

GENETIC STRUCTURE OF *Semecarpus kathalekanensis*:
A CRITICALLY ENDANGERED TREE SPECIES OF
CENTRAL WESTERN GHATS

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**GENETIC STRUCTURE OF *Semecarpus kathalekanensis*:
A CRITICALLY ENDANGERED TREE SPECIES OF
CENTRAL WESTERN GHATS**

Thesis submitted to the
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IN

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By

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CERTIFICATE

This is to certify that the thesis entitled “**Genetic structure of *Semecarpus kathalekanensis*: a critically endangered tree species of central Western Ghats**” submitted by Mr. **SURAJ P. G.**, for the degree of **Master of Science (Forestry) in Forest Biology and Tree Improvement**, to the University of Agricultural Sciences, Dharwad is a record of research work carried out by him during the period of his study in this University, under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship, or other similar titles.

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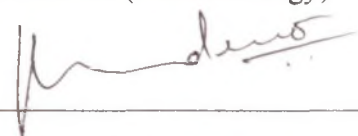


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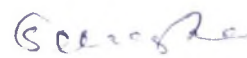
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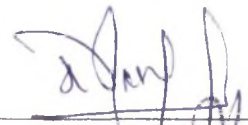


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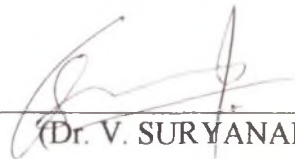
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CONTENTS

Chapter No.	Title	Page No.
I	INTRODUCTION.....	1-3
II	REVIEW OF LITERATURE..... 2.1 Floral richness of the Western Ghats 2.2 Tropical fresh water swamp forests: An endangered habitat 2.3 Rare and threatened plants of the Western Ghats 2.4 Studies on genetic diversity of tree species 2.5 Correlates of genetic diversity with life history traits 2.6 Identification of sex specific markers using biochemical tools	4-19
III	MATERIAL AND METHODS 3.1 Study site and populations 3.2 Plant material collection 3.3 Isozyme analysis 3.4 Randomly Amplified Polymorphic DNA (RAPD) analysis 3.5 Data analysis 3.6 F-statistics 3.7 Cluster analysis 3.8 Binary data analyses	20-37
IV	EXPERIMENTAL RESULTS..... 4.1 Standardization of enzyme extraction protocols 4.2 Genetic interpretation of isozyme variations 4.3 Analysis based on genetic interpretation 4.4 Analysis of eletromorph data 4.5 RAPD Analysis 4.6 Genetic differences between male and female individuals	38-61
V	DISCUSSION 5.1 Distribution and breeding system of <i>S. kathalekanensis</i> 5.2 Genetic structure of populations of <i>S. kathalekanensis</i> 5.3 Lack of population differentiation in <i>S. kathalekanensis</i> 5.4 Consequences of lower genetic diversity to the fitness 5.5 How could the ill-effects of low heterozygosity be averted 5.6 Genetic differences between male and female individuals	62-74
VI	SUMMARY.....	75-78
VII	REFERENCES	79-94
	APPENDICES.....	95-109

List of Tables

Table No.	Title	Page No.
3.1	Enzyme names, quaternary structure, sub cellular distribution, function and possible number of loci of some important Isozymes of plant system.	24
3.2	Random primers screened against the DNA samples of two populations of <i>Semecarpus kathalekanensis</i> and their nucleotide sequences	30
4.1	Allele frequency at nine loci for two populations of <i>S. kathalekanensis</i> (based on isozyme data).	40
4.2	Mean allele number per locus, mean effective allele number per locus, number of polymorphic loci, per cent polymorphism and Shannon's information index for populations of <i>S. kathalekanensis</i> .	43
4.3	Observed (H_o) and expected (H_e) heterozygosity among all adult individuals (n=34) of <i>S. kathalekanensis</i> .	45
4.4	Observed (H_o), expected (H_e) heterozygosity, genetic identity and distance for all adult individuals (n=34) of two populations of <i>S. kathalekanensis</i> as revealed by isozyme analysis.	46
4.5	F-statistics for all loci identified by isozyme analysis of <i>S. kathalekanensis</i> .	48
4.6	Electromorph frequency of isozyme bands or electromorphs in two populations of <i>S. kathalekanensis</i> .	50
4.7	Genetic diversity parameters based on electromorph frequency in <i>S. kathalekanensis</i> .	51
4.8	Partitioning of total genetic diversity of populations of <i>S. kathalekanensis</i> .	54
4.9	Genetic diversity parameters based on RAPD data In <i>S. kathalekanensis</i> .	56
4.10	Partitioning of total genetic diversity computed based on RAPD data for <i>S. kathalekanensis</i> .	58
4.11	Genetic identity (above diagonal) and genetic distance (below diagonal) between sexes in two populations of <i>S. kathalekanensis</i> , a) based on isozyme data b) based on electromorph data and c) based on RAPD data	60

List of Figures

Figure No.	Title	Between pages
3.1	Map of the study area.	20-21
4.1	Principal component analysis of two populations of <i>Semecarpus kathalekanensis</i> from central Western Ghats based on genotypic data of isozymes.	48-49
4.2	Cluster analysis of two populations of <i>Semecarpus kathalekanensis</i> from central Western Ghats based on genotypic data of isozymes.	48-49
4.3	Principal component analysis(PCA) of two populations of <i>Semecarpus kathalekanensis</i> from central Western Ghats based on phenotypic data of isozymes.	54-55
4.4	Cluster analysis of two populations of <i>Semecarpus kathalekanensis</i> from central Western Ghats based on phenotypic data of Isozymes.	54-55
4.5	Principal component analysis (PCA) of two populations of <i>Semecarpus kathalekanensis</i> from Central Western Ghats based on amplification fragment data.	59-60
4.6	Cluster analysis of two populations of <i>Semecarpus kathalekanensis</i> from Central Western Ghats based on amplification product data.	59-60
5.1	Dendrogram based on Nei's genetic distance showing clustering of sexes of two populations of <i>S. kathalekanensis</i> .	74-75

List of Plates

Plate No.	Title	Between pages
1	Habitat of <i>kathlekan</i> swamp.	20-21
2	General habit of <i>Semecarpus kathalekanensis</i> .	20-21
3	Cohort of young seedlings of <i>S. kathalekanensis</i> .	67-68
4	(a) Attempted clearing of seedlings and (b) bark removal in <i>Semecarpus kathalekanensis</i> .	67-68
5	<i>In situ</i> conservation measures taken by Karnataka Forest Department.	71-72
6	Growth of reintroduced <i>S. kathalekanensis</i> seedling showing good growth after 16 months.	73-74

List of Appendices

Appendix No.	Title	Page no.
I	Extraction buffer used and there composition. Composition of gel and electrode buffers. Stain buffer recipes Enzyme staining recipes used in the experiment	95-98
II	RAPD Protocol for the PCR amplification of <i>S. kathalekanensis</i> . Random primers screened against the DNA samples of two populations of <i>S. kathalekanensis</i> and their nucleotide sequences.	99-101
III	Banding pattern of Maleic enzyme (ME). Banding pattern of Isocitrate dehydrogenase (IDH). Banding pattern of Phosphogluco isomerase (PGI). Banding pattern of Malate dehydrogenase (MDH). Banding pattern of Phosphogluco mutase (PGM). Banding pattern of Phosphogluco dehydrogenase (PGDH).	102-104
IV	Amplification profiles of two populations of <i>S. kathalekanensis</i> with different primers.	105-109

Introduction

I. INTRODUCTION

Western Ghat forests are one of the 'hottest of the hot spots' for endemic and rare flora among the 25 hotspots recently identified world over (Myers *et al.*, 2000). An estimated 350 plant species are said to be endemic to this region. A few rare species are restricted specifically to certain unique swampy habitats called *Myristica* swamps. These fresh-water habitats harbor rare-relic floristic composition comprising of a large number of threatened plants (Chandran *et al.*, 1999). These fresh-water habitats once formed an extensive network along the streams of the Western Ghats, are now reduced to highly fragmented pockets and appear to be one of the most threatened ecosystems in India (Chandran and Mesta, 2001). Since swamps are considered to be highly productive habitats, they have been subjected to extensive destruction through human intervention for shifting cultivation, raising of monoculture plantations of commercial crops, submersion under hydroelectric power projects, mining *etc.*

Recently, a new tree species has been described from the *Myristica* swamps of Uttara Kannada district by a team of scientists of the Karnataka Forest Department (Dasappa and Swaminath, 2000). It has been named as *Semecarpus kathalekanensis* Dasappa and Swaminath, based on endemic swampy habitat of its occurrence in Uttara Kannada, *i.e.*, *Kathlekan* (meaning *Dark Forest*). *S. kathalekanensis* is a large dioecious tree possessing a few unique characters such as modified root system with prominent lenticels, the production of highly corrosive acrid juice, *etc.* Due to high allergy inducing nature of this juice, local people selectively cut these trees. Only four populations of *S. kathalekanensis* have so far been identified from Siddapura range, which collectively have less than 50 breeding individuals. Hence this species could be critically endangered in nature (Vasudeva *et al.*, 2001). The populations are distributed at around 14° North latitude and 74° East longitude in three different places in Uttara

Kannada district within a radius of 10 km. Since, *S. kathalekanensis* is a newly described species, virtually no information is available regarding the genetic structure of these populations.

Maintenance of genetic variation is considered essential for the long-term survival of any species (Frankel and Soule, 1981). It is believed that the small populations are mostly associated with lower levels of genetic diversity, which might also reduce the average fitness of constituent individuals. On a long run, loss of genetic variability reduces the raw material for evolutionary changes within the population in order to respond to changing environment. Small populations might also lose a large amount of genetic variability due to genetic drift jeopardizing the very survival of any species (Franklin, 1980). Hence the loss of genetic diversity can lead to local extinction of a species (Saccheri *et al.*, 1998).

Further, smaller population sizes can have consequences that lead to rapid extinction before genetic drift has had a chance to operate. There could be a complete failure of pollinators, or a small population of a dioecious species could consist completely of one gender and fail to reproduce. Direct human disturbances can destroy the habitat and the populations causing immediate disappearance of the populations (Savolaninen and Kuittinen, 2000). Hence, loss of habitats throughout the world due to various reasons has made conservation of rare and threatened species a real challenge.

For these reasons understanding genetic diversity of small populations has become an important emerging area in the field of conservation biology. Measuring the amount and spatial distribution of inter and intra population variability will help determining a management strategy for *ex-situ* and *in-situ* conservation. For instance, the concept of "Forest Gene Banks" proposed by Uma Shaanker and Ganeshaiah

(1997) entails working out population level genetic diversity such that populations with highest genetic diversity could be identified and the variation crucial for the survival could be conserved. Further, Vasudeva *et al.* (2001) have suggested that 'reintroduction' of the nursery grown seedlings into type localities is the most important and effective way for the restoration of critically endangered species in the wild. Since *S. kathalekanensis* is a dioecious tree, it is also imperative to know the sex of the seedling at nursery stage itself in order to construct founding populations with equal sex ratios and plant them at appropriate spatial scale.

Traditionally, researchers have employed allozyme markers to understand genetic diversity and structure of plant populations (Hamrick and Godt, 1990). With the advent of DNA based techniques, several robust tools have been developed, which essentially consider synthetic loci. RAPD based assessment can yield an abundance of markers particularly when allozyme polymorphisms are few. RAPD markers help in determination of genetic diversity (Stewart and Porter, 1995), genetic relationship among individuals (Van Hudson and Bachman, 1992) and provide an estimate of population structure (Martin *et al.*, 1997).

In this background the present study was carried out with the following objectives:

- a) to estimate genetic diversity both at within and among two populations of *Semecarpus kathalekanensis*, using allozymes and RAPD markers and
- b) to identify sex specific molecular markers, if any.

Review of Literature

II. REVIEW OF LITERATURE

Biological diversity can be conceptualized at three levels, viz., genes, species and ecosystems on the basis of cellular/molecular, taxonomic and geographic criteria, respectively. Tropical forests harbor more than half of all species. The remainder is distributed in other ecosystems across the planet, from deserts to high mountains, from deep volcanic vents in seafloors to the shallow bays of Antarctica. Tropical forests of the world are the richest repositories of diversity of plants and animals in the world. They contain more than 50 per cent of all the known species, though they cover a mere 7 per cent of the earth's land surface (Myers, 1988). Prance (1978) has estimated that about 1,55,000 out of 2,40,000 angiosperm species are restricted to the tropics. Of these, a staggering 90,000 are found in tropical America; 35,000 in the tropical Asia and 30,000 in tropical Africa. Even the lower plants such as ferns and mosses are rich in the tropics. For instance, 11,000 out of 12,000 ferns and 9,000 out of 12,000 mosses are found in the tropics. Hence the tropical forests are the seats of floral diversity. From this perspective, the genetic organization and breeding structure of tropical trees are central to an understanding of tropical diversity at all levels, as well as to the long-term conservation of that diversity.

The loss of biological diversity is the most critical environmental issue facing our planet at the dawn of the new millennium. The problem at times seems overwhelming. The solution is to identify strategies that will prevent further loss of the species, ecosystems, and ecological processes that comprise the earth's biological wealth.

Deforestation and fragmentation of tropical forests continues at alarming rates, resulting in problems ranging from species extinction to global environmental changes. Central to conservation sciences is the use of population biology studies for management and preservation of specific species (Oyama, 1993). In the light of such

rapid deforestation, active genetic management is required for conservation and to promote efficient utilization.

Since, *Semecarpus kathalekanensis* is a newly discovered tree species in the Western Ghats, literature regarding studies on population genetics is virtually nil. Hence, in this chapter, literature generally covering studies on genetic diversity of rare/endangered or commercially important species is presented. General aspects of floral richness and diversity of the Western Ghats is also included.

2.1 Floral richness of the Western Ghats

Among the 25 hotspots of endemic flora recognized in the world, two are in India viz., the eastern Himalayas and the Western Ghats (Myers *et al.*, 2000). In fact, forests of the Western Ghats along with those of Sri Lanka have been recognized as one of the "hottest of the hotspots" in the world for endemic and rare plants in the world.

The Western Ghats comprise a total area of 1,70,000 sq. km spread over Gujarat, Maharashtra, Goa, Karnataka, Kerala and Tamilnadu (Collins *et al.*, 1990). The Western Ghats region comprise a mountain range running parallel to the west coast of peninsular India from 8° N to 21° N latitude for a distance of around 1,600 km (Myers, 1988). The diversity of climatic, edaphic and biotic regions of the Western Ghats have shaped the evolution of some 3,500 taxa of angiosperms, 177 amphibians, 150 reptiles, 508 birds, 79 mammals and from the less studied groups of organisms (Narendra Prasad, 1998). Many plants new to science have been discovered from Western Ghats, most of them either being narrow endemic or with limited range. Extremely restricted areas have been reported as type localities for a number of plants species, many of which are endangered or rare (Ramesh *et al.*, 1997).

2.2 'Tropical fresh water swamp forests': An endangered habitat

Fresh-water swamps are low-lying poorly drained depressions that often open into a river/riverlet. They serve as channels for the run-off of excess water thus acting as natural flood control devices. Some times the ground water level in the swamps lies very close to the ground surface. However, scientists differ in their opinion regarding what all should be called as swamps. For instance, Gore (1983) classified mires, marshes and wetlands as swamps, while Cunningham and Saigo (1990) considered only those wetlands with trees as swamps. Davis *et al.* (1934), reported about 'swampy evergreens' with *Myristica sp.* confined to low lying poorly drained areas in the Western Ghats.

Fresh water swamps are found in many parts of the world, especially in the valleys of Mississippi and its tributaries, in Sweden, Odzala National park, Congo, in the Malaysian region, Papua New Guinea, *etc.* Swamp forests fringe the amazon and its tributaries (Clark and Benforado, 1981; Whitmore, 1993). In India, fresh water swamps are reported from the Siwalik and Doon Valley and the Bramhaputra Valley. The Western Ghats, which is one of the global centers of biodiversity and endemism (Myers, 1988; Nayar, 1996) is home to the *Myristica* swamps. Hubbel and Foster (1986) are of the opinion that some of the rarest plants occur along stream banks and swamps, although most of the swamps are altered by human activity in south India.

The swamps of Uttara Kannada are popularly known as *Myristica* swamps because of the unique floristic composition. Talbot (1911) mentions occurrence of *Myristica fatua* var. *magnifica* in Uttara Kannada. Ramesh *et al.* (1997) have viewed *Myristica* swamps as unique habitats at "ecosystem level". *Myristica* swamps at *Kathlekan* was perhaps for the first time described by Gadgil and Chandran (1989).

Very recently, Chandran *et al.* (1999) in their fine work inventoried *Myristica* swamps in four forest ranges and identified 51 swamps covering an area of 98,175 sq. meters. Recently Vasudeva *et al.* (2001) have studied the population biology of one of the rare plants of these swamps.

2.3 Rare and threatened plants of the Western Ghats

What exactly is a rare species? - is an interesting question that ecologists have often asked but seldom have agreed to a common definition (Vasudeva, 1998). The criteria used by scientists to assess and categorize plant species into classes of rarity vary considerably. For instance, Harper (1981) has considered rarity as a phenomenon in both time (related changes in the population size across time) and space (related to the sites on which the populations occur). In India, botanists have classified plants as rare, endangered and threatened based on their experience alone.

In one of the finest treatment of concept of rarity, Rabinowitz (1981) recognized that geographic range, habit specificity, and population density are the three axes on which botanical rarity can be defined. Species belonging to either small geographic range, occupying narrow habitat range or having lower population size merit to be called rare. Plants with small geographic range, high habitat specificity and low population densities characterize species exhibiting the most vulnerable form of rarity.

According to the recent Red List of Threatened species, released by the International Union for the Conservation of Nature, nine plant species are extinct (or extinct in wild), about 49 species critically endangered, and 200 in vulnerable/endangered category in India. Some of the extremely threatened plant species include *Hubbardia heptaneuron* (a monotypic genus restricted to the spray zones of Jog water-falls), *Viscum mysorense*, *Leea talbotti*, *Leuca angustissima*,

Cynoglossum ritchiei, etc.; the first two species are feared to be already extinct in the wild (Nayar, 1996). Only 10-15 populations of *Frerea indica* are reported from the wet rocky habitat in the northern Western Ghats. Similarly, *Uleris salicifolia*, a shrub occurring in the ecotone of rocky crevices of southern high altitude Western Ghats, is also highly threatened.

2.4 Studies on genetic diversity of tree species

Assessment of genetic diversity is essential in developing effective conservation strategies and sustainable forest management guidelines (Loveless, 1992). Working out genetic diversity of trees is of particular importance, since the tree flora represents the structural habitat within which other species exists. The persistence of evolutionary viable population of tropical trees is crucial to the preservation of the tropical forest habitat. From this perspective, assessing genetic organization and breeding structure of tropical trees are central to the understanding of tropical diversity at all levels, as well as to the long term conservation of the diversity (Loveless, 1992).

Rare and endangered plant species often occur in small disjunct populations with reduced genetic diversity due to increased habitat fragmentation and inbreeding (Stebbins, 1980; Simberloff, 1998; Barrett and Khon, 1991; Godt et. al., 1996; Sun, 1996). Hence, loss of biodiversity occurs not only through the loss of these species, but also through the loss of genetic diversity within these species. Hence, knowledge of how the genetic variation of a species is partitioned within and among population should play an important role in designing effective *in-situ* and *ex-situ* conservation strategies (Hamrick et. al., 1991; Maxted and Hawkes, 1997; Meffe and Carroll, 1997).

Traditionally, researchers have employed allozyme markers to understand some of the issues like, genetic diversity, population structure and aspects of ecological

genetics (Hamrick and Godt, 1990). With the advent of DNA based techniques, several robust markers have been developed which essentially constitute the synthetic loci. Randomly Amplified Polymorphic DNA (RAPD) based assessment can yield an abundance of markers. Particularly, when allozyme polymorphisms are few, RAPD markers help in determination of genetic diversity (Stewart and Porter, 1995), genetic relationship among individuals (Van Hudson and Bachman, 1992) and provide an estimate of the population structure (Martin *et. al.*, 1997).

Patterns of genetic diversity among temperate tree species have been studied to a greater depth than among tropical ones. Until the mid-1980's, most studies of genetic variation in trees focused on conifers. As of today, data on genetic diversity exists on roughly over 1000 temperate trees; only half this number of tropical species has been worked. In the following section, case studies in genetic diversity of hardwood trees are presented.

2.4.1 Case studies from Australian tree species

Recently, Moran (1992) has reviewed the pattern of genetic diversity among Australian tree species. The analysis was based on survey of genetic diversity studies done predominantly on two important genera *viz.*, *Eucalyptus* and *Acacia*. Considering isozyme studies of 25 species of *Eucalyptus*, 10 species of *Acacia* and two species of *Casuarina*, the authors have concluded that these species have higher levels of allozyme variation with most of the variation "within" rather than "between" populations. The expected heterozygosity (H_e) ranged from 0.167 to 0.239. However, *Eucalyptus* species with localized distribution showed lesser H_e ranging from 0.084 to 0.278; the species showing only regional distribution also recorded lower levels of H_e . Among the *Acacias*, *A. mangium* showed lower levels of polymorphic loci and H_e (0.017).

Moran *et al.* (1989) have observed high level of polymorphic loci as well as H_e in a wide spread *Casuarina cunninghamiana*; thus geographical range appears to partly explain the current pattern of genetic diversity among Australian species. Isozyme analysis of 18 natural populations of *A. auriculiformis* from Queensland and Northern Territory in Australia and Papua New Guinea revealed a mean expected heterozygosity (H_e) of 0.081 with values ranging from 0.002 to 0.180 (Wickneswari and Norwati, 1993). The genetic differentiation between populations was largely due to differences among the three geographic regions with very little 'between population' variation.

Acacia mangium has been considered a genetically depauperate species among the Acacias (Moran *et al.*, 1989). Estimates of genetic diversity for 10 natural populations of *A. mangium* from Australia and Papua New Guinea, based on both isozyme and RFLP analysis were higher when compared to Indonesian populations. It is interesting that provenances with higher genetic diversity were better performers (Hardwood and Williams, 1992).

2.4.2 Case studies from European tree species

Muller-Starck *et al.* (1992) compared the genetic diversity among European coniferous as well as angiosperm tree species based on allozyme, DNA, terpenes and polyphenol markers. They classified them into following four categories based on geographical range and levels of genetic differentiation among populations.

The first category consisted of species with large geographical range that show little genetic differentiation among populations. The examples include species such as *Pinus abies* ($G_{st}= 4.0$), *P. sylvestris* ($G_{st}= 1$ to 16) and *Fragus sylvatica* ($G_{st}= 3.6$ to 5.2). The second category consists of species with large geographical ranges but subdivided into different sub species with little inter-population variation such as *P. nigra* ($G_{st}= 9.8$ to 13.5), *P. halepensis* ($G_{st}= 1.3$) and *Larix decidua* ($G_{st} = 2.60$).

