APPLICATION OF BIOTECHNOLOGY IN THE CONSERVATION OF ENDANGERED PLANT SPECIES FOR GENETIC ENHANCEMENT

(BT/TF/19/02/91)

FINAL REPORT

Submitted to
Department of Biotechnology
Govt. of India

M.S. Swaminathan Research Foundation
III Cross Street, Institutional Area,
Taramani, Chennai - 600 113

PROJECT INFRASTRUCTURE & APPROVED OBJECTIVES

Title of the project	:	Application of biotechnology in the conservation of endangered plant species for genetic enhancement
Date of sanction	:	February 1992 (BT/TF/19/02/91)
Date of Implementation	:	March 1992
Duration of the project	:	5 years
Total approved cost	:	99.00 lakhs

Approved objectives

- Collection, propagation, field evaluation of endangered species listed in the Red Data Books of the Botanical survey of India, RFLP Studies of mangroves, generic DNA library.
- Probes labelled and tested for polymorphism.
- Non-Radioactive detection methods including chemifluorescence.
- PCR, RAPD - in genetic diversity measurement.
- Preparation of training manual.
- Bioindicator studies - ex situ or lab conditions.
- Green House and laboratory evaluation of species for their sensitivity to organochlorines, heavy metals, trace elements, So2, fluroides.
- Titre levels of pollutants in eliciting a response.
- Bioindicator in relation to forest disturbance, water and air pollution and land quality.

Project Personel

Principal Coordinator	Prof. M. S. Swaminathan
Principal Scientific Officer	Dr. R.J. Ranjit Daniels
Sr. Scientific Officer Gr - I	Dr. Suresh Mathews* Dr. Ajay Kumar Parida Dr. C.S. Anuratha
Sr. Scientific Officer Gr - II	Dr. M.S.S. Mohan Dr. Sudha Nair Dr. P. Balakrishna* Dr. Hemal Kanvinde* Dr. H.D. Subhasini Dr. Nivedita Ram
Sr. Research Fellow	Mr. S. Elango Mr. K.V. Ramesh* Mr. N. Mathan Mr. Vishwanath Patil* Ms. M. Lakshmi
Technical Assistant	Mr. K.M. Kadirvelu
Laboratory Assistant	Mr. Muralidharan* Mr. M.M. Saravanan Mr. Ravi Kannan
Office Attendant	Mr. Purushothaman

*** Resigned during the project period**

ACKNOWLEDGEMENTS

We are to the Department of Biotechnology, Ministry of Science and Technology, Govt. of India for financial support for undertaking the project. We are particularly thankful to the members of the Project Management Committee (Prof. T.N. Khoshoo, Prof. Kunthala Jayaraman, Dr. Joseph Thomas, Dr. S. L. Govindwar, Dr. J. R. Arora, Dr. P.K. Hajra), for their support and advice from time to time. During this project, we have received support and help from a number of individuals and also a number of organisations. We are in particular indebted to the following:

Chief Conservators of Forest, Tamil Nadu, Kerala, Andhra Pradesh and Orissa,
Director, Central Leather Research Institute, Chennai,
Director, SPIC Science Foundation, Chennai,
Director, Tropical Botanical Garden and Research Institute, Trivandrum,
Director, Forest Research Institute, Trichur, Kerala,
Director, Indian Institute of Technology, Chennai.

We also greatly appreciate the generous help and support from many of our staff members of the Foundation during the implementation of the project. Above all, we are deeply indebted to Dr. (Mrs.) Manju Sharma, Secretary, Department of Biotechnology, Govt. of India for her advice and encouragement throughout the duration of this project.

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ABOUT THE PROJECT

MEMORANDUM OF UNDERSTANDING

Memorandum of Understanding made at New Delhi through Department of Biotechnology, Ministry of Science and Technology, New Delhi (hereinafter referred to as DBT) which expression shall include its successor and its assignees on one part and centre for Research on sustainable Agrciultural and rural Development established by M.S.Swaminathan Research Foundation as a Society registered under the Tamil Nadu Societies registration Act, 1975 (hereinafter referred to as Institute) which expression shall include its respective heirs, administrators, executors, successors and assignees on the other part.

Whereas the Institute has requested for the funding of its project on APPLICATION OF BIOTECHNOLOGY IN THE CONSERVATION OF ENDANGERED PLANT SPECIES FOR GENETIC ENHANCEMENT at a total cost of Rs.246.15886 lakhs (Rupees Two crore forty-six lakhs fifteen thousand eight hundred eighty-six only) (Copy attached as Annexure-II) to achieve the following objectives.

1. This will include collection, propagation, field evaluation of endangered species listed in the Red Data Books of the Botanical survey of India, RFLP Studies of mangroves, generic DNA library.
2. Probes labelled and tested for polymorphism.
3. Non-Radioactive detection methods including chemifluorescence.
4. PCR, RAPD - in genetic diversity measurement.
5. Preparation of training manual.
6. Bioindicator studies - ex situ or lab conditions.
7. Green House and laboratory evaluation of species for their sensitivity to organochlorines, heavy metals, trace elements, So2, fluroides.
8. Titre levels of pollutants in eliciting a response.
9. Bioindicator in relation to forest disturbance, water and air pollution and land quality.

Whereas the DBT has agreed to provide, grant of Rs.91.37 lakhs (Rupees Nineteen lakhs and thirty-seven thousand only) including cost towards salaries of staff etc. for 5 (Five) years (as detailed in Annexure - II) for this project.

Now it is hereby agreed as follows :-

1. The project shall be implemented strictly in accordance with the programme outlined in the project document in Annexure-I and the terms and conditions mentioned in Annexure-III and no deviations shall be made except with the prior approval of DBT and in the event of failing to comply with the terms and conditions or committing breach of this M.O.U., the Institute will be liable to refund to the DBT the entire amount of grant with interest.
2. The Institute/Investigators wishing to publish any paper based on the research work done under this project shall acknowledge the financial support received from the DBT.
3. The know-how generated from the project shall be the property of Government of India and any receipt by way of sale of know-how, royalties etc. shall accrue to Government of India. The Government of India may, in its discretion, allow a portion of such receipts to be retained by the Institute.
4. The Institute shall utilise the grants-in-aid as expeditiously as possible for the purpose for which it has been sanctioned. No request of the Institute will ordinarily be considered for escalation of costs of equipments, works, consumables etc. The Institute3 shall be liable to refund the grants-in-aid along with due interest if substantial progress is not made in purchase of equipments within 6 months of the release of first instalment. Amount of interest earned on unutilised amount of grant shall be reported to DBT every six months so as to enable the Department to adjust it from next instalment. DBT shall have

the right to terminate/foreclose the project at any stage if convinced that the grant has not been properly utilised or appropriate progress has not been made.

