DNA and use of RNase to remove RNA. SDS (Sodium dodecyl sulfate) is used in cleaning procedures, and is commonly used as a component for lysing cells during DNA extraction. The DNA obtained using this extraction protocol is suitable for polymerase chain reaction (PCR) genotyping, which can be employed for the identification of alleles in diverse genetic and breeding approaches, such as marker assisted selection, genetic fine mapping, mutant introgression, etc. Therefore, this method should be recognizing as an efficient, rapid and inexpensive method for DNA extraction from the silkworm.

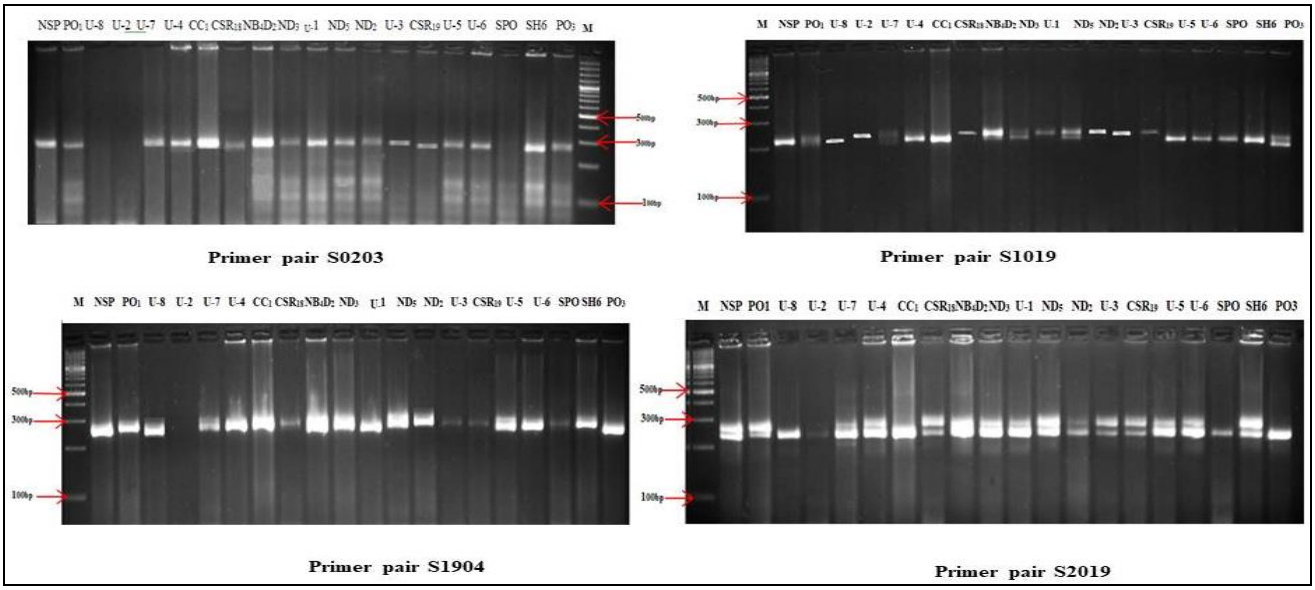
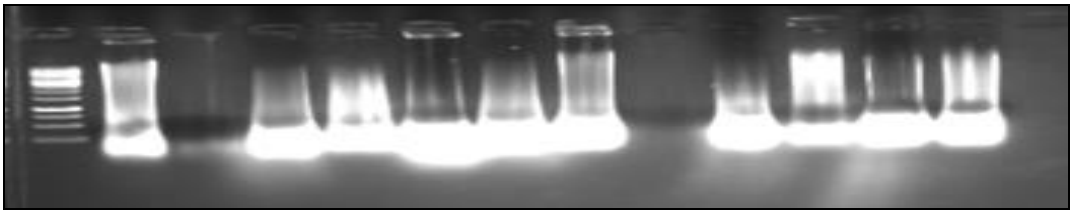
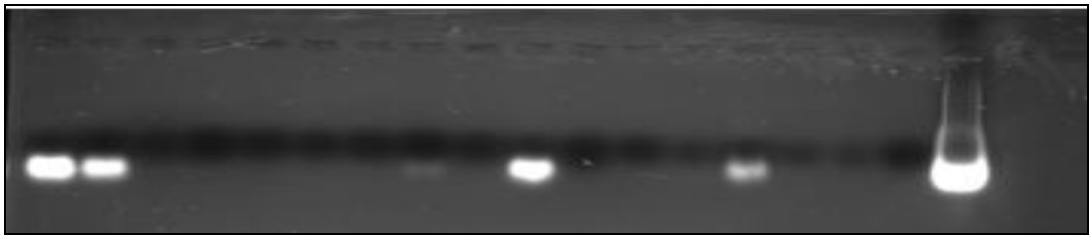


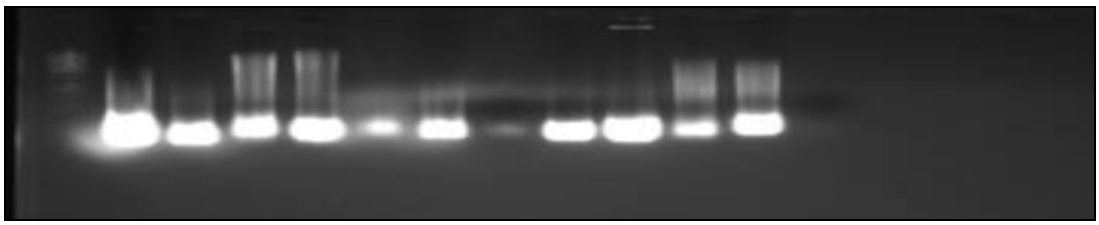
Fig 3: PCR amplification of DNA isolated from silkworm parental breeds



DNAzol Reagent Method



Sethuraman *et al*. (2002) Method



Buhroo *et al*. (2014) Method

Fig 4: These are the gel images which were obtained after following methods

Conclusion

Genomic DNA extracted with present protocol from Silkworm samples yielded good amplifiable DNA bands which were found intact and suitable for genomic studies and revealed high polymorphism among the genotypes using the SSR-PCR technique.

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