ISOLATION AND CHARACTERIZATION OF MICROSATELLITES FROM THE WILD SILKMOTH *Antheraea mylitta*

By

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B.Sc.(Ag.)

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CERTIFICATE

This is to certify that **Mr. A. Ravi Kumar** has satisfactorily prosecuted the course of research and that the thesis entitled **"Isolation and characterization of microsatellites from the wild silkmoth *Antheraea mylitta***" submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any university.

Date : (**Dr. J.NAGARAJU**)

Place: Hyderabad Major Advisor

CERTIFICATE

This is to certify that the thesis "**Isolation and characterization of microsatellites from the wild silkmoth *Antheraea mylitta***" submitted in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE IN AGRICULTURE** (**Agricultural Biotechnology**) of the Acharya N.G Ranga Agricultural University, Hyderabad, is a record of the bonafide research work carried out by Mr.A.Ravi kumar under our guidance and supervision. The subject of the thesis has been approved by the Student Advisory Committee.

No part of the thesis has been submitted by the student for any other degree or diploma or has been published. The published part has been fully acknowledged. All the assistance and help received during the course of investigation have been duly acknowledged by the author of the thesis.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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(A.Ravi Kumar)

DECLARATION

I, **A. RAVI KUMAR**, hereby declare that the thesis entitled **"ISOLATION AND CHARACTERIZATION OF MICROSATELLITES FROM THE WILD SILKMOTH *Antheraea mylitta* "**submitted to the Acharya N.G. Ranga Agricultural University for the degree of **MASTER OF SCIENCE IN AGRICULTURAL (AGRICULTURAL BIOTECHNOLOGY)** is the result of the original research work done by me. It is further declared that the thesis or any part there of has not been published earlier elsewhere in any manner.

Date: 18-03-2003

Place: Hyderabad (**A. RAVI KUMAR)**

LIST OF SYMBOLS AND ABBREVATIONS

ABI	Applied Biosystems
AFLP	Amplified Fragment Length Polymorphism
bp	Base Pair
BSA	Bovine serum albumin
CIP	Calf Intestinal Phosphatase
dd	Double distilled
DNA	Deoxyribo Nucleic Acid
dNTPs	Dinucleotide tri-phosphates
ds	Double stranded
EDTA	Ethylene Diamine Tetra Acetic acid
g	Grams
hr	Hour
ISSR	Interspersed Simple Sequence Repeats
LB	Luria-Bertani
LB^{+amp}	Luria-Bertani with Ampicillin
M	Molar
mA	Milli Ampere
mbar	Milli bar
min	Minutes
ml	Milli litre
mM	Milli molar
ng	Nanogram
nm	Nanometer
PCR	Polymerase Chain Reaction
p moles	Picomoles
PNK	Poly nucleotide kinase

QTL	Quantitative Trait Loci
RAPD	Randomly Amplified Polymorphic DNA
RE	Restriction enzyme
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulphate
SSC	Saline Sodium Citrate
SSR	Simple Sequence Repeat
STR	Short Tandem Repeat
STS	Sequence Tagged Sites
TE	Tris EDTA
U	Units
V	Volts
VNTR	Variable Number of Tandem Repeat
v/v	Volume by volume
w/v	Weight by volume
^{0}C	Degree Celcius
%	Per cent
λ	Lambda
µg	Microgram
µl	Microlitre
µM	Micromolar
5’	Five prime
3’	Three prime

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ABSTRACT

The study is aimed at establishing a standard method for the isolation of Microsatellites from the wild silkmoth *Antheraea mylitta*, ecorace Daba trivoltine. Microsatellites were isolated by the selective hybridization method in which DNA fragments containing repeat motifs specific to repeat probe used in hybridization were captured.

In the present study six biotinylated oligos of the Di-, Tri- and Tetranucleotide repeat types were used as probes in individual hybridization reactions. Genomic DNA was digested with *Sau*3A, dephosphorylated, size selected from 100-1000 bp to which Sau3A ds linkers were ligated. The linker ligated genomic digest was hybridized against biotinylated repeat oligo and eluted microsatellite enriched DNA fragments were subjected to linker digestion with *Sau*3A, purified and cloned into *Bam*H I digested pBluescript (KS^+) plasmid and transformed into INVαF competent cells.

Totally 110 white colonies were screened and the clones having insert size above 100 bp were selected and sequenced. Totally 81 clones were sequenced of which 18 were found to be positive for microsatellites. Out of 18 microsatellites 15 are dinucleotide repeats and 3 are trinucleotide repeats and no tetranucleotide repeats were captured.

CHAPTER I

INTRODUCTION

Man is always inquisitive about silk products. *SILK - The Queen of Textiles*, spells luxury, elegance, class and comfort. It withstood many a daunting challenges from other natural and artificial fibres and yet, remained the undisputed Queen of Textiles since centuries. Exquisite qualities like the natural sheen, inherent affinity for dyes and vibrant colours, high absorbance, light weight, resilience and excellent drape etc, have made silk, the irresistible and inevitable companion of the eve, all over the world.

Silk is a high value but low volume product accounting for only 0.2 % of world's total textile production. Silk production is regarded as an important tool for economic development of a country as it is labour intensive and high income generating industry that churns out value added products of economic importance.

Metaphorically, the term 'Sericulture' is synonymous with the rearing of the domesticated mulberry silkworm *Bombyx mori.* But realistically, this term encompasses the unexplored and little understood groups of sericigenous Saturniid insects in the wild backlands of India, shrouded in myths and folklore. Maintained by adivasis/ tribals around the forest ranges, this group of insects has been beckoned into the national and international markets for their commercial viability. Commonly known as vanya/wild silks, these silks are represented by the Eri, Muga and Tasar silkworms.

Wild silk generally comes in natural colours - cream, brown and gold. They are not only user-friendly but also healthy owing to their porous texture and thermal properties. All production processes are eco-friendly and do not at any stage produce chemical effluents. Tasar silk (Tussah) is coarse and copperish in colour and mainly used for furnishings and interiors. It is less lustrous than mulberry silk, but has its own feel and appeal. Tasar silk is generated by the silkworm, *Antheraea mylitta*

which mainly thrive on the food plants Asan and Arjun. In India, tasar silk is mainly produced in the states of Bihar, Jharkhand, Chattisgarh and Orissa, besides Maharashtra, West Bengal and Andhra Pradesh. Tasar culture is the mainstay for many of tribal communities in India.

The tropical tasar predominates the tasar culture in India and is represented by the wild silkworm *Antheraea mylitta* Drury. Distributed across the forest ranges of India, this species has defined itself into definite populations giving rise to distinct ecoraces. Approximately 28 such ecoraces have been identified. Earlier reports on other species have been shown to have populations with unique levels of genetic structuring (Tiedemann *et al.,* 2000). Environment, life histories, mating systems, geographical barriers seem to have a cumulative effect in shaping the genetic structure of each ecorace of the species.

One of the most recent advances in molecular genetics has been the introduction of microsatellite markers to investigate the genetic structuring of natural populations (Balloux and Moulin, 2002). Molecular markers have become essential tools for conservation biology, evolutionary and population studies as well as for mapping projects (Queller and Hughes, 1993, Jarne and Lagoda, 1996).

Microsatellites are sequences made up of a single sequence motif (1-6 bp) which is repeated tandemly. Historically, the term microsatellite has been used to describe only repeats of the dinucleotide motif CA/GT (Litt and Luty 1989, Weber and May, 1989). They are also called simple sequences repeats (Tautz, 1989) and short tandem repeats (STRs) (Edwards *et al.,* 1991). If these repeats are long enough and uninterrupted, they are excellent genetic markers due to their high level of polymorphism (Powell *et al.,* 1996).

Microsatellites are present in all organisms studied so far. Their even distribution among the genomes (Dietrich *et al.,* 1996) has one characteristic feature, that they are rarely found in the coding regions (Hancock, 1995). There are two

potential mechanisms which can explain the high mutation rates of microsatellites. The first is recombination between DNA molecules by unequal crossing-over or by gene conversion (Smith, 1976 and Jeffreys *et al.,* 1994). The second mechanism involves DNA slippage during replication (Levinson and Gutman, 1987a). Studies using yeast and *E. coli* as model organisms have shown that replication slippage seem to be the main mechanism generating length mutations in microsatellites (Levinson and Gutman, 1987b, Henderson and Petes, 1992).

The length of the microsatellite repeats may have an effect on the mutation rate such that longer repeats are more polymorphic than shorter ones (Weber, 1990; Chakraborty *et al.,* 1997; Sia *et al.,* 1997; Primmer and Ellegren, 1998; Ellegren, 2000). Microsatellite markers were first used for genetic mapping (e.g. Weissenbach *et al.,* 1992). Microsatellites can also be used to study the effects and level of inbreeding (Sweigart *et al*., 1999). The average heterozygosity of an individual measured from microsatellite data should realistically reflect the level of inbreeding.

The major challenge of microsatellites is that they need to be isolated *de novo* from most species being examined for the first time. There are several methods proposed for the isolation of microsatellites of which selective hybridization (Karagyozov *et al.,* 1993; Armour *et al.,* 1994; Kijas *et al.,* 1994) is the most successful method till now.

Silkworm strains are described based on their morphological characteristics that are highly variable and environment dependent. These limitations call for the use of reliable DNA based techniques for varietal characterization. A few studies have been carried out in *Bombyx mori* using RAPDs (random amplified polymorphic DNAs, Nagaraja and Nagaraju, 1995; Promboon *et al*., 1995; Yasukochi, 1998), RFLPs (Restriction fragment length polymorphisms, Shi *et al*., 1995), inter-SSR (Reddy *et al*., 1999a) and SSRs (simple sequence repeats Ready *et*

al., 1999b). Among all these DNA markers, SSR markers are highly informative and polymorphic.

There is no data available in wild silkmoth *Antharea mylitta* for genetic characterization. Cross amplification study using *Bombyx mori* SSR primers gave amplicons but on sequencing no repeats were found (Nagaraju *et al*., unpublished data).

From above it is evident that there is no report regarding microsatellite markers in *Antheraea mylitta* and *Bombyx mori* SSR primers did not amplify any SSR repeats although amplicons were got, despite the fact that *Bombyx mori* and *Antheraea mylitta* are supposed to be closely related insects. This indicates the different origin of *Antheraea mylitta*. This information along with the economic importance of *Antharea mylitta* makes it interesting to find new microsatellite markers.

Hence, the present study was undertaken with the following objectives:

- Isolation of microsatellites from *Antharea mylitta*
- Characterization of microsatellites.

CHAPTER II

REVIEW OF LITERATURE

In the last few years microsatellites have become one of the most popular molecular markers used with applications in many different fields. Extensive polymorphism and the relative ease of scoring are the two major features that make microsatellites of large interest for many genetic studies.

Microsatellites or simple sequence repeats (SSRs) are tandemly repeated motifs of 1-6 bases. Microsatellites are densely distributed throughout eukaryotic genomes, making them the preferred marker for very-high resolution genetic mapping (Dib *et al.,*1996 and Dietrich *et al*., 1996). Mostly they are present in coding and rarely in noncoding regions (Hancock, 1995) and are characterized by a high degree of length polymorphism. The origin of such polymorphism is still under debate though it appears most likely to be due to slippage events during DNA replication (Levinson and Gutman, 1987a; Schlötterer and Tautz, 1992). SSRs found wide acceptance and uses in diverse fields (Litt and Luty, 1989; Tautz, 1989; Weber and May, 1989) soon after their first description. Microsatellites have proven to be an extremely valuable tool for genome mapping in many organisms (Schuler *et al*., 1996 and Knapik *et al*., 1998), but their applications span over different areas ranging from ancient and forensic DNA sample studies, to population genetics and conservation/management of biological resources **(**Jarne and Lagoda, 1996).

