"We often discover what will do, by finding out what will not do; and probably he who never made a mistake never made a discovery"

Samuel Smiles

Chapter - 2

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2.1 INTRODUCTION

Defoliation caused by insect outbreaks has been the focus of concern for many biologists because of its massive impact on the country’s economy. Though only very few insect species shows irruptive population dynamics, which have attracted a disproportionate degree of attention from the biologist. Population biologists have more often focused towards understanding the traits of irruptive species or the features of their environments that made them so unusual. Ever since forest started to serve as the large scale timber extraction unit, it has become a duty to mankind, to protect them and monitor them for their economical value. Therefore, spatial and temporal dynamics of the forest pests has to be studied in detail for its proper control and management.

2.1.1 TEAK AND ITS IMPORTANCE

Teak (*Tectona grandis* L.) is a member of the moist deciduous and dry deciduous forest types. It belongs to a genus of tropical hard wood trees which falls in the family Verbenaceae. Teak has its natural habitat in tropics of south and southeast Asia. In addition, it is also one of the most important plantation trees in these regions because of its economic significance. Natural distribution of teak is found in parts of India, Myanmar, Thailand, Laos and Indonesia. In India, teak forest occurs naturally in Kerala, Tamil nadu, Karnataka, Madhya Pradesh, Maharashtra, Andhra Pradesh, Gujarat, Rajasthan, Manipur, parts of Uttar Pradesh and Orissa. It is also planted and grown in many parts of Uttar Pradesh, Orissa, West Bengal, Assam, Bihar and Andaman. The first teak plantation in India was raised during the 1840’s at Nilambur in Kerala. In India, teak is spread around 9 million hectares as natural forest and 1.4 million hectares are raised in plantations (Pandey, 1983).
Teak is a highly priced, constructional as well as multipurpose timber species. The most significant advantage of teak wood is for its resistance to the attack of termites. Its wood is well known for its strength, durability, workability and hence its timber is used in the manufacture of outdoor furniture, boat decks and indoor flooring. Teak is extensively used in India to make doors and window frames, furniture, columns and beams in old type houses. Teak has natural oils that enhance the aesthetic sense of the timber.

2.1.2 Pests of Teak Plantation

Teak plantations are threatened by two major pests: *Hyblaea puera* (Cramer, 1777) and *Eutectona machaeralis* (Walker) belonging to order Lepidoptera. *H. puera* commonly known as teak defoliator feeds on the entire leaves, leaving the midribs and other major veins intact. While *E. machaeralis* feeds only on the green matter leaving all the veins intact, qualifying its name as teak skeletonizer. The teak defoliator is of a major concern since it is involved in complete defoliation of trees during the early growing season. Defoliation does not kill the trees as such, but does lead to a huge amount of timber loss. It feeds on the tender leaves during the early growing season which results in the bifurcation of the tip in younger saplings and in older ones it causes wood decrement in the girth of the stump. In 1985 it has been experimentally demonstrated that the defoliation leads to an average loss of 44% of the potential volume increment in four to nine year-old teak plantations while the *E. machaeralis* had no significant effect on the growth (Nair et al., 1985). Teak skeletonizer usually feeds on the mature leaves before it naturally falls from the trees. *H. puera* is widely distributed in the tropics: in Oriental and Australian regions (India, Sri Lanka, Burma, Java, Papua New Guinea, Northern Queensland in Australia, Solomon Island); in Central America (West Indies); and in Africa (South Africa and parts of East Africa) (Browne, 1968).
2.1.3 Biology of Teak Defoliator

*H. puera* is a nocturnal moth with a wingspan of 3 to 4 cms. It is oligophagous, but still has 29 selective host plants of which 13 belong to Verbenaceae including teak. It has been experimentally demonstrated that *H. puera* prefers teak in presence of 18 other alternate host plants (Mohanadas, 1986).

The total life cycle of *H. puera* completes within 19 to 24 days and it is merely controlled by temperature (Figure 2.1). The larval stages of *H. puera* are the most notorious in causing defoliation. These stages last for 10-12 days, which includes five instars. The neonate larva eats a shallow depression on the surface of the tender leaf and protects itself with strands of silk. The first and second instar feeds on the leaf surface; third instar cuts out the leaf flap at the edge and folds it over, fastens it with silk and feeds from inside. Fourth and fifth-instar larvae also feed from within the shelter of leaf folds. The entire leaf except the midribs and other prominent veins are left out. The fifth instar larva descends to the ground on the silken threads and pupates under a thin layer of leaf litter or soil, within loosely built cocoon made of dried leaves. The average pupal period lasts for 6-8 days at optimal conditions. The adult moth emerges and migrates to new breeding sites.

2.1.4 Teak Defoliator Outbreaks in Nilambur

Teak defoliator outbreaks are a regular annual feature in the teak plantations of Kerala, India. It is difficult to predict the exact time and place of these outbreaks. The ecological evidence gathered from the past decade on the population dynamics of *H. puera* indicates habitual, short range movements of emerging moth populations, suggesting that these spread to larger areas, generation after generation, affecting entire teak plantations (Nair and Sudheendrakumar, 1986).
Figure 2.1 - Schematic representation of life cycle of *H. puera*.
From left, Adult female moth, eggs, early larval instars, mature larva, defoliation of teak tree and pupa.
The preliminary information on the life history of *H. puera* and the nature of its damage were published by Bourdillon in 1898. It has been estimated that during the study period in Nilambur teak plantation, protected trees increased by an annual increment of 6.7 m$^3$/ha compared with 3.7 m$^3$/ha for unprotected trees, a gain of 3 m$^3$/ha per annum (Nair *et al.*, 1985). Earlier studies also indicated that the outbreaks begin as small epicenters during the pre-monsoon season then spread out (Nair and Mohandas, 1996). It was then suspected that population build-up in the early epicenters might account for the subsequent widespread epidemic. However, a study using the time lapse (developmental time) between two epidemics to determine whether an earlier epidemic was responsible for causing the subsequent outbreak showed that all subsequent outbreaks could not be attributed to previous outbreaks, thereby indicating the possibility of migrant populations being involved (Nair *et al.*, 1998). In order to address this issue the populations were classified as 'endemic', 'epicenter' and 'epidemic', based on their time of occurrence and the density of the population as represented by the area of infestation. Endemics are insects belonging to the low-density population level; epicenters are patchy, medium density outbreaks that occur during the pre-monsoon season, while epidemic represents large area, high-density outbreak population. In order to develop an appropriate control strategy it is important to understand the relationship between these population groups. If progeny of the epicenter population causes the larger epidemics, then control of these populations could prevent major outbreaks. On the other hand, if immigrant moths were involved, it would be difficult to control major outbreaks as the reasons of migration or causative factors for migration will need to be understood. Thus, understanding the cause and effect relationship between initial small outbreaks and large outbreaks that occur later in the year is crucial for the management of the pest.
2.1.5 ECOLOGICAL HYPOTHESIS ON POPULATION DYNAMICS

