Mulberry (*Morus* spp.) is cultivated as an economic crop for its foliage and, is the only natural food for the silk producing worm, *Bombyx mori* L. Mulberry is basically a dioecious tree species but, normally cultivated in the form of low bush or dwarf tree by repeated pruning. Constraints associated with tree crops viz., perennial nature, heterozygosity and out breeding behavior are impediments in mulberry improvement program. Crop germplasm are the important source of novel genes/trait, and need to be well characterized for their desirable planta and genetic variability for identification of putative genes/markers associated with the traits of interest before they could be effectively utilized in breeding programs.

Genetic characterization of mulberry germplasm by traditional marker systems poses great deal of problem. In comparison, the molecular markers, specifically based on DNA are abundant, neutral to environmental effect; follow the Mendelian genetic principles, amenable to automation, easily generated in quick time and highly reproducible. PCR based marker technique like RAPD, ISSR, AFLP, SSR etc., have proved to be highly successful in plant genetic analysis. The present study utilizes few of these techniques in addressing the important issues in mulberry genetics.

### 6.1 DNA fingerprinting of mulberry cultivars

The main aim of the study was to develop a mulberry cultivar identification tool based on molecular analysis and a statistical approach. Mulberry genetic identification is the primary requirement for all crop improvement efforts, germplasm conservation and utilization. Even though, many of the mulberry cultivars exhibit distinct morpho-agronomical features, it is cumbersome to delineate them under different agro-climatic conditions.

In the first study, a total of 15 RAPD primers were tested against 12 collections each of Mysore Local and V-1. All the random primers produced distinct polymorphic banding pattern between the two cultivars. Band scores did not differ between repeat assessments or between the gels for any of the primers used. Besides, there was no variation in banding pattern within the cultivar/s. A total of 104 markers were generated of which 51 were monomorphic and the rest 53 were polymorphic (51 %). Among the variable markers, 47.2 % (25 band positions) was contributed by Mysore Local and the rest 52.8 % (28 band positions) was contributed by V-1 towards the polymorphism between two cultivars. All the collections under Mysore Local and V-1 were represented by a typical banding pattern, which clearly distinguished the cultivars.
Yet another study was undertaken involving two traditional cultivars – Mysore Local and Kanva-2 and two of the improved cultivars namely, V-1 and S-36 to demonstrate the effectiveness of the DNA fingerprinting for mulberry genetic identification using larger clonal population. DNA fingerprinting was carried out with 16 random primers from OPC and OPD series and a total of 185 markers were generated of which 104 (56.2 %) were polymorphic. All the four pooled replicates of each cultivar had identical DNA fingerprints (100 % similarity) suggesting the absence of intra-cultivar variation. Almost all the primers gave unique fingerprints for each of the four cultivars analyzed.

Resolving Power of the primers ranged from minimum of 0.5 (OPC-17) to maximum of 6.5 (OPC-15). The primer with a Resolving Power (Rp) > 1.5 could discriminate all the four cultivars analyzed. Prevost and Wilkinson (1999) used Marker Index (MI) and Resolving Power for assessing the ability of the primers for identification of cultivars. A strong linear relationship ($r^2 = 0.98$) was observed between the Rp of the hypothetical primers and the proportion of the 34 cultivars that each primer was able to distinguish. The present experiments have shown that RAPD-PCR analysis is quick, reproducible and generates sufficient polymorphism to have potential for large-scale DNA fingerprinting purpose along with use of Rp and MI for assessing the primer informativeness in mulberry identification.

6.2 Identification of duplicate collections in the mulberry germplasm

Conservation efforts by countries involved in sericulture have resulted in the accumulation of approximately 5000 accessions in the field gene banks all over the world. According to a rough estimate based on passport information, a large portion of these collection are suspected to be duplicates or redundant. Management of large mulberry germplasm collections in the field gene bank is complex and a costly task. An efficient strategy is required to establish the genetic identity of the collections/accessions and reduce the redundancy for minimization of management and operational cost.

A set of two true duplicates along with four groups of suspected duplicates and a closely related genotype of suspected duplicate group were considered based on the passport and morphological data. A total of 357 markers were generated by amplification with 31 primers out of which, 228 (63.9 %) were polymorphic. Cluster analysis performed based on the similarity matrix of RAPD markers showed that the two pairs of true duplicates (of Mysore Local and V-1) and three sets of suspected duplicates clustered together at 100 % similarity. However, two other collections (Kousen and Xuan-10) of suspected duplicates could be differentiated from each other at 96.6% similarity. Further, the closely related collections namely, RFS-135 and RFS-175 also could be
discriminated unambiguously at 94.4% similarity. Based on the result obtained, the collections in suspected duplicate group I (Anantha and RFS-175); group II (S-146, Check Majra and DD) and group IV (M. multicaulis 235 and M. multicaulis 212) are confirmed duplicates. The comparison of relative effectiveness of RAPD and morphological markers is useful when considering the strategy to be adopted before full-scale molecular marker analysis. A significant correlation (r = 0.66 at p = 0.01) suggests a close association between the two matrices based on morphological and RAPD similarity indices.