The major challenge of microsatellites is, prior information of DNA sequence for designing primers to the flanking regions of the repeat motifs required. In the absence of such information, one has to isolate repeat units *de novo* from most of the species, as DNA sequences are not yet known for designing the primers.

Strategies for microsatellite isolation

There are different strategies of microsatellite isolation reported from the time they were isolated first with improvements in isolation protocols (Zane *et al.,* 2002). The first strategy followed for isolation was traditional method.

Traditional method

Traditional method was first reported by Rassmann *et al*., (1991), which involves the isolation of microsatellite loci from partial genomic libraries (selected for small insert size) of the species of interest, screening several thousands of clones through colony hybridization with radioactively labeled repeat-containing probes. The number of positive clones (containing microsatellites) that can be obtained by means of this traditional method usually ranges from 12% to less than 0.04%. It can be effective only in taxa with a high frequency of microsatellites.

Traditional strategies are less useful when dealing with taxa having a very low frequency of microsatellites such as birds or plants, or when a large number of microsatellites is required as in the case of studies on genetic distances between populations **(**Zhivotovsky and Feldman, 1995 and Cooper *et al*., 1999**)** or when constructing a genetic map (Liu, 1997). A number of new protocols, overcoming these limitations, have appeared in the literature in the last few years. These methods often present differences from one another with slight improvement but they have not been extensively tested. Therefore, several alternative strategies have been devised in order to reduce the time invested in microsatellite isolation and to significantly increase yield.

Primer extension method

A different strategy has been proposed for the production of libraries enriched in microsatellite loci based on primer extension (Ostrander *et al*., 1992). This method was reported to be very efficient for the enrichment of AC repeats, yielding from 40 - 50% and up to 100% of positive clones (Paetkau, 1999). One report has employed a very similar enrichment method (Takahashi *et al*., 1996).

Modifications of RAPD

To avoid library construction and screening, some authors have proposed modifications of the randomly amplified polymorphic DNA (RAPD) approach (Williams *et al*., 1990). Wu *et al*., (1994) suggested modified RAPD approach, for the amplification of unknown microsatellites, by using repeat-anchored random primers. Cifarelli *et al*., (1995) and Richardson *et al*., (1995) suggested that since RAPD fragments seem to contain microsatellite repeats more frequently than random genomic clones, unknown microsatellites could be amplified using RAPD primers followed by southern hybridization with microsatellite probe.

These methods inspired alternative strategies for the identification of single microsatellite loci. For eg; Ender *et al*., (1996), developed PCR-based strategy for finding microsatellite loci in anonymous genomes, which avoids genomic library construction, screening, and the need for larger amounts of DNA. In the first step, parts of a genome are randomly amplified with arbitrary decamer primers (RAPD) followed by Southern hybridization using radiolabeled SSR-oligonucleotides used as probes to detect complementary sequences in RAPD products. Subsequently, positive RAPD fragments of suitable size are cloned and sequenced to confirm presence of microsatellites.

Lunt *et al*., (1999), isolated microsatellites by means of Southern hybridization of RAPD profiles with repeat-containing probes, followed by the cloning of all the RAPD products and the screening of arrayed clones. This is an

improvement over the previous protocol in the sense that here they cloned all the RAPD products and not just the positive clones as in Ender *et al*., **(**1996).

Fisher *et al*., (1996), developed 'Nonlibrary' PCR-based strategies to isolate and then sequence one region flanking microsatellite repeats based on the use of repeat-anchored primers. Lench *et al*., (1996) and Cooper *et al*., (1997), developed 'Nonlibrary' PCR-based strategies to isolate and then sequence both regions flanking microsatellite repeats based on the use of repeat-anchored primers.

Selective hybridization

A further class of isolation methods is based on selective hybridization. The basic protocol proposed by Karagyozov *et al*., (1993); Armour *et al*., (1994); Kijas *et al*., (1994), is relatively straightforward. In these studies, enrichment efficiency ranges from 20% to 90% and has been successfully used in a large variety of taxa, from plants to vertebrates, using di-, tri-, and tetranucleotide probes.

Like in traditional method, the first step is DNA fragmentation followed by vector or adapter ligation. DNA is fragmented either by sonication or by digestion with restriction enzymes. The length of DNA fragments produced by sonication (Karagyozov *et al*., 1993 and Kandpal *et al*., 1994) is less dependent on genomic nucleotide composition, but requires an additional step to obtain blunt-end fragments. This is achieved by either filling overhangs with T4 DNA polymerase (end filling) or removing them with mung bean nuclease. While with restriction enzymes, the average fragment length depends on genome base composition and restriction endonuclease recognition sequence. Moreover, differences in nucleotide composition within genomes might determine unequal sampling of genomic regions. This problem seems to be negligible as several protocols ignore it (Armour *et al*., 1994; Kandpal *et al*., 1994; Kijas *et al*., 1994; Refseth *et al*., 1997). Other authors, proposed to overcome this limitation by using multiple restriction enzymes to digest genomic DNA (Hamilton *et al*., 1999). In this case, multiple digestions can be

carried out either simultaneously, which results in a smaller average size of fragments, or performed separately and then pooled together thereby producing longer fragments. The latter option is useful in obtaining fragments in the range of 200-1000 bp, ideal for successful cloning and in recovering enough flanking regions to design primers for the amplification of individual microsatellites. Size selection can be performed after the digestion step (Kijas *et al*., 1994), or after the ligation step (Kandpal *et al*., 1994). In this latter case size selection is useful in removing free linkers, if needed.

As in any ligation, optimal experimental conditions should be found in order to maximize efficiency and to minimize unwanted concatamerization (Sambrook *et al*., 2001). To this end, many possible variants have been described, ranging from cohesive-end ligation to a dephosphorylated plasmid (Kijas *et al*., 1994) to linker-mediated ligation of blunt-ended sonicated DNA into a dephosphorylated gt10 vector (Kandpal *et al*., 1994).

PCR amplification is a convenient way to obtain sufficient amount of DNA for selective hybridization, when the starting amount of material is limited. In this case however, care must be taken to avoid overamplification of digested genomic DNA, which may lead to unequal representation of genomic fragments. Before proceeding with selective hybridization, size selection of genomic DNA has to be carried out, after ligation or after ligation-amplification.

Selective hybridization is performed by using an oligonucleotide containing several tandem repeats of the motif to be enriched as a probe. The probe can be cross-linked to a nylon membrane (Karagyozov *et al*., 1993 and Armour *et al*., 1994) or can be biotinylated at the 5' end, so that DNA hybridized with the probe, can be selectively removed by using streptavidin-coated paramagnetic beads (Kandpal *et al*., 1994 and Kijas *et al*., 1994). The use of a biotinylated probe is generally preferable because in the liquid medium the probe is fully available for

hybridization. In contrast, the nylon bound probe is partially cross-linked to the membrane, and therefore hybridizes less efficiently with the target DNA. Different probe length, different hybridization and washing conditions are reported in the literature, but the effect of these differences on microsatellite enrichment efficiency has not been extensively investigated. Only one study has standardized temperatures for stringency washes (Kandpal *et al*., 1994). Both for nylon bound and for biotinylated probes a further optimization involves the use of multiple probes in the hybridization step (Edwards *et al*., 1996), which seems to increase the overall enrichment efficacy.

After selective hybridization and several washes to remove nonspecific binding, bound DNA fragments are eluted and recovered by PCR and cloned using standard methods.

Finally recombinant clones are directly sequenced or Southern blotted and probed. Alternatively, PCR screening of recombinants seems to be a good approach for mildly enriched libraries (Waldbieser, 1995). This approach involves two PCR reactions for every clone, using one primer for the vector and a second repeat-containing oligonucleotide.

CHAPTER III
MATERIAL AND METHODS

3.1 Material

3.1.1 experimental material

For the present study of microsatellite isolation from *Antheraea mylitta* ecorace Daba trivoltine, moths were obtained from Central Tasar Research and Training Institute(CTRTI), Ranchi, Bihar.

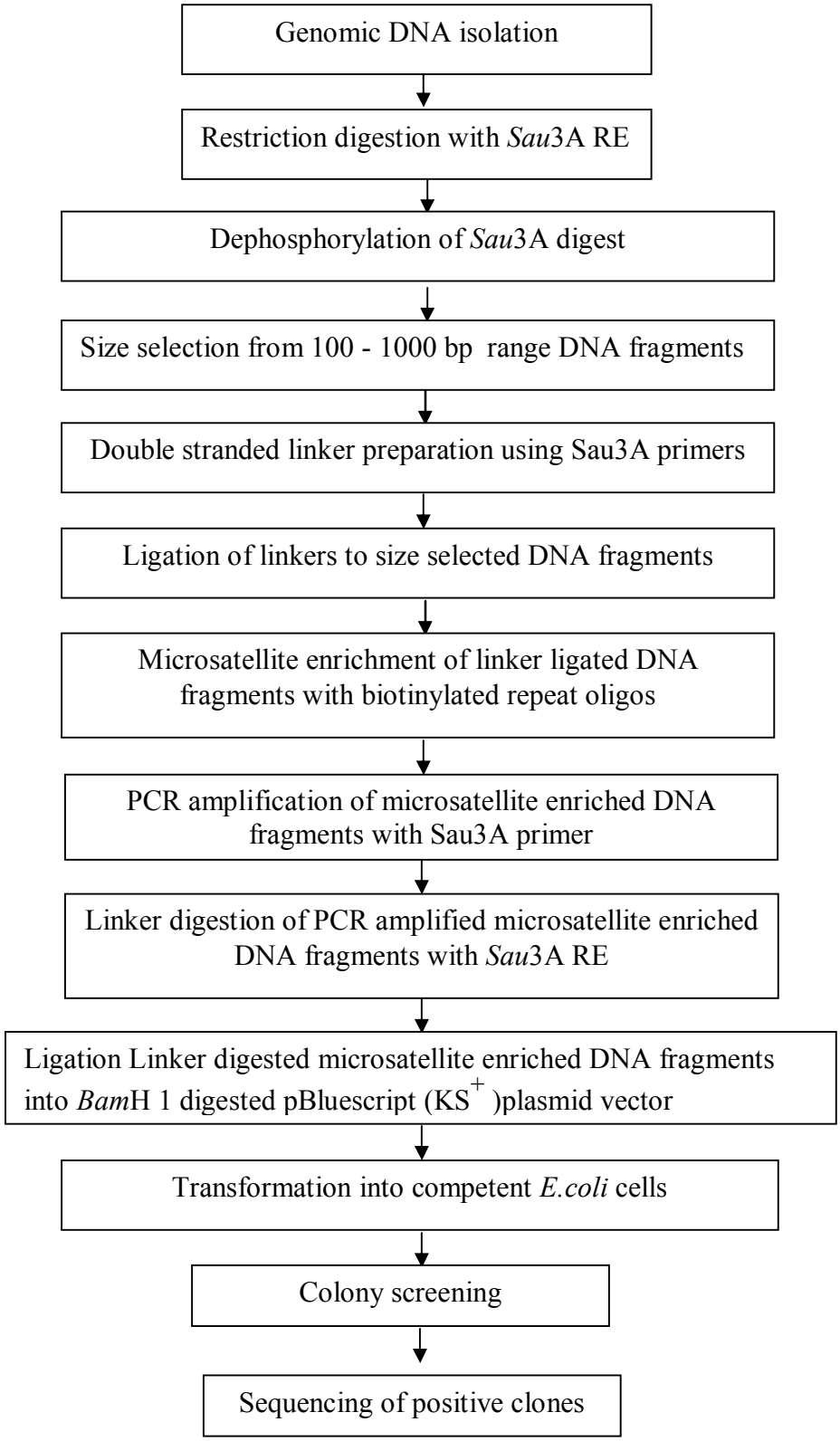
3.1.2 Primers

In the present study of microsatellite isolation two primer sets were selected, one set for preparation of ds linker named as Sau3A and Sau3B and another set of M13 universal primers for confirming the presence of insert. All these primers were obtained from Sigma. Along with these six biotinylated repeat primers of ATT, ATGA, CA, GA, GATA, CAC which are frequent in *Bombyx mori* and other insects are selected for isolation of microsatellites. The primer sequences were listed in table 1.