Past two decades of ecological observations on the migratory aspects of *Hyblaea* populations has enabled the ecologists to put forth an explanatory hypothesis. The proposed model accommodates all the observed facts on the population dynamics of the *H. puera* resulting in queries on the origin of outbreaks. During the non-outbreak period, appearance of non migratory population with continuous generations survives in the natural forest throughout the year. This endemic population accumulates and emerges into outbreak population during favorable conditions (Figure 2.2). This myth of endemic population building up into future outbreaks has not been supported by experimental data. If this concept had proven to be true, then the future outbreak in the teak plantations could be contained by controlling the endemic population. The other view is that most of the adult moths emerging from a defoliated center migrate and en masse to another site (breeding ground) where they breed and causes the outbreak. It has been experimentally estimated that at least a minimum of 4 kms distance is maintained by the moths, from the site of emergence to the site of next infestations during the outbreaks. Ecologists proposed that the sudden appearance of outbreaks and disappearance of emerged new moths from the site without producing any offsprings are considered as strong evidence to support migration. The migration occurs in *H. puera* after mating, unlike in the noctuid moths, *Spodoptera exempta*, which migrates while females are immature and unmated (Riley *et al.*, 1983). The mystery behind the migration of *H. puera* remains dark; even though suitable host trees are available in near vicinity, moth populations definitely prefer to migrate from the site of emergence. Migration seems to be a major factor in the spatial distribution of *H. puera* outbreaks.
Figure 2.2 - Schematic diagram showing the existing model of population dynamics of *H. puera* based on ecological data.

Courtesy from Nair *et al.*, 1986.
2.2 AIM OF THE STUDY

2.2.1 UNDERSTANDING GENETIC STRUCTURE OF *H. PUERA* POPULATIONS

Nilambur teak plantations often face the threat of defoliation during the pre monsoon months. These defoliations are caused by a pest, named teak defoliators (*H. puera*). What causes the accumulation of teak defoliators during specific season is not well understood. Therefore, the primary objective was to understand the genetic relationship of *H. puera* populations that are spread around the Nilambur teak plantation during the pre and post outbreak season. Subsequently, this genetic data will be validated with the ecological data collected during the teak defoliator outbreaks. To address this issue *H. puera* populations were classified into 'endemic', 'epicenter' and 'epidemic' based on their time of occurrence and the density of the population as represented by the area it infests. Genetic data regarding these three populations can provide the key to unlock the queries regarding the dynamics of the pest in the teak plantations.

2.2.2 ESTABLISHING NOVEL MOLECULAR MARKER SYSTEM (RAGEP)

There was a need for a rapid and reliable method that could discriminate the individuals based on the genomic content in a given insect population. Therefore, a novel marker system was developed having the power to differentiate the individuals from a given population that are spatially separated. This novel marker system was coined as "Randomly Amplified Gene Encoding Primers" (RAGEP). It will be used to extract mitochondrial and nuclear genomic level information. RAGEP marker system will be used to test its effectiveness in discriminating the various population groups of *H. puera*. The RAGEP markers will be experimentally demonstrated for its reproducibility, species specificity, heritability and stringency in the insect population.
2.3.1 MATERIALS

2.3.1.1 CHEMICAL AND BIOCHEMICAL REAGENTS

The chemicals and biochemicals used in the study were of molecular biology grade or extra pure analytical grade. Trizma base, Ethylene diamine tetra acetic acid (EDTA), Sodium acetate, Potassium acetate, Sodium Dodecyl Sulphate (SDS), Calcium chloride, Isopropylthiogalactoside (IPTG) were obtained from Sigma Chemicals, USA. Sucrose, Phenol, Chloroform, Isopropanol, Isoamyl alcohol, Acetic acid were obtained from SRL chemicals, India and Qualigens, India. Taq DNA polymerase was obtained from Bangalore Genei (BG), India. Big Dye Terminator DNA sequencing mixture was obtained from Perkin Elmer, Applied Biosystems, USA. The Insect Mitochondrial DNA Primer Oligonucleotide Set and Insect Nuclear DNA Primer Oligonucleotide Set were purchased from the University of British Columbia (UBC). GFX™ PCR DNA Gel Band Purification Kit, Hybond-N, dNTPs, Size standards like 100 bp ladder, 1 kb ladder were obtained from Amersham Biosciences, USA, New England Biolabs Inc., USA. Ethidium Bromide, RNase A, Proteinase K, Ampicillin, X-gal, LB broth, LB agar and Bromophenol blue were purchased from United States Biochemicals (USB), USA. UltraPure™ Agarose from Invitrogen life technologies, USA was used. Radioactive labeled $\alpha^{32}$P dCTP used in the study were obtained from Bhabha Atomic Research Centre (BARC), India.

2.3.1.2 GLASSWARES AND PLASTICWARES

Glasswares were purchased from Borosil, India and Schott-Duran GmbH, Germany. Plasticwares including centrifuge tubes (BD Falcon, USA), disposable petri plates (Axygen Scientific, Inc., USA). PCR tubes, 1.5 mL, 0.6 mL thick walled tubes (Eppendorf, Germany) and Storage bottles were from Tarsons products Pvt. Ltd., India.
2.3.1.3 INSTRUMENTS

Laboratory instruments employed for the study were Fine weighing Balance (Sartorius, Germany), High speed cooling centrifuge model Himac CR21E (Hitachi, Singapore), Cooling tabletop centrifuge model Rota 4R-V/Fm (Plastocrafts, India), Water bath (Julabo, Germany), -80°C Deep freezer (Thermo Electron Corporation, USA) and -20°C freezer (Bluestar, India). Quantification of DNA was carried out in UV-Biospec-1601 (Shimadzu, USA), Microfuge (Banglore Genei, India), Vortex mixer (SP Scientific Instruments, India), Eutech® CyberScan pH 510 (Eutech Instruments Pte Ltd., Singapore) Polymerase Chain Reaction machine (PCR) model iCycler, Power pack 300 and 200 (Bio-Rad, USA), Laminar airflow (Alpha Linear, India) Microwave oven (BPL India Ltd. India) UV Transilluminator (BG), Gel apparatus (Brovega, Balaji Scientific Services, India), Shaker cum incubator (Lab-Therm, Kühner, Switzerland). Sequencing was done in ABI 3730 DNA Analyzer (Applied Biosystems, Perkin Elmer, USA). Southern hybridization was done in XTRON HI2002 (Bartelt instruments), UV cross linker (Hoefer, USA), Geiger-Müller counter (Ludlum measurements Inc., USA). Molecular Imager®FX and Personal Fluor-S™Multilmager are the imaging system from (Bio-Rad, USA).

