Chapter 1: Introduction
Introduction

Natural silk producing insects are reared in traditional method since ancient days, which used to provide a profitable occupation to the rural masses. Chinese literature written before the birth of Christ recorded that Eri and Muga silk culture were indigenous to the valley and hills of Assam. A good number of traditional silkworm races are multivoltine in nature are available in India (Dutta 1984). These silk producing insects have been exploited as a source of viable economy and the *Bombyx mori* has been proved to be the most extensively cultured variety in India, China and Japan. On the other hand, India is unique in having three other non-mulberry silk producing insect viz. *Antheraea mylita*, *Antheraea assama* and *Philosamia ricini*, the last being given the status of poor man’s silk. Further, the N.E. region of India being subtropical and humid could support the wide range of biological diversity in general and the silkworm diversity in particular enriching this region with all these four types of silkworms.

*Philosamia ricini* Boisd known as “Eri silkworm” is very common in NE region, being reared indoor primarily on Castor leaves (*Ricinus communis*). It completes its life cycle within two months. The cocoon has the characteristic of spinning. Eri culture was scattered in the dense forest of North Eastern Region in wild and gradually it becomes a cottage industry of the tribal folk of this region. Unfortunately this industry remains unrecognized practice among the
poor section of this region. Although the products of this industry has good
demand due to various reasons, yet there are defuse information about the
biology referring to the quality and quantity of silk.

*Philosamia ricini* Boisd the domesticated eri silkworm rank next to *Bombyx mori* L for production of natural silk. The eri silk has wool like finish with a look of
cotton and the softness of silk. It is a holometabolous polyphagus and
multivoltine insect, which can be reared all the year round. The important food
plants are castor (*Ricinus communis*), Kesseru (*Heteropnase fragrans*),
Tapioca (*Monihat utilissina*), Payam (*Evodia flaxinifolia*), Borkesseru (*Alianthus*
*sp*), Gulancha (*Plumeria acutifolia*) and Gamari (*Gmelina aroborea*). The quality
of eri cocoons depends on the type of host plants and the best quality of eri
cocoons is produced when eri worms are fed on castor leaves (*Ricinus*
*communis*). Its wild counter part *P. cynthia* on the other hand either uni or
bivoltine and exhibits pupal diapuse.

Production of eri raw silk shows an increasing trend over the last few years.
During 1996-97 the total production of raw silk in India was 745 MT registering
a growth rate of 12% from base year 1987-88. Out of the 745MT, the North
Eastern region accounted for 734.64 MT amounting 98%, of which Assam also
accounted for 55% followed by Manipur 21%, Meghalaya 20% and Nagaland
2%. The amount of eri silk production has attained at a growth of 1089 MT of which 1079 MT produced by NE region (Suryanarayana et al., 2003).

Out of 256,76,700 ha of land available in this region, it is estimated that about 18,052 ha of land available under eri host plants. Of this 2575 ha in Assam, 300 ha in Manipur, 1052 ha in Nagaland, 1019 ha in Meghalaya, 63 ha in Mizoram and 34 ha in Arunachal Pradesh. Approximately 1.70 lakh families have been engaged in Eri-culture as their secondary income source. Of these 1,00,220 from Assam, 4000 from Meghalaya, 5000 from Manipur, 3000 from Mizoram, 7000 from Nagaland and 1750 from Arunachal Pradesh. Women folk are engaged mostly for rearing, spinning and weaving of the eri silk.

The stock and race differences in various biological features are considered to be the result of adaptation during long genetic process (Murakami, 1994). The existence of high genetic variability in determining the economic characters has been considered as source of resource breeding (Frankel and Brown, 1983; Frey et al., 1983). Improvement of races is only possible through the hybridization with the exotic races with proper evaluation and characterization (Tazima 1958). For such kind of improvement the conventional methods of breeding need a perfect selection procedure combined with the selection of desired traits for identifying the race, if any. The theory of selection indices is helpful for combining various attributions in such a fashion that selection on the
basis of the resulting index give the best possible gain at the genetic level (Smith, 1936). Thus Kumaresan et al. (2000) were able to establish economic quantitative traits in *Bombyx mori* L.