5. The Institute shall submit a quarterly progress report on the implementation of the project or any other information concerning the project which may be called for by DBT from time to time. The Institute shall submit annual report and audited statement of amounts within 5 (six) months of the close of the financial year of the grantee organisation.
6. The Institute shall follow the purchase and stores procedure outlined in Annexure-IV.
7. The project Managing Committee shall be formed with the following composition (including two representatives of DBT).

(i) Dr. M.S. Swaminathan	-	Chairman
(ii) Prof. T.N. Khoshoo	-	Member
(iii) Mr. John Joseph Retd. Principal Chief Conservator of Forests, Tamil Nadu	-	Member
(iv) Prof. Kunthala Jayaraman Director, Centre for Biotechnology, Anna University, Madras.	-	Member
(v) Director, Botanical Survey of India, Calcutta	-	Member
(vi) DBT Representative (Technical)	-	Member
(vii) DBT Representative (Finance)	-	Member

The Managing Committee shall meet twice a year (in June and December). The functions of the committee shall include.

i) To evaluate the progress of the project and to suggest corrective measures, where considered necessary, with a view to ensuring successful and timely implementation of the project.
ii) To consider the Annual budget estimate and revised estimates of the project and make recommendation thereon to DBT.
iii) To consider and recommend proposals for incurring expenditure on account of major works and purchases, where expenditure exceeds Rs. One Lakh.
iv) To review progress of expenditure with reference to sanctions accorded by DBT.
v) Any other important matter concerning the project. Reports of the Managing committee shall be submitted to DBT within one month of the holding of its meetings.

8. All appointments of scientific and technical staff shall be based on the recommendations of the selection committees consisting of Principal Investigator, experts from DBT, other organisations and the Institute itself and headed by the Chairman of the M.S. Swaminathan Research Foundation.
9. Appointments to non-scientific and non-technical posts shall be made on the recommendations of the selection committees consisting of Principal Investigator and other staff members of the Institute and outside experts and headed by the Chairman of M.S. Swaminathan Research Foundation.
10. The duration of this agreement is for a period of five years from 1992.

IT WITNESS WHEREOF THE PARTIES HAVE SIGNED THIS MEMORANDUM ON

FOR DEPARTMENT OF BIOTECHNOLOGY
NEW DELHI.

FOR THE INSTITUTE

WITNESSES.

File No. BT/TF/19/02/91
Department of Biotechnology,
Block No.2, 7th & 8th Floor
CGO Complex, Lodhi Road,
New Delhi - 110 003.

The Pay & Accounts Officer,
Department of Science & Technology, New Delhi - 110016.

Subject : **Environmental Biotechnology (Biodiversity)** - Financial support for the project of "Application of Biotechnology in the Conservation of Endangered Plant species for Genetic Enhancement." Project Investigator : Dr. M.S. Swaminathan, Centre for Research on Sustainable Agricultural Development., M.S. Swaminathan Research Foundation, 14, II Main Road, Kottur Gardens, Kotturpuram, Madras.

Sir,

I am directed to convey the approval of the President for the financial support from the Department of Biotechnology, Ministry of Science & Technology at a **total cost of Rs. 91.37 Lakhs (RUPEES NINETY ONE LAKH AND THIRTY SEVEN THOUSAND ONLY)** including Salaries of approved staff as detailed in Annexure-I for a period of 5 years (February, 1992 to February, 1997) to Dr. M.S. Swaminathan, Centre for Research on Sustainable Agricultural Development., M.S.Swaminathan Research Foundation, 14 II Main Road, Kottur Gardens, Kotturpuram, Madras for the above mentioned project. The details of the budget break-up is given in Annexure-I.

2. The objectives of the project are :
    1. This will include collection, propagation, field evaluation of endangered species in Red Books in Botanical Survey of India, RFLP Studies of mangroves, generic DNA library.
    2. Probes labelled and tested for polymorphism.
    3. Non-Radioactive detection methods including chemiflurescence.
    4. PCR, RAPD - in genetic diversity measurement.
    5. Preparation of training manual.
    6. Bioindicator studies - ex situ or lab conditions.
    7. Green House and laboratory evaluation of species for their sensitivity to organochlorines, h.metals, trace elements, So2, fluorides.
    8. Titre levels of pollutants in eliciting a response.
    9. Bioindicator in relation to forest disturbance, water and air pollution and land quality.

3. I am also directed to convey the Sanction of the President to the payment of **Rs.20.30 Lakhs (RUPEES TWENTY LAKH AND THIRTY THOUSAND ONLY) as first instalment for the financial year 1991-92.** The details are given below (For detailed heads of Accounts please see Annexure-I) :-

Non-Recurring Grants	
1. Equipments	15.80
Total (NR)	Rs. 15.80 Lakhs
Recurring Grants	
1. Consumables 2. Staff Salaries 3. Travel & Training 4. Others	0.50 3.00 0.50 0.50
Total (R)	Rs. 4.50 Lakhs
Total (NR + R)	**Rs.20.30 lakhs**

4. Full details of the budget in respect of the salaries of the sanctioned staff, indicating pay scale and salary break-up, for each year of the approved project period should be sent by the Institution to DBT within one month of the issue of the sanction letter to enable us to regulate the release of grant for this purpose for the subsequent period.

5. Other terms and conditions governing the financial sanction and list of equipments are given at Annexure-III and Annexure-II respectively.

6. The expenditure involved is debitable to Demand No. '72' Department of Biotechnology, Major Head '3425' : B. Other Scientific Research B. 1 (1) (2) (3) Grants in aid for the New Projects and Research Proposals B. 1 (1) (2) (3) (10) - Misc. R&D Projects for the year 1991-92 (Plan).

7. The amount in question will be drawn by the Drawing and Disbursing officer, Department of Biotechnology from the Pay and Accounts officer, Department of Science & Technology and disbursed to The Director, M.S. Swaminathan Research Foundation, Kotturpuram, Madras 600 085 by Account Payee Demand Draft/Cheque.

8. This issue under the power delegated to this Department and with the concurrence of IFD vide their Dy. No. 2060/91 Dir(F) dt. 30/12/91.

Yours faithfully,
(Senior Scientific Officer-I)

Copy forwarded for information/necessary action to :-

1. The Principal Director of Audit (Scientific Departments), AGCR Building, New Delhi 110 002.
2. Cash Section DBT (2 copies)
3. IFD, DBT.
4. Accounts Section, DBT, New Delhi 110 016.
5. Sanction Folder.
6. Dr. M.S. Swaminathan, Centre for Research on Sustainable Agricultural Development., M.S. Swaminathan Research Foundation, 14, II Main Road, Kottur Gardens, Kotturpuram, Madras.
7. The Director, M.S. Swaminathan Research Foundation, Kotturpuram, Madras 600 085.