2.3.1.4 BACTERIAL STRAINS

- JM109: (endA1, recA1, gyrA96, thi, hsdR17 (rK-,mK+), relA1, supE44, Δ(lacproAB),[F',traD36, proAB, lacFZΔM15]) from Promega, USA.

2.3.1.5 VECTORS

The pGEM®-T Easy vector from Promega was used to clone the species-specific bands from the RAGEP fingerprints. pGEM®-T Easy vector system consists of ready to use linearized pGEM®-T Easy vector
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DNA, T4 DNA ligase (3 U/µL), 2 X Ligation buffer (30 mM Tris-HCl pH 7.8, 10 mM MgCl₂, 10 mM DTT and 1 mM ATP).

2.3.2 METHODS

2.3.2.1 STUDY AREA

Nilambur teak plantation is the oldest teak plantation in the country which is known to be established in the year 1842. The study covers an area of around 1000 hectares which is surrounded by natural borders like hillocks and streams. Based on the spatial pattern of infestation in the past, the teak plantation area was divided into convenient observation units of approximately 50 ha, separated by natural boundaries of streams, roads and footpaths. The canopy of teak is continuous within in the observation area. Each area was monitored every 15 days, precisely based on the life cycle of *H. puera*.

2.3.2.2 SAMPLE COLLECTION

Larval samples were collected from the infestation sites. Whenever fifth instar larvae were available, ten larvae were preserved in 70 % alcohol and stored in deep freezer at -20°C. The remaining larvae were reared to next generation for monitoring future outbreaks. If only early instars were available, i.e., third or fourth instar, they were reared up to 5th instars in the laboratory. Each sample was assigned a code number containing the relevant details of Year / Month / Date / Block / Grid / Generation for future reference. Using the duration of each instar (egg - one day; 1st and 2nd instars - two days each, 3rd to 5th instars - three days each; pre-pupa - one day and pupa - four days), the temporal data on outbreaks were examined to see whether each subsequent epidemic could be explained on the basis of a previous outbreak. The details of location of pest incidences and extent of infestation were later transferred to the field map in order to understand the spatial pattern of infestation (Figure 2.3).
Figure 2.3 - Map of Nilambur teak plantation showing distribution of the endemic, epicenter and epidemic population of *H. puera*. 
2.3.2.3 Suspect Categorization

The suspect populations were categorized based on the moth's lifespan i.e., 21 days ± 2 days. One part of the field collected larval samples was stored for current study and the other part was reared in the laboratory by giving an artificial diet for forecasting future outbreaks. Five endemic populations, twenty-six epicenter populations and seven epidemic populations from the year 2002 outbreaks were included in the study (Table 1.1). Earlier ecological studies had indicated that outbreaks begin as small epicenters in February during the pre-monsoon season and ends by June. Endemic samples were collected throughout the year based on their stray occurrences in various life stages, while epicenter samples from each aggregated patch were collected only from the insects that attained the same stage of its life cycle at the time of collection in that patch. Similarly, the epidemic samples were also collected from insects representing the same life stages at the time of collection from each aggressive patch. The temporal relationship between the endemic population and the epicenter populations with the large scale epidemics were first worked out. The larval samples that were geographically close and had a difference of one complete life cycle stage between the population groups were subjected to molecular studies to evaluate their relatedness.

2.3.2.4 Genomic DNA Isolation

Genomic DNA was isolated from individual larval samples which were preserved in 75% ethanol. DNA isolation was carried out with minor modification in rapid isolation of total DNA from individual insects for restriction digestion according to Andrew and Gary (1995). The insect samples were ground in presence of Liquid nitrogen in 1.5 mL eppendorf tube using sterile micro pestle (Tarson). To this powdered samples 500 µL Lifton buffer [(0.2 M sucrose, 0.05 M EDTA, 0.1 M Tris, 0.5% SDS (pH 9.0)]
and Proteinase-K (20 mg/mL)] was added and incubated for 2 hrs at 55°C in water bath by inverting the tubes every 30 mins. A volume of 75 µL of 8 M potassium acetate was added to each tube, vortexed, briefly spun and incubated in ice for 30 mins. The samples were spun at 14,000 rpm for 15 mins at 4°C in refrigerated microcentrifuge and the supernatant was collected in a fresh eppendrof tube. To this supernatant 10 µL of RNase A (10 mg/mL) was added and incubated for 42°C for 1 to 2 hrs in water bath. Post RNase incubation, the DNA was isolated by phenol-chloroform method. An equal volume of 300 µL of Tris-saturated phenol (pH 7.5) and 300 µL of 24:1 chloroform isoamyl alcohol was added to each tube and the tubes were gently inverted several times. The tubes were spun for 5 mins at 12,000 rpm. The upper aqueous phase was removed carefully without disturbing the lower organic phase to a fresh eppendrof tube. To this tube, again 400 µL of 24:1 chloroform-isoamyl alcohol was added. The mixture was gently inverted several times and spun at 12,000 rpm for 5 mins. The aqueous phase was removed to another fresh tube and 50 µL of 3 M sodium acetate (pH 4.8) was added. To this aqueous phase, 95% of ice cold ethanol was added and kept overnight at -20°C. The precipitated DNA was spun down at 14,000 rpm for 15 mins and the supernatant was decanted. The pellet obtained was washed twice with 70% ethanol by centrifuging at 14,000 rpm for 5 mins. The DNA pellet was air-dried and resuspended in TE buffer and stored in -20°C.