The genus *Samia* (1972) earlier known as *Philosamia* (1874) comprises only one well-defined species *Samia cynthia* with 16 forms, variants or races (Reddy, 2000). Eri silkworm population is available in heterozygous forms in the N.E. region, which are also known as stockes, or ecoraces (Choudhury, 1992). The races surveyed in different places of North East India have been identified so far; known has been designated as white plain (Wp), white zebra (Wz), blue plain (Bp) and blue zebra (Bz) (Dipali and colleague, 2003).

Though the eri silkworm biology has been made available yet its racial distinction and following hybridization is not yet known. Earlier Koidzumi (1917, 1923) and Katsumata (1972) characterized different species of mulberry with the help of morphological characters. However recent survey work has revealed that there are noticeable morphological differences on the body of the larva of the *P. ricini* collected from different localities within the territorial jurisdiction of the NE region (Dipali and her colleges 2003). Tikader (2000) with morphological description identified species of mulberry (*Morus* spp) at different phase of characterization. But systematic and scientific information is not available till to-date.
Tissue and stage specificity of various isozymes in silkworm have widely been investigated (Nagaoka et al., 1997; Stoykova, 2001). The haemolymph proteins serve as an important source for synthesis of proteins of the adult. It has now been clear that using biotechnology as tool there are tremendous scope for the development of this silk. However, before entering into this field sufficient information relating to their status, genetic variation, economic viability etc. are the most pertinent questions. Thus the breeding programme has been appeared as essential for the improvement of the silk using morphological traits. Most often the morphological or quantitative traits of commercially important silkworms are shown to have different heritability estimate due to environmental effects (Gamo and Hirabayashi, 1983; Nino et al., 1990). Marker assisted selection (MAS), which is fast getting ground in other field of breeding ( Tanksley et al., 1982; Bulkfield et al., 1988; Jung et al., 1989), could be an effective tool for silkworm breeding to combat with the environmental effect. Thus the idea of identification of some suitable and reliable biochemicals especially isozyme as well as DNA markers have been developed.

It has been recognized that detailed study on the phenotypic variation in mulberry could be of help in selecting the parents for high yielding varieties Tikader and Rao (2002). Wagner and Selander (1974) have clearly stated the significance of isozymes in insect acts as a valuable tool for study of gene duplication among the eukaryotes and probably represents an important
process in the evolution of genome. The genetic variability of various enzymes has been studied in mulberry silkworm *B. mori* (Yoshitaken, 1963; Tazima, 1978) while Gamo (1983) attempted to correlate specific biochemical and/or genetical parameters with those contributing to yield of such biochemical traits, and the amylase has been considered as one of the most exhaustively studied enzyme system in *B. mori* (Hara et al., 1984, 1986), but with little information on the possible relationship between the amylase activity and the glycogen metabolism. Earlier, Matsumura (1951) studied the functional difference of the digestive amylase of different strains of the silkworm *Bombyx mori*, and this has been proved to be effective in sorting out the high yielding races and amylase is one of this example (Chatterjee et al., 1992). Application of zymogram technique to taxonomic problems have in general confirmed previous classifications based on morphological, cytological, behavioural and other more conventional characters (Nair et al., 1971; Richmond, 1972). In a representative study, Lakovaara et al., (1973) compared 19 species of the *D. obscura* group at 21 enzyme loci. Allozymes may prove to be especially valuable in the analysis of kin selection and other genetic aspects of sociality in insects (Hamilton, 1972).

Estimation on the genetic diversity and relationship between the germplasm collections has been proved to be useful for facilitating efficient germplasm collection and management with the help of isozymes and various molecular
markers (Rabbani et al., 1998). However, phenotypic characterization is the first step in the description and classification of germplasm (Smith and Smith, 1989).

The zymogram technique permits a new extensive examination of genetic differences between individuals, populations and species. Efficient use of the technique involves the scoring of alleles at loci encoding particular enzymes rather than the mere comparison of numbers and positions of bands of enzyme activity on gels. This approach may require formal progeny studies to establish the number of loci and to support genetic interpretations of zymogram patterns. Other applications of the zymogram technique are notably the identification of geographic sources of introduced or otherwise colonizing species, taxonomic discrimination of sibling species and host races, studies of migration measurement of introgression through zones of hybridization and the estimation of population size (Wyatt, 1961).