Species such as *A. alba*, and *P. cembra* ($G_{st}= 32$) constituted the third category and were characterized by disjunct populations that were distributed over smaller ranges with higher inter population differentiation. Extremely small geographical ranges with great inter-population differences characterized species (*P. pumila*) of the fourth category.

2.4.3 Case studies from Malaysian tree species

Wickneswari *et al.* (1995) have shown, based on isozyme analysis, that mean expected heterozygosity for six populations of *Hopea odorata* ranged from 0.137 to 0.236 with a average of 0.190. The RAPD analysis of two planted populations also revealed similar trend suggesting that the populations have sufficient genetic diversity to support a selective breeding programme.

Lee *et al.* (2000) studied *Shorea leprosula* populations of Malaysia and reported that mean species and population level heterozygosity was high ($H_e= 0.369, 0.406$, respectively), however the inter-population differentiation was lower. However, the RAPD data suggested a lower level of diversity (both for per-cent polymorphic loci and H_e) than that identified by the allozyme data.

2.4.4 Case studies from Indian forest species

Several economically important tree species have been studied in India with regard to their genetic diversity. A team of scientists headed by Uma Shaanker at the University of Agricultural Sciences, Bangalore has done pioneering studies on several important tree, bamboo and rattan species of south India. In one of the earliest reports, Uma Shaanker and Ganeshaiah, (1997) studied genetic diversity of four populations of *Phyllanthus emblica* in South India using isozymes. Populations from Petchiparai in Kerala was identified to be best in terms of allelic diversity followed by that from Biligiri Rangan Temple (BRT) hills. Based on their data the authors have also proposed a new

concept called "Forest Gene Banks" in order to conserve highest genetic diversity. Padmini *et al.* (2001) have shown that anthropogenic disturbances can potentially reduce genetic diversity and regeneration of *P. emblica* populations.

Investigating the impact of harvesting and other anthropogenic pressures on the regeneration and genetic diversity of a few NTFP species, Uma Shaanker *et al.* (1998) identified that both allelic richness and diversity index of *Kydia calycina* decreased under disturbance. The pattern was similar in the sites studied viz., BRT hills and Mudumalai wildlife sanctuary in south India. In the same study, it was also shown that in a timber species (*Terminalia crenulata*) allelic diversity and richness marginally decreased under disturbance compared to control sites.

Uma Shaanker *et al.* (2001) have reported that populations of *Terminalia bellarica* and *T. chebula* suffer from low levels of heterozygosity. It is alarming that a few populations of *T. chebula* are virtually devoid of heterozygosity. This perhaps explains the lower levels of regeneration of this species in a few localities. *Santalum album* is one of the prime commercial species, which is severely affected due to anthropogenic pressures. After examining 19 populations from southern India, Nageswara Rao *et al.* (2001) reported that *S. album* exhibits a relatively higher levels of heterozygosity ($H_o = 31\%$) at population level. Populations from southern Karnataka showed a higher H_o than those from northern Karnataka/Tamil Nadu/Kerala/Andra Pradesh. Based on the data the authors have suggested that the populations of *S. album* at the Deccan plateau region could serve as centers where forest gene banks could be established.

By mapping genetic diversity of an economically important dioecious rattan (*Calamus thwaitesii*) from the central Western Ghats, Ravikanth *et al.* (2001), perhaps for the first time, have shown that observed overall populational level heterozygosity was relatively lower (6.62 - 15.56 per-cent) compared to 37 per-cent reported by Bon *et*

al., (1995) for a related species. Constructing GIS based contours of species diversity the authors have also identified that Kodagu district in Karnataka, regions north as well south of the Palghat gap in Kerala act as epi-centers of species diversity for rattans.

Cheluvaraju *et al.* (2001) studied genetic diversity of two economically important bamboo species of the Western Ghats. Working with 22 populations of *Bambusa arundinacea* the authors have reported that populations of southern Karnataka region showed higher Nei's gene diversity compared to that in the northern region. However, a contrasting pattern for the distribution of genetic diversity was observed for *Dendrocalamus strictus* for which 16 populations were studied. Perhaps for the first time, the study provided evidence for the existence of distinct genetic provenances of the two species. The authors opine that these two regions could constitute hot spots for the conservation of genetic resources.

Another team from Institute of Forest genetics and Tree Breeding, Coimbatore have concentrated on studies on *Acacia nilotica* (Nagarajan *et al.*, 2001). Five distinct sub-species of *A. nilotica* were identified using RAPD analysis from Asian and African sources. However, the spatial distribution of genetic diversity of Asian sub-species did not match with the geographical groupings, which was attributed to unique mating system of the species.

Scientists at Tata Energy Research Institute (TERI), New Delhi have used RAPD as a tool to develop markers to delineate different genotypes of two species of poplar and of neem. The authors have identified 40 distinct rare bands in poplar, which can be effectively used in clonal identity. Adapting RAPD and AFLP techniques, it was shown that the genetic diversity of neem was highest at provenance level followed by that at intra-population level. Finally, family of a mother-tree showed least diversity. The study

also revealed that Indian accessions showed a greater diversity than those from Thailand.

Teak is one of the very important commercial timber tree species of south Asia. Working with three populations from Karnataka, Kertadikara and Prat (1995) compared the genetic diversity, as measured through isozyme data, of these Indian populations with those from Indonesia, Thailand and Africa. They concluded that Indian populations had higher expected and observed heterozygosity than other populations. The study also suggested that there is an excess of homozygosity among most of the provenances indicating a possible sib mating.

2.5 Correlates of genetic diversity with life history traits

The accumulation of information on genetic diversity of temperate plant species has provided insights into the relationships between diversity at gene level and life history traits. Hamrick *et al.* (1992), based on literature survey of 662 gymnosperm and angiosperm species have reported that long-lived woody species have a higher proportion of polymorphic loci, more alleles per locus, higher effective number of alleles per locus and more genetic diversity than other life-forms. They have reported that genetic diversity for trees was 38 per cent higher than that for annuals and 51 to 80 per cent higher than that of the other life forms. Ledig (1986) is also of the opinion that heterozygosity among tree species generally exceeds those in herbaceous plants by two folds.

Multilocus electrophoretic surveys conducted on hundreds of plants and animal species has revealed that levels of genetic variation were high but also variable among species. For example vertebrates have significantly less variation (mean heterozygosity

per individual, $H=0.036$) than invertebrates ($H=0.112$, Nevo, 1978) and plant populations may contain even higher levels of variation ($H=0.19$, Hamrick *et. al.*, 1979).

Loveless and Hamrick (1984) demonstrated that, species with large and contiguous populations, high fecundities, high rate of out-crossing, wind pollination, long generation times, and occurrence in late successional phases maintains high genetic diversity. The endemic Torrey pine and the wide spread Douglas fir represent opposite extremes of genetic diversity. Average individual H_o is 0 in the two known populations of Torrey pine and 0.33 for Douglas fir, which ranges through Canada, the USA and Mexico and from the pacific coast to the rocky mountains (Ledig and Conkle, 1983). On the other hand, Monterey cypress (*Cupressus macrocarpa*), which like Torrey pine, is restricted to only two small populations, has an average heterozygosity of 0.16 and red pine (*Pinus resinosa*), with a latitudinal range of about 12 degrees and a longitudinal range of 42 degrees, has almost no variability (Flower and Morris, 1977). Hence, the level of genetic diversity is more likely a reflection of recent historical events, life history parameters, and the mating system. Generalizations derived from the allozyme literature provide a basis on which strategies to conserve genetic diversity of threatened species can be formulated.

2.6 Identification of sex specific markers using biochemical tools

2.6.1 Dioecy and its evolution

Dioecy, a condition wherein the male and female flowers are borne on separate plants, is one of the most striking examples of evolutionary specialization. Approximately 10% of the genera of higher plants are dioecious (Yampolsky and Yampolsky, 1922). It has been argued that such extreme specialization of sexes into separate plants may have evolved as a mechanism to ensure out breeding (Darwin,

1871; Baker, 1959; Bawa, 1980) or as an optimal allocation of resources between the sexes (Bawa, 1980). Available evidence suggests that dioecy is more prevalent, in tropical than temperate ecosystem (Ashton, 1969); on islands than mainland communities and among trees than shrubs or herbs. Dioecy is also unevenly distributed across taxa. Certain families, where dioecy is particularly prevalent are Menispermaceae, Myristicaceae, Monimiaceae, Euphorbiaceae, Moraceae, Cucurbitaceae, Anacardiaceae and Urticaceae, and appears to be rather more common among dicot genera than among monocot genera (Renner and Ricklefs, 1995).

Several models have been proposed for the possible evolutionary patterns of dioecy. There are three such routes to dioecy. Charlesworth and Charlesworth (1978) postulated the process of evolution of unisexual flowers, as two independent events. That, dioecy has evolved through gynodioecy as an intermediate (the existence of females and hermaphrodites) or through androdioecy (the existence of males and hermaphrodites). In both cases, the initial step is the establishment of a single mutant.

The second evolutionary route to dioecy is through monoecy. A monoecious population could evolve into a dioecious one through a series of mutations, which alter the ratio of male to female flowers (Charlesworth and Charlesworth, 1979). The third possible route to dioecy is via distyly which describe the condition where individuals are polymorphic for style and anther positions as two different hermaphroditic floral types. Specialization of one type for maleness and the other for femaleness may progress to full dioecy (Lloyd, 1979).

2.6.2 Mode of sex determination in dioecious plants

Heteromorphic sex chromosomes are rarely found in angiosperms; some of the genera that have been reported to contain them are *Rumex*, *Cannabis*, *Humulus*,

Asparagus, *Melandrium* and *Silene*. Hirata (1924) has reported that *Cannabis sativa* is a dioecious plant with two heteromorphic sex chromosomes. Yamada (1943) revealed that the female plants have two X chromosome and one Y chromosome, with the latter being much larger than the X chromosome and the autosomes. The roles of the sex chromosomes in sex determination have been studied in haploid, diploid and triploid plants (Warmke and Davidson 1944).

Studies conducted by Bracale *et al.* (1990), on *Asparagus*, revealed that the sex is determined by homomorphic sex chromosomes in which the males are heterogametic (XY). Genetic evidence suggests that *Asparagus* is "male dominant" and contains male activator – female suppresser genetic determinants.

2.6.3 Use of Isozyme markers in sex-determination of plants

In *Asparagus officinalis*, a dioecious plant, the extracts from the phylloclads were electrophoretically analysed by Maestri, *et. al.*, (1991), for isozyme polymorphism. Out of 14 enzyme systems examined, seven exhibited a clear and consistent banding pattern. Isozyme polymorphism was studied in seven pairs of male and female doubled haploid. Segregation of polymorphic loci was examined in the back cross progenies and was found to be consistent with a simple Mendelian inheritance in all the cases, except for 3 anodal peroxidases. They demonstrated a linkage between malate dehydrogenase locus and the sex determining genes. They argued that the availability of isozyme markers may be useful in breeding and in particular, in mapping the sex genes.

2.6.4 Use of RAPD markers for sex determination

After the discovery of thermally stable DNA polymerase from *Thermus aquaticus* (i.e. Taq DNA polymerase) and automated thermal cyclers (PCR machines), extensive work has been carried out in the area of sex-determination using randomly amplified

polymorphic DNA technique. Randomly amplified polymorphic DNA technique has been used in recent years, in identification of various characters including sex determination in plants as well as in animals.

Shibu *et al.* (2000) identified sex-specific DNA markers in Nutmeg (*Myristica fragrans*) a dioecious tree valued for its Nutmeg (kernel of the seed) and mace, the dried aril surrounding the seed. Out of 60 random primers screened using RAPD-PCR, one primer, OPE 11, elicited a female sex-specific DNA amplification product of approximately 416 base pair length, while none of males tested gave this product. Their result suggests that in nutmeg, the female could be the heterogametic sex.

Hormaza *et al.* (1994) amplified the DNA segments with the objective of identifying markers linked to sex determination in *Pistachio vera*, a commercially important dioecious plant. Progenies from two female parents were pollinated with a common male parent. Two bulks of DNA were made in each cross by pooling an equal weight of fresh leaves from each individual. DNA was extracted from each bulked sample and also from seedlings of unknown sex. Seven hundred different decamer oligonucleotide primers were used to perform DNA amplification. Only one among these, OPO -08, produced a 945 bp amplification band which was present only in the bulked female samples and was absent in the bulked male samples. Therefore they proposed that the band is tightly linked to the genes that control the sex determination in *Pistachio*, before they reach the reproductive maturity.

Sakamoto *et al.* (1995), analyzed the male associated DNA sequences in *Cannabis sativa*, a dioecious plant. The DNA isolated from male and female plants were subjected to PCR amplification using random primers. Two out of 15 primers, yielded fragments of 500 bp and 700 bp which were detected in all male plants but not in any of the females. These two DNA fragments were cloned and used as probes in

gel blot analysis of genomic DNA. Then the male and female DNAs were allowed to hybridize with 500bp probe, no differences in patterns were observed between male and female plants. By contrast when these DNAs were allowed to hybridize with 730bp probe, much more intense bands specific to male plants were detected. The 730 bp DNA fragment was named as MADC-1 (Male associated DNA sequence in *Cannabis sativa*). They suggested that the 730bp DNA probe can be used to identify the sex of the plant, in its early seedling stages to facilitate screening and selection for various purposes.

Ruas *et al.* (1998) reported male specific DNA in a dioecious species *Atriplex garrettii*. DNA samples extracted individually from ten male and ten female plants were bulked by sex. RAPD fragments were generated in the two bulks in order to identify markers that were polymorphic between male and female plants. A total of 158 decamer primers were tested, a 2075 base-pair (bp) male-specific DNA fragment generated with the OPAF-14 primer was identified. The fragment was cloned and partially sequenced and 24-mer primers that exclusively amplified this fragment were constructed. When 124 male plants, 126 female plants, and one hermaphroditic plant were tested individually, the male-specific 2075-bp DNA fragment was present in the hermaphrodite and all but one of the male plants, and was absent in all female plants.

Material and Methods

III. MATERIAL AND METHODS

3.1 Study site and populations

The present study was carried out in the central Western Ghats in Siddapura range of Uttara Kannada in Karnataka (Fig. 3.1), where the hill range rarely rises over 600 m above mean sea level. The hills are steep towards the west, with deep widening valleys. The study area has a tropical climate with a well-defined rainy season between June and November. Isolated showers may also occur during December and May. The annual average rainfall is about 2,500 mm. The temperature is generally warmer during March to May, reaching a maximum of 38°C. Winters are generally not very cold. The soils of the site, derived from metamorphic rocks, are rich in iron and manganese.

The vegetation varies from evergreen to semi-evergreen forests. These forests are unique for the distribution of endemic plants species like *Arenga wightii*, *Dipterocarpus indicus*, *Pinnanga dicksonii*, *Polyalthia fragrans*, *Myristica fatua* var. *magnifica*, *Gymnacranthera canarica* (Ramesh and Pascal, 1997). The region is also well known for the occurrence of endemic mammals such as rufous-bellied hawk-eagle, blue-winged parakeet, ceylon frog-mouth, white-bellied treepie, grey-headed bulbul, wynad laughing thrush, and black headed babbler (Daniels, 1989).

Two populations of *S. kathalekanensis* found in the swampy habitat of "kathlekan" in the Siddapura range of Sirsi forest division were used as focal populations for the study (Plate 1 and 2). These populations are separated by 100 m along a stream that feeds the swamp. All adult flowering individuals were sampled for their leaf in both the populations. In these two populations only flowering individuals were selected for enzyme extraction and DNA extraction.

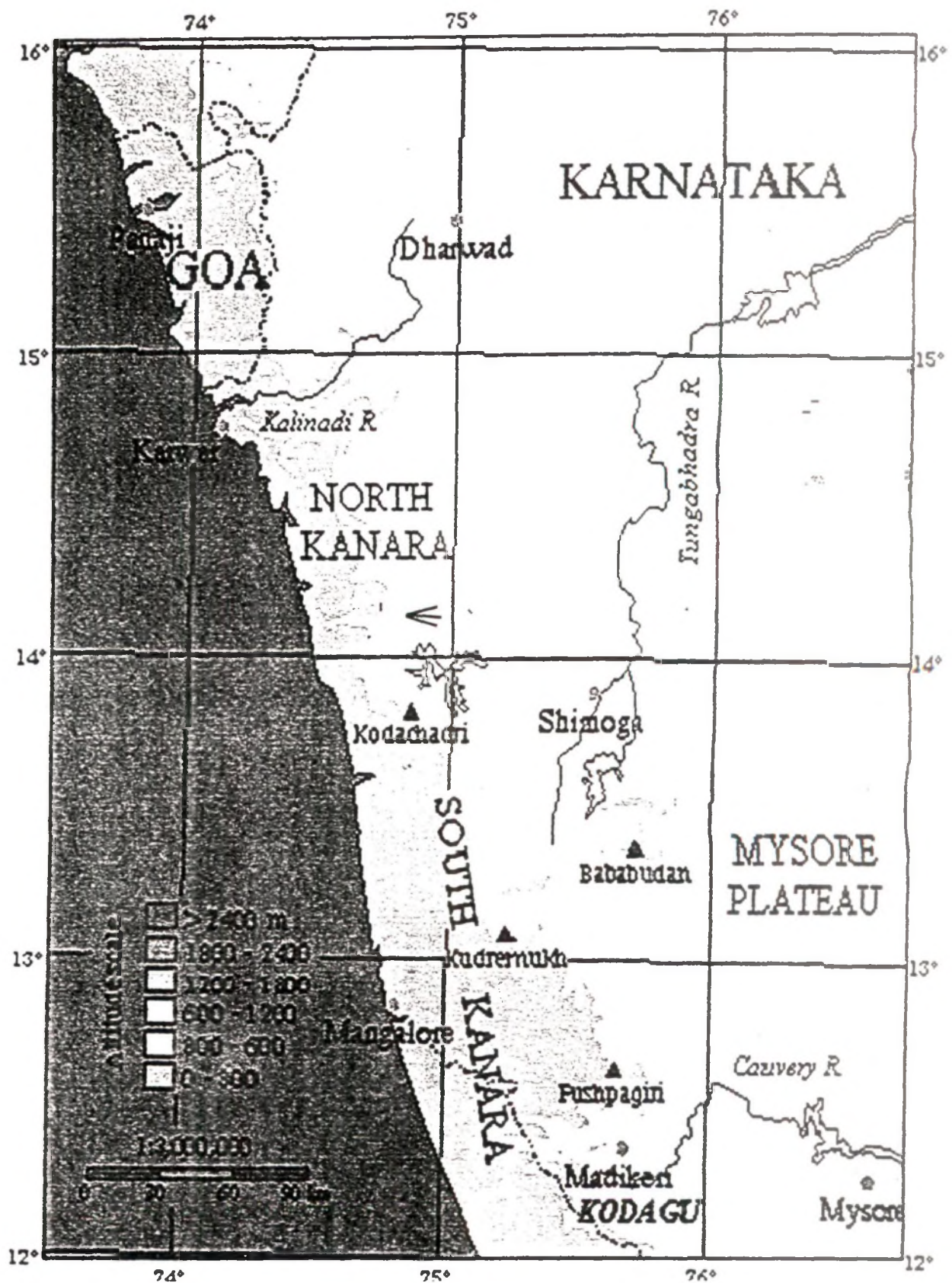


Fig. 3.1 Map showing study area.



Plate 1 Habitat of *kathlekan* swamp



Plate 2 General habit of *Semecarpus kathalekanensis*

3.2 Plant material collection

From each of the identified populations, the leaf materials were collected from flowering individuals (total of 34 individuals from both the swamp), snap frozen in liquid nitrogen and brought to the laboratory. The material thus collected was used to study the population genetic parameters following isozyme and Randomly Amplified Polymorphic DNA (RAPD) analysis. The details of protocols for isozyme analysis are given below.

3.3 Isozyme analysis

Starch gel electrophoresis for isozyme analysis was conducted following standard procedure (Leingsiri *et al.*, 1990).

3.3.1 Extraction of enzyme

The extraction of samples was done using Chase extraction buffer (Appendix-I). The ratio of tissues to volume of extraction buffer was maintained in the ratio of 1:2 (weight:volume). Extraction was done in cold using pestle and mortar and the extract was centrifuged at 10,000 rpm for 10 min at 4° C. The supernatant was collected in eppendorf tubes and filter paper wicks (Whatman# 3, size 20 mm x 1.5 mm) saturated with supernatant were used for electrophoresis.

3.3.2 Preparation of starch gel

A 11 per cent starch gel was prepared in different gel buffer system which was standardized (Appendix-I). Two hundred and fifty ml of the buffer solution was heated till boiling in a Buchner flask. Thirty three grams of starch dissolved in 50 ml of buffer was added to the contents of the Buchner flask and boiled with continuous stirring until the suspension thickened. Then the gel mixture was degassed and poured into the glass gel mould (15x10x20 cm). Care was taken to remove air bubbles if any. A plastic

sheet was then placed on top of the gel to prevent it from drying. The gel was then allowed to set at room temperature and placed at 4° C to cool one hour before loading.

3.3.3 *Loading and running the gel*

The gel thus cast was cut lengthwise about 1.5 cm from the cathodal end and the cathodal and anodal ends were separated to leave a distance of about 1cm. Filter paper wicks saturated with tissue extract were placed vertically on the cut edge of the anodal end. A distance of nearly 1.5mm was left between two consecutive wicks. A wick containing the marker dye bromophenol blue was also placed to track the sample movement. After all the wicks had been placed, the cathodal section was firmly pushed against the anodal section such that there was good contact between the two gel sections.

The loaded gel was placed in an electrophoretic chamber filled previously with electrode buffer (Histidine buffer). Using absorbent material contact was established between the two buffer tanks via the two ends of the gel. The gel was covered with a plastic wrap and electrophoresis carried out in cold (4°C) by providing necessary power source. Current of 50 mA was given. The run was stopped 1hour after overrun when the dye had reached the cathodal end. This was done to improve the mobility and resolution of the enzyme.

3.3.4 *Slicing and staining the gel*

Once the run was over, the gel was removed from the electrophoretic apparatus and placed on a glass plate. Using a thin copper wire the gel was sliced horizontally to 1.5mm thickness and placed on petridish for staining. About 6-8 slices were obtained per gel. Before slicing, staining solutions were prepared freshly for various enzymes to be stained. Of the 20 enzymes initially assayed only six enzymes showed clear and good stainability. The compositions of staining solutions for these are given in

Appendix-I. Staining was done following standard procedures of Cheliak and Pitel (1984) with an agar overlay technique. Staining solution for a particular enzyme, with 1 per cent agar solution was poured evenly on the gel slice kept in a petridish and allowed to solidify. The gels were incubated at 37°C in dark for varying periods until the bands developed. Once clear and sufficiently intense bands were obtained the gels were fixed in a fixative solution containing acetic acid, ethanol and water in the ratio of 1:4:5.