The results obtained from the study was utilized to predict the approximate number of primers, total number of markers and number of polymorphic markers needed to detect a difference between a suspected duplicate pair of mulberry collections. The number of primers (n) needed to detect at least one difference between a pair of suspected duplicate with 99% confidence is calculated as (19/31)^n = 1-0.99, which is about 9. Similarly, the total number of markers (m) and the number of polymorphic markers (p) needed for the purpose is calculated as (341/357)^m = 0.01 (100) and (212/228)^p = 0.01(64), respectively.

6.3 Molecular characterization of important mulberry varieties of India
The study was planned to investigate and establish accurate genetic identity of 18 important mulberry varieties of India which include 11 improved and 7 promising varieties by DNA fingerprinting with RAPD and ISSR markers, and to compare the relative information generated by these two marker systems in terms of genetic identity, diversity and relatedness. A total of 22 informative RAPD primers were utilized for screening 18 mulberry varieties which generated 282 markers. Eight ISSR primers produced varying number of PCR products depending on their SSR motifs resulting in a total of 102 amplified markers. The genetic similarity obtained by combined analysis of RAPD and ISSR marker was more similar to the one obtained through RAPD marker analysis alone and ranged from 0.689 (between G. No. 4 and Mysore Local) to 0.896 (between RFS-135 and RFS-175). Clustering obtained by UPGMA analysis of the combined marker data was identical to the one obtained through RAPD analysis alone. In all the three analyses, AR-11, S-1635, G. No. 2 and G. No. 4 clustered distinctly. AR-11 was considered as an out-group in all the three dendrograms. The correlation between the matrices of cophenetic values (Mantels test) for the dendrograms based on RAPD and ISSR marker data was good (r=0.69, p=0.01).

The RAPD primers, OPC-05 and OPC-06 were able to discriminate all the 18 genotypes whereas; the ISSR primer UBC-814 was able to identify only one genotype. There was very good linear relationship between both MI and the number of genotypes identified (r^2 = 0.70) and almost
similar relationship was observed between Rp and the number of genotypes identified ($r^2 = 0.69$). A strong relationship meant it was possible to estimate the number of genotypes that could be identified simply by calculating the MI or Rp of a primer.

6.4 Assessment of genetic diversity and interrelationship among mulberry germplasm
CSRTI, Mysore and CSGRC, Hosur have assembled large number of germplasm of indigenous origin through survey and exploration and exotics from different sericultural countries adding rich variability and diversity to the collection. Unless essential genetic information is simultaneously generated, mulberry breeders will not be able to utilize them directly in the crop improvement program. In this context, the study attempted to address the task using three sub-groups of mulberry germplasm from the gene banks through PCR based DNA marker techniques.

6.4.1 Indigenous and exotic collections:
The study included both indigenous and exotic collections and in general these are widely used as parental materials in mulberry breeding program in India. A total of 391 markers were amplified by 31 RAPD and ISSR primers of which 89.5 % (350 markers) were polymorphic. The Dice dissimilarity coefficient calculated for the two marker systems indicated that, least was between Himachal Local and Punjab Local (0.120). But, the maximum dissimilarity (0.424) recoded was between MS-3 and BR-2 and hence the most divergent pair among the collections. UPGMA clustering based on the dissimilarity coefficients resolved into eight recognizable clusters.

Absence of relationship between geographic origin and the DNA marker based clustering of germplasm suggests that mulberry during different historical periods have spread from its centre of origin to the Indian sub-continent. Further, continuous hybridization and introgression between the materials have also probably erased the distinctness due to different origin. Overall, on the basis of diversity indices, the out breeding plants like cultivated mulberries are much less differentiated than autogamous species because of the occurrence of gene flow. They contain low-frequency alleles which contribute little to diversity but promote differentiation between them (Brown, 1989). Even though, cultivated mulberries present a continuous genetic background, some materials show interesting characteristics e.g., China White, which needs to be utilized in mulberry improvement program.

6.4.2 Representative germplasm from morphological based clusters:
A total of 30 mulberry germplasm utilized in the study were selected from phenotypic based clusters obtained from two independent evaluations conducted on germplasm collections (Rajan et al., 1997; Rajan and Sarkar, 1998). These germplasm are also highly diverse in terms of
morphological, growth and yield parameters which are indicative of being represented in different clusters.