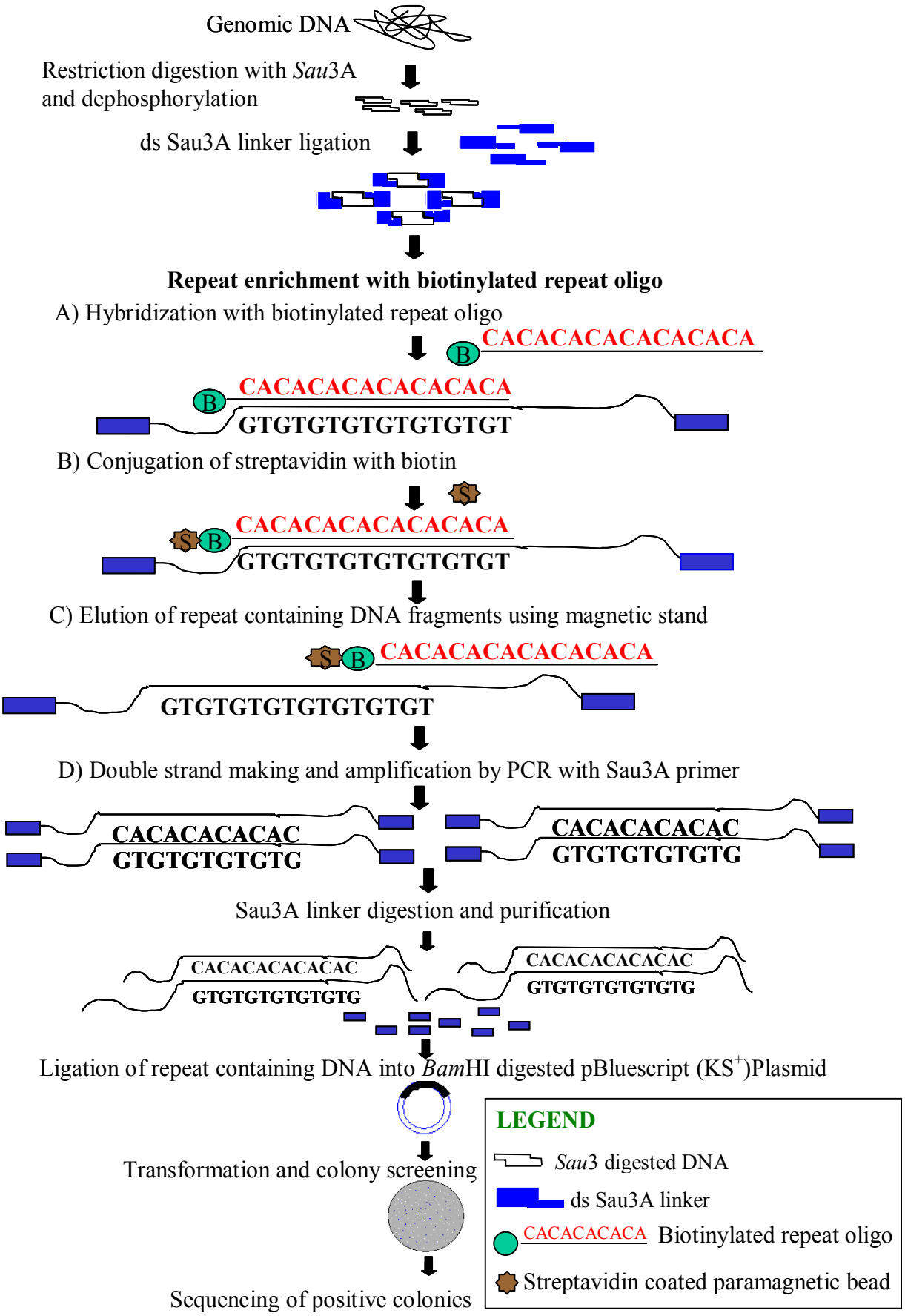
Table1: Table showing the primer sequences used in the present study

S.NO	Primer Name	Primer Sequence (5'→ 3')
1	Sau3A	GGCCAGAGACCCCAAGCTTCG
2	Sau3B	GATCCGAAGCTTGGGGTCTCTGGCC
3	M13 F	CCCAGTCACGACGTTGTAAAACG
4	M13 R	AGCGGATAACAATTTCACACAGG
5	(ATT)8	ATTATTATTATTATTATTATTATT
6	(ATGA)7	ATGAATGAATGAATGAATGAATGAATGA ATGA
7	(CA)10	CACACACACACACACACACA
8	(GA)10	GAGAGAGAGAGAGAGAGAGA
9	(GATA)7	GATAGATAGATAGATAGATAGATAGATAG
10	(CAC)7	CACCACCACCACCACCACCACCACCAC

3.1.3 Experimental design



o1.3.4 pictorial representation of Microsatellite isolation



3.2.2 METHODS

3. 2. 2 ISOLATION, DIGESTION AND SIZE SELECTION OF DIGESTED GENOMIC DNA

3.2.2.1 Isolation of genomic DNA

The silkmoths were frozen in liquid Nitrogen, ground using mortar and pestle to which extraction buffer was added. The resulted slurry was transferred into a oakridge tube to which proteinase K was added to a final concentration of 100 ug/ ml. The contents were mixed by swirling and inverting the tube and incubated at 37^0C waterbath for 2 hrs with occasional mixing of sample for every 15 min. Then equal volume of Tris-saturated phenol was added and contents were gently mixed by inverting the tube for 10 min and spun at 8000 rpm for 20 min. The clear aqueous phase separated was transferred to a new okaridge tube using cut tips to which equal volume of Phenol : Chloroform : Isoamyl alcohol (25 : 24 : 1) was added and spun at 8000 rpm for 20 min. The aqueous phase formed was transferred to a new tube to which equal volume of chloroform was added and spun at 8000 rpm for 20 min. The aqueous phase containing DNA was precipitated by adding $1/10^{th}$ volume of 3M Sodium acetate (pH 5.2) and 2 volumes of absolute ethanol. The precipitated DNA was spooled out using pipette tip and washed once with 70% ethanol, air dried and resuspended in $T_{10}\,E_1$ buffer. RNase was added to a final concentration of 100ug/ ml and incubated at 37^0C for 1 hr and stored at 4^0C. The DNA was purified by Phenol: Chloroform method as mentioned above. Finally DNA was precipitated by absolute ethanol , washed once with 70% ethanol and finally dissolved in $T_{10}\,E_1$ buffer.

3.2.2.2. Quality and quantity of extracted genomic DNA

5 ul of DNA was mixed with 1 µl of 6X loading dye and loaded on to a 1.0% agarose gel along with 300 ng of lambda *Hind* III (MBI Fermentas) digest marker

and electrophoresed at 90V for 30 min. The quantity of extracted DNA was estimated based on the intensity of lamda *Hind* III digest marker bands as the top band accounts half amount (150 ng) of total loaded amount. The quality of genomic DNA was confirmed for its integrity.

3.2.2.3 Restriction digestion with *Sau*3A restriction enzyme

For *Sau*3A digetion, 8.0 ug of genomic DNA was digested to generate DNA fragments with 5' GATC overhangs for subsequent ligation reactions. The reaction was set up for a reaction volume of 100 µl having final concentration of 1X *Sau*3A digestion buffer (NEB), 0.3U/µl of *Sau*3A, distributed 25 ul each into four 0.2 ml microfuge tubes and incubated at 37^0C for 2 hrs. After 2 hrs 5.0 µl reaction mix having a final concentration of 1.5 U/µl of Sau3A enzyme, 1X *Sau*3A digetion buffer was added and incubated at 37^0C overnight. Samples were stored at -20^0C.

3.2.2.4 Checking the digestion of genomic DNA on agarose gel

5 ul of digested sample was mixed with 2 ul of 6X loading dye loaded on 1.5% agarose gel and electrophoresed at 100 V till the bromophenol blue moves to 2/3rd along with 300 ng of 100 bp marker (MBI fermentas). The digestion was confirmed by identifying the dense smear at 100-1000 bp range.

3.2.2.5 Quantification of digested genomic DNA fragments

The quantity of digested genomic DNA/ µl in 100 µl reaction was estimated from the initial quantity of genomic DNA used in digestion.

3.2.2.6 Dephosphorylation of digested genomic DNA fragments

Dephosphorylation of digested genomic DNA fragments was done to prevent the self ligation of digested genomic DNA fragments. To carry out dephosphorylation, amount of 5' ends of DNA fragments was calculated according to the formula :

$$\text{Amount of 5' ends (picomoles)} = \frac{X}{Y \times 660 \text{ g/mole/bp}} \times \frac{10^{12} \text{ p mole/ mole}}{} \times \frac{2 \text{ ends/ molecule}}{}$$

where :

X- Mass of digested DNA fragments in grams

Y – Length of digested DNA fragments in bp

According to amounts of 5' ends obtained, 3.3 picomoles of *Sau*3A digested genomic DNA was incubated with 0.3 U/µl of calf intestinal phosphatase (NEB, USA) in a reaction volume of 60 ul having a final concentration of 1 X CIP dephosphorylation buffer in a 0.2 ml microfuge tube at 37^0C for 30 min. After 30 min, second aliquot of CIP enzyme (2.0 µl) was added and continued incubation at 37^0C for additional 30 min. Finally the enzyme was heat inactivated by adding SDS and EDTA to a final concentration of 0.5% and 5 mM respectively. The contents were mixed and proteinase K was added to a final concentration of 100 ug/ µl (by adding 0.3 ul of 20 mg/ml stock proteinase K) and incubated at 56^0C for 30 min and cooled down to room temperature. DNA was purified by extracting once with Phenol: Chloroform and once with Chloroform:IAA (24:1). DNA fragments were precipitated by adding $1/10^{th}$ volume of 3 M Sodium acetate and 2 volumes of 100% ethanol. 70% ethanol wash was given and the pellet was dried completely in vacutherm (Hereaus) at pressure of 50 mbar and at a temperature of 50^0C and dissolved in 20 ul of sterile dd H_2O and stored at - 20^0C.

3.2.2.7 Size selection of 5' dephosphorylated, digested DNA fragments

The dephosphorylated *Sau*3A digests were size selected to get fragments in the range of 100 - 1000 bp range for sequencing. Dephosphorylated DNA fragments were run on 1.5% agarose gel along with a size selection marker (Gene ruler 100 bp marker). The fragments in the 100 - 1000 bp range were cut with a sterile scalpel blade and used for gel extraction.