(Ira Bhattacharya)
Senior Scientific Officer-I

Report of the Review Committee 11:00 a.m. to 5.30 p.m. on 18 December, 1992

Meeting of the Management Committee of the Project entitled "Applications of Biotechnology in Conservation of Endangered Plant Species for Genetic Enhancement" held at Madras on December 18, 1992. The following attended the meeting held at the Centre for Research on sustainable Agricultural and Rural Development, Madras from 11:00 a.m. to 5.30 p.m. on 18 December, 1992

Dr. T.N.Khoshoo - Chairman
Dr. M.S.Swaminathan
Prof. Kunthala Jayaraman
Dr. U.M. Narayanan
Mr. John Joseph

Dr. Manju Sharma and Dr. B.D. Sharma could not attend the meeting on account of the suspension of flights by Indian Airlines. Shri. R. Rajamani, Secretary, Ministry of Environment and Forests kindly attended the afternoon presentations, as a special invitee.

The Committee members went round the laboratories to see the facilities created and equipment's already bought during the period under report (March - November, 1992). Apart from the above project, the committee also evaluated the progress made under another project of DBT entitled "Genetic Engineering and establishment of Genetic Resource Centre for Adaptation to Sea Level Rise" whose duration expires in April, 1993. Detailed presentations were made by Drs. S. Sanjay V. Deshmukh, N. Subramaniam, Balaji, Mohan, Balakrishna and Hemal Kanvinde. After in-depth discussions with the concerned scientists the following points emerged which are being submitted for the consideration and approval of DBT.

Recommendations :

1. Considering the excellent work done and practical and theoretical results obtained and the international interest created by the project, the Committee strongly recommends continuation of the project "Genetic Engineering and Establishment of Genetic Resource Centre for Adaptation to Sea Level Rise" during the VIII Plan period, i.e., upto March 31, 1997.

2. The Committee recommends that the components dealing with Molecular Biology, Micropropagation and Bioindicators be funded by DBT. This amount may be added to the larger scheme entitled. "Applications of Biotechnology in Conservation of endangered plant species for genetic enhancement". Such integration of the two projects would be helpful both scientifically and administratively.

3. It is recommended that the component dealing with conservation, cultivation, restoration and evaluation of Mangrove Genetic Resources may be forwarded to the Ministry of Environment and Forests for financial support.

4. Regarding the project "Application of Biotechnology in conservation of endangered plant species for genetic enhancement : the progress made during the last 10 months is indeed impressive. Work initiated on the extent and nature of genetic diversity and multiplication through tissue culture technique using cambium as the explant is indeed a novel approach. The future plans were discussed and suggestions made by members of the committee. Based on the indepth review, the following needs of this project indicated in the Report submitted to the Review Committee (copy enclosed) are recommended for approval.

Taking the advice of experts like Prof. D.L. Hawksworth of U.K. and others in the Marine Biology institutions in India, the work at M.S.Swaminathan Research Foundation hitherto concentrated only on lichens and benthic invertebrates. Lichens are presently universally accepted as Bioindicators. However, a study of laborious and expensive. Also, the organism *Perna viridia* (a bivalve) which is being presently studied is much harvested by humans all over the Indian coast and hence complicates interpretation of observed distribution/population patterns.

Against this background the need to look for other organisms especially faunal has been felt. Without deviating much from the original proposal and ongoing work, it is proposed to continue work on lichens and invertebrates. However, amongst invertebrates, the group has chosen ants and butterflies; the study of which is less expensive and less laborious. Further, to identify rough and ready organisms for practical manual it was felt essential that the group also includes some vertebrates such as fish (benthic), amphibians and birds. The justification is that these organisms have been used efficiently elsewhere. Fishes have local names - easy to communicate to lay-men. Amphibians are global concern presently and birds are recommended by the Bird Life International (ICBP).

As unicellular microorganisms are found in large numbers, they respond directly to the environment changes. Growth conditions will influence the expression of cellular functions including enzymes, affecting the levels and extent of activity. The structure of microbial community will alter rapidly as microbial populations respond to changes externally to maximise the chance of survival and to optimise their growth rate. In the natural environment even when microorganisms grow slowly, their generation time will still be within hours or days, which is rapid when compared with plants and animals. Thus the microorganisms with their morphological, physiological and genetic characteristics make a very good Early Warning System for pollution since they will signal changes which may still be reversed.

Based on an in-depth review, the following needs of this project indicated in the report submitted to the Review Committee (Appendix 1) are recommended for approval. An up-to-date statement of expenditure ending on 30th Nov.1993 for both the projects are enclosed (Appendix 2a & 2b). The complete list of staff as on date for both the projects are also enclosed (Appendix 3a and 3b).

(a) Last year (Dec. 1992), the Review Committee recommended grant of Rs.25.1 lakhs towards cost escalation of equipment's. This grant has not been released so far. However, the first batch of equipments has already been procured. Orders are being placed for the remaining equipments sanctioned under the project. The total cost escalation for all the 31 equipments sanctioned in the project now comes to Rs.21,87,013/-. The details of the same are given in Appendix 4 and 5. DBT may kindly sanction the same at the earlier.

(b) The Committee noted that a sum of Rs.16.23 lakhs sanctioned under Recurring Costs has also not been released so far. DBT is requested to do the same for efficient functioning of the project.

c) The equipments were recommended by the Review Committee in December 1992, but no sanction letter has been received from DBT so far.

(d) For efficient functioning of the project some additional equipments have been identified and are listed below. These are recommended for sanction by DBT.

(e) Tissue culture forms one of the major components in the conservation of endangered plant species. Only one research Assistant is currently employed for Tissue culture work in this project. This is inadequate. The Committee recommends that two additional posts of Research Assistant be created for Tissue Culture work.

(f) Due to the escalation of price of fine chemicals and enzymes etc. and also maintenance costs, the following additional amount is recommended for sanction. The Committee examined in depth the fiscal requirements and recommends a grant of Rs.37.00 lakhs (Rupees thirty seven lakhs only) towards recurring expenditure for the total tenure of the project.

The Committee visited the CD-ROM facility created at M.S. Swaminathan Research Foundation and recommends the subscription of DERWENT Biotech Abstracts on CD, enabling access to about 30,000 records every year on conservation and biodiversity. Online information retrieval includes costs of connect time on VSNL-based gateways.

(g) In view of increased field work, it has become imperative to have a Jeep (with driver) for collecting live material in the field and bringing the same expeditiously for cultivation in Green-house and culture in the laboratory. The Committee feels that this would enhance the efficiency of work of the project. An amount of Rs.6,50,000/- therefore, needs to be sanctioned towards the cost of the jeep (Rs.3.00 lakhs), maintenance and fuel (Rs.3.00 lakhs), and the salary of the driver (Rs.50,000) for the entire tenure of the project.