2.3.2.5 Quantitation of DNA

DNA was quantitated by spectrophotometric analysis. The stock DNA was quantified using a spectrophotometer at 260 nm (Shimadzu). The concentration of DNA in 1 µL of the DNA solution was calculated using the following equation.

\[
\text{Concentration (µg/mL)} = \frac{50 \times \text{Optical Density (OD)} \times \text{dilution factor}}{1000}
\]
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The quality of the DNA was checked spectrophotometrically by taking the absorbance ratios at 260/280 nm. The absorbance ratios of 1.6 - 1.8 were considered good quality DNA, free from RNA and protein contamination. The main stock was diluted to make a working stock of 100 ng/µL concentration.

2.3.2.6 POLYMERASE CHAIN REACTION

Good quality DNA was subjected to both mitochondrial and nuclear RAGEP PCR amplifications. A preliminary RAGEP screening with 50 insect specific nuclear primers (UBC) and 37 insect specific mitochondrial primers (UBC) were carried out. Primers were evaluated for their extent of polymorphism and reproducibility. Only 11 nuclear (Table 1.2) and 11 mitochondrial primers (Table 1.3) were selected for the final study based on their reproducibility. Each PCR was performed in a total volume of 30 µL. Each reaction consisted of 1 X Taq buffer with 1.5 mM MgCl₂, 1.2 U of Taq polymerase (BG), 0.25 mM of dNTPs (Amersham) and 12 pmols of primer (UBC) per reaction. Amplifications were performed in a thermocycler (Bio-Rad) and programmed as follows: initial denaturation at 95°C for 5 mins, followed by 45 cycles of cycle denaturation at 94°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 2 mins, and final extension at 72°C for 5 mins and held at 4°C.

2.3.2.7 AGAROSE GEL ELECTROPHORESIS

The RAGEP amplicons were separated using 1.2% agarose gel in 0.5 X TBE buffer. Gel loading dye (10 X contain 0.25 gms of Bromophenol blue and Xylene cyanol in 30% glycerol in water) 3 µL was added to the 30 µL PCR product of which 15 µL was loaded in agarose gel. The electrophoresis was carried out at constant 80 volts. When the bromophenol blue reached 3/4th of the gel, the electrophoresis was stopped and stained with ethidium bromide (0.5 µg/mL). The stained gel
was imaged in Fluor-S™ multi imager system (Bio-Rad) using Quantity one software module. The molecular sizes of each band were estimated by comparing with a co-migrating 100 bp ladder (Amersham).

2.3.2.8 Cloning Species-Specific Bands

The RAGEP fingerprints were separated in 1.2% agarose gel and the bands were excised from the agarose gel using a sterile scalpel blade under a low intensity UV light. The DNA fragment was eluted from the gel using GFX gel purification column (Amersham) according to manufacturer’s instructions given below.

2.3.2.8.1 Elution of DNA from Agarose Gels

The GFX™ PCR DNA and Gel Band Purification Kit (Amersham) was used for the purification. Elution was done as per the manufacturer's instructions. The DNA band to be eluted was excised out from the agarose gel with a scalpel blade under a low intensity UV light. The excised band was made into smaller pieces and transferred into a 1.5 mL eppendorf tube. The weight of the gel band was measured and Capture buffer was added in the ratio 10 µL of buffer for each 10 mg of gel slice. The sample was mixed vigorously by vortexing and incubated at 60°C until the agarose dissolved completely (approximately 15 minutes). The tube was then centrifuged briefly and the supernatant was transferred to a GFX column placed inside a collection tube. This was incubated at room temperature for 1 min and then centrifuged at maximum speed in a microcentrifuge for 30 secs. The flow through was discarded from the collection tube and 500 µL of wash buffer was added to the GFX column. This was centrifuged for 3 mins at maximum speed in a microcentrifuge. The column was transferred to a fresh 1.5 mL tube and 30 µL of sterile de-ionised water was added onto the glass fibre matrix. The sample was incubated at room temperature for 1 min and centrifuged at maximum speed for a min. The concentration
of the eluted DNA was checked by agarose gel electrophoresis and quantified using spectrophotometer.

2.3.2.8.2 LIGATION

The purified RAGEP products were ligated into pGEM®-T Easy plasmid vector according to manufacturer’s instructions. The ligation was carried out in a volume of 10 µL with 1 X ligation buffer, 50 ng of PCR product, 50 ng of linearized pGEM®-T Easy vector DNA and 3 U of the T4 DNA ligase enzyme. Ligation mix was incubated overnight at 4°C and products were transformed into competent JM109 strain of *E. coli* cells.

2.3.2.9 COMPETENT CELL PREPARATION

Competent JM109 strain of *E. coli* cells were prepared by Calcium chloride method as described by Sambrook *et al.* (1989). A single bacterial colony was inoculated into 5 mL of LB broth and incubated overnight at 37°C. From the overnight culture, 500 µL was inoculated into 50 mL of fresh medium and grown at 37°C in a shaker cum incubator till the optical density reached OD₆₀₀. The log phase culture was pelleted at 5000 rpm for 10 mins at 4°C. The supernatant was decanted and the cell pellet was resuspended in 10 mL of freshly prepared ice-cold CaCl₂ (100 mM) solution. The cell suspension was pelleted again at 5000 rpm for 10 mins at 4°C. The supernatant was decanted and the cell pellet was once again resuspended in 5 mL of ice cold CaCl₂ (100 mM) and incubated for 30 mins. This cell suspension was pelleted and this pellet was once again resuspended in 1 mL of CaCl₂ (100 mM) and 666 µL of 50% glycerol. The resuspended cells were aliquotted into 100 µL and stored at -80°C. The cells were then checked for viability, contamination and competency before use.
2.3.2.10 Transformation

A single vial of competent cell was thawed on ice for 5 mins. A 5 µL of ligation mix was added into the vial, mixed gently by flicking and kept on ice for 20 mins. The cells were given heat-shock at 42°C for 60 secs and immediately snap cooled. The cells were kept for recovery by adding 400 µL of LB medium and were incubated at 37°C with 150 rpm shaking for 1 hr. Around 150 to 200 µL of culture were plated in duplicates on LB agar plates supplemented with ampicillin (100 mg/mL), X-gal (40 µg/mL) and IPTG (0.1 mM). The plates were incubated at 37°C overnight in an incubator. The positive clones were selected by blue-white screening and the plasmids were isolated from the white colonies.

2.3.2.11 Screening of Recombinants

White colonies where picked from the transformed plate and inoculated in LB broth containing ampicillin (100 mg/mL) medium and grown for 4 hrs at 37°C with 200 rpm in a shaker cum incubator. The cultures were screened by PCR with the T7 and SP6 universal primers which flank the multiple cloning site of the pGEM®-T Easy vector. The negative colony i.e., the blue colony was also taken as a positive control for PCR. The colony PCR using T7 and SP6 primers yield a 150 bp PCR product in negative clones and in the positive clones size will be insert + 150 bp product of MCS. PCR positive clones were used for plasmid DNA preparation.