3.2.2.8 Gel extraction of dephosphorylated genomic DNA fragments

(AUPREP™ GEL EXTRACTION KIT METHOD)

The sliced gel containing the DNA fragment was weighed and placed in a sterile 2-ml eppendorf tube to which 0.5 ml GEX Buffer was added (0.5 ml/100mg). Gel was incubated at 60^0 C for 5 to 10 minutes until the gel got dissolved. GEL column was placed onto a collection tube to which dissolved gel solution was added centrifuged at 13K rpm for 1 min and flow -through was discarded. Column was washed once with 0.5 ml of WF Buffer, once with 0.7 ml WS Buffer by centrifuging at 13 K rpm for 60 seconds and flow-through was discarded. Column was centrifuged at full speed for another 3 min to remove residual ethanol. Then the column was placed onto a new 1.5 ml centrifuge tube and DNA was eluted from the column by adding 30 µl of dd H_2O (preheated to 90^0C) at centre of the membrane allowing it to stand for 2 min and centrifuging for 3 min at 13K rpm. Samples were stored at -20 C.

3.2.3 PREPARATION AND LIGATION OF ds Sau3A LINKERS TO SIZE SELECTED *Sau*3A DIGEST

3.2.3.1 Preparation of ds Sau3A linkers

Double stranded linkers were prepared by using Sau3A and B primers for attachment to digested and dephosphorylated DNA fragments. Sequences of Sau3 primers used in this study were,

Sau3A: 5'GGCCAGAGACCCCAAGCTTCG 3'

Sau3 B: 5'GATCCGAAGCTTGGGGTCTCTGGCC 3'

3.2.3.1 Phosphorylation of 5' end of Sau3B primer having overhang

As the primers obtained were 5' dephosphorylated, it is necessary to phosphorylate 5' end of Sau3B primer as it forms the 5' overhang and it will form the phosphodiester bond with the 3' OH group of digested genomic DNA fragment. 600 picomoles of Sau3B primer was phosphorylated in a reaction volume of 50 µl having

final concentration of 1 X T4 PNK buffer (NEB), 0.4 U/µl of T4 PNK enzyme (NEB) and incubated at 37^0 C for 2 hrs. Then finally heat killed the enzyme by incubating at 65^0 C for 20 min. Samples were stored at 4^0C.

3.2.3.2 Annealing of Sau3 A and Sau3 B primers

Double stranded linker was prepared by adding equimolar concentrations of 600 pico moles phosphorylated Sau3B primer and 600 picomoles of Sau3A primer. The reaction was carried out in 0.5 ml eppendorf tube for a total volume of 200 µl containing NaCl at a final concentration of 100 mM and heated to 95^0C for 5 min and gradually cooled down to room temperature to result in double stranded linker.

3.2.3.3 Concentration of double stranded linker

The concentration of double stranded linker was calculated according to the amounts Sau3 A and Sau3B primers (picomoles) used in linker preparation to the total volume and expressed in picomoles/µl.

3.2.3.4 Ligation of ds linker to digested DNA fragments

Ligation of ds linkers to digested DNA fragments for enrichment of DNA fragments was performed in the molar ratio of 1:10 (DNA fragments : ds linkers). The reaction was carried out in a 0.2 ml microfuge tube containing 6.0 picomoles of dephosphorylated DNA fragments, 60.0 picomoles of double stranded linker in a total reaction volume of 50.0 µl having final concentration of 1 X ligase Buffer (NEB), 50 U/µl of High conc T4 DNA ligase enzyme (NEB) and incubated at 14^0 C overnight. The samples were stored at -20^0C until further use.

3.2.3.5 Checking ligation of ds linkers by PCR with Sau3-A primer

Ligation of ds linkers to digested genomic DNA fragments was confirmed by setting PCR reaction with Sau3A primer in a reaction volume of 20 µl containing 1.0 µl of linker ligated DNA as template, 1 x PCR buffer (Perkin Elmer), 1.5 mM $MgCl_2$ (Perkin Elmer), 0.1 mM dNTPs (Amersham), 0.5 picomoles of Sau3A primer, 0.3

units of *Taq* polymerase (in house prepared *Taq* polymerase, CDFD, Hyderabad) and were run on a Gene Amp 9700 (PE) thermal cycler for 35cycles with the following conditions.

Conditions	**Temperature**	**Time**	
Initial denaturation	94^0 C	2 minutes	
Cycle denaturation	94^0 C	30 seconds	35 cycles
Cycle annealing	55^0 C	30 seconds	
Cycle extension	72^0 C	2 minutes	
Final extension	72^0 C	10 minutes	

3.2.3.6 Checking the PCR amplification by agarose gel electrophoresis

10 µl of PCR product was run on a 1.5% agarose gel along with negative control (only ligation mix without PCR) and 100 bp marker (MBI) to check the amplification and further use in enrichment.

3.2.3.7 Gel elution of PCR amplified linker ligated DNA fragments

Gel slice containing smear between 200 and 1000 bp was cut by using a sterile scalpel blade. Weight of the gel slice was measured and placed it into a sterile 2-ml centrifuge tube and eluted by Auprep protocol as mentioned above .

3.2.3.8 Quantification of gel extracted sample by spectrophotometer

The concentration of linker ligated DNA fragments was quantified by spectrophotometer (Ultraspec 2100 pro) at 260 nm wavelength.

3.2.4 REPEAT-ENRICHMENT WITH BIOTINYLATED OLIGOS

Repeat enrichment of genomic DNA fragments was done using biotinylated repeat oligos. In the current study six biotin labeled primers (listed in table2) were used and hybridization reactions were carried out individually with the six oligos at their respective hybridization temperatures (listed in Table 3).

Table 2: List of biotinylated repeat oligos used in this study

S.NO	Biotinylated repeat oligo
1	(ATT)8
2	(ATGA)7
3	(CA)10
4	(GA)10
5	(GATA)7
6	(CAC)7

Table 3: Table showing hybridization temperatures for different biotinylated oligos

Biotinylated repeat oligo	Tm (^{0}C)	Hybridization temperature (^{0}C)
(ATT)8	48	38
(ATGA)7	70	60
(CA)10	60	50
(GA)10	60	50
(GATA)7	84	74
(CAC)7	70	60

Repeat enrichment was carried out in 5 steps

3.2.4.1 Preparation of beads

20 µl of homogenized Dynabeads M-280 Streptavidin (10ug/µl) was taken in a 1.5 ml tube to which 300 µl of bead washing buffer was mixed and spun at 13 k rpm for 1 min. This step was repeated thrice and finally the beads were resuspended in 50 µl of bead washing buffer and stored at 4^0C until used**.**

3.2.4.2 Hybridization of linker-ligated genomic DNA with biotinylated repeat oligo

Hybridization reaction was carried out in a 0.2 ml microfuge tube having final concentration of 0.25 ng/µl of linker ligated DNA fragments, 1.0 pmol/µl of biotinylated repeat oligo, 0.06 X SSC, 0.001% SDS (hybridization buffer) in a total reaction volume of 100 ul, incubated at 95^0C for 5 min and quick chilled on ice for 2 min. Then it was incubated at respective hybridization temperature of biotinylated probe (Table 2) for 1 hr in thermal cycler (Perkin elmer 2400).

3.2.4.3 Conjugation of biotin - streptavidin beads

The hybridization mix was transferred into 2 ml eppendorf tube and to which 50 µl of Dynabeads M-280 Streptavidin were added and incubated at room temperature for 30 min with constant gentle agitation.

3.2.4.4 Washing

After conjugation, eppendorf tube was kept in magnetic stand and supernatant was removed. Then the bead-hybridized fragment complex was washed 3 times each for 5 min by adding 400 µl non-stringent washing buffer (1X TE + 1M NaCl). The complex was further washed with 400 µl of stringent buffer (0.2X SSC + 0.1% SDS) for 3 times each for 5 min at room temperature. After final wash, the washing solution was completely removed by keeping the eppendorf tube in magnetic stand.

3.2.4.5 Elution

After washing, 40 µl of sterile water was added, tapped gently and incubated at 95^0C for 5 min. The eppendorf tube was kept immediately in magnetic stand, supernatant (which are enriched with repeats) was pipetted out and stored at 4^0C.

3.2.4.6 Amplification of repeat- enriched DNA

A total of 5 PCR reactions for each of the six eluted samples were carried out in a reaction volume of 20 µl containing 8.0 µl of eluted DNA, 1 x PCR buffer (PerkinEelmer),1.5 mM $MgCl_2$ (Perkin Elmer), 0.1 mM dNTPs (Amersham), 0.5 p moles of Sau3A primer, 0.3 units *Taq* polymerase (in house prepared *Taq* polymerase, CDFD, Hyderabad) and were run on a Gene Amp 9700 for 35 cycles with the following thermal conditions.

Conditions	Temperature	Time	
Initial denaturation	94^0 C	2 minutes	
Cycle denaturation	94^0 C	30 seconds	35 cycles
Cycle annealing	55^0 C	30 seconds	
Cycle extension	72^0 C	2 minutes	
Final extension	72^0 C	10 minutes	

All the 5 PCR reaction products were pooled and stored at 4^0C until further use.

3.2.4.7 Removal of linkers

50 µl of post hybridized PCR product containing Sau3A linkers on both sides were digested with *Sau*3A restriction enzyme to generate 5' GATC overhangs for ligation into *Bam*HI digested pBluescript (KS^+) vector. A total of 80 µl reaction volume having final concentration of 1 X *Sau*3A digetion buffer (NEB), 1 X BSA (NEB), 0.06 U/µl of *Sau*3A restriction enzyme (NEB, USA) and incubated at 37^0C for 2 hr.

3.2.4.8 Checking the digestion

Digestion was checked by running 5 µl of digested sample along with undigested sample and lambda *Hind* III digest marker on 1.5% agarose gel.

3.2.4.9 Purification of linker digested DNA fragments

Digested samples were purified from linkers by running 80 µl on a 1.5% low melting agarose gel. The gel was cut above the thick band of adapters (40 and 80 bp) and purified by silica slurry method as explained in previous experiments.

3.2.4.10 Checking the concentrations of linker digested and purified DNA fragments

The concentration of gel purified *Sau*3A digested enriched genomic DNA was calculated spectrophotometrically at 260 nm.

3.2.5 SSR LIBRARY CONSTRUCTION AND SCREENING

3.2.5.1 pBluescript (KS^+) plasmid isolation by alkaline lysis

pBluescript (KS^+) plamid colony was picked up with a sterile toothpick, inoculated into a 200 ml LB^{+Amp} medium and incubated overnight at 37^0C incubator shaker at 250 rpm speed . The overnight grown culture was transferred into 30 ml sterile oakridge tube and spun at 4K rpm for 5 min at 4^oC. Supernatant was discarded and tube was inverted on paper towel to remove the entire supernatant. Pellet was resuspended in 1 ml of Solution I, squirted with pipette tip, vortexed thoroughly and kept in ice for 5 min, to which 2 ml of freshly prepared Solution II was added, mixed contents by gently inverting the tube for 5-6 times and kept in ice for 5 min. Then 1.5 ml of Solution III was added, inverted the tube 5-6 times gently and kept the tube in ice for 3 min. Then the tubes were centrifuged for 15 min at 13 K rpm. Supernatant was transferred into a oakridge tube to which equal volume of isopropanol was added, mixed by inverting and incubated for 60 min at room temperature. Then the contents were spun for 30 min at 13 K rpm. Supernatant was

discarded and the pellet was washed once with 70% ethanol. Pellet was dried in vacutherm and dissolved in 30 µl of dd H_2O.