RAPD analysis involved a total of 21 informative primers which generated 249 markers out of which 220 (88.4 %) were polymorphic. Almora Local and Punjab Local were the closest with a similarity of 89.9 % and Shin Ichinose is equi-distant from Kokuso-13 and Papua New Guinea with the least similarity at 57 %. Average genetic distance among these 30 mulberry genotypes was calculated as 0.265.

The UPGMA grouping of genotypes based on marker similarity coefficient values resolved into 10 clusters. The study confirms the earlier view held by Sharma et al. (2000) in mulberry, that relationship based on geographical origin could not be established. They explained that most of the mulberry genotypes were domesticated far from their initial place of origin, where they established, adapted and persisted. The authors also suggested a “possible” route of domestication of the most widely grown mulberry species M. alba, a native of China to areas as far apart as India, Europe and America. The present study confirms the finding of Rajan et al. (1997) and Rajan and Sarkar (1998) that the criteria for selection of parents in hybridization program should be more based on the genetic diversity rather than the geographical distance. The assessment of genetic interrelationships among the genotypes is more dependable based on the molecular marker data if one takes into consideration of the few known relationships amongst the materials studied.

6.4.3 Indigenous germplasm collections assembled from survey and exploration:

The study included some of the collections having economically important attributes and may be considered for release for cultivation after systematic evaluation trials. The dissimilarity coefficient based on the morphological markers indicated high variability. Genetic distances between four pairs of accessions were the least (0.154) namely, Acc. 1 and Reshammajri-6, ERRC-71 and Guhanathapuram, ERRC-71 and Sultanpur and Hariharpur. But, the maximum genetic distance (0.923) was recorded between Kalimpong Local and Seekapuri. The UPGMA analysis of all the thirty-six germplasm collections based on dissimilarity coefficient resulted in 11 clusters, of which II and III are the largest comprising of 6 collections each.

RAPD analysis (15 primers) generated a total of 94 markers with a polymorphism of 93.6 %. The calculation of Dice dissimilarity coefficient resulted in a diagonal matrix which indicated genetic distance values ranging from 0.049 (ERRC-101 and ERRC-71) to 0.500 (Guhanathapuram and Barbat Farm). Clustering based on RAPD marker Dice dissimilarity coefficients matrix resulted in 15 recognizable groups of which cluster II is the largest having 11 collections.

ISSR primers (17 Nos.) generated a total of 235 markers; with 202 polymorphic (85.95 %) markers. Dice dissimilarity coefficient indicated that the mulberry germplasm collection ERRC-71
and ERRC-101 were the most similar (0.044) whereas, Krishnaswami-2 and Acc. 1 were the most diverse (0.431). Mean genetic distance among the thirty-six germplasm collections was calculated as 0.304. UPGMA clustering based on SHAN routine, using ISSR marker dissimilarity coefficient values resulted in 15 clusters.

A total of six microsatellite markers were utilized for DNA profiling and assessment of allelic variability. The number of alleles per locus varied from 3 - 11 with a mean of 5.2. The expected heterozygosity \( H_e \) varied from 0.34 - 0.87, whereas the observed heterozygosity \( H_o \) ranged from 0.85 to 0.96 with a mean of 0.93. Based on the microsatellite allele sharing, the least genetic distance was observed in case of two pairs of collections namely, ERRC-71 and ERRC-101 (0.00) and Michlal Farm and Dudhia Piasbari and maximum (0.81) was calculated between Jodhpur and Acc. 16. The UPGMA clustering based on the SSR marker data indicated a different grouping compared to that of RAPD or ISSR. A total of 12 distinct clusters could be visualized and the cluster V was the largest having 9 entries.

The assessment of genetic distance based on all the marker systems, viz., RAPD, ISSR and SSR unambiguously projected ERRC-101 and ERRC-71 as genetically closest except that based on the morphological data. The clustering based the four marker systems showed different dendrogram topologies. The correlations between the matrices of cophenetic values for the dendrograms based on different marker data were low. Absence of congruence in the interrelationship established by morphological vs. other molecular markers is expected in the background of complexities of phenotypic expression of heterozygous, perennial tree crop.