3.3.5 Scoring the gels

After fixation, the gels were washed in water to remove the fixative and the staining solution. Then the gels were blotted using tissue paper. The banding patterns were then traced onto a transparency sheet. Gel photographs were taken and the gels packed in polythene covers and stored at 4° C for future reference.

3.3.6 Enzymes studied

Out of the twenty enzymes initially examined six enzymes namely Phosphoglucumutase (PGM), Phosphoglucoisomerase (PGI), Phosphoglucose dehydrogenase (PGDH), Malate dehydrogenase (MDH), Isocitrate dehydrogenase (IDH) and Maleic enzyme (ME) were studied for two *Semecarpus* populations (Table 3.1). The genetic interpretations of the six enzyme systems studied were done based on the patterns that were observed in the two populations of *S. kathalekanensis*.

3.4 Randomly Amplified Polymorphic DNA (RAPD) Analysis

3.4.1 Isolation of genomic DNA of *S. kathalekanensis*

Thirty four individuals were selected from the two populations for DNA extraction and for further analysis. Since the leaves of this plant are very rich in phenolics and

Table 3.1 Enzyme names, quaternary structure, sub cellular distribution, function and possible number of loci of some important Isozymes of plant system initially screened in *S. kathalekanensis*.

Sl. No.	Abbreviation	Enzyme Name	E.C.No.	Quaternary Structure	Subcellular Distribution	Function	No. of loci
1	ACP	ACID PHOSPHATASE	3.13.2	Monomer/ Dimer	Varies	Hydrolysis of phosphate esters	2 - 4
2	AK	ADENYLATE KINASE	2.7.1.3	Monomer	c.p	Phosphorylation	1-2
3	ADH	ALCOHOL DEHYDROGENASE	1.1.1.1	Dimer	C	Fermentation	1-3
4	ALD	ALDOLASE	2.1.2.13	Tetramer	C,p	Glycolysis, calvin cycle	2
5	DIA	DIAPHOROSE	1.8.1.4	Monomer	c,p,mt	Oxidative pathways	1-4
6	EST-D	ESTRASE-D	-	-	-	-	-
7	G6PDH	GLUCOSE 6 PHOSPHATE ISOMERASE	1.1.1.49	Dimer	c,p	Calvin cycle, ppp	2
8	PGI	PHOSPHOGLUCOISOMERASE	5.3.1.9	Dimer	c,p	Glycolysis	2
9	G3PDH	GLYCERALDEHYDE-3 PHOSPHATE DEHYDROGENASE	1.1.1.8	Tetramer	c,p	Glycolysis	3
10	HK	HEXOKINASE	2.7.1.1	Monomer	c,p,mt	Glycolytic enzyme	
11	IDH	ISOCITRATE DEHYDROGENASE	1.1.1.41	Dimer	c,p	TCA cycle	1(2)
12	MDH	MALATE DEHYDROGENASE	1.1.1.37	Dimer	c,mt,mb	TCA cycle, photo respiration, C4 photosynthesis	3
13	ME	MALIC ENZYME	1.1.1.4	Tetramer	C	TCA cycle	1
14	MR	MENADIONE REDUCTASE	1.6.99.2	-	-	-	-
15	PER	PEROXIDASE	1.11.1.7	-	c,cw	Oxidative pathways	2-13
16	PGM	PHOSPHO GLUCO MUTASE	5.4.2.2	Monomer	c,p	Converts starches to sugars	2
17	6PGDH	6 PHOSPHO GLUCONATE DEHYDROGENASE	1.1.1.43	Dimer	c,p	ppp	2
18	SKDH	SHIKIMIC ACID DEHYDROGENASE	1.1.1.25	Monomer	c,p	Synthesis aromatic Compounds	1 - 2
19	SUDH	SUCCINIC DEHYDROGENASE	1.3.99.1	Monomer	-	-	-
20	GDH	GLUCOSE DEHYDROGENASE	-	-	-	-	-

(C=cytosol;mt = mitochondria ; mb = microbodies; p = plastid ; cw = cell wall ; PPP = Pentose Phosphate Pathway ; TCA = Tricarboxylic acid cycle)

polysaccharides, which interfere with DNA isolation, CTAB extraction method was employed (Saghai-Marooof *et al.*, 1984).

3.4.2 DNA isolation by cetyl (hexadecyl) tri-methyl ammonium bromide (cTAB) method.

3.4.2.1 Reagents used

Extraction buffer:

- a) The extraction buffer contained 20 mM Na EDTA in 100 mM TRIS HCl (pH 8.0). To this 1.4 M NaCl and 2% w/v cTAB were added. The cTAB was dissolved by heating to 60° C and stored at 37° C. One percent β -Mercapto-ethanol (50 μ l/10 ml extraction buffer) was added just before use.
- b) Poly vinyl pyrrolidone phosphate (PVPP) added just before grinding the sample.
- c) Chloroform; Isoamyl alcohol (24:1 v/v) (wash solution)
- d) NaCl (5M)
- e) TE buffer: contained 10 mM Tris HCl and 1 mM EDTA of pH 8.0.
- f) 76 % Ethyl alcohol (wash solution)
- g) Chilled absolute Ethanol (double distilled)
- h) RNase (10 mg/ml): RNase was dissolved in 10 mM Tris HCl and 15 mM NaCl of pH7.5; it was then boiled for 5 minutes and cooled to room temperature.

3.4.2.2 Protocol followed

Ten ml of extraction buffer was taken into a sterile oak-ridge centrifuge tube, heated to 60° C and incubated for 30 minutes at room temperature. One per cent β -mercaptoethanol was added to it just before use. One gram of leaf tissue (stored at -70° C) was ground to fine powder using liquid nitrogen, in a sterile mortar and pestle. After mixing the powder with approximately 70-mg PVPP, the powder was transferred into the centrifuge tube containing the extraction buffer. The contents were thoroughly mixed by inverting the tubes several times and incubated at 60° C for one hour with

intermittent shaking. After the incubation, it was cooled to room temperature and 10 ml of Chloroform: Isoamyl alcohol (24:1 v/v) was added and mixed gently by inverting the tubes 20-25 times until it formed an emulsion.

The mixture was centrifuged at 8000 rpm for 15 minutes and the clear aqueous phase was transferred to a new sterile tube. Centrifugation was repeated twice by adding Chloroform: Isoamyl alcohol (24:1 v/v) of about 1/7th volume of the supernatant. To this further, 2.5 ml of 5M NaCl and 10 ml of chilled ethanol were added and gently mixed and kept overnight for incubation at -40°C . After the incubation, it was subjected to centrifugation at 8000 rpm for 10 minutes at room temperature. The supernatant was poured-off and the pellet was washed with 76 per-cent ethanol and spun at 5000 rpm for 5 minutes. The wash was repeated once again to completely remove the salts and polysaccharides. The supernatant was then drained out and only the pellet was retained. Ethanol was completely removed without drying the DNA completely by leaving the tubes uncovered at 37°C for 30-40 minutes.

After drying, the pellet was re-suspended in 200 μl of TE buffer. The pellet was completely dissolved in it. Using a sterilized cut tip it was pooled, treated with RNase at the final concentration of 10 μl and incubated at 37°C for 30 minutes. Further using a sterile cut tip, it was transferred to a sterile microfuge tube and stored at 4°C for future use (only ethanol precipitated DNA was stored at -20°C).

3.4.2.3 Purification of DNA

Since the DNA preparations obtained by the above method may contain considerable amounts of RNA, polysaccharides, phenolics and other impurities, the extract was subjected to further purification. To the DNA samples, one tenth of the volume of 3-M sodium acetate of pH 5.5 was added and mixed gently. Double the volume of cold ethanol was added to it, mixed gently and kept on ice for further

precipitation for about two hours. This was centrifuged at 6000 rpm for 5 minutes to pellet out the DNA. It was washed with 70 % ethanol and the traces of ethanol were removed by air drying for one to two hours. The pelleted DNA was re-dissolved in 100µl of sterile TE buffer, treated with 10ul RNase and incubated at 35° C for 30 minutes. For longer use it was stored at -20° C after ethanol precipitation.

3.4.2.4 Quantification of DNA

The method adopted for the quantification of DNA is based on the spectrophotometric measurement of U-V absorbance at 260 nm. A major disadvantage of this approach is that, RNA, oligonucleotides, proteins and other contaminants, interfere with the measurement. Further the technique requires microgram amounts of DNA to ensure reliable readings.

10µl of DNA aliquot was diluted in 990 µl of 1X TE (in the ratio of 1:100) in a microcuvette. The optical density was determined at 260nm and 280nm against a blank of 1X TE. The DNA concentration in the sample was calculated using the formula:

$$\text{DNA } (\mu\text{g}/\mu\text{l}) = \frac{\text{OD}_{260} \times 50 \times \text{Dilution factor}}{1000}$$

The ratio of OD₂₆₀ to OD₂₈₀ provides information about the purity of DNA sample. Pure DNA preparations show a ratio between 1.8 to 2.00. (The factor 50 is derived from OD at 260nm = 50µg /µl under standard conditions *i.e.* 1 cm light path.)

3.4.2.5 Evaluation of DNA quality

The reagents used:

1. 0.5x Tris boric acid EDTA buffer (TBE)
2. 0.8% Agarose
3. Ethidium bromide (10mg/ml stock)

4. Loading buffer: contained 0.5 % Bromophenol blue 75%Glycerol.

To examine the extent of contamination by RNA and the intactness of genomic DNA, the samples were subjected to simple agarose gel electrophoresis followed by staining with ethidium bromide. The dye intercalates into the DNA double helix and gives fluorescence when observed under UV light on a trans-illuminator in a gel documentation system. Agarose gel (0.8%) in 1X TBE was prepared with ethidium bromide (0.5ng /ml). About 2.5 µl of loading buffer was mixed with 10 µg of sample and loaded on to the gel. This was then subjected to electrophoresis at 50V for 2 hours and observed in a UV trans-illuminator.

3.4.3 Polymerase chain reaction (PCR) based randomly amplified polymorphic DNA (RAPD) analysis

Polymerase chain reaction (PCR), introduced in 1985, revolutionized the methodological repertoire of molecular biology. By this technique one can amplify any DNA sequence of interest to high copy numbers, thereby circumventing the need of molecular cloning. In order to amplify a particular DNA sequence, two single stranded oligonucleotide primers are designed, which are complementary to the specific sequences on the template DNA. Addition of a thermostable DNA polymerase in a suitable buffer system and cyclic programming of denaturation, primer annealing and polymerization steps result in the exponential amplification of the sequence between the primer sites.

Principle of PCR

The PCR is based on the enzymatic *in-vitro* amplification of DNA. Starting from a very low amount of template DNA (in nanograms), millions of copies of one or more particular target DNA fragments are produced, which can be electrophoresed and visualized by staining or autoradiography. PCR is characterized by its high speed,

selectivity and sensitivity. In a typical PCR, three temperature controlled steps can be discerned, which are repeated in a series of 25 to 50 cycles.

A single cycle in PCR is composed of the following three steps:

- a) Thermal denaturation of DNA
- b) Primer annealing.
- c) Polymerization.

3.4.3.1 Standardization of RAPD protocol

A "master mix" of reaction mixture for each primer, sufficient for all the samples is prepared. Master mix was prepared for the total number of tubes and primer was added separately. In case of primer screening and DNA was added separately in case of sample screening. Master mix preparation facilitates easier pipetting as the individual reaction tubes require very low quantity of the reagents. The components of the amplification reaction were optimized and a typical 25 μ l PCR mixture comprised of 2.5 μ l (= 75ng) of DNA, 2.5 μ l of 10X assay buffer for Taq polymerase with 15mM MgCl₂, 2.5 μ l of dNTP's of 1mM stock, 0.6 units of Taq DNA polymerase and 2.5 μ l of 0.3 μ M of primer.

The master mix was divided into equal volumes into labeled sterile microfuge PCR tubes and 2.5 μ l Primer (0.3 μ M) was added at last while standardizing. The content was mixed by a brief centrifugation. PCR reaction was carried out on a PTC-100 MJ research thermal cycler. The reaction started by setting the standardized protocol, (Appendix-II). Totally 65 primers were used to screen the *S. kathalekanensis* genomic DNA. The random sequence primers are obtained from Operon Technologies Inc. USA. The primer code number and sequences are given in the Appendix-II. Of the 65 random primers tried 10 primers (Table 3.2) were selected for screening DNA from all the population.

Table 3.2 Random primers standardised for the DNA samples of *S. kathalekanensis* and their nucleotide sequences.

Sl. No.	Oligo Name	Primer sequence	Molecular weight(MW)
1	KIT W 6	5'-AGG CCC GAT G-3'	3053
2	PRIMER 2	5'-TGC CGA GCT G-3'	3044
3	PRIMER 3	5'-AGT CAG CCG C-3'	2997
4	KIT X 2	5'-TTC CGC CAC C-3'	2924
5	KIT X 12	5'-TCG CCA GCC A-3'	2973
6	KIT X 13	5'-ACG GGA GCA A-3'	3086
7	KIT X 14	5'-ACA GGT GCT G-3'	3068
8	KIT X 15	5'-CAG ACA AGC C-3'	3006
9	KIT X 17	5'-GAC ACG GAC C-3'	3022
10	KIT X 18	5'-GAC TAG GTG G-3'	3108

3.4.3.2 Separation and detection of amplified PCR products

The method adopted for the separation and detection of amplified PCR product was agarose gel electrophoresis with ethidium bromide staining. This enables the detection of DNA fragments as small as about 10ng per band. Staining was done by adding ethidium bromide (0.5µg/ml) to pre-cooled 1.5 % agarose in 1X TBE buffer.

3.4.3.3 Agarose gel electrophoresis

1.5 % agarose in 1X TBE is casted into gel mould and allowed to solidify. The amplified DNA product was loaded to the well in the gel and a constant electric field is imposed. Under neutral or slightly alkaline conditions, DNA migrates towards the anode.

Since the agarose matrix acts as a molecular sieve with pore sizes depending upon the concentrations, DNA fragments upto about 20 kb are separated according to the size. After brief spinning of PCR tubes, 4 µl of loading buffer was mixed with amplified PCR products and was subjected to electrophoresis in 1.5 % agarose gel containing ethidium bromide stain, at 70 volts for 3 hours; 0.5X TBE was used as a running buffer. After the electrophoration, the gel was observed under U-V trans-illuminator in Alpha innotech gel documentation system.

3.5 Data analyses

3.5.1 Genetic interpretation of isozyme variation:

The interpretations of the six enzyme systems were based on the banding patterns observed in the two populations of *S. kathalekanensis*. The interpretations of the banding patterns were done and checked if they conform to the quaternary structure of the protein. Individual isozymes were designated by a number referring to

its relative position on the gel, with the lowest number corresponding to the most anodally migrating loci. Alleles within a locus were designated in a similar manner.

3.5.2 Scoring the gels of both Isozymes and RAPDs

The isozyme data from six enzyme systems was scored following the method of Uma Shaanker and Ganeshaiah (1997). Presence of a band was scored as 1 and absence as 0. Binary coding was used to score the gels (Wendel and Weeden, 1989). Considering the bands of DNA as an allele, each allele was given a score of '0' (for absence) or '1' (for presence). All the distinct bands were designated with numbers, starting from the lowest migrating (cathodal) to the fastest migrating (anodal) bands. Each distinct band was referred to as an electromorph and its presence or absence was used in all the analysis relating to the genetic diversity. The analysis was done following the POPGEN (version 1.32) Software (Yeh and Boyle, 1997).

3.5.3 Population genetic analysis

The data was subjected to population genetic analysis following the software POPGENE (version 1.32) (Yeh and Boyle, 1997). The following genetic parameters were estimated/ determined.

a) Allele and genotype frequencies: At each loci, the frequency of each allele and the genotype frequency was computed.

b) Mean allele number per locus: The mean number of allele per locus for each population was determined.

c) Effective number of alleles: The number of alleles needed within a locus to maintain the current level of heterozygosity was determined as

$A_e = 1/\sum p_i^2$, where p_i is the frequency of the i^{th} allele in each population.

The proportion of loci polymorphic was estimated for each population. A locus was considered polymorphic only if the frequency of the most frequent allele was below 95%.

3.5.3.2 Shannon's Information Index

The diversity of allele within a locus was estimated as the Shannon's diversity index ($SI = -\sum p_i \log p_i$), where p_i is the frequency of the i^{th} allele at a locus. The SI was averaged over loci to refer to the diversity of alleles at all loci.

3.5.3.3 Per cent observed heterozygosity

For each locus the number of heterozygotes were recorded and expressed as per cent observed heterozygosity. The per cent heterozygosity was then pooled and averaged over all loci to arrive at the per cent observed heterozygosity for a population. The per cent heterozygosity was computed

- a) Over all loci
- b) Only for polymorphic loci.

3.5.3.4 Per cent expected heterozygosity

The expected heterozygosity for each locus and over all loci for each population in Hardy-Weinberg equilibrium was computed as $H_e = 1 - \sum p_i^2$, where p_i refers to the allele frequency.

3.5.3.5 Population differentiation statistics

Differentiation of populations was analyzed following Wright's (1978) F-statistics and Nei's (1973) genetic diversity statistics.

3.6 F-statistics

The F-statistics measures the degree of genetic differentiation among populations relative to the total population variance. This statistics is sensitive to the total number of populations included in the analysis. F-statistics is given by,

$$(1-F_{is}) (1-F_{st}) = (1-F_{it})$$

Where,

F_{it} = is the total deviation from expected frequencies under Hardy-Weinberg Equilibrium.

F_{st} = is the deviation due to population subdivision and

F_{is} = is the deviation within population subdivisions.

3.6.1 Gene flow (N_m) among populations

Gene flow of a population or the estimated number of migrants exchanged between populations per generation was calculated by using Wright's method (1951).

This method is influenced by the distribution of common alleles.

$N_m = (1-F_{st})/4 F_{st}$ where F_{st} = mean proportion of total gene diversity at polymorphic loci due to differences between populations.

3.6.2 Genetic distance among populations

Inter populational relationships were established by computing the unbiased genetic identity and distance coefficients (Nei, 1978) for all possible pairwise comparison.

3.7 Cluster analysis

Based on the genotypic data of the allozymes, Squared Euclidean distance between all pairs of individuals over all the populations was computed using the statistical package, Statistica ver. 4.5. A Dendrogram of the clustering of the individuals was then constructed using Ward's clustering algorithm.

Principal component analysis: A PCA based on the genotype frequencies was performed using Statistica ver. 4.5 program. In all the input data contained 34 individuals over 9 loci for 2 populations. The proportion of variation extracted by the first two axes was recorded and the principal loading of the variables determined. Based on the two axes, a 2-D plot was developed to examine the dispersion of the individuals from the two populations.

3.8 Binary data analyses

3.8.1 Mean allele number

Based on the frequency of the presence and absence of the band, the mean allele number for an electromorph was computed. The mean allele number was averaged over all electromorphs for a given population and the standard deviation was computed.

3.8.2 Per cent polymorphism

For each population the proportion of electromorph that exhibited polymorphism was computed and expressed as percent polymorphism. An electromorph was considered polymorphic if the frequency of occurrence (presence) that electromorph was not equal to one or the frequency of non-occurrence (absence) was not equal to zero.

3.8.3 Gene diversity

Nei's gene diversity (Nei. 1973) which is equivalent to the total population differentiation for an effectively infinite population was computed as:

$$h = 1 - \sum P_i^2$$

Where, P_i is the frequency of the occurrence of the i^{th} electromorph.

The Gene Diversity was calculated for each electromorph and then averaged over all the electromorphs for given populations. The Standard Deviation of the gene diversity was computed for all electromorphs and for only those which are polymorphic.

3.8.4 Shannon's Information Index

For each electromorph the presence and absence of the band were considered as two different alleles as it is usually followed in RAPD analysis (Yeh, 1997). Based on the frequency of occurrence of those two alleles (presence and absence) for a given electromorph, we computed Shannon's Information Index as:

$$I = - \sum_{i=1}^2 P_i \times \log P_i$$

Where, P_i is frequency of the i^{th} band.

The Shannon's information index overall the electromorph was then averaged and standard deviation was computed to indicate the overall density or heterogeneity of the populations. The 'I' was computed for all electromorphs and polymorphic electromorphs.

3.8.5 Mean polymorphic index

The presence and absence of the bands was used to calculate a polymorphic index based on the frequency of occurrence of each band as:

$$P_i = \sum_{i=1}^n p \times (1-p)$$

Where P_i is frequency of i^{th} band and 'n' is the total number of bands.

The number of polymorphic electromorphs weighted the P_i as: $P_i / \text{Polymorphic electromorphs}$

3.8.6 Population differentiation statistics

Genetic differentiation of populations was investigated by computing Nei's genetic distance among all pairs of populations and by partitioning the total variation into those within and among populations.

3.8.7 Nei's genetic distance among population

Nei's genetic distance was computed between all possible pairs of populations. Based on these distances, cluster analysis was performed following UPGMA method (Sneath and Sokal, 1972) and a dendrogram was developed.

3.8.8 Partitioning of variation

The total diversity (H_T) was calculated by averaging allele frequencies across all populations for each electromorph. H_S , the heterozygosity for all the sub population was derived from the average value of H_e . G_{st} , which compares the ratio among population component of diversity to total, was then computed.

3.8.9 Gene flow among population

The average gene flow among population [the number of migrants per generation (N_m)] was estimated following (Wright, 1951) as

$$N_m = (1 - G_{st}) / 4G_{st}$$

Where, G_{st} = proportion of variation to among populations.

Based on the presence and absence data of the allozymes and amplified products, cluster analysis and PCA were done as described in the section 3.7.

Experimental Results

IV. RESULTS

4.1 Standardization of enzyme extraction protocols

Assessment of genetic variability by isozyme electrophoresis depends critically on the resolution of the enzyme activity. Therefore it is imperative that the protocols employed to examine the isozyme polymorphism capture as great a detail as possible and resolve the enzymes unambiguously. Attempt has been made to standardize the type of tissue most suitable for extraction and the various buffer systems that yield consistent and good stainability of the enzyme.

Leaf samples showed consistent enzyme activity with good staining intensity for most of the enzymes with selected extraction gel and electrode buffers. Among the extraction buffers, Chase buffer gave a good extraction and resolution of the polymorphic enzymes in leaf samples. Among several other extraction buffers tried, borate, vegetative and sandal buffers gave good resolution but only for a few enzymes.