3.2.5.2 Digestion of pBluescript (KS^+)vector DNA

pBluescript (KS^+)plasmid vector was digested with *Bam*H I (NEB) restriction enzyme to generate 5' GATC overhangs complementary to 5' GATC overhangs of repeat enriched DNA fragments. Plasmid DNA digestion was carried out in a 0.2 ml microfuge tube in a total reaction volume of 30.0 µl containing 3.75 ug of plasmid, having final concentration of 1 X *Bam*H I restriction enzyme buffer (NEB), 1 X BSA (NEB), 1.3 U/µl of *Bam*H I restriction enzyme (NEB). The reaction mix was incubated at 37^0C waterbath for 3 hrs.

3.2.5.3 Checking the digestion and quantity of plasmid DNA by agarose gel electrophoresis

The digestion of plasmid was checked by loading 2 µl of digested sample and undigested plasmid along with lambda *Hind* III digest marker on 1.5% agarose gel.

3.2.5.4 Purification of digested plasmid DNA

Total volume of digested sample (30.0 µl) was loaded on to a 1.0% low melting agarose gel and electrophoresed at 100 V for 30 min. The single band corresponding to the digested plasmid was cut with a sterile scalpel blade and extra gel was trimmed off. Weight of the gel slice was measured and placed in 1.5 ml eppendorf tube to which GEX buffer (AuPrep™) was added at a ratio of 1ml/500mg of gel slice. Eppendorf tube was kept in 55^0C waterbath for 5-10 min until gel got melted completely. To the melted gel 15.0 µl of silica slurry was added, mixed and kept at 55^0C for 5 min. Contents were mixed by smooth vortexing and repeated the steps of incubation at 55^0C for 5 min and vortexing three times. The contents were spun at 13 K rpm for 1 min. Pellet was resuspended in 500 µl of wash buffer, mixed, spun at 13 K rpm for 1 min and discarded the supernatant (washing step repeated

once). Pellet was air dried and resuspended in 50 µl of sterile distilled water (pre heated to 70^0C) and kept at 37^0C waterbath for 15 min with occasional mixing of pellet for every 5 min. Then spun at 13 K rpm for 1 min. Supernatant was taken out into a fresh eppendorf tube without disturbing the pellet. It was spun again at 13 K rpm for 1 min and taken out supernatant into a fresh eppendorf tube.

3.2.5.5 Checking the quality and quantity of purified plasmid DNA by agarose gel electrophoresis

The quality and quantity of purified plasmid was checked by loading 2 µl of purified sample along with lambda *Hind* III digest marker on 1% agarose gel.

3.2.5.6 Dephosphorylation of pBluescript KS $^+$ vector DNA

Dephosphorylation of digested genomic DNA fragments was done to prevent the self ligation of digested plasmid DNA. To carry out dephosphorylation, amount of 5' ends of DNA fragments was calculated according to the formula as mentioned in section 3.2.2.6.

According to calculated values, 4.0 picomoles of *Bam*HI digested plasmid DNA was incubated with 0.25 U/µl of calf intestinal phosphatase (NEB) in a reaction volume of 40 µl having a final concentration of 1 X CIP dephosphorylation buffer (NEB) in a 0.2 ml microfuge tube at 37^0C for 30 min. After 30 min, second aliquot of CIP enzyme (10 U) was added and continued incubation at 37^0C for additional 30 min. Finally the enzyme was heat inactivated by keeping at 65^0C for 15 min.

3.2.5.7 Purification of dephosphorylated plasmid DNA

To the 40 µl of dephosphorylated mixture 7.0 µl of silica slurry was added and purified by the same method as mentioned above.

3.2.5.8 Ligation of pBluescript (KS^+) vector and enriched Sau3A digest

Ligation reaction was performed individually for all the repeat enriched DNA fragments obtained by using six biotinylated repeat oligos at vector : insert molar ratio of 1**:**5 for successful ligation of all DNA fragments. The following quantities of vector and DNA fragments corresponds to 1:5 molar ratio of vector and DNA fragments .

Digested and dephosphorylated vector : 100 ng

Enriched DNA fragments : 200 ng

Ligation reaction was set up for 10 µl reaction volume having final concentration of 10 ng/µl dephosphorylated vector, 6.5 ng/µl enriched DNA fragments, 1 X T_4 DNA ligation buffer (NEB), 2.4 U/µl of T_4 DNA ligase (NEB) and incubated at 14^0C overnight. The samples were stored at - 20^0C until use.

3.2.5.9 Transformation of competent INVαF cells (Invitrogen)

Preparation of ligation mix

6 µl of ligation mix from each of six individual ligation reactions was pooled and from which 6 µl was taken for transformation into 100 µl of invitrogen competent cells.

Procedure

Ultra competent cells were thawed on ice, to which 6.0 µl of pooled ligation mix was added, tapped gently and incubated in ice for 30 min. After that the cells were given heat shock at 42^0C water bath for 80 sec, kept in ice immediately and incubated for 3 min. After that to the transformed mixture 0.9 ml of SOC medium was added and incubated at 37^0C incubator shaker for 1 hour at 250 rpm.

3.2.5.10 Preparation of LB agar plates

200 ml of LB agar was melted in microwave and allowed to cool to 50^{O}C. Ampicillin was added to a final concentration of 100 µg/ml, then media was poured into 150 mm gridded petri-dishes and allowed to solidify. Plates were stored at 4^{0}C until use.

3.2.5.11 Plating of transformation mix

Before plating of transformation mix 20 µl of 1 M IPTG and 100 µl of 100 mg/ml X-gal was spreaded over the surface of an LB Amp^{+} agar plate and allowed to absorb for 30 min at 37^{0}C prior to use. The transformation mix was spun at 6K rpm for 3 min and 800 µl of SOC medium was removed and the pellet was suspended in remaining 200 µl of SOC medium and entire 200 µl was used for plating.

3.2.5.12 Library screening

White colonies obtained were picked up with a sterile toothpick and suspended in 20 µl of dd H_2O, out of which 5 µl was used as template for colony PCR and 15 µl was used for primary culture inoculation.

3.2.5.13 Setting up Colony PCR

PCR reaction was performed in a total reaction volume of 10 µl having 5 µl of colony suspension as template, 1 x PCR buffer, 1.5 mM $MgCl_2$ (Perkin elmer), 0.1 mM dNTPs (Amersham), 0.5 picomoles of forward and reverse primers, 0.3 units *Taq* polymerase (in house prepared Taq polymerase, CDFD, Hyderabad) and were run on a Gene Amp 9600 for 35 cycles with the following conditions.

Conditions	Temperature	Time	
Initial denaturation	94^0 C	2 minutes	
Cycle denaturation	94^0 C	30 seconds	35 cycles
Cycle annealing	55^0 C	30 seconds	
Cycle extension	72^0 C	2 minutes	
Final extension	72^0 C	10 minutes	

3.2.5.14 Inoculation for primary culture

The 15 µl of colony suspension was added to 300 µl of LB Amp medium and kept at 37^0C incubator shaker at 250 rpm overnight. The colonies which are having inserts according to colony PCR results were subcultured in 5 ml of LB Amp^+ medium by adding 20 µl of primary culture and kept at 37^0C incubator shaker at 250 rpm overnight.

3.2.5.15 Plasmid isolation

Plasmids were isolated from secondary cultures by alkaline lysis method as explained in previous experiments.

3.2.5.16 Checking the quality and quantity of plasmid

Plasmids were run on a 1.0% agarose gel along with lambda *Hind* III digest marker to check the quality and quantity of plasmid.

3.2.5.17 Preparation of plasmids

Plasmids should be free from RNA for sequencing as it will interfere in sequencing. RNase A was added to a final concentration of 100 µg /ml and incubated at 60^0C for 15 min, given Phenol : Chloroform treatment and precipitated with 2 volumes of 100% ethanol and $1/10^{th}$ volume of 3M sodium acetate, washed with 70% ethanol and suspended in 20 µl of dd H_2O.

3.2.5.18 Insert PCR with M 13 universal primers

PCR reaction was performed in a total reaction volume of 10 µl having 10 ng plasmid DNA, 1 x PCR buffer (Perkin elmer), 1.5 mM $MgCl_2$ (Perkin elmer), 0.1 mM dNTPs (Amersham), 0.5 picomoles of forward and reverse primers, 0.3 units CDFD*Taq* polymerase (in house prepared *Taq* polymerase, CDFD, Hyderabad) were run on a Gene Amp 9600 (PE) for 35 cycles with the following conditions.

Conditions	**Temperature**	**Time**	
Initial denaturation	94^0 C	2 minutes	
Cycle denaturation	94^0 C	30 seconds	35 cycles
Cycle annealing	55^0 C	30 seconds	
Cycle extension	72^0 C	2 minutes	
Final extension	72^0 C	10 minutes	

3.2.5.19 Checking insert PCR products by agarose gel electrophoresis

The PCR products were run on 2.0% agarose gel at 100 V along with a 50 bp size selection marker (MBI fermentas). The sizes of the inserts were checked based on marker.

3.2.6 SEQUENCING OF POSITIVE CLONES AND SCREENING FOR MICROSATELLITES

3.2.6.1 Sequencing

Plasmids containing insert sizes of above 100 bp according to insert PCR results were selected and diluted to 100 ng/ ul and sequenced using automated sequencer (ABI prism 3700).

3.2.6.2 Screening for microsatellites

The sequences were screened for the presence of microsatellites using the software MICAS.

CHAPTER IV

RESULTS

4.1 Isolation of genomic DNA

Genomic DNA was isolated from the wild silkmoth strain Daba trivoltine and the quantity of DNA extracted estimated was approximately 600ng/ µl (Figure 1).

4.2 Restriction digestion with *Sau*3A restriction enzyme

*Sau*3A being a 4 base cutter, on an average cuts every 256 bases, if we take 6 base cutter that will cut every 4096 bases which creates problems while cloning and sequencing. That is why we chose a 4 base cutter. Secondly, this is also the expected fragments to be ligated into vector. The digestion of genomic DNA was found to be uniform smear on 1.5% agarose gel, which indicated the successful digestion of genomic DNA. As 8.0 ug of genomic DNA was used for digestion in a final volume of 100 µl, the concentration of digested DNA was approximately 80 ng/µl (Figure 2).

4.3 Dephosphorylation of digested genomic DNA fragments

4.3.1 Calculation of amount of 5' ends in DNA sample

5' ends in DNA sample were calculated using the formula as mentioned in previous section 3.2.2.6 as below

Concentration of digested DNA fragments = 80 ng/µl
Total volume of digested genomic DNA taken for dephosphorylation = 50 µl

Therefore, total quantity of digested genomic DNA in 50 µl = 50 x 80 = 4000 ng
= 4.0 ug

$$\text{Amount of 5' ends} = \frac{4000 \times 10^{-9}}{800 \times 660} \times 10^{12} \times 2$$

= 15.0 picomoles

Therefore, 50 µl of digested genomic DNA contains 15.0 picomoles of 5' ends.

4.4 Preparation of double stranded Sau3A linkers

Sau3A and Sau3B primers were used for ds linker preparation and the structure of ds linker formed with 5' GATC overhang as shown in the (Figure 3)

4.4.1 Concentration of double stranded linker

200 µl of ds linker reaction mix contains 600 picomoles of each primer. Therefore, final concentration of ds linker was 3.0 picomoles/ µl.