6.5 Genetic polymorphism and interrelationship among wild mulberry collections of India and management

The total of seventy-two collections of two wild species of mulberry (M. laevigata and M. serrata), which is naturally distributed in India, were from diverse origin, distribution and probably representing the entire diversity available in the country. Both RAPD and ISSR primers (27 Nos.) amplified 409 DNA markers of which 378 (92.4 %) were polymorphic. The extent of polymorphism shown by RAPD (93.5 %) was slightly more than that of ISSR marker analysis (88.5%). Based on the combined analysis of both the marker systems, the mean genetic similarity was 0.699 in the case of M. laevigata compared to 0.785 in the case of M. serrata collections, signifying higher variability in the former. When both the species were analyzed together, the collections of M. laevigata from Ranchi (Ranchi-1 and Ranchi-4) were genetically close \((s = 0.953)\) and, a M. laevigata collection from Andaman Is. namely, Chidia Tapu-4 and a collection of M. serrata –
UP(R13B10) were genetically the most distant (s = 0.529). These findings are concurrent with known geographical origin and taxonomic status.

The results obtained on UPGMA analysis indicate beyond doubt that the Andaman Is. collections are very unique in their genetic constitution and significantly differs from the mainland types. The natural population of *M. laevigata* occurring in the Island are truly unique in terms of important morphological traits such as smooth, glossy leaves and the largest leaf area ever recorded (Ravindran et al., 1997) among the mulberry species. One can hypothesize that most of the collections from North-Eastern and North-Western India may represent the fraction of natural population which is in the verge of slow loss from the forest areas due to human activities. However, those collections from Central India including the state of Jharkhand, Madhya Pradesh, Chattisgarh etc., and all the South Indian collections are introduced from the natural sources within the country for cultivation for fruits and for providing shade in coffee estates.

These two species, which are truly wild, harbors some of the important genes for diseases and pest resistance, saline tolerance (*M. laevigata*), and frost tolerance (*M. serrata*) and can be exploited in mulberry improvement program. *M. serrata* also possess some of the desirable characteristics viz., thicker leaves and greater moisture retention capacity. Recent studies have shown that some of the collections of *M. laevigata* (unpublished data) and *M. serrata* (Tikader and Dandin, 2005) can be utilized for introgression with cultivated species. Japan is the only sericulturally advanced country where wild mulberry genetic resources have been exploited for developing new improved mulberry varieties (Sharma et al., 2000). There is a need for serious effort towards *in situ* besides, *ex situ* conservation of these wild mulberry species before they are lost from their habitat.

### 6.6 Important outcome of the study

i. Protocol of DNA fingerprinting by PCR based marker techniques (RAPD and ISSR) for mulberry cultivar identification was standardized. A linear correlation of MI or Rp of a primer with the number of mulberry genotypes that can be distinguished was established. MI or Rp can be used to predict the performance of different primers in large-scale DNA fingerprinting experiments in mulberry.

ii. Molecular IDs' of important mulberry cultivars of India and germplasm collections were developed and can be used for protection of Plant Breeders Rights (PBRs') and germplasm registration.
iii. A strategy for identification of duplicate/redundant collections in the gene bank by RAPD analysis along with passport and morphological data was developed for rational and economic management of mulberry gene bank.

iv. DNA profiling of large number of mulberry germplasm by RAPD, ISSR and SSR markers was undertaken for assessment of genetic variability, diversity and interrelationships. The molecular diversity information generated can be used for selecting promising parents for breeding program for exploitation of hybrid vigor.

v. Genetic polymorphism and diversity existing in the population of two wild mulberry species of India viz., *M. laevigata* and *M. serrata* were studied and concluded that wild mulberry of Andaman Island represents a unique gene pool. The study suggests the utilization of wild mulberry species in broadening the genetic base of cultivated mulberries and for mining of agronomically important genes. The present study on genetic analysis of wild mulberry population of India, recommends immediate conservation measures by both ex-situ and in-situ approaches.

### 6.7 Conclusion and future prospects

A lot of DNA marker systems have been developed including RFLP, RAPD, ISSR, AFLP and SSR. Markers are predicted to find widespread application in plant identification, assessment of diversity and interrelationship among crop germplasm, genome tagging, linkage mapping, marker assisted selection (MAS), quantitative trait loci (QTL) analysis etc. Yet, the actual benefit in developing plants with new traits by the aid of these techniques is limited. There is a continuous requirement for highly saturated linkage maps with marker tightly linked to the gene of interest. This can be achieved through better marker systems and accurate phenotyping. Marker systems need refinement to make them more user friendly, effective and economical; but at the same time ensuring high reproducibility across laboratories. At the moment, SSRs possess the best properties for application by plant breeders, but a big step forward will be done when DNA sequencers are made available for all the concerned researchers. Despite the problems and difficulties, it seems convincing that PCR based DNA markers and associated techniques can supplement the conventional methods to make the future plant breeding more effective. Markers can especially find application in areas where conventional methods are not sufficient like utilization of quantitatively inherited characters and agronomically interesting genes from wild species. Once the linkage maps have been created, MAS can help to speed up the process of breeding. Together with marker techniques, conventional methods can accelerate the adaptation through genetic change to stabilize the yield.