Among the gel and electrode buffers tried, Histidine (H) gel and electrode buffer gave good resolution. TEB and CTC produced considerable resolution for a few enzymes. Lithium borate, Histidine citrate and B buffer systems showed poor resolution and staining. Of the twenty enzymes that were tried, only six enzyme systems viz., Phosphoglucose isomerase (PGI), Phosphoglucose dehydrogenase (PGDH), Phosphoglucose mutase (PGM), Isocitrate dehydrogenase (IDH), Maleic enzyme (ME) and Malate dehydrogenase (MDH) showed good stainability.

Based on these results, isozyme analysis of the species and populations was restricted to the above six enzyme systems only, using leaf as the source of enzymes. The extraction was done using Chase extraction buffer and the electrophoresis run using histidine gel and histidine electrode buffer systems.

4.2 Genetic interpretation of isozyme variations

The interpretations of the six enzyme systems studied were based on the banding patterns that were observed in the two populations of *S. kathalekanensis* from the *Myristica* swamps of Uttara Kannada in the central Western Ghats. The interpretation of the banding pattern was checked for consistency with the known quaternary structure of the enzymes. Further, the interpretation was also done by comparing the banding pattern with those reported for the enzymes in other species. Of the 20 enzymes initially screened, six enzymes namely PGM, PGI, ME, IDH, MDH and PGDH exhibited good resolution, staining and polymorphism. A locus was considered polymorphic only if the most frequent allele was represented in less than 95% of the individuals sampled. The banding patterns for each of the six enzyme systems are presented below.

Maleic enzyme (ME): Among all the enzymes studied, only ME was tetrameric. Electrophoresis of the tissue samples revealed a single staining zone corresponding to a single locus (Appendix-III). This enzyme was monomorphic in both the populations and did not exhibit polymorphism.

Isocitrate dehydrogenase (IDH): This dimeric enzyme exhibited a single staining zone with only one allele (Appendix-III). Further, the banding patterns showed a significant absence of heterozygotes. In fact, the frequency of this allele was consistently greater than 0.95 in both the populations (Table 4.1).

Phosphoglucosomerase (PGI): This dimeric enzyme exhibited two distant zones of activity corresponding to two loci (Appendix-III). The fast moving anodal loci was designated as PGI-2 and the slow moving cathodal loci as PGI-1. There were two alleles for PGI-1 (a and b). Only PGI-1 was considered for further analysis because of

Table 4.1 Allele frequency at nine loci for two populations of *S. kathalekanensis* (based on isozyme data).

Sl. No.	Loci	Alleles	<i>Kathlekan-I</i> population	<i>Kathlekan-II</i> population	Pooled over populations
1	ME	a	1	1	1
		b	-	-	-
2	IDH	a	1	1	1
		b	-	-	-
3	PGI	a	0.63	0.56	0.59
		b	0.38	0.44	0.41
4	MDH 1	a	1	1	1
		b	-	-	-
5	MDH 2	a	1	1	1
		b	-	-	-
6	PGM 1	a	0.19	0.22	0.21
		b	0.81	0.78	0.79
7	PGM 2	a	0.94	0.97	0.96
		b	0.06	0.03	0.04
8	PGDH 1	a	1	1	1
		b	-	-	-
9	PGDH 2	a	0.75	0.89	0.82
		b	0.25	0.11	0.18

its polymorphic nature in both the populations as well as because of the inconsistency of PGI-2.

Malate dehydrogenase (MDH): Two zones of activity corresponding to two loci (Appendix-III) characterized this dimeric enzyme. Only one allele was distinguishable in each locus of the two populations examined. This enzyme was monomorphic in both the populations. In both the populations studied, the frequencies of the allele (MDH) were consistently greater than 0.95 (Table 4.1).

Phosphoglucumutase (PGM): This monomeric enzyme system stained for two clear zones indicative of two loci (Appendix-III). The faster anodally moving loci (PGM 3) was characterized by one allele. The slower migrating zone (designated as locus PGM1 and PGM2) is characterized by two alleles (a and b). The locus was found to be polymorphic in both the populations studied. However, the allele PGM 2 occurred at frequencies greater than 95% only in *kathlekan-II* population (Table 4.1).

Phosphoglucose dehydrogenase (PGDH): Three distinct zones of activity were observed in this enzymes corresponding to two loci of this enzyme (Appendix-III). While the slowest moving loci (PGDH1) exhibited only one allele, the fastest and the intermediate loci (PGDH2 and PGDH3) showed two alleles. The slowest moving loci PGDH1 was not polymorphic for any of the populations, but PGDH2 showed polymorphism.

4.3 Analysis based on genetic interpretation

4.3.1 Genetic diversity parameters

Allele frequency

The frequency of the alleles at a given locus varied between populations. Not all loci were polymorphic at both the populations (Table 4.1). Variation in allele frequency was observed only for four loci namely, PGI, PGM1, PGM2 and PGDH2, except PGM2

in *kathlekan-II* whose frequency was consistently greater than 0.95. The loci pertaining to the enzymes ME and IDH were monomorphic in both the populations.

Allele number

The mean allele number per locus for two populations was 1.44 ± 0.53 when all loci were considered for analysis (Table 4.2). Pooled over both the populations, the mean number of alleles per locus was also 1.44 ± 0.53 . However, considering polymorphic loci only, the mean allele number per loci reached its upper limit of 2.0 (Table 4.2).

Effective allele number

The mean effective allele number per locus required to maintain the current level of heterozygosity calculated considering only polymorphic loci for analysis in *kathlekan-I* population was 1.51 ± 0.31 and was 1.58 ± 0.37 for *kathlekan-II*. When all loci were considered, the effective allele number in the two populations, was 1.23 ± 0.33 (*kathlekan-I*) and 1.20 ± 0.34 (*kathlekan-II*), respectively (Table 4.2). The effective allele number over all the populations was highest for the loci PGI with a mean of 1.98 and least for PGDH 1 with 1.25 when only polymorphic loci was considered.

Shannon's information index

Estimate of Shannon's information index, a measure of genetic heterogeneity was computed based on the relative allele frequencies at each locus and then averaged over all loci. Considering data of all the loci, the Shannon's index was 0.216 ± 0.28 for *kathlekan-I* and 0.188 ± 0.27 for *kathlekan-II* population. However, when only polymorphic loci was considered, the Shannon's index was more in *kathlekan-II* population (0.522 ± 0.17) than that of *kathlekan-I* (0.485 ± 0.18). Overall index, considering all 34 adult individuals for all loci, was 0.204 ± 0.27 ; it was higher (0.551 ± 0.11) considering only the polymorphic loci (Table 4.2).

Table 4.2 Mean allele number per locus, mean effective allele number per locus, number of polymorphic loci, per cent polymorphism and Shannon's information index for populations of *S. kathlekanensis* (considering all loci identified by isozyme data)

Sl. No.	Population	Allele number		Effective allele number		Shannon's index		No. of polymorphic loci	Per cent polymorphism
		Mean	± SD	Mean	±SD	Mean	±SD		
1	Kathlekan-I	1.44	0.53	1.23	0.33	0.22	0.28	4	44.44
2	Kathlekan-II	1.44	0.53	1.20	0.34	0.19	0.27	4	44.44
3	Pooled over populations	1.44	0.53	1.21	0.33	0.20	0.27	4	44.44

Polymorphism

The per cent polymorphism for the two populations did not vary (44.4%); and hence, the over all value was also 44.4%.

Observed heterozygosity

The observed heterozygosity (H_o) calculated for the data pooled over both the populations and over all the loci was 9.48 ± 16.62 per cent, which was less than the expected based on Hardy Weinberg equilibrium ($H_e = 13.38 \pm 18.88$ per cent) indicating an overall deficiency of heterozygosity (Table 4.3). Interestingly, the SD values associated with these means were higher suggesting a higher variation among individuals. Similar trend was also seen when polymorphic loci were considered. However, the values of H_o and H_e were, as expected higher ($H_o=25.49$ and $H_e=37.28$).

The values of H_o and H_e (based on all the loci studied) were also computed separately for individual population and is shown in the Table 4.4. Both the populations showed deficiency in heterozygosity levels than that expected from Hardy-Weinberg equilibrium. Among the two populations, *kathlekan-I* showed lesser level of heterozygosity (8.33 ± 14.52) per cent than *kathlekan-II* (10.49 ± 12.47 per cent). This pattern was also true when only polymorphic loci were considered (Table 4.4b). The deficiency of heterozygotes persisted even considering only polymorphic loci.

4.3.2 Genetic distance based genetic interpretation

The extent of divergence among the two populations of *S. kathalekanensis* was analyzed computing genetic distances, genetic identity and extent of gene flow among populations. Further, partitioning of genetic variability within and among populations was also done to estimate the within and between population diversity.

The genetic distance between two populations was almost negligible (0.0033), concomitantly, the two populations were almost identical to the tune of 99.67 % (Table

Table 4.3 Observed (H_o) and expected (H_e) heterozygosity among all adult individuals ($n=34$) of *S. kathalekanensis* (based on isozyme data pooled over populations).

Sl. No.	Locus	Over all loci		Polymorphic loci	
		H_o	H_e	H_o	H_e
1	ME	0	0	-	-
2	IDH	0	0	-	-
3	PGI	0.412	0.492	0.412	0.492
4	MDH 1	0	0	-	-
5	MDH 2	0	0	-	-
6	PGM 1	0.353	0.332	0.353	0.332
7	PGM 2	0.088	0.086	0	0.295
8	PGDH 1	0	0	-	-
9	PGDH 2	0	0.295	-	-
	MEAN	0.095	0.134	0.255	0.373
	$\pm$ SD	0.166	0.188	0.223	0.105

Table 4.4 Observed (H_o), expected (H_e) heterozygosity, genetic identity and distance for all adult individuals ($n=34$) of two populations of *S. kathlekanensis* as revealed by isozyme analysis.

a. Considering all loci

Sl. No.	Locus	<i>Kathelekan-I</i>		<i>Kathelekan-II</i>	
		H_o	H_e	H_o	H_e
1	ME	0	0	0	0
2	IDH	0	0	0	0
3	PGI	0.375	0.484	0.444	0.508
4	MDH 1	0	0	0	0
5	MDH 2	0	0	0	0
6	PGM 1	0.25	0.314	0.444	0.356
7	PGM 2	0.125	0.121	0.056	0.056
8	PGDH 1	0	0	0	0
9	PGDH 2	0	0.387	0	0.203
	MEAN	0.083	0.145	0.105	0.125
	$\pm$ SD	0.140	0.196	0.193	0.190

b. Considering only polymorphic loci.

Sl. No.	Locus	<i>Kathelekan-I</i>		<i>Kathelekan-II</i>	
		H_o	H_e	H_o	H_e
1	PGI	0.375	0.4839	0.4444	0.5079
2	PGM 1	0.25	0.3145	0.4444	0.3556
3	PGM 2	0.125	0.121	---	
4	PGDH 2	0	0.3871	0	0.2032
	MEAN	0.1875	0.3266	0.2963	0.3556
	$\pm$ SD	0.1614	0.1536	0.2566	0.1524

c. Genetic identity (above diagonal) and genetic distance (below diagonal)

Sl. No.	Population	<i>Kathlekan-I</i>	<i>Kathlekan-I I</i>
1	<i>Kathlekan-I</i>	-	0.9967
2	<i>Kathlekan-II</i>	0.0033	-

4.4c). Analysis of the partitioning of the genetic variability based on F-statistics indicated that genetic diversity among populations was equivalent to that within the populations (Table 4.5). Only 1.10% of the total genetic variability was due to differences among populations ($F_{st} = 0.011$; Table 4.5). The amount of total genetic variation that can be ascribed to population differentiation varied among loci; the highest of 3.26% for PGDH2 and lowest of 0 % for IDH, MDH1, MDH2 PGDH1 and ME. In general all the estimates indicated a poor differentiation of the populations of *S. kathalekanensis*.

4.3.3 *Principal component and cluster analysis based on genetic analysis of allozyme data*

The individuals of the two populations were ordinated using cluster analysis and Principal Component Analysis (PCA) based on the allozyme data. The PCA provides a visual representation of the dispersion of the individuals on two axis hence it is popular ordination tool among ecologists. The first three PCA components extracted about 85% of the total variation in the data, which is quite substantial. As shown in the Fig. 4.1, there was a lack of clear differentiation between two populations; further several individuals had clustered at a single point. This strongly suggests that there was very less inter as well as intra-population differentiation.

Cluster analysis did not reveal clear-cut separation of the two populations (Fig. 4.2). However, two major clusters were identified, although they consisted of individuals from both populations.

Table 4.5 F-statistics for all loci identified by isozyme analysis of *S. kathalekanensis* (data pooled over both the populations).

Sl. No.	Loci	Sample size	F_{is}	F_{it}	F_{st}	N_m
1	ME	68	-	-	0	-
2	IDH	68	-	-	0	-
3	PGI	68	0.1487	0.1529	0.005	49.9
4	MDH 1	68	-	-	0	-
5	MDH 2	68	-	-	0	-
6	PGM 1	68	-0.0678	-0.0658	0.0019	134.86
7	PGM 2	68	-0.0546	-0.0473	0.007	35.5
8	PGDH 1	68	-	-	0	-
9	PGDH 2	68	1	1	0.0326	7.42
	MEAN	68	0.281	0.2859	0.011	22.42

F_{is} = Deviations within population sub-division

F_{st} = Deviations due to population sub-division

F_{it} = Total deviation from expected frequencies under Hardy-Weinberg equilibrium.

N_m = Gene flow estimated using the formula $F_{st} = 0.25 (1 - F_{st})/F_{st}$.

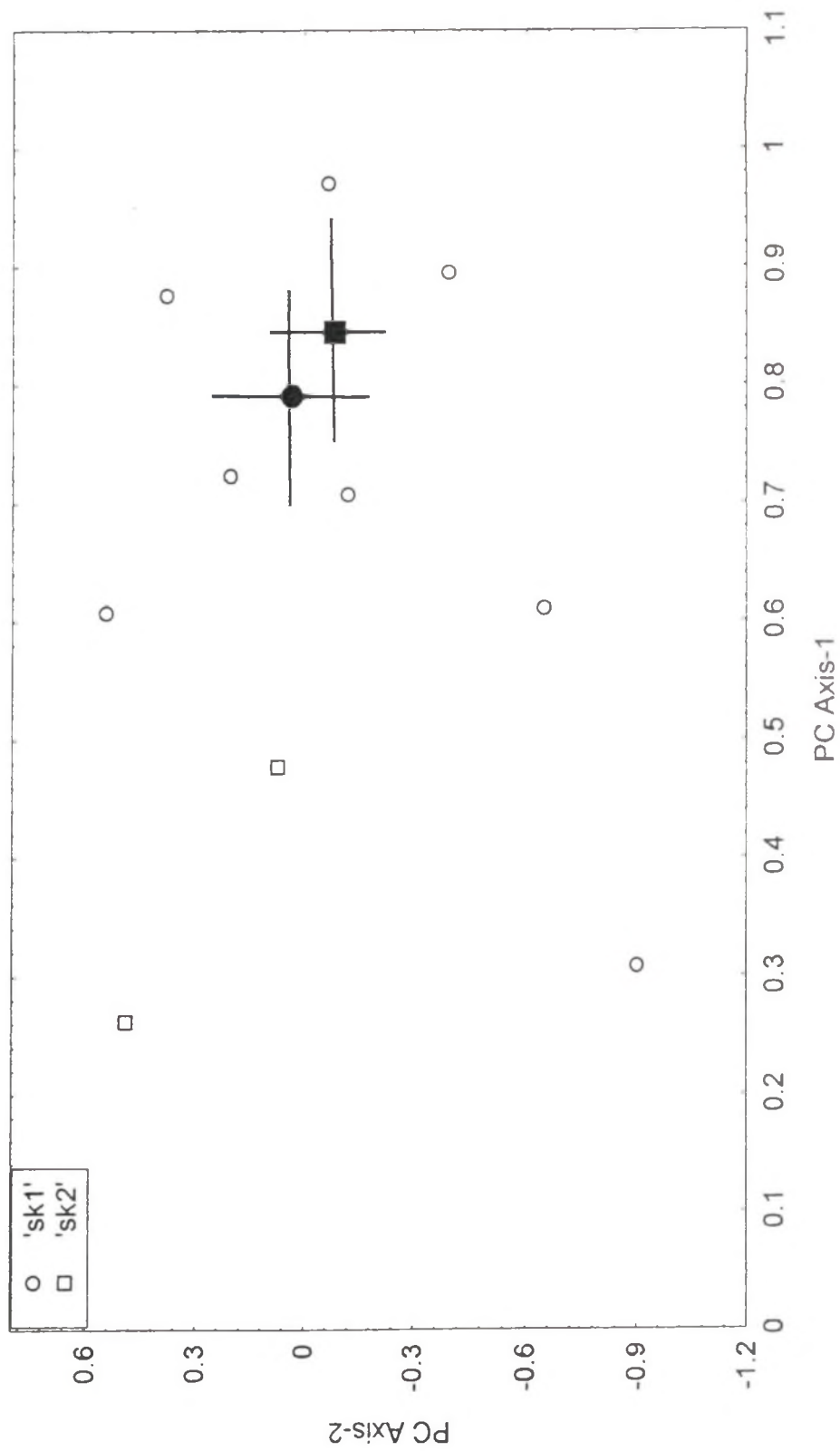


Fig 4.1 Principal component analysis of two populations of *Semecarpus kathalekanensis* from central Western Ghats based on genotypic data of isozymes. The standard deviations from the mean for both axes for two populations are depicted. sk1= population-I and sk2 =population-II.

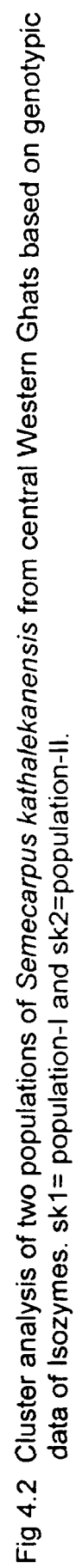


Fig 4.2 Cluster analysis of two populations of *Semecarpus kathalekanensis* from central Western Ghats based on genotypic data of Isozymes. sk1= population-I and sk2=population-II.

4.4.1 Based on all loci

For this analysis every band was treated as an electromorph. Fourteen distinct electromorphs from six enzyme systems were recovered from the two populations. The electromorph frequency of all the six enzymes is presented in the Table 4.6. Of the six enzymes, fourteen loci were recovered. Electromorphs ME, IDH, MDH1, MDH2, PGM3 and PGDH 1 were found to be monomorphic in these populations. PGM2 and PGDH 3 were recovered in low frequency hence can be designated as rare alleles.

Based on the presence or absence of a band, several population diversity parameters namely mean eletromorph number per locus, per-cent polymorphism, Nei's diversity and Shannon's information index, were computed for the two populations of *S. kathlekanensis*. The analysis was done over all loci, irrespective of whether they were monomorphic or polymorphic. The data are shown in the Table 4.7a.

Mean electromorph number per locus: The mean electromorph number per locus was slightly higher in *kathlekan-I* (1.57), compared to *kathlekan-II* (1.50). Over both the populations, the mean number per locus was 1.57 (Table 4.7a).

Percent polymorphism: A locus is considered polymorphic if both the bands (presence and absence) were scored in a population. Further the allele was considered polymorphic only if the most frequent allele (0 or 1) was < 95% frequency. *Kathlekan-I* population exhibited a slightly higher polymorphism of 57.14%, when compared to *kathlekan-II* (50.00%).

Electromorph diversity (*h*): Electromorph diversity was estimated following Nei's (1973) index of diversity for each locus and then averaged over all loci. *Kathlekan-I* was found to have a higher electromorph diversity of 0.189 ± 0.204 than that of *kathlekan-II* (0.168

Table 4.6 Electromorph frequency of isozyme bands or electromorphs in two populations of *S. kathalekanensis*.

Sl. No.	Loci	<i>Kathalekan-I</i>	<i>Kathalekan-II</i>	Pooled over populations
1	ME	1	1	1
2	IDH	1	1	1
3	PGI 1	0.57	0.53	0.55
4	PGI 2	0.21	0.26	0.23
5	PGI 3	0.34	0.43	0.38
6	MDH 1	1	1	1
7	MDH 2	1	1	1
8	PGM 1	0.17	0.26	0.21
9	PGM 2	0.75	1	0.83
10	PGM 3	1	1	1
11	PGM 4	0.07	0.03	0.05
12	PGDH 1	1	1	1
13	PGDH 2	0.50	0.67	0.58
14	PGDH 3	0.13	0.057	0.09

Table 4.7 Genetic diversity parameters based on electromorph frequency in two populations of *S. kathlekanensis*.

a. Considering all loci

Sl. No.	Population	Sample size	Gene diversity		Shannon's index		Electromorph Number	
			Mean	±SD	Mean	±SD	Mean	±SD
1	<i>Kathlekan-I</i>	16	0.199	0.204	0.299	0.292	1.571	0.514
2	<i>Kathlekan-II</i>	18	0.168	0.213	0.249	0.302	1.500	0.519
3	Pooled data	34	0.192	0.293	0.289	0.293	1.571	0.514

b. Considering only polymorphic loci*

Sl. No.	Population	Sample size	Gene diversity		Shannon's index	
			Mean	±SD	Mean	±SD
1	<i>Kathlekan-I</i>	16	0.348	0.133	0.523	0.157
2	<i>Kathlekan-II</i>	18	0.383	0.144	0.561	0.176
3	Pooled data	34	0.386	0.127	0.567	0.146

* the electromorph number is 2.0 since only polymorphic loci are considered.

c. Genetic identity (above diagonal) and genetic distance (below diagonal)

Sl. No.	Population	<i>Kathlekan-I</i>	<i>Kathlekan-II</i>
1	<i>Kathlekan-I</i>	-	0.9901
2	<i>Kathlekan-II</i>	0.0099	-

± 0.213). When the analysis was done over both the populations the electromorph diversity was 0.192 ± 0.293 .

Shannon's information index (I): Estimate of Shannon's information index was computed as a measure of electromorph diversity for each locus and then averaged over all loci. Shannon's information index was 0.299 ± 0.292 for *Kathlekan-I* and 0.249 ± 0.302 for *kathlekan-II*. When analysis was done over both the populations the index was found to be 0.289 ± 0.293 .

In summary, it appears that based on several measures of electromorphic diversity the *kathlekan-I* population is more diverse than *kathlekan-II* population, although the magnitude of difference was narrow.

4.4.2 Based on all polymorphic loci

The electromorphic diversity parameters of the two populations were also analyzed considering only polymorphic loci. The results are shown in the Table 4.7b.