4.4.2 Checking ligation of ds linkers by PCR with Sau3A primer

Ligation of ds linkers to size selected *Sau*3A digest was confirmed by PCR amplification with linker specific primer Sau3A. A thick smear was formed between 200-1000 bp region which indicated the successful ligation of ds linkers to all size selected *Sau*3A digested DNA fragments (Figure 4).

4.4.3 Quantification of gel extracted linker- ligated DNA fragments by spectrophotometer

The linker ligated DNA fragments were purified and quantified and the results are shown in table 4.

Table 4: Table showing the OD value at 260 nm and the concentration of linker ligated DNA in nanograms.

OD at 260	260/280	ng/ul
0.06	1.58	25

4.5 Repeat-enrichment with biotinylated repeat oligo

The hybridization of DNA fragments with biotinylated repeat oligos was confirmed and enriched by PCR using linker specific Sau3A primer. Smear formed between 200-1000bp region indicated the successful hybridization of repeat containing DNA fragments.

4.6 Removal of linkers

Linkers were removed from repeat enriched DNA fragments using *Sau*3A restriction enzyme, a thick band was formed between 40 and 80 bp indicating the successful digestion of linkers (Figure 5).

4.6.1 Quantification of repeat enriched linker digested DNA

Linker ligated repeat enriched DNA with six repeat oligos were digested with *Sau*3A, purified and quantified by spectrophotometer. The spectrophotometer readings are given in the table 5.

Table 5: Table showing OD values and concentrations of linker digested repeat enriched DNA

S.NO	Enriched with repeat oligo type	OD at 260	260/280	ng/ ul
1.	(ATT)8	0.011	1.72	39
2.	(ATGA)7	0.010	1.61	38
3.	(CA)10	0.021	1.79	69
4.	(GA)10	0.013	1.54	44
5.	(GATA)7	0.010	1.53	38
6.	(CAC)7	0.011	1.58	39

4.7 Digestion of pBluescript (KS^+) vector DNA

pBluescript (KS^+) Plasmid was digested with *Bam*H I restriction enzyme, which linearized the plasmid giving a single band of 3.0 kb in 1% agarose gel, which indicated successful digestion (Figure 6).

4.8 Purification and quantification of Digested plasmid

Digested plasmid was purified by silica slurry method and the quantity of purified plasmid was approximately estimated to be around 125 ng/µl according to lambda *Hind* III digest marker (Figure 7).

4.9 Library screening

Library was screened for the presence of inserts by colony PCR. Totally 110 colonies were screened and among which 81colonies were found to be positive for inserts. The amplification profiles of colony PCR results were shown in figure 8.

4.10 Sequencing:

Of 81 clones sequenced 18 were found to be positive for microsatellites of di- and tri-nucleotide repeats but no tetra- nucleotide repeats were detected. The types of di and tri nucleotide repeats captured are listed in Table 6.

Table 6: Table showing the list of types of microsatellite repeat motifs captured by different types of biotinylated repeat oligos and insert sizes of clones.

S.NO	Clone name	Insert size (bp)	Repeat motif	Captured using biotinylated oligo
1.	B4	153	(TG)19	(CA)10
2.	E3	128	(GA) 29	(GA)10
3.	F6	103	(AG) 24	(GA)10
4.	H1	129	(GA) 20	(GA)10
5.	H6	----	(GTG) 9	(CAC)7
6.	I2	303	(CT) 26	(GA)10
7.	P4	123	(GT) 15	(CA)10
8.	Q8	121	(TG) 26	(CA)10
9.	S2	140	(TG)12--(TG)5	(CA)10
10.	T4	175	(CAC) 6	(CAC)7
11.	T7	152	(CAC)3---(CAC)2---(CAC)3	(CAC)7
12.	U5	----	(AG)7-----(AG)38	(GA)10
13.	V10	149	(TG) 18	(CA)10
14.	W3	146	(GA) 41	(GA)10
15.	W4	151	(TC) 23	(GA)10
16.	W5	185	(AG)16-----(AG)24	(GA)10
17.	X3	129	(TC) 26	(GA)10
18.	X9	106	(TG)23--(TG)2	(CA)10

Note : ----- : The sequencing of the clone has to be repeated to get second flanking region

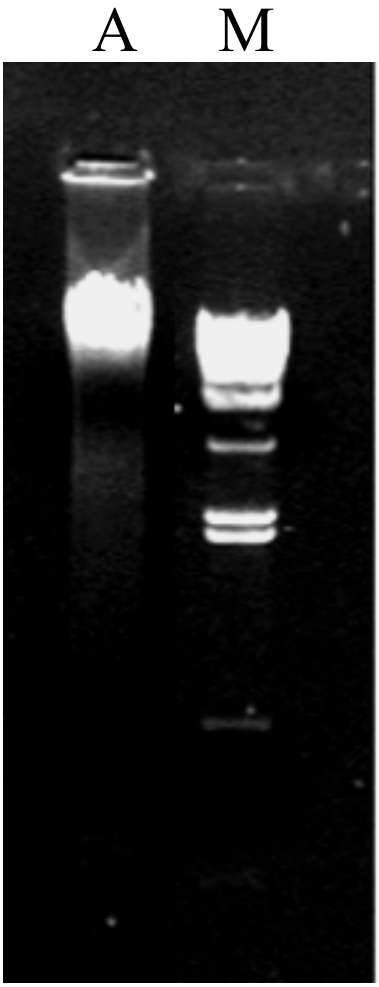


Figure1: Genomic DNA of *Antheraea mylitta*.

A: Genomic DNA (0.5 ul)

M: Lambda *Hind* III digest marker (400 ng)

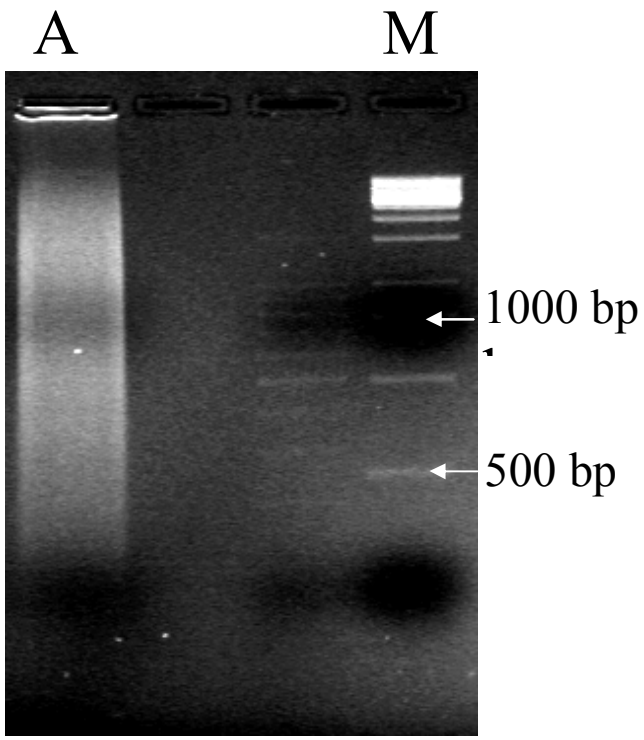


Figure 2: *Sau*3A digested genomic DNA of *Antheraea mylitta*.

A: Digested genomic DNA

M: 1kb marker (Invitrogen)

Figure 3: Double stranded Sau3A linker having 5' GATC overhang

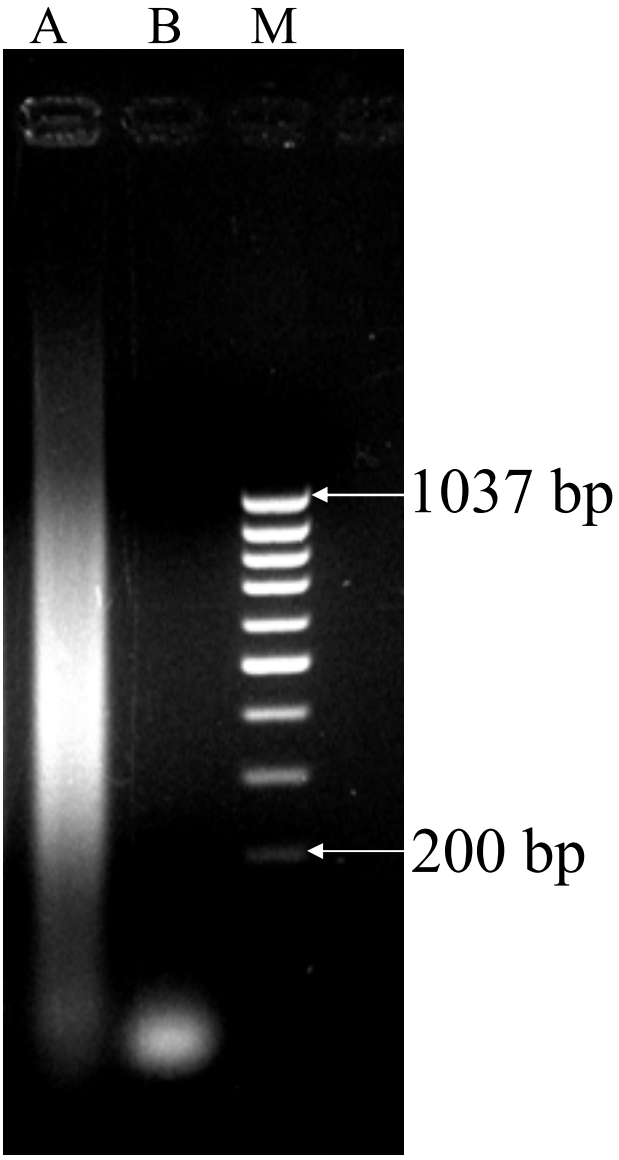


Figure 4 : Amplification of linker ligated size selected *Sau3*A digest.