(h) A consolidated statement of accounts to be released by DBT is given below.

Amount to be released for 1993-94

	Amount in Rupees
Non-recurring (Balance of budget amount not released till Mar.94 :	66,000
Non-recurring (a) Cost escalation for equipment already procured	5,96,513
(b) Cost escalation for equipments ordered	15,90,500
(Table I & II)	21,87,013
Recurring amount not released as per budget till Mar.94 : (excluding salary)	7,00,000
Staff salaries as per budget till Mar.94 :	9,23,000
	38,76,013
Additional recurring grant requested for 93-94 :	12,00,000
	50,76,013

JOSEPH THOMAS
MEMBER

DR.J.R.ARORA
MEMBER

T.N.KHOSHOO
CHAIRMAN

Report of the Review Committee on Dec. 17 and Dec. 18, 1993

Meeting of the Management Committee of the Project entitled "Applications of Biotechnology in Conservation of Endangered Plant Species for Genetic Enhancement" and "Genetic Engineering and Establishment of Genetic Resources Centre for adaptation to Sea Level rise", constituted by the Department of Biotechnology met at the M.S. Swaminathan Research Foundation, Madras on Dec. 17 and Dec. 18, 1993.

The following attended the meeting :

Dr. T. N. Khoshoo	- Chairman
Dr. Joseph Thomas (SPIC Science Foundation)	- Member
Dr. Arora (DBT)	- Member

Dr. Manju Sharma could not attend the meeting on account of unavoidable reasons. The Committee also had detailed discussions with Dr. M.S.Swaminathan. Earlier the Committee members went around the laboratories to see the facilities created and equipment's bought during the period under report (March to November, 1993).

The duration of the project "Genetic Engineering and Establishment of Genetic Resource Centre for Adaptation to Sea Level Rise" was extended by DBT till April 1995. Detailed presentations were made by Dr. Ranjit Daniels, Dr. Suresh T. Mathews, Mr.P. Balakrishna, Dr. M.S.S. Mohan, Dr. P.C.Jose Kutty and Dr. Sanjay V.Deshmukh.

After in-depth discussions with the concerned scientists, the following points emerged which are being submitted for the consideration and approval of DBT.

Recommendations :

1. Some excellent work has been done during the period under report. This work is of practical and theoretical significance leading to the generation of considerable international interest in the projects. The Committee is fully satisfied with the progress of the work, not withstanding the time taken in shifting of the institute from rented buildings to its permanent premises.

2. Under the project " Genetic Engineering and Establishment of Genetic Resource Centre for Adaptation to Sea Level Rise", the work related to RFLP and RAPD of 4 species of *Rhizopora* from various localities in India has been completed. It has helped to understand the nature and extent of genetic diversity within and between the populations of species. This is an excellent piece of work which has been submitted by Mr.N.Subramanian for the award of Ph.D. degree to the University of Madras.

3. The Project entitled "Application of Biotechnology in the Conservation of Endangered Plant Species for Genetic Enhancement: has made impressive progress. All the positions sanctioned have been filled following proper recruitment procedures. Thirteen endangered plant species have been under investigation (page 3 of Appendix 1). Work on the extent and nature of genetic diversity and multiplication through Tissue Culture technique involving axiliary bud culture have been initiated. The future plan was discussed and suggestions were made by the members of Committee.

Furthermore, it was thought desirable that the programme on Bioindicators be widened as given below. This would not need any additional expenditure :

a) In account of the steep cost escalation the price of equipment's has indeed risen (Page 7 of the report). It is recommended that an additional sum of Rs.25.1 lakhs be made available to the Centre to enable the purchase of the equipment which are indeed critical for the progress of the work.

b) The additional equipment requested in page 8 of the report be also provided. These are essential for deriving optimum benefit from the extensive new laboratories being built for this Project and which will become available for use in April, 1993.

c) The budget proposed for 1992-93 may be made available immediately.

d) The budget for 1993-94 is recommended.

e) Scanning, Tunnelling Microscope/Scanning Electrochemical Microscope is recommended for Biological studies. The above instrument will provide real time vision on molecular reactions and biological activities. For example, DNA distortion or DNA contamination studies can be carried out without restoring to inference techniques.

f) More funds are to be allocated for the purchase of chemicals, enzymes etc., and also for the maintenance of buildings and instruments.

g) A clean room is recommended where the contamination of external origin can be avoided while carrying out sensitive and fragile experiments involving specimens susceptible to external influences. One of the rooms in the main building can be earmarked for the clean room.

h) The facilities available in laboratories in close proximity to the M.S.Swaminathan Research Foundation Laboratory can be made use off to avoid the delay in getting the equipment's as and when funds are available for carrying out special experiments. For example, NMR facilities existing at Central Leather Research Institute can be made use of.

i) Atomic Absorption Spectrophotometer with Electro thermal graphite furnace and zeeman background correction be purchased as AAS without the above facility may become obsolete soon.

j) The civil engineering work in the first floor, to house the laboratories, is completed. But now the infrastructural facilities like electrical connections, laboratory furniture, provision of clean rooms (0.3 u) are to be provided. These facilities are a must for the laboratory to become functional. The necessary funds for the above facilities are requested from DBT. Laboratory furniture, according to M/s. Labmate (Asia) Limited, Madras is likely to cost Rs.7,63,013 for the minimum furnishings. For the provision of clean rooms it was estimated that the cost will be around Rs.15 Lakhs. It would be helpful if DBT could kindly provide the necessary grants for the above set-up, which is an essential for the laboratory to become functional.

k) Consumables : Keeping in view of the costs of the consumables for the kind of work proposed, it is recommended to provide the necessary amount of Rs.200 lacs requested for the purchase of consumables for 1992-1993.

(T.N. Khoshoo)
Chairman

MINUTES OF THE FOURTH REVIEW MEETING OF THE PROJECT MANAGEMENT COMMITTEE HELD AT THE M.S.SWAMINATHAN RESEARCH FOUNDATION, MADRAS ON 16.2.95 BETWEEN 1130 A.M. AND 4.30 P.M.

Project Reviewed : Application of biotechnology in the conservation of endangered plant species for genetic enhancement (BT/II/19/02/91)

Management committee Members Present

Dr. Joseph Thomas (Chairman)
Dr. (Ms) Manju Sharma (Member)
Dr. M.S. Swaminathan (Member)
Mr. John Joseph IFS (Member)

Dr (Ms) Kunthala Jayaraman (Member) could not attend the meeting due to her proposed visit to USA. She however held detailed discussions on 2.9.95 with Drs Ajay Parida and P. Balakrishna of the MSSRF about the progress of work in the project. Dr (Ms) Kunthala Jayaraman expressed her satisfaction over the progress made and indicated that she will personally visit the Foundation as soon as she is back from USA.