Mean electromorph diversity: The mean Nei's electromorph diversity over all populations was 0.386 ± 0.127 . On individual population basis, the electromorphic diversity was highest for *kathlekan-II* (0.383 ± 0.144), when compared with that of *kathlekan-I* (0.348 ± 0.133).

Shannon's information index (I): The over all mean Shannon's information index was 0.567 ± 0.146 . The index was higher in *kathlekan-II* (0.561 ± 0.176) than that of *kathlekan-I* (0.523 ± 0.157).

4.4.3 Population differentiation in *S. kathalekanensis*

The extent of population differentiation among the populations of *S. kathalekanensis* was assessed by estimating the inter-population electromorphic distance, gene-flow among populations and by partitioning the electromorphic variation due to that between and within populations (Table 4.7c).

The pairwise Nei's electromorphic distance and electromorphic identity among the population was computed (Table 4.7c). The average Nei's electromorphic distance and electromorphic identity between pairs of population was 0.0099 and 0.9901 respectively. Gene flow between the two populations was 14.34 (Table 4.8). It is suggested that estimates of gene flow below one indicates a restricted exchange of gene pool leading to electromorphic separation of populations while that above one actually indicates an extensive exchange of gene pool. All the three estimates indicated a poor differentiation of the populations. In other words, there appears to be a high extent of gene flow between the two populations leading to perhaps a low level of electromorphic differentiation between the populations.

Partitioning of electromorphic variation (G_{st}): The total electromorphic diversity (H_t) was partitioned into diversity within population (H_s) and among population (D_s). G_{st} , proportion of total diversity found among populations was calculated. These estimates were calculated for the species as a whole by using data from the two-populations. The average G_{st} was 1.71% indicating that a substantial proportion of the total diversity is in fact explained due to within population variation (Table 4.8). This again indicates poor differentiation of the two populations.

4.4.4 Principal component and cluster analysis based on electromorph data

The principal component analysis and cluster analysis of the electromorph data is shown in the Fig. 4.3 and 4.4. As in the case of genotypic data, here also, there was no clear separation of individuals belonging to *Kathlekan-I* and *Kathlekan-II* populations. However, there was larger scatter of the points. The first three components of the PCA accounted for 70% of the total variation, which is lesser/higher than PCA based on genotypic data of allozyme. The cluster analysis also did not show clear differentiation of the two populations.

Table 4.8 Partitioning of total genetic diversity of populations of *S. kathalekanensis*.(sample size = 34 matured trees)

Sl. No.	Loci	Total genetic diversity (H_t)	Within population diversity (H_s)	Proportion of total diversity (G_{st})	N_m^*
1	ME	0	0	-	-
2	IDH	0	0	-	-
3	PGI1	0.496	0.495	0.002	130.15
4	PGI2	0.358	0.355	0.007	36.35
5	PGI3	0.473	0.468	0.010	24.87
6	MDH1	0	0	-	-
7	MDH2	0	0	-	-
8	PGM1	0.338	0.332	0.019	13.09
9	PGM2	0.208	0.186	0.097	2.33
10	PGM3	0	0	-	-
11	PGM4	0.087	0.088	-0.015	1000
12	PGDH1	0	0	-	-
13	PGDH2	0.484	0.472	0.025	9.67
14	PGDH3	0.169	0.170	-0.004	1000
	MEAN	0.187	0.183	0.017	14.34
	S. D.	0.041	0.040		

N_m = Gene flow estimated using the formula $N_m = 0.25 \times (1 - G_{st})/G_{st}$

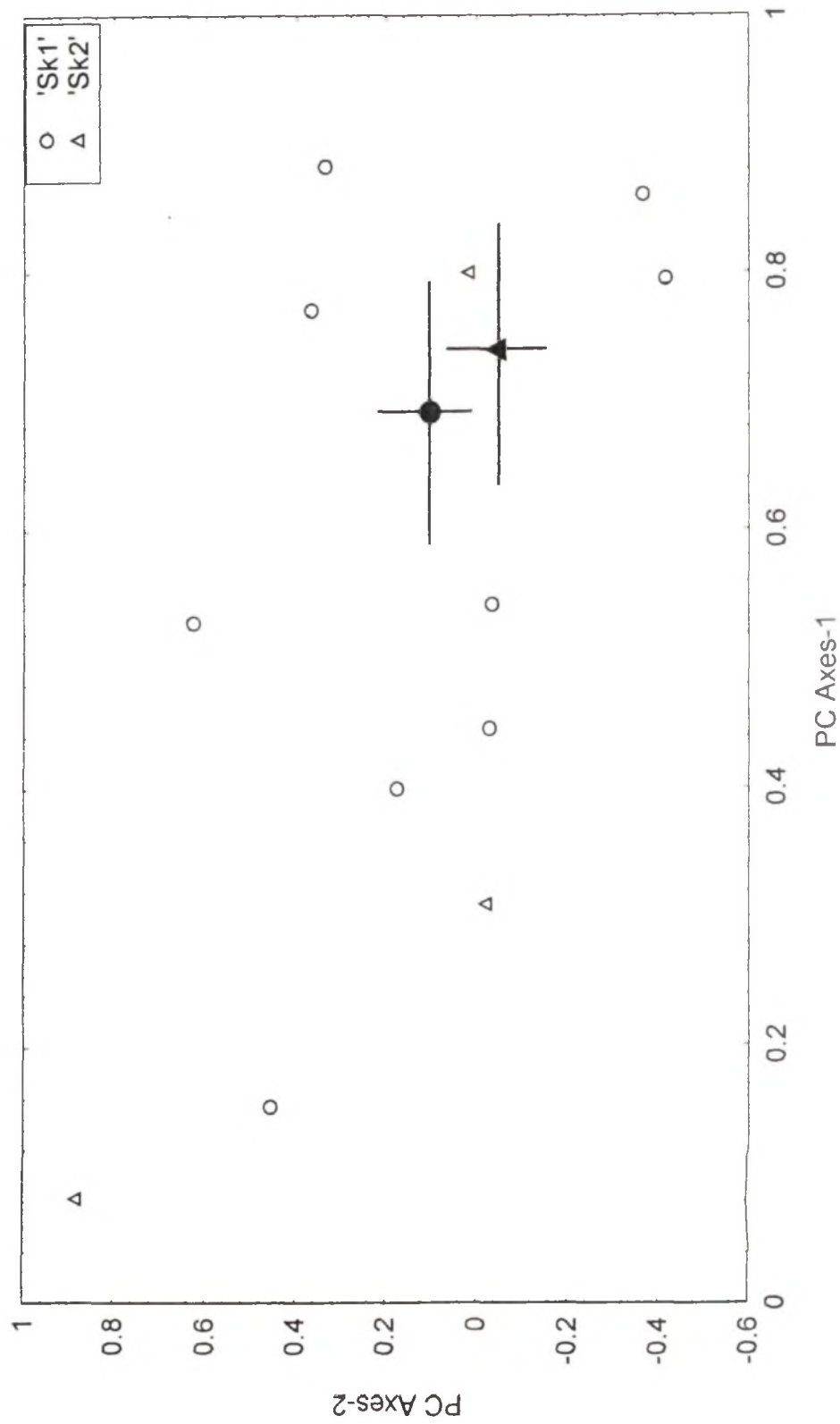


Fig 4.3 Principal component analysis of two populations of *Semecarpus kathalekanensis* from central Western Ghats based on phenotypic data of isozyms. The standard deviations from the mean for both axes for two populations are depicted. sk1=population-I and sk2= population-II.

4.5 RAPD Analysis

The genomic DNA obtained from the 33 individuals from the two populations were subjected to RAPD-PCR using 10 random primers and a total of seventy-six loci were generated. There was low amplification of DNA in one of the individual of *Kathlekan-I*, hence that data could not be used. The amplification fragments were scored as present (1) or absent (0). Based on this binary scoring, population genetic analysis was done using the software POPGENE ver 1.32 (Yeh and Boyle, 1997). Amplification profiles of two populations of *S. kathalekanensis* with different primers is provided in the Appendix-IV.

4.5.1 Amplification fragment diversity parameters based on all loci

The following parameters were analyzed: mean allele number per locus, per cent polymorphism, Nei's gene diversity, Shannon's information index, The analysis was done considering all the loci, irrespective of whether they were monomorphic or polymorphic. The data are presented in the Table 4.9a.

Mean amplification product number per locus: The mean amplification fragments per locus was higher (1.71 ± 0.46) in *kathlekan-I* than in *kathlekan-II* (1.62 ± 0.49). For data pooled over both the populations, it was 1.75 ± 0.44 (Table 4.9a).

Per-cent polymorphism: A locus is considered polymorphic if either the presence or absence (1 or 0) of an amplification fragment was at least 95 % frequent in the population. *Kathlekan-I* population exhibited the higher per cent polymorphism (71.05%), when compared with population at *kathlekan-II* (61.84%).

Amplification fragment diversity (*h*) Amplification fragment diversity was estimated following Nei's (1973) index of diversity for each locus and then averaged over all loci. *Kathlekan-I* was found to have highest amplification diversity ($0.316 \pm 0.0.208$) when

Table 4.9 Genetic diversity parameters based on RAPD data in two populations of *S. kathlekanensis*

a. Considering all loci

Sl. No.	Population	Sample Size	Gene diversity		Shannon's index		Electromorph Number	
			Mean	±SD	Mean	±SD	Mean	±SD
1	<i>Kathlekan-I</i>	15	0.316	0.208	0.451	0.294	1.711	0.457
2	<i>Kathlekan-II</i>	18	0.241	0.204	0.354	0.294	1.618	0.489
3	Pooled data	33	0.323	0.200	0.464	0.281	1.750	0.436

b. Considering only polymorphic loci

Sl. No.	Population	Sample Size	Gene diversity		Shannon's index	
			Mean	±SD	Mean	±SD
1	<i>Kathlekan-I</i>	15	0.444	0.056	0.635	0.060
2	<i>Kathlekan-II</i>	18	0.396	0.081	0.582	0.094
3	Pooled data	33	0.437	0.065	0.627	0.070

c. Genetic identity (above diagonal) and genetic distance (below diagonal)

Sl. No.	Population	<i>Kathlekan-I</i>	<i>Kathlekan-I I</i>
1	<i>Kathlekan-I</i>	-	0.9073
2	<i>Kathlekan-I I</i>	0.0972	-

compared to that of *kathlekan-II* (0.241 ± 0.204). The amplification diversity over all the 33 individuals was 0.323 ± 0.20 .

Shannon's information index (I): Estimate of Shannon's information index was computed as a measure of amplification diversity for each locus and then averaged over all loci. Shannon's information index was highest for *kathlekan-I* (0.451 ± 0.294), compared with *kathlekan-II* (0.354 ± 0.294). When the analysis was done over both the populations, the Shannon's information index was found to be 0.464 ± 0.281 .

In summary, it appears that based on several measures of amplification diversity the *kathlekan-I* population in general had a higher level of polymorphism and was genetically more diverse, when compared with that of *kathlekan-II* population.

4.5.2 Amplification fragment diversity parameters based on polymorphic loci only

Mean amplification product diversity: The mean Nei's gene diversity over all populations was 0.437 ± 0.065 (Table 4.9b). On an individual population basis, the amplification diversity was highest for *kathlekan-I* (0.444 ± 0.056), when compared with that of *kathlekan-II* (0.396 ± 0.081).

Shannon's information index (I): The over all mean Shannon's information index was 0.627 ± 0.070 . The Shannon's information index was 0.635 ± 0.060 in *kathlekan-I* when compared with *kathlekan-II* (0.582 ± 0.094).

4.5.3 Population differentiation in *S. kathalekanensis*

The extent of population differentiation among the populations of *S. kathalekanensis* was assessed by estimating the inter population amplification fragment distance, estimating the gene-flow among populations and by partitioning the amplification fragment variation due to that between and within populations. The data are presented in the Table 4.9c and Table 4.10

Table 4.10 Partitioning of total genetic diversity computed based on RAPD data for *S. kathalekanensis* considering all loci.

Parameter	Total genetic diversity (H_t)	Within Population diversity (H_s)	Proportion of total diversity (G_{st})	N_m^*
Mean	0.3084	0.2782	0.0979	2.3033
$\pm$ SD	0.0386	0.0333	-	-

Total number of loci considered = 76; number of individuals = 33

* N_m = Gene flow estimated by the formula, $N_m = 0.25 \times (1 - G_{st}) / G_{st}$

The pair-wise Nei's amplification product distance and amplification product identity among the population was computed (Table 4.10). The average Nei's amplification fragment distance and amplification identity between pairs of population was 0.0972 and 0.9073 respectively (Table 4.9c). Gene flow between the two populations was 2.3033. All the three estimates indicate a poor differentiation of the populations.

Partitioning of Genetic variation (G_{st}): The total genetic diversity, H_t was partitioned into diversity within population (H_s) and among population (D_s) These estimates were calculated for the species as a whole by using data from two-populations. The average G_{st} was 9.79% indicating that a substantial proportion of the total diversity is in fact explained due to within population variation (Table 4.9c). This again indicates poor differentiation of the two populations.

4.5.4 PCA and cluster analysis of the amplification fragment data

The data on amplification fragments of both the populations were subjected to principal component analysis and cluster analysis and the results are shown in the Fig. 4.5 and 4.6. As in the case of allozyme analysis, there was no clear separation of individuals belonging to *Kathlekan-I* and *Kathlekan-II* populations. However, there was larger scatter of the points. The first three components of the PCA accounted for 51 % of the total variation, which is higher than that based on the allozyme data. The cluster analysis also did not show clear differentiation of the two populations.

4.6 Genetic differences between male and female individuals

For this analysis, data pooled over both the populations were considered. Genetic distances were calculated based on isozyme, electromorph as well as PCR products. The results are summarized in the Table 4.11. As shown in the Table 4.11a-

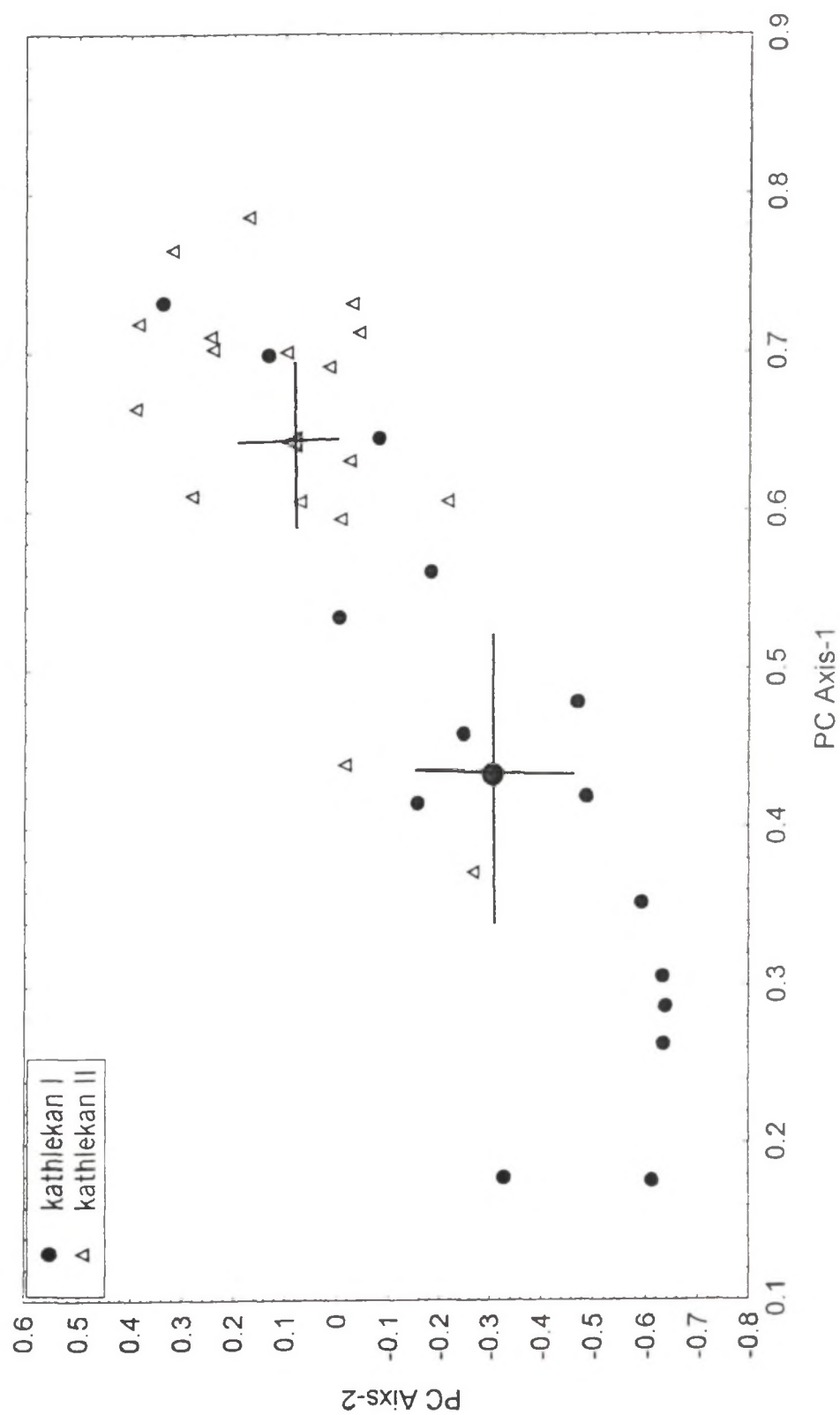


Fig 4.5 Principal component analysis of two populations of *Semecarpus kathalekanensis* from central Western Ghats based on amplification fragment data. The standard deviations from the mean for both axes for two populations are depicted. kathlekan I=population-I and kathlekan II=population-II.

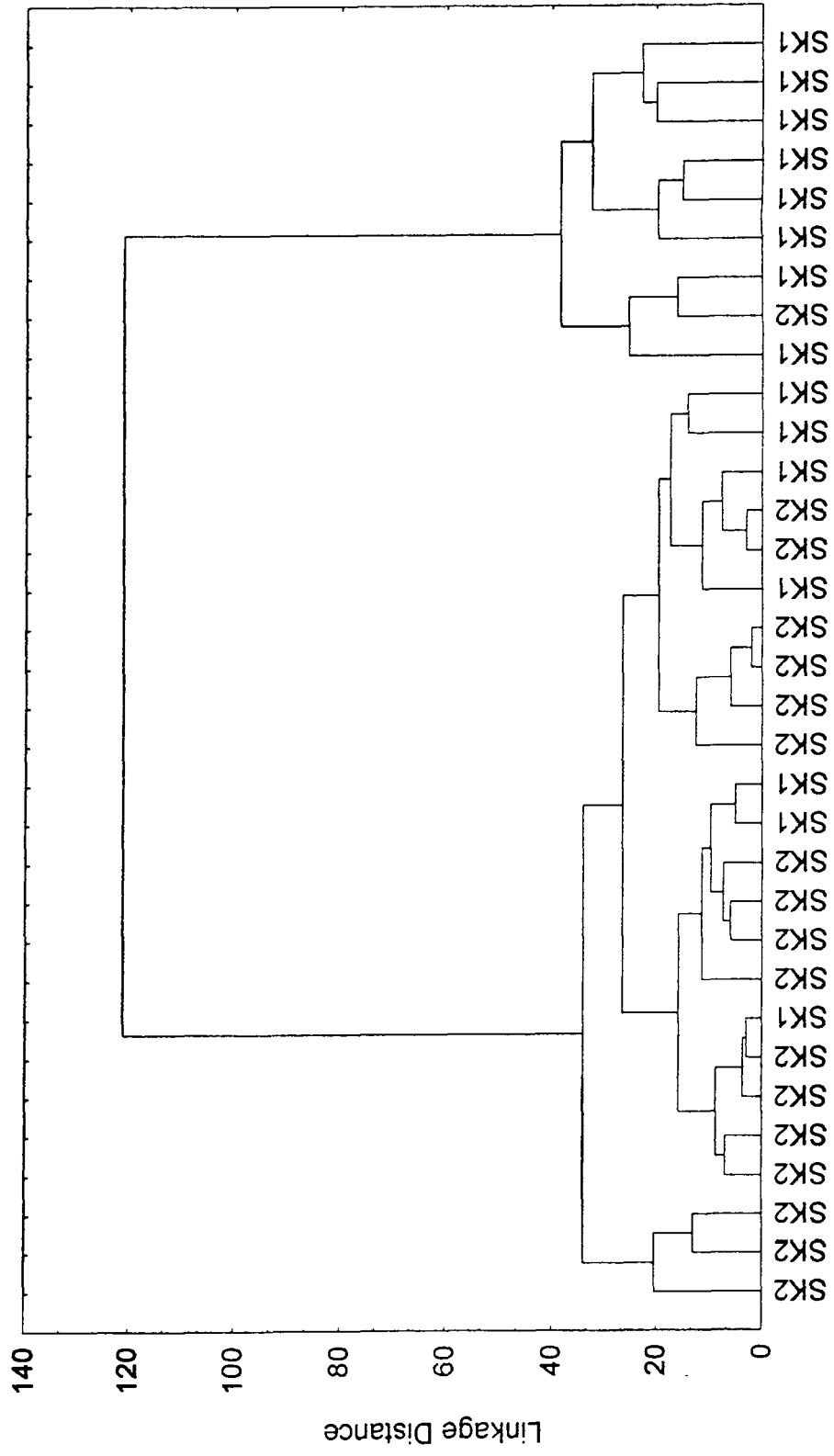


Fig 4.6 Cluster analysis of two populations of *Semecarpus kathalekanensis* from central Western Ghats based on amplification fragment product data. sk1=population-I and sk2=population-II.

Table 4.11 Genetic identity (above diagonal) and genetic distance (below diagonal) between sexes in two populations of *S. kathalekanensis*

a. Based on isozyme data (genetic interpretation)

Population	Female of population I	Male of population I	Female of population II	Male of population II
Female of population I	-	0.9816	0.9943	0.981
Male of population I	0.0186	-	0.9942	0.9987
Female of population II	0.0057	0.0058	-	0.9954
Male of population II	0.0192	0.0013	0.0046	-

b. Based on electromorph data

Population	Female of population I	Male of population I	Female of population II	Male of population II
Female of population I	-	0.9699	0.9876	0.9729
Male of population I	0.0305	-	0.9841	0.9498
Female of population II	0.0125	0.0161	-	0.9843
Male of population II	0.0274	0.0515	0.0159	-

c. Based on RAPD data

Population	Female of population I	Male of population I	Female of population II	Male of population II
Female of population I	-	0.8777	0.8897	0.8875
Male of population I	0.1305	-	0.9607	0.9557
Female of population II	0.1168	0.0401	-	0.9728
Male of population II	0.1193	0.0453	0.0276	-

c, the two sexes were differentiated to a lesser extent. Interestingly, the distance between females of *kathlekan-I* and *kathlekan-II* were always higher than between males of the two populations.