A: PCR product of linker ligated DNA fragments

B: Linker ligated DNA fragments with out PCR (-ve control)

M: 100 bp marker (MBI)

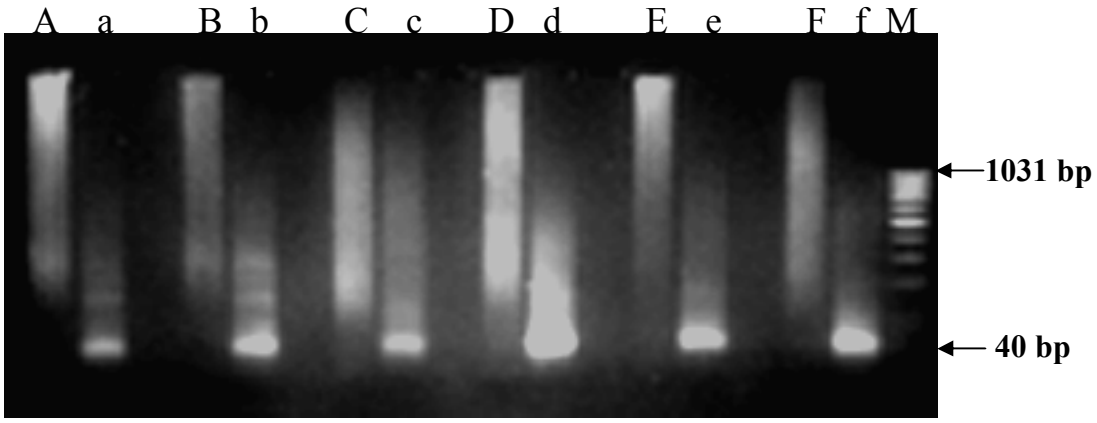


Figure 5: Digestion of ds linkers

A: DNA fragments enriched with (ATT)8 repeat oligo

a: Linker digested DNA fragments enriched with (ATT)8 repeat oligo

B: DNA fragments enriched with (ATGA)7 repeat oligo

b: Linker digested DNA fragments enriched with (ATGA)7 repeat oligo

C: DNA fragments enriched with (CA)10 repeat oligo

c: Linker digested DNA fragments enriched with (CA)10 repeat oligo

D: DNA fragments enriched with (GA)10 repeat oligo

d: Linker digested DNA fragments enriched with (GA)10 repeat oligo

E: DNA fragments enriched with (GATA)7 repeat oligo

e: Linker digested DNA fragments enriched with (GATA)7 repeat oligo

F: DNA fragments enriched with (CAC)7 repeat oligo

f: Linker digested DNA fragments enriched with (CAC)7 repeat oligo

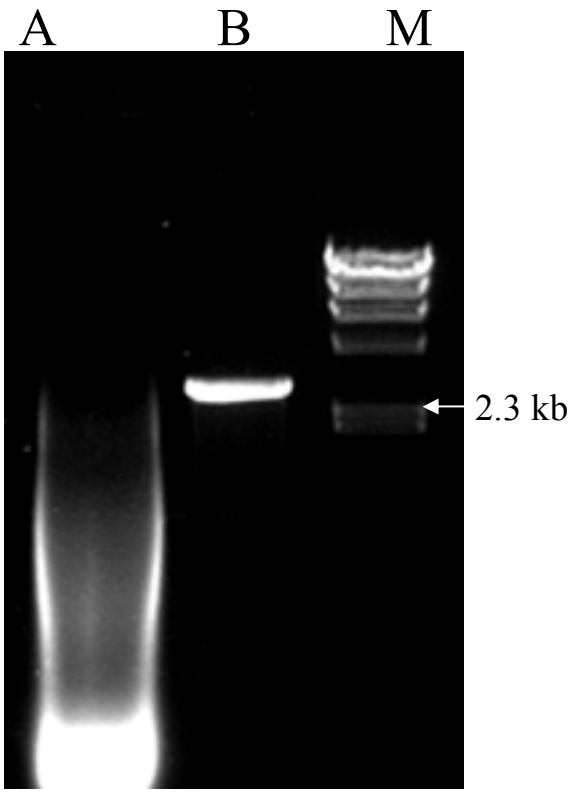


Figure 6: Restriction digestion of pBluescript (KS^+) plasmid.

A: digested plasmid

B: undigested plasmid (control)

M: lambda *Hind* III digest marker

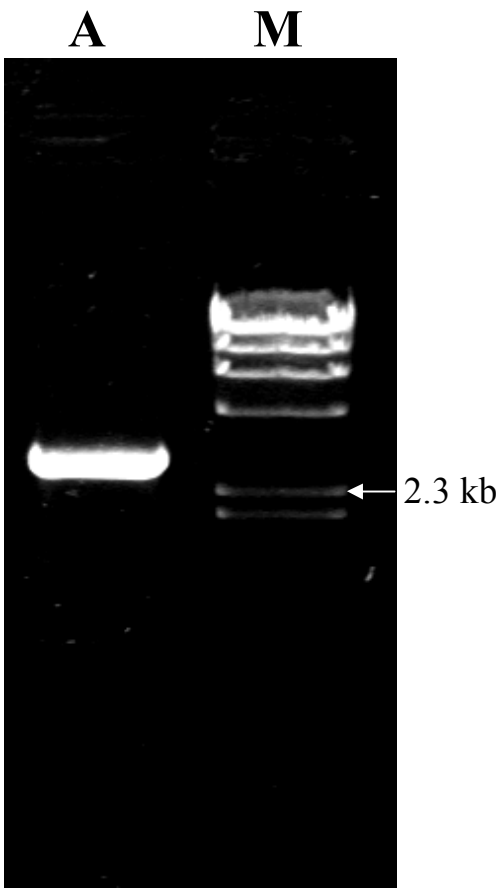


Figure 7: Purified *Bam*H I digested plasmid

A: Purified digested plasmid

M: Lambda *Hind* III digest marker

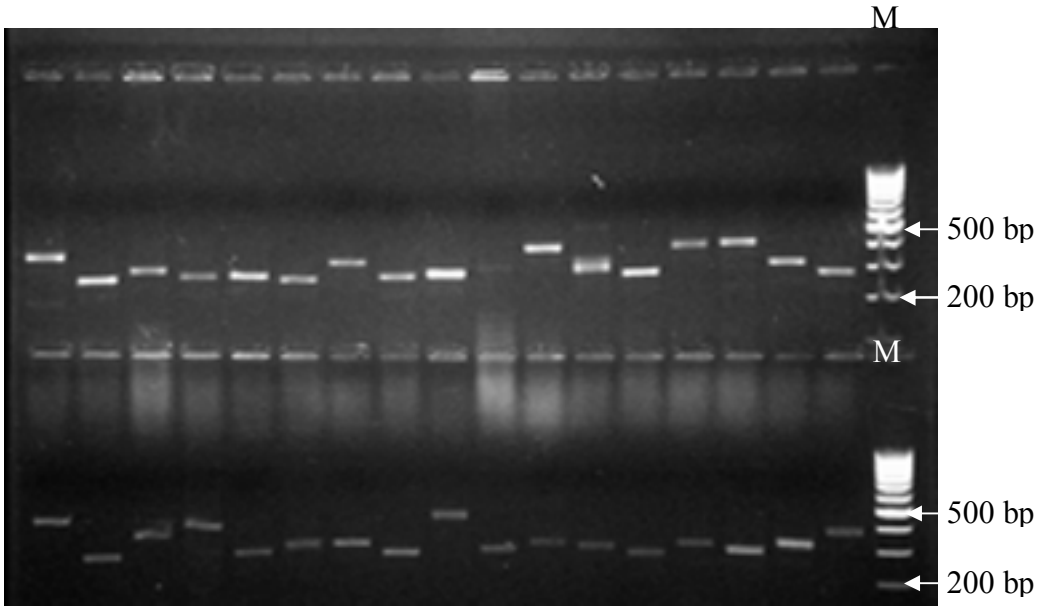


Figure 8: Amplification profile of colony PCR

M: 100 bp marker (MBI)

CHAPTER V
DISCUSSION

Marker based characterization of genomes is generally done for phylogenetic studies, gene discovery etc. In Lepidoptera, the well characterized genome of *Bombyx mori* with 1018 markers (Yasukochi, 1998) shows how marker based analysis is important in the absence of whole genome sequence information, for the same.

Towards that end, isolation of microsatellite markers, which are markers of choice in an yet another economically important Saturniid moth *Antheraea mylitta* was taken up. Studying the evolutionary dynamics of microsatellite loci between *Bombyx mori* and *Antheraea mylitta* would throw light upon the phylogenetic relationship between the two species. With the *Antheraea* species being endemic to the Indian subcontinent and different ecoraces evolving at different parts of the subcontinent, the ancestry and the relationship among the different ecoraces could be established using the microsatellite markers.

Choosing a method for microsatellite isolation is very challenging because the efficiency varies with protocol. Among the number of protocols developed on microsatellite isolation, selective hybridization is found to be most effective method. (Karagyozov *et al*., 1993; Armour *et al*., 1994 and Kijas *et al*., 1994).

Genomic DNA was extracted from silkmoth according to Sambrook *et al.,* (2001). This method is based on Phenol: Chloroform extraction method and it was found that approximately 150 ug of DNA was extracted from a single pupae and the concentration of DNA/ul was found to be 600 ng/ul.

To digest genomic DNA a four base cutter *Sau*3A was selected as it cuts on an average every 256 bases and this will aid in easy cloning and sequencing. If we take 6 base cutter that will cut every 4056 bases which will create problems while cloning and sequencing. Genomic DNA was digested and it gave a uniform smear without any discrete bands, which indicated the effective digestion of genomic DNA. The digested genomic DNA fragments were 5' dephosphorylated to prevent self-ligation of genomic digest and for effective ligation of ds linkers to both ends of digested genomic DNA fragments (Sambrook *et al.*, 2001). Dephopsphorylated *Sau*3A digest was size selected for fragments between 100-1000 bp by gel extraction. This is based on the fact that fragments of mean size of 500 bp could be easily cloned and sequenced.

In ds linker preparation linker forming primers Sau3A and Sau3B obtained were 5'dephosphorylated. The 5'GATC overhang forming primer Sau3B was 5' phosphorylated using T_4 PNK enzyme to form a phosphodiester bond between 5' phosphate group of ds linker overhang and 3' OH group of genomic DNA digest in a ligation reaction. In double stranded linker preparation equimolar concentrations of Sau3A and Sau3B primers were used in the presence of NaCl at a final concentration of 100 milli molar as it enhances the annealing between the nucleotides.

According to Fleischer and Loew (1995), sticky end ligation of ds linker to the Sau3A digest was done at equimolar concentrations of both the components. But the efficient attachment of ds linkers to dephosphorylated *Sau*3A digest was attained at a molar ratio of 1:10. The ligation of ds linker can be confirmed by PCR with linker specific primer Sau3A. The ligation of ds linker gave a thick smear between 100-1000bp. This indicated the successful ligation of ds linkers to all size selected *Sau*3A digest (Figure 3).

As both *Bombyx mori* and *Antheraea mylitta* are distantly related species and non availability of data on genome of *Antheraea mylitta*, 6 microsatellite repeat

probes were selected which are frequent in *Bombyx mori* and other insects such as ATT, ATGA, CA, GA, GATA, CAC.

The hybridization of biotinylated repeat oligo against Sau3A linker ligated genomic digest was carried out separately as it increases the efficiency of isolation at a specific hybridization temperature for each probe. This is to ensure a higher efficiency of isolation at a specific hybridization temperature for each probe. This also allows tighter control over hybridization and wash temperatures and will give more product for repeats with very different melting temperatures or repeats that are rare (Fleischer and Loew, 1995).

After hybridization and elution, the repeat containing DNA fragments with linkers obtained were single stranded. They were made into ds and enriched by PCR with linker specific primer Sau3A, for further use in linker removal, purification and ligation into vector. The enrichment of repeat containing DNA was confirmed by an intense smear on an agarose gel after PCR amplification, which indicated the successful enrichment. It was shown that all PCR products of repeat enriched DNA with six repeat probes gave intense smear.

The enriched fragments were subjected to linker removal with *Sau*3A digestion to generate 5'GATC overhangs for ligation into *Bam*H I digested pBluescript KS^+ plasmid as both *Bam*H I and *Sau*3A overhangs are compatible. Before ligation the *Bam* HI digested pBluescript KS^+ plasmid was dephosphorylated with alkaline CIP to prevent self-ligation of plasmid vector and for effective ligation of insert DNA.