Shri. S.B. Krishnan, JS and Financial Adviser to the Ministry of Science and Technology and Dr. P.K.Hazra, Director Botanical Survey of India, could not attend the meeting.

Review and the Committee's Recommendations :

Dr (Ms) Manju Sharma visited the laboratories and other facilities including the glass house, database centre and gene bank at the MSSRF between 11.30 am and 1.00 pm.

The Review Meeting started with an address of welcome by Dr. M.S. Swaminathan, Chairman of the MSSRF. Detailed presentations on the work completed till this time, the work proposed till the end of the project in 1997 and future plan of work till 2002 were made by Drs.R.J.Ranjit Daniels, Ajay Parida, Sudha Nair and P. Balakrishna between 2.00 and 3.00 pm.

Following the scientific presentation, Mr.C.K.Ramachandran (Manager Budget and Accounts, MSSRF), gave a detailed account of the immediate and future financial requirement (till the end of the project) before the Management Committee.

The Chairman, Dr. Joseph Thomas, Dr.(Ms) Manju Sharma and the Management Committee expressed their satisfaction and commented that extremely good work has been done during the project period. The Committee specifically recommended the following:

1) The project cost of Rs.25.00 lacs was due to the deficit incurred in meeting the cost escalation on equipments already purchased. This escalation cost, despite the recommendation of the previous Management Committees, has so far not been sanctioned. The result is that one of the most important of sanctioned equipments viz.. the ultracentrifuge has not been purchased till date. The Committee strongly urged that the ultracentrifuge be purchased without any further delay and that the Department of Biotechnology may consider the urgent release of the above amount.

2) That the travel grant per year to be raised to Rs.1.00 lac. This is being made in the light of the inadequacy of previous travel grants and that the project's travel needs were largely met with grants from other projects related to biodiversity. Travel requirements during the following years will be higher due to the proposed field trials and evaluation of the micropropagated plants.

3) The projected immediate requirement of Rs.10.00 lacs (as per the break up below) placed before the Committee by the Manager Budget and Accounts be released at the earliest. This comprises consumables Rs.4.15 lacs; salaries Rs.3.48 lacs; travel and training Rs.1.37 lacs; contigencies Rs.1.00 lacs.

4) An overhead cost of 15% of the total budget be provided to the MSSRF immediately as this was not originally provided in the project.

5) Considering the requirements and cost escalation of chemicals (especially imported) the originally sanctioned amount of Rs.0.75 lacs towards consumables per year is well below the actual expenditure. This may therefore be raised to Rs.4.5 lacs per year, so that the project objectives can be fully realised.

6) In addition to the ongoing work relating to bioindicators, emphasis be given to identifying organisms and tools by which the local school children can be educated. Also some periodical training programmes be conducted with the prior experience gained from this project the financial support for such training programmes may be separately provided by DBT.

7) The micropropagation work should concentrate on the mass multiplication of a few selected endangered plants for field trails and demonstration during the following years.

8) As a next phase of the above project, it was suggested that 'Genetic Enhancement Centre' be considered for the set up at the MSSRF for providing material for practical breeding with support of the DBT for a period of 5 years. It was recommended that a project formulation Committee under the Chairmanship of Dr. Joseph Thomas (SPIC Sci. Foundation) be set up. The other members of the committee will include Prof. Deepak Bastia (Duke University Medical Centre, North Carolina, USA). Prof. B.B.Chatoo (Dept. of Biotechnology, M S Baroda University), Dr. K K.Narayanan (SPIC Sci. Foundation, Madras) and Drs. Ajay Parida and P. Balakrishna of the MSSRF. Besides the expert Committee it is proposed that Dr. S.Jayaraj, Shri. John Joseph IFS and Dr.R.J.Ranjit Daniels be included as special invitees in the programme. The committee will meet during November-December 1995 to formulate the project.

The project Management Committee was closed at 4.30 pm with a vote of thanks to all the members, Chairman and other participants.

MINUTES OF THE FIFTH REVIEW MEETING OF THE PROJECT MANAGEMENT COMMITTEE HELD AT THE M.S.SWAMINATHAN RESEARCH FOUNDATION, MADRAS ON 29.8.96 BETWEEN 3.30 TO 5.30 P.M.

Project Reviewed : Application of biotechnology in the conservation of endangered plant species for genetic enhancement (BT//19/02/91)

Management committee Members Present

Prof. M.S. Swaminathan (Member)
Dr. Joseph Thomas (Chairman)
Dr. S.L. Govindwar (Member and Representative DBT)
Mr. John Joseph IFS (Member)

Prof. Kuntala Jayaraman (member) could not attend the afternoon meeting. However, she along with Dr. S.L.Govindwar visited the laboratories and held detailed discussions with the project personnel in the forenoon.

Dr. T.N. Khoshoo expressed his inability to attend the meeting. The Director, Botanical Survey of India did not attend the meeting.

Review and Committees recommendations :

The review meeting started with welcome by Prof. M.S.Swaminathan.

Dr. Ajay Parida gave a broad overview of the fiscal and technical aspects of the project. He particularly expressed appreciation to Mr. S.B. Krishnan, Joint Secretary and Financial Advisor, Dept. of Science and Technology and Dr. S.L. Govindwar, PSO, DBT for timely release of the approved funds from Department of Biotechnology.

Following this brief presentations were made by Drs. M.S.S.Mohan, C.S.Anuratha and P.Balakrishna, who highlighted on the work done in the areas of bioindicators, molecular biology and tissue culture during the last year.

The members expressed overall satisfaction for the work done in the project and commented that extremely good work has been carried out by the groups with respect to the approved objectives. The Committee suggested exploring possibility for establishing linkages with industries in areas of using potential beneficial microbial strains as well as the established tissue culture protocols for rare/endangered plant species with medicinal property. They also suggested intensification of work related to the salt tolerant studies in the mangroves.

The project comes to an end in February 1997. In the line of the recommendations of the Committee last year, two projects have been formulated and submitted to the Department of Biotechnology for financial support. Dr. Ajay Parida gave a detailed presentation on the project "Establishment of a Genetic Enhancement Centre : Application of Biotechnology in the Conservation and Genetic Improvement of Coastal Agro-biodiversity". He outlined the rationale, objectives and anticipated outcome of the proposed project. The Committee felt that the project is a timely one and also falls among the identified priority areas

of the Department of Biotechnology by both the working group for Ninth Plan and the Scientific Advisory Committee of DBT. The Committee strongly recommended this project for support from DBT.