Discussion

V. DISCUSSION

The last few decades have seen an enormous concern and interest in the conservation of biodiversity worldwide. While these concerns have emphasized the conservation at the species level, they do not as much address the conservation of genetic resources both within the species and populations. In practice, conservation programs usually ignore genetic diversity and substitute 'number of individuals' or 'sub species' as an assumed surrogate of genetic diversity (Boyle, 2001).

Maintenance of genetic variation is considered essential for the long-term survival of any species (Frankel and Soule, 1981). The loss of genetic diversity can lead to local extinction of a species (Saccheri *et al.*, 1998). Hence, it is necessary to understand how the diversity of life particularly at the genetic level is maintained under natural conditions. Based on this knowledge, one can suggest appropriate strategies and policies for its conservation. Measuring the amount and spatial distribution of inter and intra population variability will help determining a management strategy for *ex-situ* and *in-situ* conservation. Therefore as in case of biodiversity, emphasis should also be given to study of genetic diversity.

Genetic diversity, which exists below the species level, has long been recognized in forest research (Hamrick *et al.*, 1979; Hamrick, 1983; Loveless and Hamrick 1984; Ledig, 1986; Libby and Critchfield, 1987). Genetic variation in rare species, its partitioning within and among populations, and its ecological and evolutionary significance are crucial to the formulation of sound strategies for their conservation (Falk, 1990a). However, there is a general paucity of published literature on the biology of rare species. With few exceptions, rare plants are poorly understood in terms of life history characteristics (Falk, 1990b). Thus, in case of rare species information may lack in precisely those cases where it is most needed.

Forest tree populations are often considered large and continuous, but many tree species occur as very small populations in nature. It is well known that large populations generally maintain ample genetic variability, and are 'equipped' to respond to selection pressures. One of the main features of the rare and endangered species is their small population sizes; it is believed that small populations are known to have little genetic variation and many a times have undergone genetic bottleneck. Because small populations can potentially lose a large amount of genetic variability due to genetic drift, they may have reduced chance of long-term survival (Franklin, 1980; Soule, 1980; Lande and Barrowclough, 1987). Consequently, the rate at which genetic variation is lost in isolated populations is of great concern to conservation biologists who must decide how to apportion limited resources to protect rare or endangered species.

One such case is that of *Semecarpus kathalekanensis* which is a large sized tree species occurring in the fresh-water swampy habitat at geographical area between 14° 16' N latitude and 74° 44' and 74° 46' E longitude. The species was described recently by Dasappa and Swaminath (2000). They named the species based on the place of the swamp (*i.e.* *Kathlekan*) where a good population of this species occurs. The species is found as mixed stands along with *Myristicaeae* family members restricted to the fresh water swamps of the central Western Ghats. However, the extent of geographic distribution, and abundance of the populations are still poorly defined. The occurrence of its closest relatives *i.e.* *S. travencorica* and *S. auriculata* is reported only from the state of Kerala (Chandran *et al.*, 1999). So far only four populations of *S. kathalekanensis* are reported in this region.

Vasudeva *et al.* (2001) have recently identified that three distinct life-history features *viz.*, high habitat specificity (restricted only to a few *Myristica* swamps), small population size (less than 50 breeding individuals) and dioecious nature of this species

have complicated and constrained its conservation. They are of the opinion that introduction of founding populations into newer type habitats is one of the important ways to recover this critically endangered species. Such reintroduction is dependent on understanding of the genetic structure of the existing populations. Further, since *S. kathalekanensis* is a dioecious species, it is also imperative to identify male and female plants at the nursery stage itself so that sex ratio could be maintained in the newer populations. With this background, an attempt has been made to generate baseline information on inter and intra-population genetic variability of *S. kathalekanensis*.

The results of the present study on *S. kathalekanensis* are discussed below in the context of estimating genetic diversity both within and among two populations of *S. kathalekanensis*, and to identify sex specific markers if any, using both allozymes and RAPD markers and proposing strategies for the conservation. Details of the distribution and breeding system of the species are also presented.

5.1 Distribution and breeding system of *S. kathalekanensis*

Of the four populations identified in the Western Ghats, adult individuals were found in only three populations. The study was conducted in the two populations of 'kathlekan' swamp, separated by 100 m from each other along the stream. The boundary of the swamp was demarcated by the presence of *Pinnaga dicksonii*. *S. kathalekanensis* was generally found to occur towards the boundary, while trees of *Gymnocranthera canarica* a dominant canopy tree occupied the center of the swamp. A rusty brown layer of oily substance was generally found on the water surface near the edges. Clear signs of disturbance to the habitat were found. Exotic weeds such as *Chromolaena* occurred in abundance at the ecotone regions of the swamp. The 'kathlekan-I' population had 40 individuals with 16 in flowering while the 'kathlekan-II'

population had 38 individuals with 18 flowering trees. Since all these populations together consist of less than fifty breeding individuals, the species may be designated as 'critically endangered' (Frankel *et al.*, 1995; Vasudeva *et al.*, 2001).

S. kathalekanensis is dioecious but rarely monoecious individuals were also found. Male and female inflorescence are borne at the terminal branched racemes, which had many flowered panicles. In *Kathlekan-II* population, three trees were monoecious. These individuals produced very few fruits during the fruiting season. The occurrence of male, female and hermaphrodite individuals has been reported earlier as trioecious and is generally grouped under polygamous category. Presence of both monoecious and dioecious individuals suggests that *S. kathalekanensis* could well be one of the earliest species in which dioecy had evolved.

5.2 Genetic structure of populations of *S. kathalekanensis*

In the present study, genetic diversity has been estimated through isozyme analysis as well as from PCR-based RAPD markers. RAPD has been popular among PCR-based techniques for the estimation of genetic diversity mainly because of their rapidity and ability to handle large sample sizes (Wickneswari, 2001). RAPDs have been successfully used for the estimation of genetic diversity and relatedness in several tree species such as *Pinus*, *Populus*, *Glyricedia Shorea*, *Garcinia*, *Elaeis*, *Switenia*, *Eucalyptus* and *Lansium* (Wickneswari, 2001). However, RAPDs are dominant markers, they can not be effectively used to calculate the levels of heterozygosity.

Only four isozyme loci were polymorphic in the populations (Table 4.1). The mean allele number per locus for both the populations was 1.44 ± 0.53 when all loci were considered for analysis (Table 4.2). These values are quite lesser than those generally reported (Hamrick *et al.*, 1992) for other endemic species (1.82). The mean

effective allele number per locus required to maintain the current level of heterozygosity calculated considering only polymorphic loci for analysis in *kathlekan-I* population was 1.51 ± 0.31 and was 1.58 ± 0.37 for *kathlekan-II* which is also on the lower side. Estimate of Shannon's information index, a measure of genetic heterogeneity was computed considering all 34 adult individuals for all loci, was 0.204 ± 0.27 ; it was higher (0.551 ± 0.11) considering only the polymorphic loci. The per cent polymorphism for the two populations did not vary (44.4%); and hence, the over all value was also 44.4%.

RAPD profiles showed a diversity index of 0.627 proving to be a high-resolution method for the detection of genetic variation. Since conventional measures of the genetic variation such as proportion of polymorphic loci and heterozygosity cannot be calculated from RAPD, data comparisons with levels of genetic variation based on isozyme data is difficult.

This study showed that the overall heterozygosity of the populations of *S. kathalekanensis* is 9.48 ± 16.62 per cent ($n= 2$ populations, 34 adult individuals; Table 4.3), with a narrow range between 8.3 to 10.49 per cent. These estimates of heterozygosity are comparable to tropical tree species (11.1 per-cent), which show regional to widespread distribution as reported by Hamrick and Loveless (1986). Ravikanth *et al.* (2001) have shown that in a dioecious rattan, the H_o values range from 6.62 to 15.56 per cent, with a overall mean of 11.63 per cent. Based on three earlier studies Loveless and Hamrick (1984) have reported that the mean diversity within population for dioecious species was 12.1 per-cent. A few dioecious species such as *Eurya japonica* (claimed to be the most widely studied Asian tree species) has a very high population heterozygosity $H_o = 0.425$ (Chung and Kang, 1994).

The percentage heterozygosity was higher for *kathlekan-II* population (29.63 ± 25.66) than for *kathlekan-I* (18.75 ± 16.14), both of which were less than that expected

from Hardy-Weinberg equilibrium (Table 4.4). Hence it is evident that both the populations are suffering from varying extent of heterozygote deficiency. Accordingly, though levels of heterozygosity may compare well with those reported for the tropical dioecious woody species, *S. kathalekanensis* of the central Western Ghats seem to suffer from inbreeding.

This could in part be attributed to the lack of sufficient mating partners in the population, leading to the successive inter-mating between a small group of plants. Moreover, *S. kathalekanensis* may lack specialized seed dispersal mechanisms and numerous fruits have been observed near female plants (Vasudeva *et al.*, 2001). This may favor the establishment of cohorts of related individuals (Plate 3) which could lead to partial inbreeding (Hamrick, 1982), and /or create a Wahlund effect (Hartl and Clark, 1989) causing heterozygote deficiencies. Similar results have been shown in an endangered species, *Pinus rzedowski* a pine species (Delgado *et al.*, 1999), where heterozygote deficiency was around 16.2%.

Low levels of heterozygosity and high degree of heterozygote deficiency have been attributed to several reasons including a) the experience of severe bottlenecks in the evolutionary past of the species which reduces the genetic variability within the populations (Ledig, 1988) and b) due to genetic drift. Unfortunately *S. kathalekanensis* could have suffered from both the causes. Being a relic-species of highly specialized habitat, the populations of this species could have been severely reduced due to anthropogenic pressures (Plate 4a, 4b). In fact, Chandran *et al.* (1999) are of the opinion that the *Myristica* swamps, being one of the most productive ecosystems of the Western Ghats, they have been constantly converted into Areca gardens. Vasudeva *et al.* (2001) have also reported that in some populations, the number of flowering individuals are so low that there could have been a genetic bottleneck.



Plate 3 Cohort of young seedlings of *S. kathalekanensis*

a)



b)



Plate 4 (a) Attempted clearing of seedlings and (b) bark removal in *Semecarpus kathalekanensis*

The low levels of heterozygosity and heterozygote deficiency are intriguing in *S. kathalekanensis*, which being dioecious should be obligate outcrossers. Despite its dioecious nature the *S. kathalekanensis* is indeed predisposed to very limited mating opportunity. This may be due to the family structure of *S. kathalekanensis*, where inbreeding can be expected because of irregular flowering and fruiting which limits the dispersal of both pollen and seed (Vasudeva *et al.*, 2001). Moreover, low population densities, hyperdispersion of adults, habitat homogeneity, erratic and asynchronous flowering within the populations can contribute to extensive reproductive isolation, even among plants within a population (Federov, 1966).

Hence, it is reasonable to hypothesize that genetic variation in *S. kathalekanensis* is reduced with every generation due to various reasons. We tested this hypothesis by categorizing the matured individuals into two groups based on GBH. Those which had more than 100cm GBH (n=17) considered 'older' and those which had less than 100cm GBH (n=16) considered 'younger'. The observed and expected percent heterozygosity was calculated. Interestingly, H_o was significantly higher in 'older' individuals than 'younger' individuals (11.11 ± 21.3 Vs 6.67 ± 11.55 per-cent, respectively). Further, the H_e value for these two groups was 12.85 ± 19.4 for 'older' and 13.41 ± 17.95 for 'younger' individuals, suggesting that the heterozygote deficiency might increase in the 'younger' individuals. Hence, the younger generations might possess reduced genetic variation.

A significant asynchrony in flowering has been noticed among the male and female trees (Vasudeva *et al.*, 2001). Due to asynchrony in flowering, individuals may restrict random gamete exchange and may lead to further inbreeding and consequent depression. Furthermore, because of the failure to set seed, seed predation,

regeneration survival of *S. kathalekanensis* may also be severely affected leading once again to depauperation of the breeding population.

Thus it appears that *S. kathalekanensis* is suffering from almost every aspect of its life history. It would be worthwhile to examine the patterns of genetic variation in similar species that are endemic or otherwise highly threatened and compare those with the results obtained from this study.

5.3 Lack of population differentiation in *S. kathalekanensis*

The level of population differentiation in *S. kathalekanensis* is low. The mean F_{st} value, which is a measure of the proportion of total genetic diversity at polymorphic loci (which is due to differences among populations) was about one per cent (Table 4.5). In other words, nearly 100 per-cent of the total genetic variation is entirely accounted by the variation within the population. This estimates of F_{st} is compared to that obtained for 37 tropical taxa (10.9%; Loveless and Hamrick, 1984) and is comparable with estimates obtained by other workers. For instance, Hall *et al.* (1994), reported an F_{st} value of 0.046 for *Carapa guianensis*. These low levels in *S. kathalekanensis* are also indicated by binary analysis of isozymes data and RAPD data. Gene flow measured as the average number of individuals exchanged between populations per generation (N_m) for genetic interpretation, binary analysis of isozymes and RAPD data are 2.3, 14.34 and 22.42 respectively (Table 4.5, 4.8 and 4.10). RAPD analysis showed a slightly higher level of population differentiation.

F_{st} is inversely related to N_m . A low F_{st} means that populations are genetically identical. Genetic differentiation among populations is principally a function of gene flow among populations *via* pollen and seed dispersal. The seeds of *S. kathalekanensis* are very buoyant and can survive in water for substantial periods of time and germinate

even when partial submerged. As the two *kathlekan* populations are separated by about 100 m from each other along the stream, there could be a possible migration of floating seeds to adjacent population, which probably is one of the reasons for high level of gene flow indicated by the low degree of population differentiation. This could also suggest that higher level of gene flow could counter balance the effects of genetic drift and could be a major factor shaping the genetic structure of the populations.

5.4 Consequences of lower genetic diversity to the fitness

In small populations, loss of genetic variability may limit its ability to respond to new conditions and long term changes in the environment such as pollution, new disease, and global climate change (Falk and Holsinger, 1991). Genetic drift and inbreeding influence fitness through inbreeding depression, the loss of fitness with increasing homozygosity. Small populations may produce seeds of lower fitness because of inbreeding depression in recently reduced populations. O'Brien and Evermann (1988) argued that populations lacking genetic diversity are vulnerable to infectious diseases and hence may go extinct. They showed that monomorphism leads to disease and ultimately a higher rate of extinction as illustrated by two examples. The cheetah (*Acinonyx jubatus*) is usually monomorphic at almost every locus. It was observed that all cheetah in a wildlife park at Oregon, USA were infected when an epidemic of feline infectious peritonitis (FIP) swept through park suggesting the homogeneity. A second example is a naturally isolated population of lions (*Panthera leo*) of Ngorogoro crater in Africa. These lions which have undergone inbreeding for several centuries show lowered genetic variation as well as higher frequency of sperm abnormalities than those in the surrounding area.

Evidence from studies on plant populations show that fitness seems to increase with the heterozygosity levels. Increased heterozygosity has associated with increasing age, earlier sexual maturity, and increased vegetative and reproductive output in *Liatris cylindracea* (Schaal and Levin, 1977). In addition, heterozygosity and growth rate are positively correlated in some temperate tree species (Ledig, 1986). Further, some studies have suggested that highly heterozygous organisms are better able to contend with fluctuating environment (Huenneke, 1991). In *Gentianella germanica* for example, Fischer and Mattrial (1998), have showed that mean number of seeds per plant increased linearly with increase in molecular variance ($r=0.76$, $p<0.02$). Similarly they have also shown that the flowers per planted seed was also linearly related to the molecular variance ($r=0.62$, $p<0.05$).

5.5 How the ill effects of low heterozygosity could be averted in *S. kathalekanensis*?

Populations with limited genetic variation are most vulnerable to extinction, due to reduced potential for evolution in response to environmental changes (Beardmore, 1983). A three pronged strategy for protecting populations of this endangered species can be envisioned. Firstly, protecting all the existing populations. It is often necessary to do more than protection of sites where it occurs and manage the habitat on the sites. For example, in case of *S. kathalekanensis*, Karnataka Forest Department has attempted to establish an *in situ* conservation site by fencing one of the populations in *Kathlekan* (Plate 5) by effectively checking all human interference and of the invasive weeds to these populations (Vasudeva *et al.*, 2001). Such conservation plans for a species will protect the existing population and thus restoring the number in their native habitats.



Plate 5 *In situ* conservation measures taken by Karnataka Forest Department.

Secondly, a further possible step in the recovery of a species is to replenish existing population and/or to introduce material into sites where the species is likely to survive. Such recovery plans for endangered plants often call for the creation of new, self sustaining populations within historic range and characteristic habitat (Whitten, 1990; Vasudeva *et al.*, 2001).

Finally, to enrich the local variability, seedlings collected from different populations can be introduced into the already existing populations following the concept of forest gene banks (Uma Shaanker and Ganeshaiah, 1997). This method is an effective tool to maintain or to increase genetic variation within population, however, it may reduce variance among populations. Griffith *et al.* (1989) are also of the opinion that attempts to manage the species by transplanting individuals between sub-populations is an effective tool.

For some endangered plant species reintroduction of individuals grown protectively may be the best conservation strategy. Reintroduction can be done into an existing wild population to boost their numbers or to an area where species has become locally extinct. Thus species reintroduction would act as a functional bridge between *ex situ* and *in situ* conservation methods (Frankel *et al.*, 1995). Till date, several hundred species of flowering plants have been reported to be propagated and reintroduced into wild (Heywood, 1995). The case of *Schoenus ferrugineus*, a rare grass like plant growing in marshy places of England, is interesting (Brookes, 1981). During 1950 all the sites of this species were submerged by a hydroelectric scheme, but hundreds of plants were simultaneously introduced to other places. The fate of these transplants were analyzed after 30 years and found that at least in two sites the species had established well (Brookes, 1981).

Pavlik *et al.* (1993) have succeeded in founding a new population of an endangered heterostylus herb *Amsinckia grandiflora* within its historic range. In India, reintroduction of rare plant species is restricted to a few orchid species. *Paphiopedium druryi*, an awesome slipper orchid was multiplied through tissue culture and reintroduced to type localities in Agasthiyamalai hill ranges of south Western Ghats (Tiwari, 1995). However, there are no reports of the success of these programmes. Hence creating new, self-sustaining populations of rare plant species still remains a great challenge.

Fortunately, such an attempt was done for *S. kathalekanensis* (Vasudeva *et al.*, 2001). About 40 nursery raised seedlings were reintroduced into two new localities. The authors have reported that the survival was quite remarkable (Plate 6). This clearly indicates that *S. kathalekanensis* can be successfully re-introduced into the new localities. They only require little moisture during summer for their establishment.

Since the *S. kathalekanensis* populations have higher heterozygote deficiency it is essential that the following few aspects are considered to increase the same in reconstituted / founding populations.

- i) Sampling of seedlings from all the female trees pooled over both the populations.
- ii) Find and sample seedlings from nearby populations, if any.
- iii) Use vegetatively propagated saplings from non-flowering individuals from all the populations
- iv) Induce flowering through various silvicultural treatments
- v) Take-up supplemental pollination to encourage greater cross-pollination.



Plate 6 Growth of reintroduced *S. kathalekanensis* seedling showing good growth after 16 months.

5.6 Genetic differences between male and female individuals

For this analysis, data pooled over both the populations were considered. Genetic distances were calculated based on isozyme, electromorph as well as PCR products. Of the sixty-five primers employed in the study, none of them could unequivocally show differences between the two sexes. This calls for further experimentation on marker identification. As shown in the Table 4.11, the two sexes were differentiated to a lesser extent. Interestingly, the distance between females of *kathlekan-I* and *kathlekan-II* were always higher than between males of the two populations hinting a differential gene flow between the populations through male and female function. Further, the dendrogram generated based on Nei's genetic distance clearly grouped monoecious individuals as a separate cluster suggesting the sex determination in *S. kathalekanensis* could be genetically based (Fig. 5.1).

Since the overall sex ratio computed in an early study (Vasudeva *et al.*, 2001) was shown to be nearly 1:1, it can be best expected that among the introduced plants the same sex ratio would be maintained.

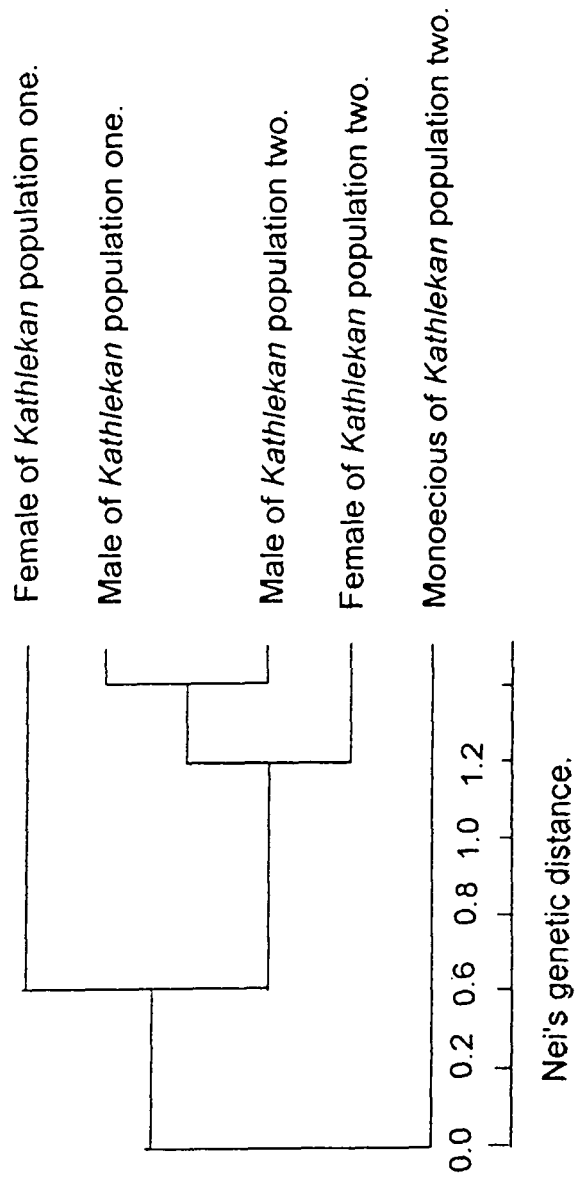


Fig. 5.1 Dendrogram based on Nei's genetic distance showing clustering of sexes of two populations of *S. kathalekanensis*.