The problem of protein and enzyme polymorphism was widely carried out by several investigators in relation to the comparative biochemistry, ecology, phylogeny, genetics and breeding of silkworm *B. mori* (Eguchi et al., 1975; Konicheva et al., 1975; Sankina et al., 1974, 1975).
Isozymes are enzymes, which have similar or identical function as catalyst, but are by one means or another identifiably different with respect to structure (Wagner and Selander, 1974). Both homo- and heteromultimeric enzymes may exist as isozymes in a variety of ways. Perhaps the most important case is a simple change in amino acid sequence in the same polypeptide involving the substitution of one or more amino acids for another or others. This results in the production of an enzyme that may or may not be detectably different from the standard. This group of isozymes, called allozymes, is ordinarily considered to be the result of mutations causing amino acid substitutions. Allozymes are “genetically segregating isozymes encoded by the same locus” or “variant proteins produced by allelic forms of the same locus” (Prakash et al., 1969). Isozymes resulting from the mutation of the α-gene will be allozymes of one another, and the same is true for the β-gene. Allozymes of homomultimers are all presumably the result of mutation in the same gene, and are therefore, produced by different alleles of the same gene. Not only do the isozyme patterns of many enzymes change during the lifecycle of an insect, but there is also a high degree of tissue and cell-type specificity. Indeed, this specificity may even be intracellular. Isozymes provide a valuable tool for the study of gene duplication, a phenomenon that is widespread among the eukaryotes and probably represents an important process in the evolution of genomes (Lefevre and Green, 1972).
On the other way, the amylases from silkworms have drawn the attention of many sericulturist since 1934 (Matsumura, 1934), and a number of studies concerning the properties, activities, and polymorphism of amylase isozymes have been reported (Hara et al., 1984; Abraham et al., 1992; Asakawa et al., 1989 and Hanang, 1989). Wyatt (1967) suggested that amylase in the insect haemolymph probably helps in the degradation of glycogen in the haemolymph. Analytical review of Patnaik and Dutta (1995) has clearly showed the importance of amylase and isozyme as marker in the process of selection. Because, the genetic factor i.e. the ultimate realization of silk yield are dependent upon the environmental condition (leaf quality, biotic and abiotic factor). Allozyme variations have been developed to analyze dispersal and divergence among population and species of North American cave cricket (Caccone and Sbordoni, 1987). Thus, it is important to short out the genes sensitive to environmental fluctuation (Wu & Chen, 1988; Dutta, 1992; Chatterjee et al., 1990). The enzyme helps in digestion of starch in the food plants (Tanaka and Kusano, 1980). Thus Promboon et al. (1993) was able to select and characterize the high amylase activity strains to be used as raw materials for the future breeding work. They also suggested the amylase in these polyvoltine insects may contribute to the hardy character of the race. Therefore, the marker assisted selection (MAS) procedure (Buifield et al., 1988; Jung et al., 1989) could be effective tool in the process of identification of all biochemical traits in the silkworms. Amylase had generated more interest
when Matsumara (1933, 1951) first demonstrated the functional difference of amylase in different strains of *Bombyx mori*. Further, Matsumara (1980) had documented that amylase activity is feeble soon after 4th moult, then gradually increases its action until the full growth of the 5th stage, while male is stronger than the female in enzyme action. Moreover, a marked racial difference in terms of amylase activity was observed between two races of *Bombyx*.

Among sericigenus lepidopteran insects digestive amylases have been studied in detail only in the monophagous silkworm, *B. mori*, however in polyphagous tasar silkworm, *A. mylita* amylase activity was detected in haemolymph and in digestive juice (Abraham et al., 1992). Amylase is one of the key enzymes involved in carbohydrate digestion and metabolism in insect (Buonocore et al., 1976; Nagaraju and Abraham, 1995). α-Amylase is an endoenzyme, which hydrolyses the α-1, 4-glycosidic bonds of starch, which is a polymer of linear amylose chain connected by α-1, 6 bonds.