The ligated products were used to transform into INVαF ultra competent cells (Invitrogen). After transformation entire 200 µl of transformation mix was plated on a LB^{+amp} agar with IPTG and X-gal plate for blue white selection (α complementation) and incubated at 37^0C for 16 hrs. Approximately 250 blue/white colonies were obtained of which 110 were found to be white colonies. These 110

white colonies were screened primarily for the presence inserts by colony PCR with M13 universal primer. Colony PCR results showed that all 110 white colonies were found to contain inserts of different sizes and 81 of which were found to be above 100 bp size. These 81 clones were selected for sequencing as sufficient flanking regions should required for primer designing. Of these 81 clones sequenced 18 were found to be positive for microsatellites. Of the 18 microsatellites captured 15 were found to be dinucleotide repeats of AG/TC, GT/CA and 3 were trinucleotide repeats of CAC/GTG and the overall efficiency of protocol was found to be 22.22%, which is quite less compared to previous reports on selective hybridization.

The following reasons could be attributed to the overall low efficiency of the protocol:

Six different repeat containing probes were used in hybridization reactions separately. Among 18 microsatellites captured all of them were captured using GA, CA and CAC repeat probes and no microsatellites were captured with the remaining ATT, ATGA and GATA repeat probes, even though PCR product of ATT, ATGA and GATA enriched DNA showed intense smear on agarose gel. This indicates the possibility of non-specific hybridization of genomic digest to ATT, ATGA and GATA repeat probes, as there is no competition between non-specific and specific DNA (repeat containing DNA) for hybridizing to repeat probe, if the genome is not having or poor in ATT, ATGA and GATA repeats.

The sequences obtained revealed several interesting features about *Antheraea mylitta* genome in comparison to available data on *Bombyx mori* genome.

a. Of the 18 microsatellites 15 were found to be dinucleotide repeats of AG/CT, GA/TC, TG/CA, indicating a possibility of the presence of higher number of dinucleotide repeats.
b. Also the microsatellites observed were predominantly of higher GC content compared to AT rich *Bombyx mori* genome.

c. The only trinucleotide repeat obtained was CAC/GTG where the probes used were CAC and ATT. This also indicates the possibility that the *Antheraea mylitta* genome is of a higher GC content compared to the *Bombyx mori* genome.
d. The above results showing a distinct difference in GC content between the distantly related species of *Antheraea mylitta* and *Bombyx mori* and it may be responsible for the poor cross amplification data on *Antheraea mylitta* using *Bombyx mori* SSR primers (Nagaraju *et al*., Unpublished data).

CHAPTER VI

SUMMARY

DNA was isolated from wild silkmoth *Antheraea mylitta* ecorace Daba trivoltine which was obtained from Central Tasar Research and Training institute (CTR&TI), Ranchi.

The genomic DNA was digested with *Sau*3A restriction enzyme, dephosphorylated and size selected from 100-1000 bp region fragments. The size selected digested DNA fragments were purified by spin column method, to which Sau3A ds linkers were ligated and used for repeat enrichment.

The linker ligated DNA fragments were hybridized with six biotinylated repeat oligos separately at their respective hybridization temperatures. The hybridized repeat containing fragments were eluted using streptavidin coated paramagnetic beads and magnetic stand.

The eluted samples were enriched by PCR with linker specific primer Sau3A. Repeat enriched and amplified DNA containing ds linkers were removed by digestion with *Sau*3A restriction enzyme and purified by silica slurry method. The purified fragments were ligated into *Bam*H I digested pBluescript (KS^+) plasmid separately.

The ligated samples were pooled in equal proportions and transformed into Invitrogen competent cells. Blue/white selection (α complementation) was done using IPTG and X-gal. White colonies were screened for the presence of inserts by colony PCR. The colonies containing inserts of above 100 bp were selected, from which plasmid was isolated and sequenced.

Totally 110 white colonies were screened, of which 81 were found to be having inserts size of above 100 bp. These 81 clones were sequenced of which 18 were found to have microsatellite inserts. Out of 18 microsatellites captured 15 were found to be dinucleotide repeats and 3 were trinucleotide repeats however no tetra nucleotide repeats were obtained. The overall efficiency of this method in this study was found to be 22.22% which is less compared to previous reports of selective hybridization.

These microsatellites have to be characterized in different ecoraces of *Antheraea mylitta* for population genetic studies for further analysis.

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APPENDIX I

LIST OF CHEMICALS

- Ethyl alcohol (Les Alcohol De Commerce Inc.)
- Agarose (USB)
- Ampicillin (Sigma)
- *BamH* I restriction enzyme (NEB)
- *Bam*H I restriction enzyme buffer (NEB)
- Bromophenol blue (Sigma)
- BSA (Bovine serum albumin) (NEB)
- CDFD *Taq* polymerase(Home made, CDFD)
- Chloroform (Qualigens)
- CIP enzyme (Calf intestinal phosphatase) (NEB)
- CIP buffer (Calf intestinal phosphatase) (NEB)
- dNTP's (Di nucleotide triphosphates) (Amersham)
- Dynabeads M-280 streptavidin (Dynal)
- EDTA (Ethylene Diamino Tetra Acetic acid) (USB)
- Ethidium bromide (USB)
- GEX gel extraction buffer (Auprep)
- Glucose (USB)
- Glycerol (Qualigens)
- IPTG (Isopropylthio-β-D-galactoside) (Sigma)
- Isoamyl alcohol (Merk)
- Isopropanol (Qualigens)
- Lamda *Hind* III marker (NEB)
- NaCl (Sodium chloride) (Qualigens)
- NaOH (Sodiun hydroxide) (Qualigens)
- Phenol (Bangalore Genei)
- Potassium acetate(Qualigens)

- Proteinase 'K' (Sigma)
- RNase A (Ribonucleases) (Sigma)
- *Sau*3A digestion buffer (NEB)
- *Sau*3A restriction enzyme (NEB)
- SDS (Sodium dodecyl sulphate) (USB)
- Silica slurry
- SOC (Invitrogen)
- T_4 PNK buffer (NEB)
- T_4 PNK enzyme (NEB)
- Tris-HCl (Sigma)
- Tryptone (Qualigens)
- WF buffer (Auprep)
- WS buffer (Auprep)
- X-gal (Sigma)
- Xylene cynol (Sigma)
- Yeast extract (Qualigens)

APPENDIX II

BUFFERS AND STOCK SOLUTIONS

Alkaline Lysis solution I

50 mM glucose

25 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Alkaline Lysis solution II

0.2 N NaOH

1% SDS

Alkaline Lysis solution III

5 M potassium acetate : 60.0 ml

Glacial acetic acid : 11.5 ml

H_2O : 28.5 ml

Ampicillin

100 mg of ampicillin was added to1 ml water and mixed.

Bead washing buffer

1 X TE

100 mM NaCl

CIP dephosphorylation buffer

100 mM NaCl

50 mM Tris-HCl

10 mM $MgCl_2$

1 mM DTT

EDTA(0.5M)

Dissolved 186.1 g of disodium EDTA .2 H_2O in 800 ml of H_2O and stirred using magnetic stirrerand adjusted pH to 8.0 with NaOH.

Ethidium Bromide

Stock 20 mg/ml can be prepared by dissolving 1 gm of ethidium bromide in 50 ml of water.

Hybridizaion buffer

6 X SSC

% SDS

IPTG: (1 M)

2.5 g of IPTG dissolved in 8 ml of distilled H_2O and. Adjusted volume to 10 ml and sterilized by passing through 0.22-um disposable filter.

LB Medium

Tryptone : 10 g

Yeast extract : 5 g

NaCl : 10 g

Adjusted pH to 7.0 with 5 N NaOH and made upto 1 litre.

Non-stringent washing buffer

1 X TE

1M NaCl

Pancreatic RNase: (20 mg/ ml)

20 mg of crude pancreatic RNase was dissolved in 1 ml of TE (pH 7.6).

Phenol :Chloroform : Isoamyl alcohol (25:24:1)

Equal parts of equilibriated phenol and Chloroform : Isoamyl alcohol (24:1) were mixed and stored at 4^{o}C.

Proteinase K (20 mg/ ml)

20 mg of proteinase K was dissolved in 1 ml of sterile 50 mM Tris (pH 8.0), 1.5 mM calcium acetate.

SOC Medium

Tryptone : 20 g

Yeast extract : 5 g

NaCl : 0.5 g

1 M Glucose : 20 ml

Made up to 1 litre with sterile water.

Stringent washing buffer

0.2 X SSC

0.1% SDS

T_4 DNA ligase buffer

50mM Tris-HCl

10mM $MgCl_2$

10mM DTT

1mM ATP

25 ug/ml BSA

TAE Buffer

For 10X stock solution

400 mM Tris base

200 mM Glacial acetic acid

10 mM EDTA

Dissolve in appropriate amount of sterile water.

TE buffer

10 mM Tris HCl

mM EDTA, pH 8.0

Tris-Cl (1 M)

121.1 g of tris base was dissolved in 800 ml of H_2O and the pH was adjusted to 8.0 and made up to 1 litre with sterile water.

Tris saturated phenol

Freezed phenol was melted at 68^oC to which equal volume of Tris-Cl (0.5 M ,pH 8.0) was added and stirred with magnetic stirrer for 15 min. The top aqueous phase was aspirated using a pipette. Then equal volume of 0.1 M Tris-Cl was added to phenol and stirred with magnetic stirrer for 15 min. The top aqueous phase was aspirated using a pipette. This was repeated till the pH reached to > 7.8. Finally the top aqueous phase was removed and to which 0.1 volume of 0.1 M Tris-Cl (pH 8.0) containing 0.2% β mercaptoethanol was added and stored at 4^oC.

X-gal solution (20 mg/ ml)

20 mg of X-gal was dissolved in dimethylformamide and filter sterilized.

3 M Sodium Acetate

408 gm of sodium acetate dissolved in 800 ml double distilled water. The pH was adjusted to 5.2 using glacial acetic acid.

6X Gel loading buffer

0.25 % (w/v) bromophenol blue

0.25 % (w/v) xylene cyanol

40 % (w/v) sucrose in water

10 X PCR buffer

500 mM KCl

100 mM Tris-Cl

10 X T4 Polynucleotide kinase buffer

700 mM Tris-Cl (pH 7.6)

100 mM $MgCl_2$

50 mM DTT

20 X SSC

175.3 g of NaCl and 88.2 g of Sodium citrate was dissolved in 800 ml of sterile water and adjusted pH to 7.4 with NaOH . Finally volume was adjusted to 1 litre.

APPENDIX - III

EQUIPMENT

- ABI Prism automated DNA sequencer (Perkin Elimer)
- Agarose Gel Electrophoresis system (Pharmacia)
- Autoclave
- Gel Documentation System (Bio-Rad)
- DNA thermal cycler (Perkin Elimer)
- Dry heating block(Bangalore Genei)
- Electronic metteler balance (Toledo)
- Freezer and refrigerator of 4^{o} C and -20^{o} C (BPL)
- Ice maker (Hoshizaki)
- Incubator shaker (37^{0}C)
- Laminar air flow (Leaf)
- Magnetic Stirrer (Janke and Kunkel)
- Magnetic strand (Dynal)
- Microwave oven (BPL)
- Microcentrifuge (Sigma)
- Mini centrifuge (Bangalore Genei)
- Pipetteman (Gilson)
- pH meter (Banglore Genei)
- UV- absorbance spectrophotometer (Unicam)
- UV- transilluminator (UVP)
- Vaccum dryer (Haraeus)
- Vaccum filter (Millipore)
- Vortex mixer (Banglore Genei)
- Water bath with thermostat (30^{o} C to 100^{o} C) (Julabo)

APPENDIX IV

SOFTWARE USED

- MICAS : Microsatellie Analysis Server (Developed by CDFD, INDIA)
  Web link : http://www.cdfd.org.in/bioinfo.html