2.3.2.12 Plasmid Isolation

Plasmid isolation was done as per Birnboim and Doly (1979) with minimal modifications. The 10 mL of overnight E. coli culture was pelleted by centrifugation at 10,000 rpm. The bacterial pellet was thoroughly resuspended in 200 µL of TEG buffer. The cells were lysed by adding 300 µL of lysis buffer (0.2 M NaOH, 1% SDS) and gently
mixed by inverting the tubes. The lysed bacterial cells were neutralized by adding 300 µL of neutralization buffer (1.32 M Potassium acetate) was added to the cell lysate, mixed and incubated in ice for 5 mins. Centrifugation was done at 12,000 rpm for 10 mins; the overlying supernatant was transferred to a new 1.5 mL tube. To the supernatant, 15-20 µL of RNase A solution (10 mg/mL) was added and incubated for 65°C for 15 mins. The solution was extracted with chloroform: isoamyl alcohol twice and the resulting supernatant was collected in fresh tubes containing equal volume of isopropyl alcohol and kept at room temperature for 5 mins. Plasmid DNA was precipitated by centrifugation for 15 mins at 12,000 rpm. The resulting pellet was washed twice with 70% ethanol and air-dried. To the dried pellet 30 µL of nuclease free water was added and the plasmid DNA was quantified visually in agarose gel.

2.3.2.13 Clone Sequencing

2.3.2.13.1 Template Preparation

The cloned plasmid DNA was used as templates for the sequencing reaction in the automated DNA sequencer. The DNA obtained after isopropanol precipitation was dissolved in 32 µL of deionised water. To this, 8 µL of 4 M NaCl and 40 µL of autoclaved 13% PEG 8000 was added. The sample was mixed thoroughly and left on ice for 20 mins. The plasmid DNA was precipitated by centrifugation at 15,000 rpm for 15 mins at 25°C. The supernatant was removed carefully and the pellet was rinsed with 500 µL of 70 % ethanol. The pellet was air-dried and resuspended in 20 µL of deionised water.

2.3.2.13.2 Sequencing Reaction

Sequencing was based on Sanger's Dideoxy Chain Termination method. It was carried out in ABI 3730 DNA Analyzer (Applied
Biosystems, Perkin Elmer) using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 3.1. (Applied Biosystems)

The Ready Reaction mix includes:

1) A-Dye Terminator labelled with dichloro (R6G)
2) C-Dye Terminator labelled with dichloro (ROX)
3) G-Dye Terminator labelled with dichloro (R110)
4) T-Dye Terminator labelled with dichloro (TAMRA)
5) Deoxynucleoside triphosphate (dATP, dCTP, dTTP, dUTP)
6) AmpliTaq DNA Polymerase, FS
7) 5 X sequencing buffer (400 mM Tris-HCl, 10 mM MgCl₂, pH 9.0)

The 10 µL sequencing reaction mixture consisted of 100 ng of template (plasmid DNA), 5 pmols of the primer, 1 X sequencing buffer [80 mM Tris-HCl, 2 mM MgCl₂ (pH 9.0)] and 1 µL of the reaction premix were mixed together for PCR reaction. The thermocycling conditions were as follows; 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds and extension at 60°C for 4 mins. The ramp rate of the thermal cycler was set at 1°C /sec.

2.3.2.13.3 POST REACTION CLEAN UP

The products obtained after sequencing PCR were cleaned up prior to capillary electrophoresis. To the sequencing PCR product 10 µL of sterile deionised water, 2 µL of 3 M sodium acetate (pH 4.8) and 2 µL of 125 mM EDTA were added and transferred to a fresh sterile 1.5 mL eppendorf tube. To this mixture, 50 µL of 95% chilled ethanol was added and mixed thoroughly. The mixture was incubated for 15 mins at room temperature. Subsequently, the sample was spun at 12,000 rpm for 20 mins at room temperature. The supernatant was discarded and the pellet was washed twice in 70% ethanol and air-dried. The pellet was resuspended in Template Suppression Reagent (TSR) and heat denatured just before the capillary electrophoresis in ABI 3730 DNA Analyzer (Applied Biosystems, Perkin Elmer).
2.3.2.14 RADIOACTIVE LABELING OF THE PROBE

For the southern hybridization the probes were radiolabeled using PCR based method. The recombinant plasmids containing the species-specific band are used as the template for the radiolabeling of the probe. PCR reactions were set in a total volume of 30 µL. Each reaction consisted of 1 X Taq buffer with 1.5 mM MgCl₂, 1.2 U of Taq polymerase (BG), 0.25 mM of dATP, dTTP, dGTP (Amersham) with α³²P dCTP (BARC) and 12 pmols of primer per reaction. The primers namely SR-J-14233, N4-N-8924, CB-N-10920 and TK-N-3785 (UBC) were used for radiolabeling their corresponding clones. The radiolabeled product was precipitated and washed. The radiolabeled probe was purified by ethanol precipitation in order to remove the unbound α³²P dCTP and other PCR components. Thus precipitated DNA was air-dried and the dried pellet was resuspended in 30 µL of nuclease free water. Around 2 µL of resuspended product was spotted on to a whatman paper. The paper was then dried and reading was taken in a Scintillation Counter. Incorporation of α³²P dCTP was also checked by loading 5 µL of labeled product in 1.2% agarose gel and the gel was dried, wrapped in cling film and exposed to phosphor imager screen for an hour. The image was captured using Molecular Imager®FX with the aid of the Quantity one software (Bio-Rad).

2.3.2.15 PCR-SOUTHERN HYBRIDIZATION

2.3.2.15.1 PRE-TREATMENT OF THE GEL

The PCR products were resolved in 1.2% agarose gel without ethidium bromide. The gel was soaked in alkaline transfer buffer (0.4 M NaOH and 0.6 M NaCl) for 15 mins. The gels were rinsed with sterile Milli-Q water before laid over the presoaked membrane.
2.3.2.15.2 **Capillary Transfer**

The DNA was transferred to Hybond N+ membrane (Amersham) by downward capillary blotting as described by Sambrook *et al.* (1989). The capillary transfer was carried out for overnight using alkaline transfer buffer. The PCR products ranging from 100 bp to 3.0 kb were efficiently transferred to the membrane. Thus transferred DNA was fixed to the nylon membrane by cross-linking using UV Crosslinker (Hoefer). The gels were cross checked by staining with EtBr after the transfer.