The association of the activity of amylase in the digestive juice with cocoon characters induced a good deal of interest in the genetic variability (Hara et al., 1984, 1986; Hirata, 1974). Hirata (1974) analyzed the relationship between amylase activities in the larval digestive juice and several quantitative characters using bivoltine strains with high and low digestive activity. Amylases with its isozymes have been established as marker in different strains of

Patnaik and Dutta (1995) reviewed the usefulness of amylase as a marker in Silkworm breeding. However, the use of allozyme marker in the process of racial distinction in *P. ricini* is not known.

The carboxylesterase represent a large and diverse group of enzyme that exhibits wide and overlapping substrate specificities and inhibition pattern. They show universal distribution in different groups of animals and plants occurring in a large number of forms determined by distinct gene loci showing tissue distribution and high degree of genetic variability. The importance of esterase in the study of field population variation in *Globodera rostochiensis* and *Globodera pallida* was well demonstrated by Fox et al. (1988). Gao and Jiaju (1988) deduced that there was a close relationship between esterase isozyme of the fox tail millet and its ancestral species. However, the physiological functions of esterase are known only in few cases despite the relative case with which esterases can be demonstrated. Kai et al. (1972) had attempted to study the mode of action in ovaries and mature eggs in relation to diapause with the help of electrophoresis of protein and esterase. The use of esterase as genetic marker has been proved to be successful in several insect species (Ogita 1968; Narang et al., 1971; Gartside, 1980). The correlation between pathogenicity and zymogram of esterase helps in establishing the isolates in rice blast fungus (Park et. al., 1986). The *Apis mellifera* esterase (EC 311) has been studied
using electrophoresis to estimate the frequencies of the known variants in European honeybees population (Del Lama et al., 1990). Further, Ruvolo-Takasusuki et al. (1997) attempted to genetically characterize *Apis mellifera* based on esterase 1a.

Esterases, many of which have unusually broad substrate specificities, are the most highly polymorphic enzymes in insects and other organism (Johnson et al., 1966). Over a dozen alleles have been identified at esterase loci in local populations of butterflies (Burns et al., 1967, 1971) and the olive fruit fly (*Dacus oleae*) (Zouros and Krimbas, 1969) but with extremely poor information on *P. ricini*. Stoykova (2001) by means of electrophoresis investigated nonspecific esterase in the silkworm fat body and identified sixteen races and eight interrace hybrids during ontogenesis.

The haemolymph protein, draw the attention of several workers due to its synthetic activity is associated with the differentiation process. Application of different method's showed that both species and stage specific proteins have been proved successful (Chen, 1966). The electrophoretic pattern of insect haemolymph protein was earlier reviewed by Wyatt (1961). In holometabolous insects the larval haemolymph protein (LHP) are synthesized in the fat body during the larval development and released into the haemolymph and are selectively reabsorbed by the fat body during pupation (Levenbook, 1985).
There is a sevenfold increased in protein concentration during larval life, at first gradual, then rapid in the last instar; little change during spinning and the first part of the pupal period with a sharp fall during development of the adult (Wyatt, 1961). The importance of haemolymph protein as a reserve is also illustrated by changes during starvation. Thus, when *C. euphorbiae*, *B. mori* or *S. lutaria* is subjected to enforced starvation, haemolymph protein falls remarkably with non-protein and remains unchanged. This presumably reflects hydrolysis of protein to maintain amino acids and thus osmotic pressure (Wyatt, 1961).

Investigation on element profile and mineral requirements is probably the most neglected area of research in insect nutrition and certainly the most difficult field (Subburathinan & Krishnan, 1992). Teuniaux (1970) stated that the phytophagus insect have higher Mg indices. According to House (1974) *B. mori* required Ca, Fe, Mg & Zn; but Na is not very much used by the silkworm. Metcalf and Flint (1967) reported the importance of Zn, Fe in insect life. The relationship between trace element content and physiological significance in the two species of whale tapeworm (*Diphyllobothrium macroovatum* & *Diphyllobothrium balaenopterae*) was discussed by Yamane et al. (1986) and the importance of Fe was discussed by Locke et al. (1992). Iron deficiency and its metabolism in brain was studied by Shukla et al. (1989). Fe an essential nutrients, but it is also a protein toxin, because it can catalyze oxidative reaction that are descriptive to cells (Law 2003). Recently, Winzerling (2002) studied Fe
metabolism in insect and its availability affects a wide range in the biological process. Kling (2003) studied Fe metabolism in insect and its effect on intracellular and the promotion of cellular oxidative damage in many species and also have isolated and sequenced insect transferrins from mosquitoes and moth. Growth rate and cell adhesion decrease due to reduction in Fe & Mg level (Walach et al., 1986), while iron and zinc has been found to play a role in growth of silkworm by influencing the cocoon yield and shell percentage (Ito and Niimura, 1966).