The Committee suggested setting up of an Institutional Biosafety Committee for proposed recombinant DNA work to be taken up in the project. For this it was also suggested to write to the Department of Biotechnology for nominating a representative to serve on the Committee.

Dr. Sudha Nair presented a detailed account of the proposed project entitled "Establishment of a Center for Biological Monitoring for the Sustainable Management of Biodiversity : Monitoring Ecosystem Health Using Microbial Biodiversity" and highlighted the need, objective, workplan and expected output of the project. The proposed project involves both basic research and a training component. The Committee felt that the project is of immediate importance as there is a big lacunae in the areas of microbial diversity and recommended for support from the DBT. The project should help school children and *Panchayats* to monitor the health of the ecosystems and take timely corrective measures where needed.

The members felt that the proposed objectives for both the projects are extensive and hence in the further refinement of these projects, prioritisation of the work plan could be done. Such refinements could be done after receiving the comments of the referees to whom DBT will be sending the proposals.

The meeting ended at 5.30 pm.

INTRODUCTION

INTRODUCTION

Earth's biological diversity - including all organisms and species, their genetic variation as well as their complex assemblage of habitat and ecosystems - is the result of several thousand years of evolutionary change. Humankind shares with all other species a genetic heritage and numerous ecological linkages that form the context within which human societies have developed a complex set of values about biodiversity.

The diversity of species constitutes a natural patrimony and life support system. Tragically this life support system is under grave threat. Human activities have severely degraded biological diversity - primarily through the profound modification of its habitats and ecosystems. There is a general scientific agreement that if the current rate and the nature of disturbances continue, future generations will confront the most severe extinction episode in the history of life on earth. Not only does humankind stand to lose numerous unseen and unknown species that will provide the necessary elements to maintain human health and well being but humankind risks losing the ecological systems which provide the vital services that make life itself possible. The conservation and sustainable utilisation of biological diversity and prevention of intraspecific genetic erosion are essential to access the variability needed to achieve sustainable advances in biological productivity.

The loss of biodiversity, particularly in the tropical regions, is occurring at an alarming rate. The Indian region, encompassing two biogeographic realms and three major biomes including 10 ecogeographic regions is one of the 18 identified megacentres of biodiversity in the world. The estimated number of plants species in this region is about 45,000 of which one third are flowering plants. As many as 1,500 plant species are under various degrees of threat and find mention in the Red Data Books published by the Botanical Survey of India of which about 171 are from the Tamil Nadu region. Table 1 lists the rare/ endangered and threatened plant speces of the Tamil Nadu region.

The present project, therefore, aims at the conservation of endangered plant species using biotechnological tools. It has three major components:

- Identification, propagation and re-introduction of endangered plant species
- monitoring community / ecosystem health using micro / macro organisms as indicators
- Utilising molecular tools for genetic characterisation and as an effective aid for conservation

In addition, particular emphasis has been given to the most productive coastal mangrove ecosystems. Mangrove species occupying the tidal estuarine regions

are of immense importance in maintaining coastal ecological and human livelihood security. Though the area under mangroves in India constitutes 7% of that of the world, in terms of species composition, they represent 60% of the known mangrove species worldwide. Tables 2 and 3 lists the mangrove species found in Pichavaram and Bhitarakanika mangrove forests. These species possess valuable genetic material for developing novel genetic combinations for tolerance to sea water intrusion and anticipated climatic changes in the coastal regions in future.

Table 1. Name of 123 species listed as extinct, possibly extinct, endangered, vulnerable or rare which occur or are known to have occured in Tamil Nadu, India.

Species	Status in India	Distribution within Tamil Nadu
Acranthera grandiflora	Endangered	Tirunelveli
Actinodaphne bourneae	Endangered	Palani hills
Actinodaphne lanata	Endangered	Nilgiris
Actinodaphne lawsonni	Rare	Nilgiris
Amomum microstephanum	Rare	Anamalais
Anoectochilus rotundifolius	Endangered/Possibly extinct	High wavies
Antinostrophe serratifolia	Rare	Anamalais
Aponogeton appendiculatus	Indeterminate	Madras
Atuna travancorice	Indeterminate	Courtallum
Belosynapsis kewensis	Endangered	Tirunelveli/Kanyakumari
Bentinckia codapana	Rare	Tirunelveli
Bulbophyllum albidum	Rare	Nilgiris/Tirunelveli
Bulbophyllum acutiflorum	Rare	Nilgiris
Bulbophyllum Elegantulum	Vulnerable	Nilgiris
Bulbophyllum catiense	Vulnerable	Nilgiris
Bunium nothum	Possibly extinct	Nilgiris
Campanula alphonsii	Rare	Nilgiris/Palani hill
Capparis diversifolia	Rare	Tirunelveli
Capparis rusifera	Rare	Tirunelveli
Capparis rheedii	Rare	Tirunelveli
Capparis shevaroyensis	Vulnerable	Ramanathapuram
Carex christii	Indeterminate/Possibly extinct	Nilgiris
Carex pseudoaperta	Indeterminate	Nilgiris
Carex vicinalis	Indeterminate	Nilgiris
Cayratia pedeta	Rare	Nilgiris
Cayratia roxburghii	Vulnerable	Tirunelveli
Ceropegia barnesii	Endangered	Nilgiries
Ceropegia decaisneana	Rare	Anamalais/Nilgiris

Ceropegia fimbriifera	Vulnerable	
Ceropegia maculata	Endangered/Possibly extinct	Anamalais
Ceropegia metziana	Rare	Western Ghats
Ceropegia thwaitesii	Vulnerable	Kodaikanal
Ceropegia omissa	Endangered	Courtallum
Ceropegia spiralis	Vulnerable	Area not furnished
Ceropegia pusilla	Rare	Nilgiris
Chrysoglossum hallbergii	Intermediate & Insufficiently known	High Wavies
Cleoxme burmanni	Indeterminate	Ramanathapuram
Coelogyne mossiae	Vulnerable	Nilgiris/Palani hills
Commelina hirsuta	Rare	Nilgiris/Palani hills
Commelina tricolor	Vulnerable	Karadimalais
Commelina wightii	Vulnerable	Nilgiris/Palani hills
Corymborkis veratifolia	Rare	Nilgiris
Cotomeaster buxifolius	Vulnerable	Nilgiris/Palani hills
Crotalaria clavata	Endangered	Coimbatore/Madurai/Salem
Crotalaria digitata	Rare	Kolli/Palani hills
Crotalaria fysonii	Endangered	Palani hills
Crotalaria globosa	Rare	Nilgiris/Courtallum/Dindigul
Crotalaria kodaiensis	Endangered	Palani hills
Crotalaria longipes	Endangered	Kolli/Nilgiris
Crotalaria preiestleyoides	Rare	Anamalais/Nilgiris
Crotalaria peduncularis	Rare	Anamalais/Palani hills
Crotalaria rigida	Rare	Nagapattinam/Coimbatore/Tirunelveli
Crotalaria scabra	Rare	Coimbatore/Kanyakumari/Salem/Tirunelveli
Cyathea nilgiriensis	Endangered	Nilgiris
Decaschistia rufa	Endangered	Chengalpattu
Desmos viridiflorus	Endangered	Anamalais/Coimbatore
Dictyospermum ovalifolium	Rare	Western Ghats
Elaecarpus venustus	Vulnerable	Kanyakumari
Elephaglossum beddomei	Rare	Nilgiris/Anamalais
Elephaglossum nilgiricum	Endangered	Nilgiris
Elephaglossum stigmatolepis	Vulnerable	Nilgiris/Palani hills
Eragrostis rottleri	Presumed extinct	Tranquebar
Eria albiflora	Rare	Nilgiris
Eriochysis rangacharii	Presumed extinct	Nilgiris
Eugenia discifera	Endangered	Sethur hills
Eugenia singampattiana	Endangered/Possibly extinct	Tirunelveli
Euonynus angulatus	Endangered	Nilgiris