2.3.2.15.3 **Hybridization**

The cross-linked membrane was pretreated with 2 X SSC for 10 mins. The nylon membranes were transferred to the hybridization barrel carefully using membrane forceps. Care should be eventually taken to avoid air bubbles forming between the membrane and barrel wall. The membrane was pre-hybridized at 60°C for 2 hrs. The probe was denatured at 95°C for 5 mins in PCR followed by snap cooling. Denatured probe was added to the prehybridization buffer and hybridization was continued for overnight (14-16hrs) at 60°C. The membrane was washed twice with 50 mL of wash buffer containing 2 X SSC with 0.1% SDS at room temperature for 10 mins. The membrane was then washed twice with 50 mL of wash buffer containing 1 X SSC and 0.1% SDS for 10 mins at 60°C. A single wash for 5 mins with wash buffer containing 0.5 X SSC with 0.1% SDS at 60°C and the radioactivity count was checked using a Geiger-Müller counter (Ludlum measurements Inc.). The membrane was exposed to phosphor imager screen for overnight and screen was scanned in Molecular Imager® FX (Bio-Rad).

2.3.2.16 **Band Analyses**

The polymorphic content for both nuclear and mitochondrial RAGEP fingerprints of each sample from different regions were
interpreted using the fingerprint type module of Bionumerics v 2.0 (Applied Maths, Kortrijk Belgium). Band search parameters were kept constant as 5% minimum profiling for all the gels. The position tolerance for selection of bands in constructing a dendrogram was kept constant at 1% throughout the interpretations. Only bands showing clear and reproducible patterns were included in the final analyses and these were scored. Real-time normalization of gel pictures was done and band position for all the gels were adjusted based on the external reference system (100 bp ladder) and monomorphic bands are kept as internal reference. Normalization aids to control the brightness and streakiness of bands without altering the faint bands and also helps to control the inter-gel mobility shifts. Subsequently a data matrix of similarity values was produced for each individual for each marker. The Dice coefficient was used to analyze the similarities of the banding patterns. Consensus similarity matrix and dendrogram based upon individual matrices from different markers were used for pair wise clustering based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) with average linkages (Avise, 1994). The UPGMA dendrogram prevails on the assumption that nucleotide substitution rates are same across all branches. It employs a sequential clustering algorithm, in which local topological relationships are identified in order of similarity, and the phylogenetic tree was built in a stepwise manner using Bionumerics v 2.0.
Table 2.1 - Details of *H. puera* population used in study.

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Table 2.2 - Insect nuclear gene specific primer sequences used in nuclear RAGEP-PCR.

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Table 2.3 - Insect mitochondrial gene specific primer sequences used in mitochondrial RAGEP-PCR.

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2.4 RESULTS

2.4.1 GENESIS OF A NEW MARKER SYSTEM

Several arbitrary primers were selected (Operon Technologies, Inc., USA) and screened for polymorphisms. Many resulted in either monomorphic bands or failed to amplify. At this juncture a need arose to develop rapid, reliable and reproducible primers which could assure polymorphic bands in a given set of insects. Therefore, some of the universal insect gene-specific primers were selected [University of British Columbia (UBC)]. UBC primer kits contain 37 mitochondrial gene specific primers and 50 nuclear gene specific primers which are universally validated for insects.

In new PCR method was developed where a single nuclear or mitochondrial primer is used at low stringency (annealing temperature at 36°C), which was named as RAGEP. It was similar to RAPD but unlike RAPD, RAGEP primers are gene-specific and longer [nuclear (21-26 nucleotides) and mitochondrial (19-26 nucleotides)]. RAGEP PCR with nuclear primers and mitochondrial primers were named as nuRAGEPs and mtRAGEP respectively. RAGEP primers are designed on promising phylogenetic genes already reported in insect systems. Many of the primers were degenerate and were validated for its reproducibility. RAGEP marker system was validated for its species specificity, reproducibility, stringency, heritability and also its utility to discriminate the endemic, epicenter and epidemic populations of teak defoliator from one another.

2.4.2 CHARACTERIZATION OF RAGEP MARKERS

2.4.2.1 REPRODUCIBILITY OF RAGEP MARKERS

Reproducibility is an important factor that decides the outcome of all the experiments. For any successful molecular marker systems, reproducibility of the fingerprints is extremely essential to prove that the experiments are reliable. RAGEP fingerprints were experimentally demonstrated for
reproducibility. The banding pattern of RAGEP fingerprints were similar for the same individual in subsequent PCRs using the same primers (Figure 2.4). The nuclear and mitochondrial RAGEP fingerprints were also observed to have highly reproducible banding patterns.

2.4.2.2 Stringency of RAGEP Markers

The stringency of the RAGEP fingerprints was evaluated by repeating the experiments with same sets of samples at variable temperatures 36°C and 42°C (Figure 2.5). It is known that increasing the annealing temperature will reduce the non-specific bands. The current observations also showed reduction in the bands. However, most of the prominent bands were reproducible at higher annealing temperature. The RAGEP fingerprints were highly reproducible at respective annealing temperature while the prominent bands could be resolved in both temperature conditions.

2.4.2.3 Species Specificity of RAGEP Markers

The species specificity for the RAGEP-PCR was evaluated by generating fingerprints with same primers in different insect species (Figure 2.6). For this experiment, some of the common agricultural pests that belong to the order Lepidoptera were selected. Species specificity was evaluated by comparing the banding patterns in *H. puera* with other insects like Teak skeletonizer [*Eutectona machaeralis* (Walker)], Leaf roller [*Sylepta derogata* (Fabricius)], Leaf folder [*Cnaphalocrocis medinalis* (Guenée)], and Silkworm [*Bombyx mori* (Linnaeus)]. The results confirmed that the RAGEP fingerprints were unique for each species. This result further authenticates that this system can be employed for inter-species diversity study.