Summary

VI. SUMMARY

Semecarpus kathalekanensis is a large sized medicinal tree species that was recently described from the *Myristica* swamps of the central Western Ghats. It is a critically endangered dioecious tree having less than fifty breeding individuals in this region (perhaps in the entire Western Ghats) occurring in fragmented populations. High habitat specificity exhibited by the species and its loss has constrained the conservation efforts. Recently suggested recovery plan for this species envisages establishment of founding populations by reintroducing the nursery grown seedlings into newer type localities. It is imperative to ensure that such founding populations possess genetic diversity sufficient enough to cope with the future challenges and an appropriate population sex ratio is maintained. For this reason, it is essential to understand the genetic structure of the existing populations and ideally to identify a sex specific markers that can be used in the nursery stage itself. Further, the allozyme diversity can also be used as a yardstick to measure the effectiveness of *in situ* and *ex situ* conservation programs.

With this background a study was undertaken to estimate genetic diversity of 34 adult individuals of *S. kathalekanensis*, from two neighboring populations adopting allozyme markers and DNA fingerprinting. In the process it was designed to identify sex specific markers, if any.

In the starch gel electrophoreses, of the twenty isozymes initially examined, standardization was possible for six isozymes viz., *Maleic enzyme (ME)*, *Isocitrate dehydrogenase (IDH)*, *Phosphoglucose isomerase (PGI)*, *Malate dehydrogenase (MDH)*, *Phosphoglucose mutase (PGM)*, and *Phosphoglucose dehydrogenase (PGDH)*. Only four isozyme loci out of 9 were polymorphic in both the populations (44.44%). The overall-mean allele number per locus was 1.44. The Shannon's information index computed

based on all loci was 0.204 ± 0.27 ; however, it was higher (0.55 ± 0.11) considering only the polymorphic loci. This study showed that the overall heterozygosity of the populations of *S. kathalekanensis*, was 9.08 ± 16.62 per cent, which is lower than the average values for species with similar life-history traits. Further, the observed mean heterozygosity ($H_o=0.095$) was substantially lower than the expected mean heterozygosity ($H_e=0.134$) based on Hardy-Weinberg equilibrium. This suggests that the populations are suffering from heterozygote deficiency. The genomic DNA obtained from the 33 individuals from two populations were subjected to PCR based RAPD using 10 random primers and a total of seventy-six loci were generated. The per cent polymorphism ranged from 71.05 to 61.84. The RAPD fingerprinting generated in the study showed a diversity index of 0.627 proving to be a high-resolution method for the detection of genetic variation.

The level of population differentiation among two populations studied in *S. kathalekanensis* is low. The mean F_{st} value, which is a measure of the proportion of total genetic diversity at polymorphic loci that is attributable to differences among populations, was about just one per cent. In other words, nearly 100 per cent of the total genetic variation is accounted entirely for by the variation within the population. This estimates of F_{st} is very low compared to that obtained for 37 tropical taxa (10.9%) by Loveless and Hamrick, (1984). This low level in *S. kathalekanensis* is also indicated by binary analysis of isozyme data and RAPD data. Gene flow measured as the average number of individuals exchanged between populations per generation (N_m) for genetic interpretation, binary analysis of isozymes and RAPD data are 2.3, 14.34 and 22.42, respectively. Since *S. kathalekanensis* is butterfly pollinated one can expect a higher gene exchange between neighboring populations. Further, the seeds of *S. kathalekanensis* are very buoyant and can survive in water for substantial periods of

time and germinate even when partial submerged. As the two *kathalekan* populations are separated by about 100 m from each other along the stream, there could be a possible migration of floating seeds to adjacent population.

Generally, low levels of heterozygosity and high degree of heterozygote deficiency observed among rare/endemic tree species have been attributed to a) severe genetic bottleneck in the evolutionary past of the species and b) due to genetic drift. Being a rare relic-species of highly specialized habitat, the populations of *S. kathalekanensis*, could have been severely reduced due to anthropogenic pressures and due to low number of flowering individuals leading to genetic bottlenecking. Further, despite its dioecious nature, *S. kathalekanensis* is indeed predisposed to very limited mating opportunity because of irregular flowering and fruiting which limits the dispersal of both pollen and seed. Hence, it is reasonable to hypothesize that genetic variation in *S. kathalekanensis* is reduced with every generation due to various reasons leading to depauperation of the breeding population. Thus it appears that *S. kathalekanensis* is suffering from almost every aspect of its life history. It would be worthwhile to examine the patterns of genetic variation in similar species that are endemic or otherwise highly threatened and compare those with the results obtained from this study.

Since the *S. kathalekanensis* populations have higher heterozygote deficiency it is essential that the following few aspects are considered to increase the same in reconstituted / founding populations.

- i) Sampling of seedlings from all the female trees pooled over both the populations.
- ii) Find and sample seedlings from nearby populations, if any.

- iii) Use vegetatively propagated saplings from non-flowering individuals from all the populations
- iv) Induce flowering by various silvicultural treatments
- v) Take-up supplemental pollination to encourage greater cross-pollination.

Of the sixty-five primers employed to study the genetic differences between male and female individuals, none of them could unequivocally show differences between the two sexes. This calls for further experimentation on marker identification. Interestingly, the genetic distance between female trees of *kathlekan-I* and *kathlekan-II* were always higher than that between males of the two populations hinting a differential gene flow between the populations through male and female function. Since the overall sex ratio computed in an earlier study was shown to be nearly 1:1, it can be best expected that among the introduced plants the same sex ratio would be maintained.

Future Line of Work

1. Identification of few more populations of *S. kathalekanensis* in historical range within Western Ghats using Geographic Information System approach.
2. To attempt creating a self-sustaining viable population using the laboratory grown seedlings.
3. Other DNA techniques could be adopted to identify sex specific markers.
4. Heterozygote deficiency at different age groups of plants in *S. kathalekanensis* needs to be assessed.
5. Effects of different silvicultural practices to enhance random mating could be tried.

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* Originals not seen

Appendices

APPENDIX-I

A. Extraction buffer used and their composition

(Based on Pitel and Cheliak, 1984; Siciliano and Shaw, 1976; Ridgeway *et al.* 1970; Schaal and Anderson, 1974)

Chase extraction buffer 0.011 M EDTA 0.001 M KCl 0.002 M MgCl ₂ 4 % PVP 40 1 % PEG 0.2 M PO ₄ buffer 0.027 mM NADP 0.003 mM NAD 0.03 M DTT 10% glycerol 10% PVPP	Wendels buffer 100 mM Tris HCl (pH 7.5) 7% Sucrose 10% PEG-20 10% PVP 40 0.1% BSA 20 MM Sodium metasilphite 200 mM Sodium tetraborate 0.3% mercaptoethanol 250 mM Ascorbic acid	Feret buffer 0.1 M Tris HCl 0.01 M Ascorbic acid 5.4 mM Cysteine HCl 1% Tween-80 0.2% MgCl ₂ 0.2% CaCl ₂ 0.5 M Sucrose 1% Mercaptoethanol
Fruit trees extraction buffer 0.05 M Tris 0.007 M Citric acid 0.1% w/v Cysteine HCl 0.1% w/v ascorbic acid 1% PEG 20 10% PVPP 1 mM mercaptoethanol	Vegetative buffer 8% PVP 0.2 M Sucrose 0.5 mM EDTA 1 mM dithiothreitol 1 mM Ascorbic acid 0.1% BSA 0.4 mM NAD 0.01 mM NADP 0.2 mM Pyridoxal 5'phosphate	Borate buffer 0.05 M Borate buffer (Sodium tetraborate) 0.02 M Cysteine HCl 0.5 M Sucrose 1% Triton-X 0.2% MgCl ₂ 0.2% CaCl ₂ 1% PEG 20 8% PVP 40 10% PVP 36 0.4 mM NAD 0.3% mercaptoethanol
Phosphate buffer 0.1 M PO ₄ buffer (pH 7.5) 0.029 M Sodium tetraborate 0.017 M Sodium metabisulphite 0.2 M ascorbic acid 0.016 M diethyl dithiocarbamate 4% w/v PVP 40 1% mercaptoethanol	Tris buffer 100 mM Tris 1% mercaptoethanol 10% PVP 40	Lithium borate buffer 50 mL lithium borate gel buffer KCl MgCl ₂ EDTA

B. Composition of Gel and electrode buffers

(Based on Market and Faulhaber, 1965; Poulik, 1957; Aashton and Braden, 1961; Stuber *et al*, 1977)

BUFFER	pH	Composition	
		Electrode buffer	Gel Buffer
Continuous Tris Citrate (CTC)	8.0	0.25 M Tris 0.075 M Citric acid	1:25 dilution of electrode buffer
Tris Electrode Buffer (TEB)	8.5	0.5 M Tris 0.24 M Boric acid 0.016 M EDTA	1:10 dilution of electrode buffer
Boric acid buffer (B)	8.5	0.3 M Boric acid 0.06 M Lithium hydroxide	0.03 M Tris 0.005 M Citric acid 1% electrode buffer pH 8.5 (10x concentration)
Histidine buffer (H)	7.0	0.125 M Tris pH 7.0	0.05 M Histidine HCl mM EDTA pH 7.0 (Dilute stock 4:1)
Lithium borate buffer	8.3		
Histidine citrate buffer	6.5	0.065 M Histidine 0.007 M Citric acid	0.016 M Histidine 0.002 M Citric acid

C. Enzyme staining recipes used in the experiment

(Based on Cheliak and Pitel, 1984; Leingsiri *et al.*, 1990)

Alcohol dehydrogenase (ADH)	Aldolase (ALD)	Adenylate kinase (AK)
5 mL 0.2 M Tris HCl pH 8.0 5.0 mL ethanol 0.5 mL NAD 0.5 mL NBT 0.5 mL PMS	5 mL 0.2 M Tris HCl pH 8.0 125 mg Fructose 1,6-diphosphate 38 mg Arsenic acid 150 units glyceraldehyde 3-phosphate dehydrogenase 0.5 mL NAD 0.5 mL MTT 0.5 mL PMS	5 mL 0.2 M Tris HCl pH 8.0 80 mg glucose 25 mg ADP 125 units hexokinase 25 units G6PDH 0.5 mL MgCl ₂ 0.5 mL NAD 0.5 mL MTT 0.5 mL PMS

Acid phosphatase (AP) 25 mL 0.2 M sodium acetate buffer pH 5.0 75 mg sodium α naphthyl acid phosphate 25 mg fast garnate GBC salt 2.5 mL 1% $MgCl_2$	Diaphorase (DIA) 5 mL 0.2 M Tris HCl pH 8.0 0.5 mg 2,6 - dichlorophenol iodo phenol 13 mg beta NADH 0.5 mL MTT	Esterase (EST) 5 mL 0.2 M Phosphate buffer 25 mg alpha & beta naphthyl acetate in 25 mL acetone 50 mg fast blue RR salt
Glucose dehydrogenase (GLDH) 5 mL 0.2 M Tris HCl pH 8.0 250 mg glucose 0.5 mL NAD 0.5 mL PMS 0.5 mL NBT	Glucose 6 phosphate dehydrogenase (G₆PDH) 5 mL 0.2 M Tris HCl pH 8.0 100 mg glucose 6 phosphate 0.5 mL $MgCl_2$ 0.5 mL NAD 0.5 mL MTT 0.5 mL PMS	Iso citrate dehydrogenase (IDH) 5 mL 0.2 M Tris HCl pH 8.0 100 mg iso citric acid 0.5 mL $MgCl_2$ 0.5 mL NADP 0.5 mL MTT 0.5 mL PMS
Glyceraldehyde 3-phosphate dehydrogenase (G₃PDH) 5 mL 0.2 Tris HCl pH 8.0 125 mg Fructose 1,6 diphosphate 38 mg Arsenic acid 180 μ l aldolase 0.5 mL NAD 0.5 mL NBT 0.5 mL PMS	Hexokinase (HK) 5 mL 0.2 Tris HCl pH 8.0 25 mg glucose 5.5 μ l G6PDH 6 mg ATP 0.5 mL $MgCl_2$ 0.5 mL NADP 0.5 mL MTT 0.5 mL PMS	Malate dehydrogenase (MDH) 5 mL 0.2 Tris HCl pH 8.0 12.5 mL 0.5 M malic acid 0.5 mL NAD 0.5 mL NBT 0.5 mL PMS
Malic enzyme (ME) 5 mL 0.2 Tris HCl pH 8.0 12.5 mL malic acid pH 7.0 0.5 mL $MgCl_2$ 0.5 mL NADP 0.5 mL MTT 0.5 mL PMS	Menadione reductase (MR) 5 mL 0.2 Tris HCl pH 8.0 25 mg menadione 25 mg NADH 1 mL NBT	Phospho gluco isomerase (PGI) 5 mL 0.2 Tris HCl pH 8.0 12.5 mg fructose 6 phosphate 200 μ l G6PDH 0.5 mL $MgCl_2$ 0.5 mL NADP 0.5 mL MTT 0.5 mL PMS

Phospho gluco mutase (PGM)	Shikimate dehydrogenase (SKDH)	Succinic dehydrogenase (SUDH)
5 mL 0.2 Tris HCl pH 8.0 150 mg glucose 1 phosphate 0.25 mL glucose 1,6- diphosphate 5.5 µl G6PDH 0.5 mL MgCl ₂ 0.5 mL NADP 0.5 mL MTT 0.5 mL PMS	5 mL 0.2 Tris HCl pH 8.0 25 mg shikimic acid 0.5 mL MgCl ₂ 0.5 mL NADP 0.5 mL NBT 0.5 mL PMS	5 mL 0.1 M phosphate buffer 75 mg succinic acid 100 mg EDTA 12.5 mg ATP 0.5 mL NAD 0.5 mL NBT 0.5 mL MTT 0.5 mL PMS
Peroxidase (PER) a) 15 mL 1% H ₂ O ₂ b) 15 mL 20% acetic acid 300 mg benzidine dehydro chloride Mix both before staining		

D. Stain buffer recipes

- 1) 0.5M malic acid
 - a) dissolve 67.2g of DL Malic acid in 800ml water
 - b) adjust pH to 7.0 with 10N NaOH
 - c) make up the volume to 1 litre
- 2) 0.2M acetate, pH 5.0

Sodium acetate-11.49g
 Glacial acetic acid-3.6ml
 Make up the volume to 1 litre
- 3) 0.2 M Tris HCl (pH 8.0)

Trizma base - 24.22 g
 Make upto 750 mL with distilled water
- 4) 0.2 M Phosphate (pH 7.0)

Na₂HPO₄ – 28.4 g
 Adjust pH with NaH₂PO₄
- 5) NAD - 10 mg/ml
- 6) NADP - 10 mg/mL
- 7) NBT - 10 mg/mL
- 8) MTT - 10 mg/mL
- 9) PMS - 5 mg/mL
- 10) MgCl₂ - 10 mg/mL

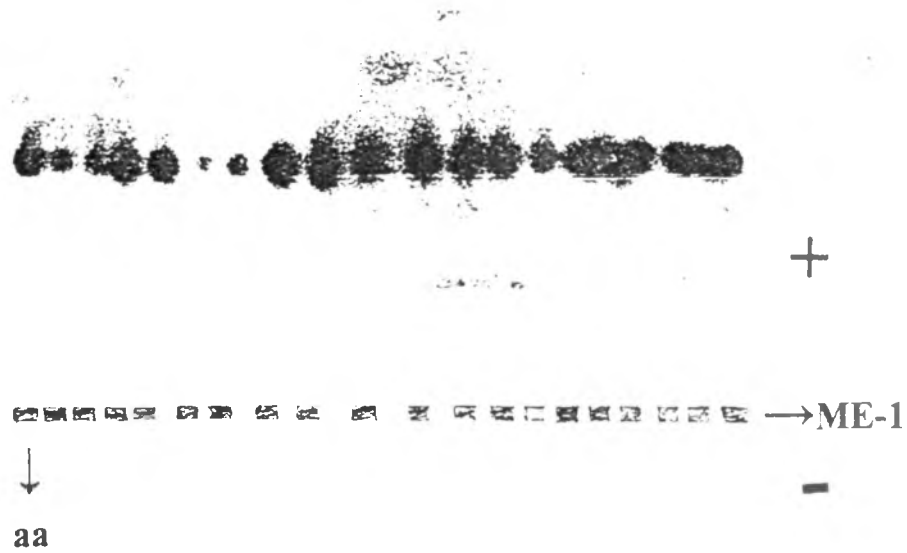
A. RAPD Protocol for the PCR amplification of *S. kathalekanensis*

Steps	Temperature	Time (in minutes)	Purpose
1	94 ⁰ C	3	Initial denaturation
2	94 ⁰ C	5sec	Cyclic denaturation
3	35 ⁰ C	5sec	Primer annealing
4	72 ⁰ C	2	Chain elongation
5	(42 times from step2 to step 4)		
6	72 ⁰ C	8	For the final elongation
7	4 ⁰ C	(till analysis)	For cooling

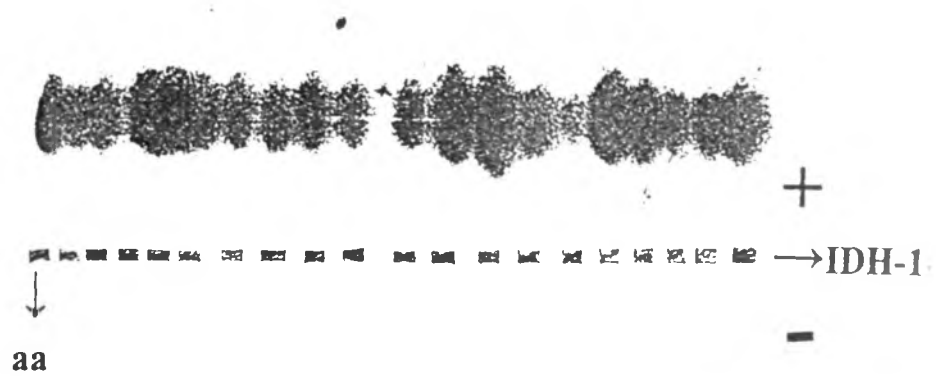
B. Random primers screened against the DNA samples of two populations of *Semecarpus kathalekanensis* and their nucleotide sequences

Sl.No.	Oligo Name	Primer sequence	Molecular weight(MW)
1	KIT V 1	5'-GTG GCA TCT C-3'	3019
2	KIT V 2	5'-CAT CGC CGC A-3'	2973
3	KIT V 3	5'-ACA GCC TGC T-3'	2988
4	KIT V 4	5'-GGC TGC AAT G-3'	3068
5	KIT V 5	5'-GGC TGC GAC A-3'	3053
6	KIT V 6	5'-AAG GCT CAC C-3'	2997
7	KIT V 7	5'-AGA GCC GTC A-3'	3037
8	KIT V 8	5'-AGG CAG AGC A-3'	3086
9	KIT V 9	5'-AGC AGC GCA C-3'	3022
10	KIT V 10	5'-CAA ACG TGG G-3'	3077
11	KIT V 11	5'-AGA CGA TGG G-3'	3117
12	KIT V 12	5'-AAG CCT GCG A-3'	3037
13	KIT V 13	5'-GGG TCT CGG T-3'	3075
14	KIT V 14	5'-GGT CGATCT G-3'	3059
15	KIT V 15	5'-AGT CGC CCT T-3'	2979
16	KIT V 16	5'-GGG CCA ATG T-3'	3068
17	KIT V 17	5'-GAC GTG GTG A-3'	3108
18	KIT V 18	5'-GTG GAG TCA G -3'	3108
19	KIT V 19	5'-TGA GGG TCC C-3'	3044
20	KIT V 20	5'-AGC CGT GAA A-3'	3061
21	KIT W 1	5'-CTC AGT GTC C-3'	2979
22	KIT W 2	5'-ACC CCG CCA A-3'	2942
23	KIT W 3	5'-GTC CGG AGT G-3'	3084
24	KIT W 4	5'-CAG AAG CGG A-3'	3086
25	KIT W 5	5'-GGC GGA TAA G-3'	3117
26	KIT W 6	5'-AGG CCC GAT G-3'	3053
27	KIT W 7	5'-CTG GAC GTC A-3'	3028
28	KIT W 8	5'-GAC TGC CTC T-3'	2979
29	KIT W 9	5'-GTG ACC GAG T-3'	3068
30	KIT W 10	5'-TCG CAT CCC T-3'	2939
31	KIT W 11	5'-CTG ATG CGT G-3'	3059
32	KIT W 12	5'-TGG GCA GAA G-3'	3117
33	KIT W 13	5'-CAC AGC GAC A-3'	3006
34	KIT W 14	5'-CTG CTG AGC A-3'	3028
35	KIT W 15	5'-ACA CCG GAA C-3'	3006
36	KIT W 16	5'-CAG CCT ACC A-3'	2957
37	KIT W 17	5'-GTC CTG GGT T-3'	3050
38	KIT W 18	5'-TTC AGG GCA C-3'	3028

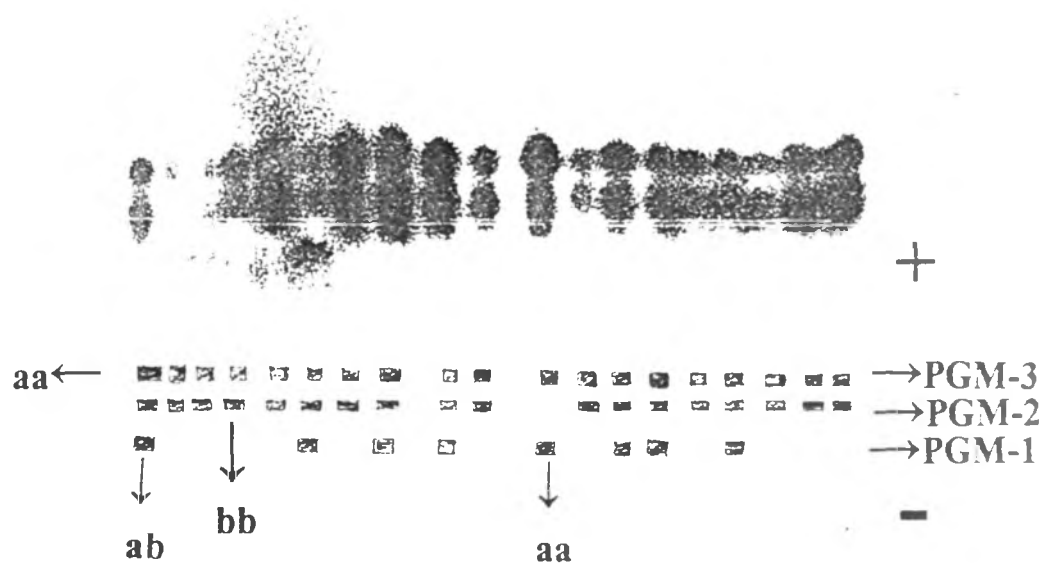
Sl.No	Oligo Name	Primer sequence	Molecular weight(MW)
39	KIT W 19	5'-CAA AGC GCT C-3'	2997
40	KIT W 20	5'-TGT GGC AGC A-3'	3068
41	KIT X 1	5'-CTG GGC ACG A-3'	3053
42	KIT X 2	5'-TTC CGC CAC C-3'	2924
43	KIT X 3	5'-TGG CGC AGT G-3'	3084
44	KIT X 4	5'-CCG CTA CCG A -3'	2973
45	KIT X 5	5'-CCT TTC CCT C-3'	2890
46	KIT X 6	5'-ACG CCA GAG G-3'	3062
47	KIT X 7	5'-GAG CGA GGC T-3'	3093
48	KIT X 8	5'-CAG GGG TGG A-3'	3133
49	KIT X 9	5'-GGT CTG GTT G-3'	3090
50	KIT X 10	5'-CCC TAG ACT G-3'	2988
51	KIT X 11	5'-GGA GCC TCA G-3'	3053
52	KIT X 12	5'-TCG CCA GCC A-3'	2973
53	KIT X 13	5'-ACG GGA GCA A-3'	3086
54	KIT X 14	5'-ACA GGT GCT G-3'	3068
55	KIT X 15	5'-CAG ACA AGC C-3'	3006
56	KIT X 16	5'-CTC TGT TCG T-3'	3010
57	KIT X 17	5'-GAC ACG GAC C-3'	3022
58	KIT X 18	5'-GAC TAG GTG G-3'	3108
59	KIT X 19	5'-TGG CAA GGC A-3'	3077
60	KIT X 20	5'-CCC AGC TAG A-3'	2997
61	PRIMER 1	5'-CAG GCC CTT C-3'	2964
62	PRIMER 2	5'-TGC CGA GCT G-3'	3044
63	PRIMER 3	5'-AGT CAG CCG C-3'	2997
64	PRIMER 4	5'-AAT CGG GCT G-3'	3068
65	PRIMER 5	5'-ACG GGT CTT G-3'	3099



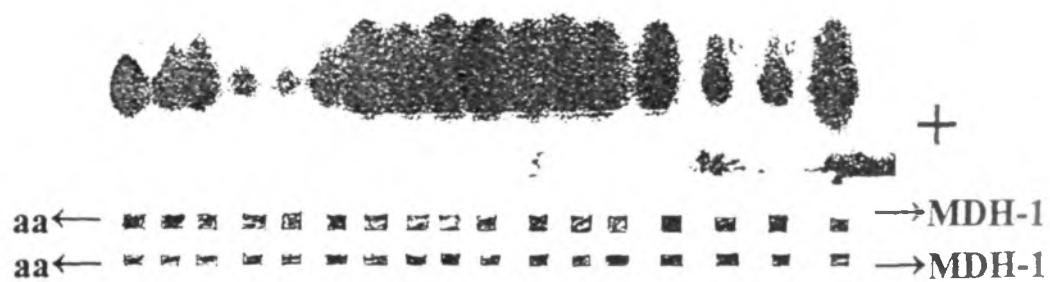
Banding pattern of Malic enzyme.