Zinc is required throughout the body to activate enzyme and to form metalloenzymes. These enzymes, which are required for metabolism and protein synthesis, are more critical in achieving maximum fertility. The relative paucity of these micronutrients, both in diet and in the body, suggests their importance in the regulation of whole body metabolism, including energy utilization and work performance. Another intracellular cation, Zn, is required for more than 300 enzymes from many species. Zn containing enzymes participate in many component of macronutrient metabolism, particularly cell replication, (Lukaski, 1995). Zinc, its application helps in increasing the pupal weight and filamentlength. Zn content of reproductive tissue is probably important for reproduction in B. mori (Friend, 1958) while, Subburathinan et al. (1992) has also comments on the importance of Zn in relation to other minerals and its importance for reproduction.
The biological importance of Mg, Zn and Cr is revealed by the various metabolic processes in which these elements regulate biological function. Mg, a ubiquitous element that plays a fundamental role in many cellular reactions, is involved in more than 300 enzymatic reactions in which food is metabolized. Mg also serves as a physiological regulator of membrane stability (Lukaski, 1995). John et al. (1969) has used AAS to study the net movement of Ca and Mg across the midgut epithelia of the American cockroach as both ions are affected by the concentration of midgut lumen contents. Magnesium and manganese activate deoxyribonuclease and this phenomenon was earlier explained by Metcalf (1946). The importance of Mg in silkworm diet was earlier stressed by Shymala & Bhat (1968), and indicated that its efficiency in better cocoon weight and yield. Subburathinan et al. (1992) observed that MgCl₂ at 1% level increased the economic characters of the larva and accelerate the growth of the silkworm through orientation of the physiological activities including reproduction in B. mori.

The functional significance of Copper is not understood, although it is essential for animal and plant metabolism in which the element is most likely to play an important role in insect nutrition (Ernest, 1992). Cu deficiency inhibits cytochrome oxidase activity. The Cu function as oxygen carrier in the haemolymph of mollusca and arthropod. It provides catalytic oxidation in the
formation of melanin pigment in insect cuticle. However, the role of Cu in the
production of silk is not known (Chapman, 1998).

Selenium has become one of the most exciting nutrients of the 1970's and
1980's. Se has been identified as toxicant and essential micronutrient in fish
(Kleinow et al., 1986). Once classified solely as a toxic mineral, has now been
regarded as an essential one (Haas, 2003). Darvish (2003) has stated that Se
being trace mineral, essential nutrient proves its powers as a natural weapon to
activate the immune system. Trumble, (2002) emphasized the impact of
selenium on plant-insect metabolism. The property that allows Se to be
biochemically active at dietary level is less than 1ppm, (Maas, 2003). Se
functions through the enzyme glutathione peroxides, which reduces hydrogen
and other organic peroxides, which can damage body tissues (Hoekstra, 1973).
Recently the Se as selinite has been introduced as micronutrient for P. ricini
and an encouraging result has been obtained in terms of silk synthesis (Deka et
al., 2003).

The role of either isozyme or the elemental profile in the racial distinction of the
species is not very much known. The racial diversity based on element has
been an extensively difficult task, despite of its significant importance in the
process of biological functions. Thus in the present investigation an attempt has
been made to characterize available ecoraces of *Philosamia ricini* in the North Eastern region of India using isozyme as marker.

**Aim and objectives:**

It is aimed to establish/identify ecoraces of *P. ricini* of North Eastern region from the allozyme variation. Therefore, the following objectives have been considered:

i. Ecorace variation at morphological level.

ii. Determination of allozyme viz. a) Amylase b) Esterase to establish ecoraces at larval stages.

iii. Haemolymph protein variation.

iv. Variation of certain element in the silk gland.