2.4.2.4 Species-Specific Tags using RAGEP Fingerprints

Some of the prominent monomorphic bands from *H. puera* were selected and sequence characterized for species-specificity. These
selected bands where again checked for the reproducibility in *Hyblaea* samples. For species specific-tagging, six representatives from *H. puera* and six other lepidopterans, Teak skeletonizer [*E. machaeralis* (Walker)], Cotton-Bollworm [*Heliothis armigera* (Hübner)], Fruit-piercing Moth [*Othreis fullonia* (Clerck)], Leaf roller [*Sylepta derogata* (Fabricius)], Leaf folder [*Cnaphalocrocis medinalis* (Guenée)] and Silkworm [*Bombyx mori* (Linnaeus)] were used for this study. The monomorphic bands from the mitochondrial primers SR-J-14233, N4-N-8924, CB-N-10920 and TK-N-3785 were selected. Both the gel image and the blots showed that the RAGEP fingerprints generated for the individual species were unique (Figure 2.7). It is obvious, from this result that the bands generated randomly at lower stringency using RAGEP-PCR were inimitable to that particular species. These species specific tags varied from 400 bp to 1.2 kb. This provided the trustworthiness of RAGEP fingerprints in developing precise SCAR markers. Four sequence-specific tags for *H. puera* species were developed in the present study.

### 2.4.2.5 Heritability of RAGEP Markers

Heritability of the RAGEP markers was tested separately for each nuclear and mitochondrial marker. The samples were collected from the field during the outbreak season and were reared in the laboratory. However, the field collected samples were not used directly for their heritability because of its heterogeneity and anonymous lineage. Therefore, these outbreak field collected samples were reared for two generations in the laboratory. These progeny generations were labeled as P (progenies of the field collected sample) and F (progenies of P). The P and F had predetermined lineage and were employed in examining the heritability of the RAGEP marker system. The heritability of the marker was estimated by comparing the banding pattern of the parent with the progenies (Figure 2.8). Heritability of the RAGEP fingerprints was found to be consistent as the band sharing index was found to be at least 50% among P and F lineage. It
was further validated by the intra population studies using this marker system suggesting its reliability in detecting heritability.

2.4.3 RAGEP MARKERS IN POPULATION DYNAMICS OF H. PUERA

In nuclear RAGEP markers, the bands scored were in the size range of 200 bp to 1.8 kb. With nuclear RAGEP markers, an average of 2 to 3 monomorphic bands were observed, except for primer CK6–5' (Figure 2.9B). In each marker, the average number of bands scored varied from 7 to 16. The maximum number of bands were detected using primer cytC-B-3', while the maximum number of monomorphic bands were detected using primer EFS599.

Each individual RAGEP marker gel was screened and a similarity matrix was generated. Similarity matrixes of all the experimental patterns were combined to generate a UPGMA tree. While evaluating the similarity matrix based on the Dice coefficient for all nuclear specific RAGEP markers and while constructing a UPGMA tree, it was observed that the various population groups of H. puera fall in two clusters, which were further divided into two major sub clusters. Average similarity between the two major clusters was 20%, while between the two sub clusters it was observed to be 34%. In one major cluster, all the endemic insects cluster together with some of the insects from the epicenter populations; however, both populations fall into two distinct sub-clusters (Figure 2.9A). Similarly, in the second major cluster, the remaining populations from the epicenter and entire epidemic insect populations were likewise seen to fall into two distinct sub-clusters.

Using the mitochondrial RAGEP markers, the average number of bands scored for each primer ranged from 6-15. All bands scored were in the size range of 300 bp to 1600 kb (Figure 2.10B). The maximum number of bands were observed using primer SR-J-14233 while the least number of bands were observed using primer N4-N-8924. Among
mitochondrial markers, an average of 1-2 monomorphic bands was observed. The maximum number of monomorphic bands was observed using primer CB-N-10920. Two distinct clusters were observed in the UPGMA dendrogram for mitochondrial markers. Similarity between the two clusters was only 20%. One of these clusters comprised the majority of the endemic samples with few insects from epicenter population, while the other cluster was comparatively larger having two sub clusters. Both these sub-clusters have insects from epicenter and epidemic populations (Figure 2.10A). From this dendrogram, it was deduced that all the seven epidemic population samples tested in the study shared the gene pool with the epicenter populations. In contrast, the endemic population was genetically distinct from the epidemic population. Mitochondrial RAGEPs were found to be more polymorphic and informative, compared to nuclear RAGEPs.

2.4.4 Molecular Hypothesis on Population Dynamics

The current hypothesis based on the molecular study using RAGEP fingerprints revealed that endemic population does not play a significant role in causing epidemic (Figure 2.11). Both nuclear and mitochondrial RAGEP fingerprints suggest that there was a minor gene flow between the immigrants and endemic moths that resulted in localized epicenter populations (Y, Z, N, W, K and L). The results from these studies also confirm that majority of the moths in epicenter population are immigrants, migrating from other teak plantations. The study hypothesized that the epicenter population is solely contributed by the immigrant insects which results in large scale infestations. The dendrogram shows that the patchy epicenters build up the large epidemics. If proper pest management strategies are implemented on the epicenters or the locations of the migrating moths, then apparently the future large outbreaks can be efficiently prevented.
Figure 2.4 - Reproducibility of RAGEP fingerprints. Mt1-3, Nu1-3 depicts reproducibility of RAGEP's using mitochondrial and nuclear primers respectively.

Figure 2.5 - Stringency of RAGEP fingerprints. P1, P2, F1 and F2 samples are tested for higher (42°C) and lower (36°C) annealing temperatures.
Figure 2.6 - Species specificity of RAGEP fingerprints.
Figure 2.7 - Species-specific tags generated for *H. puera* using RAGEP fingerprints.

Panel A shows the agarose gel and Panel B shows the corresponding PCR-southern for the respective markers and the arrows indicates the species specific bands.
Figure 2.8 - Heritability of RAGEP fingerprints.
P1 and P2 are parents F1 and F2 are their progenies respectively.
Figure 2.9 - RAGEP fingerprints generated by nuclear gene specific markers in individuals of three population (Panel B) and UPGMA dendrogram showing clustering of different insect population of *H. puera* (Panel A).
Figure 2.10 - RAGEP fingerprints generated by mitochondrial gene specific markers in individuals of three population (Panel B) and UPGMA dendrogram showing clustering of different insect populations of *H. puera* (Panel A).
Figure 2.11 - Schematic diagram showing the proposed model of population dynamics of *H. puera* based on molecular data.
2.5 DISCUSSION

Several technical advancements on the DNA fingerprinting methodologies have been established to resolve the taxonomic uncertainties and address the issue on species variability and migration (Welsh and McClelland, 1990; Williams et al., 1990; Caetano-Anolles et al., 1993; Vos et al., 1995; Schlipalius et al., 2001; Hebert et al., 2003). The RAGEP-PCR method described here uses gene-specific primers and randomly amplifies the nuclear and mitochondrial-like gene products. Longer mitochondrial (19-26 nucleotide) gene encoding primers are likely to increase the reproducibility, specificity and is more informative about the genome, when compared to RAPD technique. This method was found to be efficient, simple and highly reproducible. Here it has been effectively used to discriminate the various population groups of H. puera infesting teak plantations in South India. It can also be used to discriminate various taxonomically related moths to the species level.