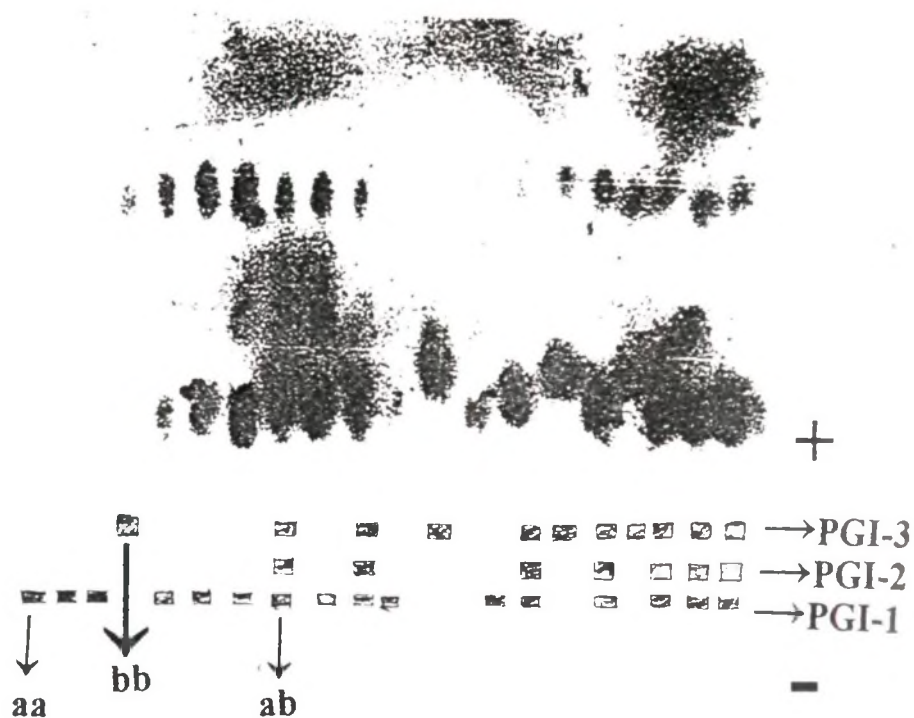
Banding pattern of Isocitrate dehydrogenase.



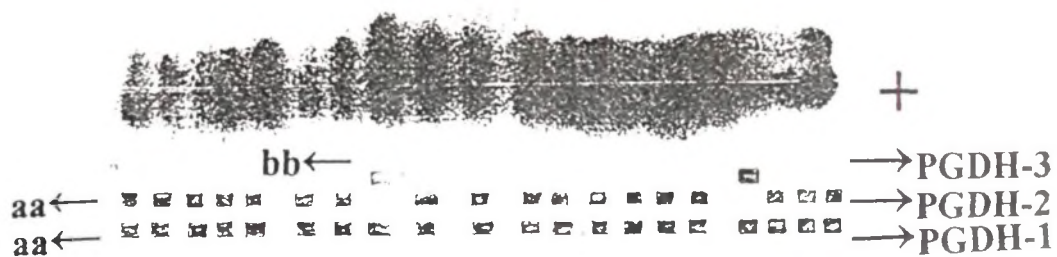
Banding pattern of Phosphoglucumutase.



Banding pattern of Malatedehydrogenase.

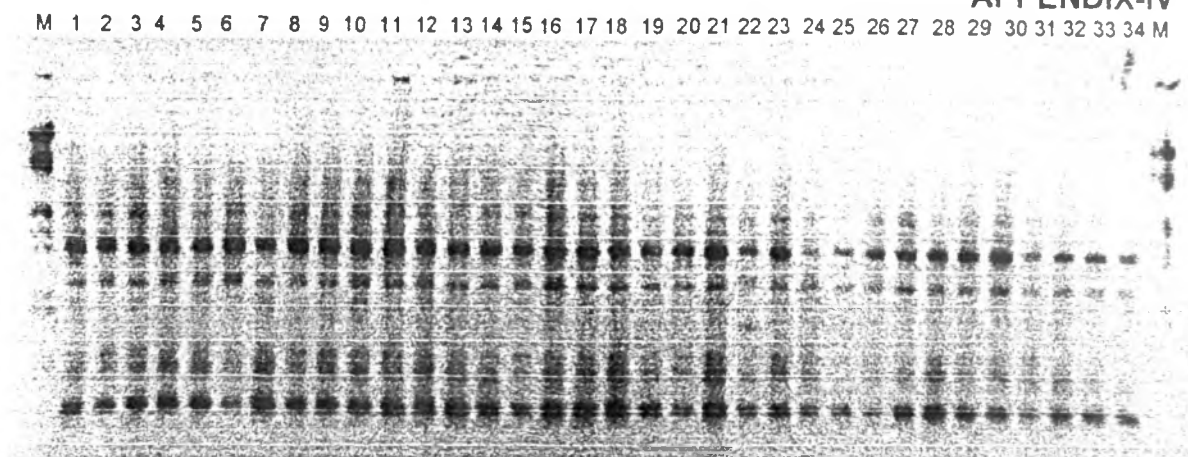


Banding pattern of Phosphoglucosomerase.



Banding pattern of Phosphoglucose dehydrogenase.

APPENDIX-IV

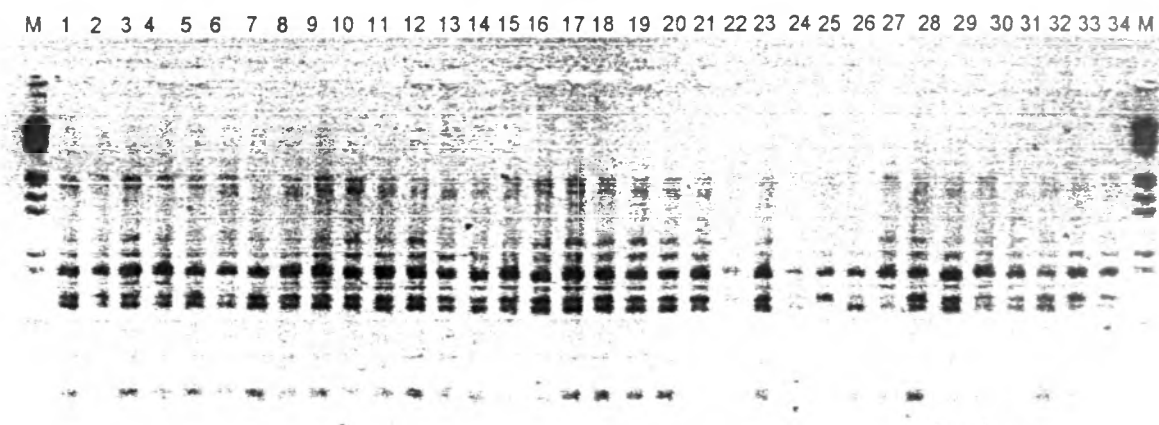


Amplification profile of two populations of *S. kathalekanensis* with the primer KITw6

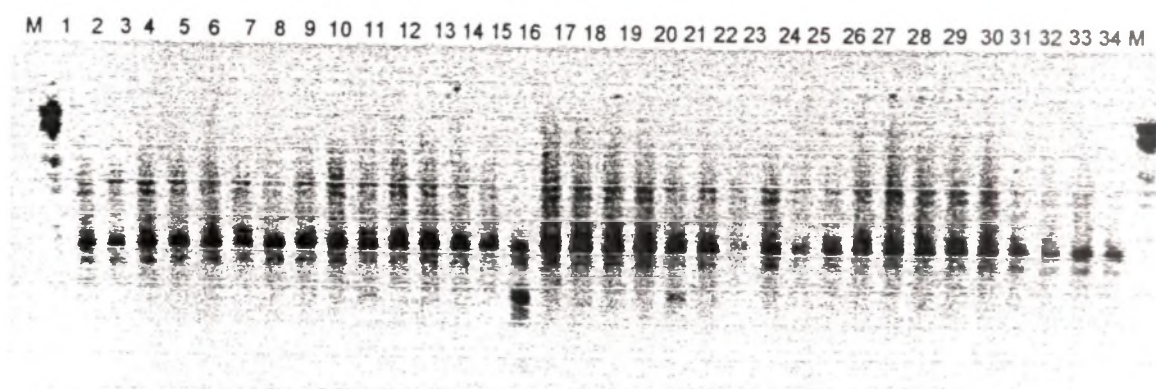
Note: M- Marker (Hind III Double digest)

Lane numbers	SEX	POPULATION
1-3	Monoecious	Two
4-11	Male	Two
12-13	Unknown	Two
14-18	Female	Two
19-23	Male	One
24-34	Female	One

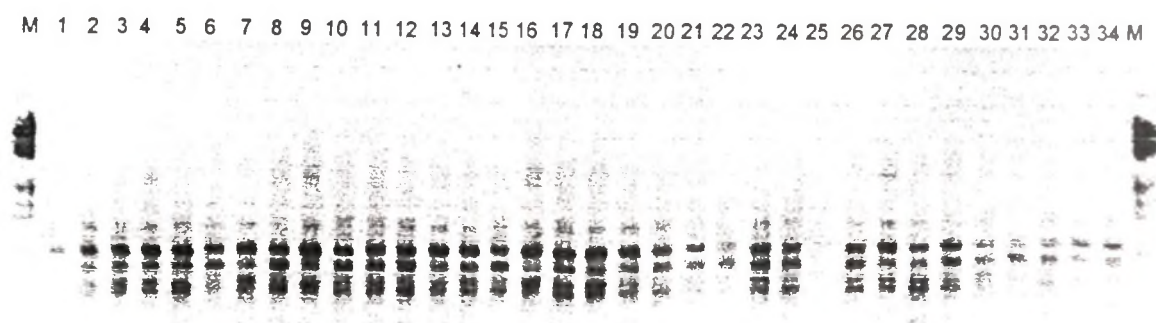
Same pattern follows for all the gel images.



Amplification profile of two populations of *S. kathalekanensis* with the primer KITx18.

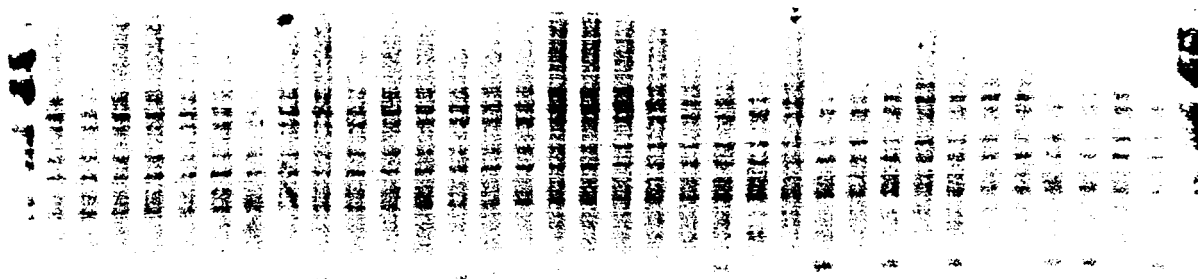


Amplification profile of two populations of *S. kathalekanensis* with the Primer p2



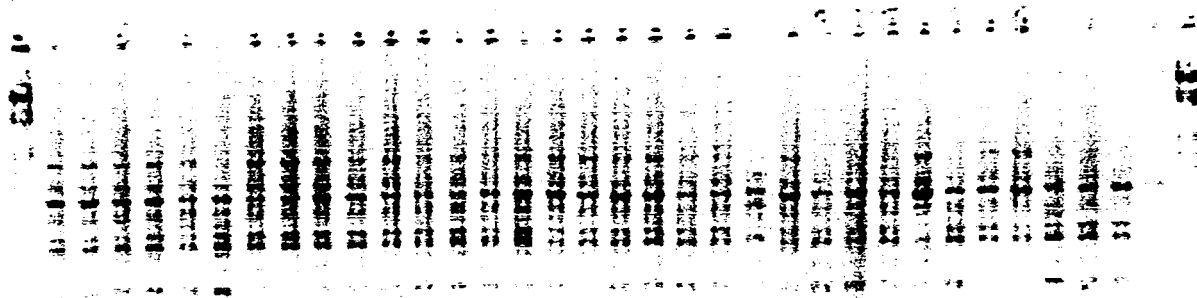
Amplification profile of two populations of *S. kathalekanensis* with the Primer KITx2.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 M

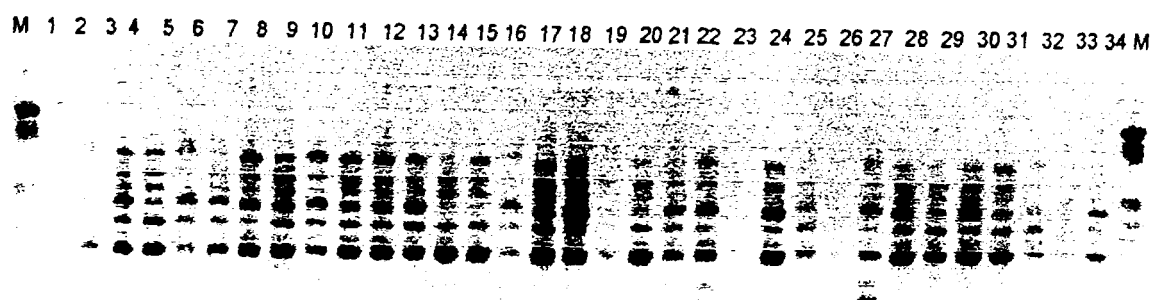


Amplification profile of two populations of *S. kathalekanensis* with the Primer p3.

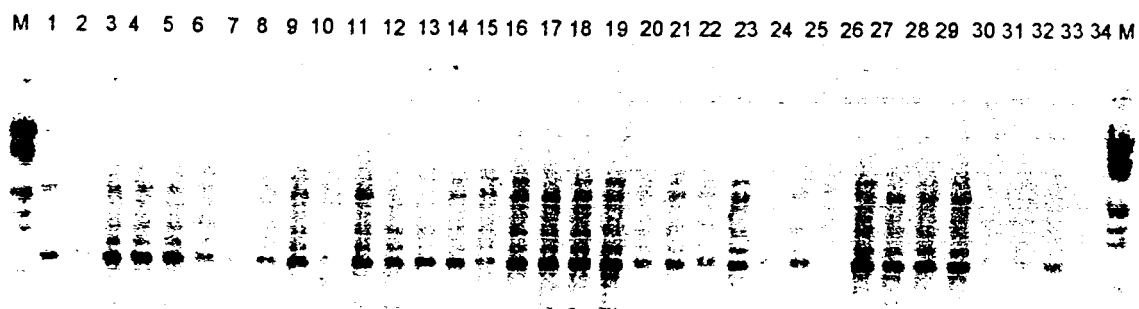
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 M



Amplification profile of two populations of *S. kathalekanensis* with the Primer KITx13.

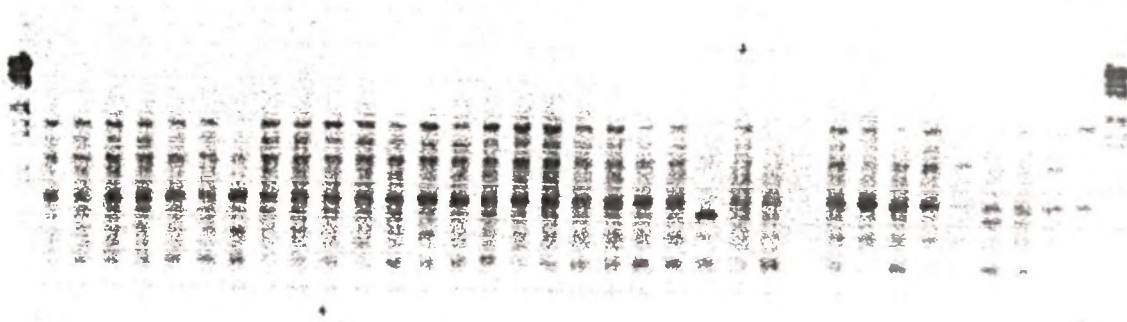


Amplification profile of two populations of *S. kathalekanensis* with the Primer KITx17.



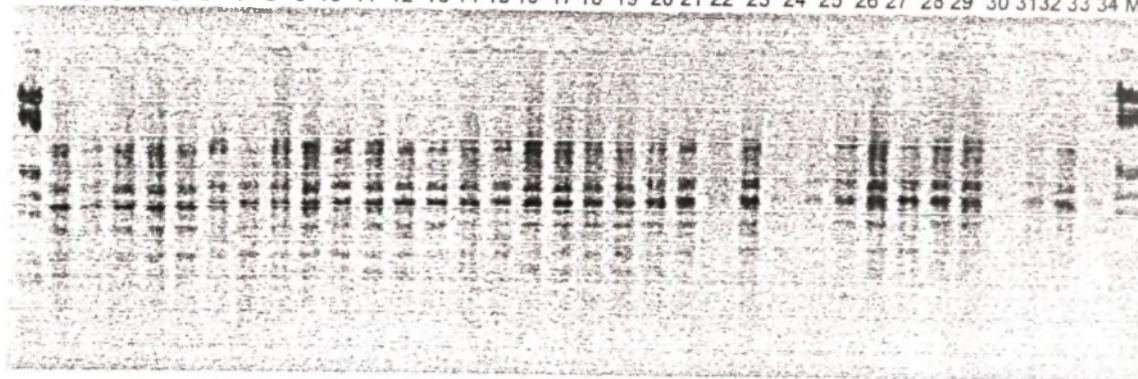
Amplification profile of two populations of *S. kathalekanensis* with the Primer KITx15.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 M



Amplification profile of two populations of *S. kathalekanensis* with the Primer KITx14.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 M



Amplification profile of two populations of *S. kathalekanensis* with the Primer KITx12.

Genetic structure of *Semecarpus kathalekanensis*: a critically endangered tree species of central Western Ghats

SURAJ P.G.

2001

Dr. R. VASUDEVA
Major Advisor

ABSTRACT Th-7014

Genetic structure of *Semecarpus kathalekanensis*, a critically endangered, dioecious medicinal tree species of the *Myristica* swamps of the central Western Ghats was studied adopting isozyme and PCR based Randomly Amplified Polymorphic DNA (RAPD) techniques. Leaf samples from 34 adult individuals of two neighboring populations were subjected to starch-gel electrophoresis after standardizing the protocol for six isozymes. Only four isozyme loci out of 9 identified were polymorphic in both the populations. The overall-mean allele number per loci was 1.44. The Shannon's information index based on all loci was 0.204 ± 0.27 ; it was 0.55 ± 0.11 based on polymorphic loci. The observed mean heterozygosity (H_o) of the populations was extremely low at 0.095 compared to the expected heterozygosity (H_e) based on Hardy-Weinberg equilibrium (0.134). This strongly suggests that the populations are suffering from heterozygote deficiency, which could be attributed to severe genetic bottlenecks and genetic drift. Rare-relic nature and severe anthropogenic pressures faced by the species might be responsible for genetic drift, while, lack of mating partners due to incomplete blooming synchrony among male and female trees could result in inbreeding. Interestingly, bigger matured individuals with GBH (>100 cm) had higher H_o (11.11%) than those which had less than 100 cm GBH (6.67%) indicating that younger generations might possess reduced genetic variation, which calls for an urgent recovery plan.

The genomic DNA were initially subjected to PCR based RAPD using 65 primers. A total of 76 loci were identified based on 10 random primer data. The polymorphism for RAPD ranged from 71.05 to 61.84 per cent and a diversity index of 0.627. The level of genetic differentiation among two populations was very low as suggested by mean F_{st} value ($<1\%$). This study, perhaps the first assessment of genetic diversity of a critically endangered tree species in India, strongly suggests that understanding genetic structure of rare/endangered species is highly essential in formulating conservation plans.