Euonynus serratifolius	Endangered/Possibly extinct	Anamalais/Nilgiris
Goniothalamus rhynchantherus	Rare	Tirunelveli
Habenaria barnesii	Rare	Nilgiris
Hedyotis albonervia	Endangered	Tirunvelvi
Hedyotis barberi	Vulnerable	Area not furnished
Hedyotis buxifolia	Rare	-----
Hedyotis cyanantha	Rare	-----
Hedyotis eualata	Rare	----
Hedyotis ramarowii	Vulnerable	----
Hedyotis swersioides	Rare	----
Hedyotis hirsutissima	Possibly extinct	Nilgiris
Helichrysum perianigerum	Rare	Coimbatore
Humboldtia decurrens	Rare	Tirunelveli
Humboldtia bourdilloni	Endangered	Courtallum
Humboldtia unijuga	Endangered	Tirunelveli
Hydrocotyl conferata	Rare	Nilgiris/Palani hills
Impatiens neo-barnesii	Endangered	Nilgiris
Indigofera barberi	Rare	S. Arcot/Shevroy hills
Indotristicha tirunelveliana	Rare & Vulnerable	Tirunelveli
Liparis biloba	Vulnerable	Nilgiris
Miliusa nilagirica	Vulnerable	Nilgiris/Anamalais
Murdannia juncoides	Rare	Courtallum
Murdannia lanceolata	Vulnerable	Red hills
Nueracanthus neesianus	Endangered/Possibly extinct	North Arcot
Ophiorrhiza brunosis	Presumed extinct	Nilgiris/Palani hills
Ophiorrhiza pykarensis	Possibly extinct	Nilgiris
Orophea uniflora	Rare	Tirunelveli
Palaquium bourdillonii	Extinct/ Indeterminate	Tirunelveli
Paphiopedilum druryi	endangered/Possibly extinct	
Peucedanum anamallayense	Indeterminate	Anamalais
Pimpinella pulneyensis	Possibly extinct	Palani hills
Piper barberi	Rare	Kanyakumari
Popowia beddomeana	Rare	Tirunelveli
Psychotri globisephala	Endangered	Courtallum
Rhynchosia velutina	Vulnerable	Tanjore/Tirunelveli/Kanyakumari
Salacia beddomei	Rare	Anamalais
Santapaua madurensis	Endangered	Madurai/Pudukottai/Tanjore
Senecio kundaices	Endangered	Nilgiris
Smilax wightii	Rare	Nilgiris
Sphaeropteris crinitu	Endangered	Nilgiris
Syzygium gambleanum	Endangered	Kanyakumari
Syzygium courtallense	Endangered	Courtallum

Teucrium plectanthoides	Vulnerable	Tirunelveli
Thottea barberi	Vulnerable	Tirunelveli
Vanasushava pedata	Rare	Anamalais/Palani hills
Vanda wightii	Possibly extinct	Nilgiris
Vanilla wightiana	Rare	Tirunelveli/Kanyakumari
Veronia pulneyensis	Endangered	Palani hills
Veronia recurva	Endangered/Possibly extinct	Anamalais
Wendlandia angustifolia	Presumed extinct	Tirunelveli
Willisia selengionoides	Rare	Anamalais
Youngia nilgiriensis	Endangered	Nilgiris

Table 3. List of mangrove and associate species of Pichavaram forest

Name of the species	
Mangroves	**Mangrove associates**
Acanthus ilicifolius	*Aeluropus lagopoides.*
Aegiceras corniculatum	*Arthrocnemum indicum*
Avicennia marina	*Clerodendrum inerme*
Bruguiera cylindrica	*Cyperus*
Ceriops decandra	*Derris heterophylla*
Ceriops tagal	*Salvadora persica*
Excoecaria agallocha	*Sesuvium portulacastrum*
Luminitzera racemosa	*Suaeda maritima*
Luminitzera littorea	*Suaeda monoica*
Rhizophora apiculata	*Salicornia brachiata*
Rhizophora mucronata	*Sonneratia apetala*
Rhizophora lamarckii	*Xylocarpus mekongensis*

The effective date of implementation of the project was March 1992. The project was being regularly monitored by the project management committee, the minutes of the mmetings are given in the previous section. For the sake of brevity, the work undertaken, and the results obtained are detailed under three sections

(a) Survey, collection and propagation
(b) Monitoring ecosystem health using bioindicators and
(c) Molecular tools as an aid to conservation of Mangrove Genetic Resources

keeping in view the approved objectives of the project. A few representative photographs and illustration highlighting the work done are annexed to this report. A list of papers presented, published/ accepted for publication is also appended.

Table 4. List of mangrove and associate species of Bhitarkanika

Acanthus ilicifolius
A. volubils
Acristichum aureum
Aegilati rotundifolia
Aglaia cucullata

Avicennia alba
A. marina
A. officinalis

Brownlowia tersa
Bruguiera cylindrica
B. gymnorhizza
B. parviflora
Caesalpinia bonduc
C. crista
Cerbera manghas
Ceriops decandra
C.tagal (Per.) Rob.
Clerodendrum
Cynometra iripa
Dalbergia spinosa

Derris scandens
Excoecaria agallocha
Fimbristylis ferruginia
Finlaysonia obovata
Heliotropium curassavicum
Heritiera fomes
H. littoralis
H. kanikkensis

Hibiscus tiliaceous
Instia bijuga.
Ipomoea tuba
Kandelia candel
Lumnitzera racemosa
Merope angulata
Mucunga gigantea.
Myriostachia wightiana
Nypa frutican
Phoenix paludosa
Porteresia coarctata
Rhizophora apiculata.