Identification of population specific tags using highly variable DNA-based genetic markers can provide valid information regarding structural dynamics, gene flow, inbreeding and migratory pattern of that population. Identification of species-specific tags is a well established protocol in economically important plants. Species-specific markers and tags are very useful in identifying and sorting out the sibling species, species isolation and addressing the queries pertaining to sub classification of a species. In entomology, SCAR markers have been employed in detecting predation on whitefly (Agusti et al., 2003), identification of isomorphic species in the Anopheles dirus complex (Manguin et al., 2002), and in the identification of commercial tasar silkworm (Saha and Kundu, 2006).

Mitochondrial and nuclear RAGEP fingerprints are derived from the randomness of RAGEP-PCR. It is difficult to predict with certainty that the bands are diagnostic features of the mitochondrial or nuclear genome. The mitochondrial DNA sequences are frequently transferred to the nucleus.
giving rise to NUMTs, which are considered to be common in eukaryotes (Richly and Leister, 2004a; 2004b). It can be predicted that the mitochondrial RAGEP PCR products which uses gene specific primers, could therefore be a result of amplification of homologous genes or pseudo genes that represent nuclear mitochondrial DNA (NUMTs). Very high rate of horizontal transfer between organellar and nuclear genomes has been reported in the brown mountain grasshopper, *Podisma pedestris* L. (Bensasson et al., 2000).

In the past several molecular methods have been developed to address various phenotypic and population dynamic parameters of insects. In insect populations the factors like age group, sex, life stages, etc. and the processes including birth, death, immigration and emigration have been very well explained using molecular markers (Roderick, 1996). Using DNA fingerprinting [(GATA)₄] and RAPDs the differentiation process of the grain aphids populations across agricultural ecosystems are elucidated. It was also possible to discriminate the micro and macro geographical heterogeneity (De Barro et al., 1994). Highly diagnostic banding patterns in individuals of *S. avenae* on wheat and cocksfoot grass, *Dactylis glomerata* (L.) were observed during the early months of infestation, which declined as the season progressed, largely as a result of genetic drift and local movement between adjacent host species (De Barro et al., 1995). The ribosomal ITS region indicates the monophyly and a strong biogeographic pattern of each biotype have been reported in whitefly, *Bemisia tabaci* populations studied throughout the world (De Barro et al., 2000). While evaluating the genetic structure in introduced population of the fire ant, *Solenopsis invicta* using different classes of markers, it was confirmed that both mitochondrial and nuclear markers display the same hierarchical structure (Ross et al., 1999). Distinct mitochondrial and nuclear DNA sequence divergence patterns for phylogenetic inference has been established among nymphalid butterflies (Brower and DeSalle, 1998). In the present study the randomness of RAGEP fingerprints from nuclear and mitochondrial genomes were
explored to address the population dynamics of *H. puera* and its involvement in causing large scale infestation in teak plantations.

Randomness of genome amplification methods have been efficiently used in constructing the phylogenetic history in the weevil, *Aubeonymus mariafrancisciae* (Roudier), which had diverged recently (Taberner *et al.*, 1997), while the origin of the Argentine stem weevil, *Listronotus bonariensis* (Kuschel) in New Zealand, was traced to the eastern coast of South America (Williams *et al.*, 1994). Use of RAPDs in examining the population structures of the saw-toothed grain beetle (Brown *et al.*, 1997) and characterization and identification of gypsy moth was explored genetically (Garner and Slavicek, 1996). The host based genotype variation in *S. avenae* (Lushai *et al.*, 2002), and genotypic variation among different phenotypes of asexual adult winged and wingless cereal aphids have been well documented (Lushai *et al.*, 1997). Earlier reports involving molecular DNA markers mention the use of these markers in the detection of sibling species of black flies, *Simulium* spp. (Brockhouse *et al.*, 1993), while the dynamics of colonization of *D. subobscura* (Collin) in the west coast of North America and its impact in the sibling species *D. athabasca* and *D. azteca* has been extensively studied by allozymes, mitochondrial DNA (MtDNA) and RAPD markers (Pascual *et al.*, 1997).

The present study using RAGEP-PCR provides a useful tool to trace the relationship of endemic, epicenter and epidemic populations of the teak defoliator in Nilambur teak plantations. The dendrogram produced from nuclear RAGEP clearly indicates that the endemic insects are not involved in causing the epidemic; however, they are apparently involved in the localized spread by building up small epicenter populations. Similarly, while evaluating the observation based on mitochondrial RAGEPs, it is further apparent that endemic populations were not involved in causing the epidemics. This suggests that all the epidemic insects, which are spatially distinct, but temporally co-occurring, share the same gene pool.
2.6 SUMMARY

Earlier studies on teak defoliator, based on temporal and spatial distribution of the larvae indicated that the epicenters were not constant over the years and did not represent highly favourable local environments (Nair and Sudheendrakumar, 1986). The present study found little evidence to show that the aggregation of moths belonging to the endemic populations cause the epicenter populations. On the other hand, the findings do suggest the alternate hypothesis, (i.e.) the immigration of moths from distant teak plantations causes the epidemic, and that there is a continuous inflow of moths during the infestation period. This suggests that under a single demographic structure, two phenotypic classes of *H. puera* coexist during the outbreak season. The degree of variability observed for RAGEPs also argues that this technique could be useful for addressing a variety of questions, including individual identification, strain identification and phylogenetics.

The present results appear to validate the hypothesis that the control of epicenter population would help to prevent large scale outbreaks *H. puera* population in teak plantations. Therefore, appropriate strategies should be adopted to control the epicenter population, which occurs in a smaller area. This appears to be a more practical and economical approach for teak defoliator management when compared with management of the pest in the total plantation area covering thousands of hectares. Thus, the molecular markers using the novel RAGEP-PCR could enhance the understanding of insect population dynamics and aid in tracing the spread and cause of epidemics.

The development and establishment of a novel marker system for insect population have been contributed by our study. RAGEP marker system has proven to be efficient in reproducibility, species-specificity, heritability and stringency.