R. mucronata
R. Stylosa
Salacia trinoides
Salvadora persica
Sarcolobus carinatus

S. globosus
Salicornia brachiata
Sesuvium portulacastrum.
Suaeda maritima
S. nudiflora
S. monoica
Sonneratia apetala
S. alba
S. Caseolaris
S. giriffithii
Tamarix troupii
T. erichoides
T. dioica
Thespesia populnea
T. populneodes
Tylophora tenuis

SURVEY, COLLECTION AND PROPAGATION

II. Survey, Collection, Propagation and Field Evaluation of Endangered Plant Species

Conservation of rare plants provide a test of our ability to maintain the quality of an ecosystem. Many species occur however in near minimum viable population size and are vulnerable to demographic, genetic and environmental variation, as well as destruction of habitat. Their very rarity and fragility reduces the margin for errors in efforts to protect them. Many of them may be edaphic, endemic or otherwise naturally restricted in distribution. Ironically until recently endangered plants as a group have been under represented in scientific literature, further complicating their recovery (Falk and Holsinger 1991).

Integrated conservation methods should be based on calculated interweaving of multiple conservation methods like land acquisition, species re-introduction and ecological restoration besides several others. From the perspectives of conservation biology, insights into species ecology provide clues that can inform strategies to preserve them. The actual mechanisms of endemism and the relationship between endemic distribution and particular edaphic factors are still matters of controversy.

Several practices in the realm of biological management are important to conservation. The simplest of them is the propagation and release of plant material for the enhancement of a damaged but extant population. Enhancement project have the benefit of working with an existing population in its natural ecological setting. Introduction and reintroduction as necessarily tools in the management of rare species (Falk and Mcmahan 1988; Falk 1987). Both the methods involve the release of plant material onto a site not currently inhabited by the target species in order to establish a breeding population. The distinction between the two approaches are different. Reintroduction denotes the release of material onto a documented location for the taxon, while introduction involves placing the taxon in areas where it had never occurred before. Reintroduction is thus more conservative of the two options. Introduction of material onto a new site, on the other hand, must always be regarded as an empirical test of understanding of biology of the species. Likewise introduction by definition involve manipulation of the distribution of genetic variation among populations.

Thus there is a need for us to distinguish between management of existing communities and establishing new ones. However, the boundary between these two is academic. Off-site management is thus far the most proven method to conserve rare and endangered plant species. This can be considered under four classes : genepool maintenance, propagation, research and education.

About 171 plant species are reported to be in threatened or rare state in Tamil Nadu. The status report of Botanical Survey of India (Red Data Books) describes the distribution of these species but further details regarding the initiative to conserve them are very rare. Also the status as mentioned in the Red Data Books are supplemented by the data given by the International Union for the Conservation of Nature (IUCN) and by the Foundation for Revitalisation of Local Health Traditions (FRLHT) of Bangalore. Detailed studies were undertaken at the M S Swaminathan Research Foundation, Madras as a part of the implementation of the current project in surveying, collecting such rare plants that can be of use for medicinal and related purposes also. This gives value addition to the entire exercise of the objective to conserve the rare and threatened plants.

Field based data retarding their distribution, sites of distribution and status were collected and were already presented in the previous reports. Extensive studies revealed that some species described as rare in Red Data Books were common in Kerala like that of *Syzygium travancoricum*. The field surveys helped us to reorganise the status of the plants we are studying.

All the plants (Table 1) where micropropagation studies are undertaken were maintained in the Green House attached to the M S Swaminathan Research Foundation in Madras. During the previous years studies were aimed at identifying suitable plants for micropropagation, standardising the protocols for micropropagation. During the current year (1995-1996), re-evaluating the efficiency of such protocols, development of protocols for multiple shoot production, hardening methodologies for such plants micropropagated for field transfer, field transfer of the micropropagated plants, field evaluation of the plants transferred and confirming the genetic fidelity of the plants were undertaken. The following sections will explain the details.

Re-evaluating the protocols for micropropagation of endangered plants

Several species including those of *Rauwolfia micrantha, Rauwolfia tetraphylla, Piper longum, Piper barberi, Ceropegia jainii, Hydrocotyl conferta, Uraria picta, Syzygium travancoricum, Freria indica, Kampheria galanga* were reassed for their potential in both multiple shoot production and hardening. Although the protocols were developed during 1994 - 1995 for some of these plants the efficiency of such protocols were tested. After repeated evaluations the protocols were communicated to the Department of Biotechnology (DBT).

Standardised protocols for *Syzygium travancoricum, Piper barberi, Ceropegia jainii* were already included in the previous report submitted to DBT (1994-1995). Protocols for other species are included here.

Rauwolfia micrantha

For *in-vitro* propagation, nodal cuttings were used from the plants maintained in the green house. Initiation was done using MS medium with (2mg/l) BA and multiplication was achieved using MS medium with (2mg/l) BA and (0.05mg/l) Adenine sulphate. Using 0.5 strength MS with (1mg/l) IBA rooting was achieved. Plants that were rooted *in-vitro* were transferred for hardening in the controlled environmental chamber and subsequently into the mist chamber before transferring them into the field. Multiple shoot production was achieved at the rate of 5-7 shoots per single explant.

Rauwolfia tetraphylla

This species belongs to the vulnerable category. Shoot initiation was successfully developed using nodal segments (1.0 - 1.5 cm) on MS medium with BA (0.5 - 2.0 mg/l) or KN (0.5 -2.0 mg/l). After initiation the plants were transferred into shoot multiplication medium containing MS with BA, KN and also with BA, KN, Adenine sulphate or with BA, KN, Adenine sulphate and IAA at (0.05-2.0mg/l) levels. Root induction was achieved using half MS basal with 6% sucrose. Multiple shoots were in the order of 7-10 per shoot initial.

Piper longum

Nodal cuttings (1.0-1.5 cm) were used for shoot initiation on MS + BA (0.5-2.0mg/l) and KN (0.5mg/l). The initials were subcultured onto MS with (0.5mg/l) BA and (0.5mg/l) KN. Rooting was achieved using half MS basal medium. High frequency shoot multiplication and root induction were achieved. Multiple shoot formation at the rate for 15-19 were observed.

Piper barberi

Being an endangered species and a relative of Piper species P. barberi micropropagation was attempted. Nodal cuttings, apical and axillary meristems were used as explants in addition to leaf discs. Shoot initiation was observed in plants grown on MS + BA(1.0mg/l) + KN (0.5mg/l). Shoot multiplication was achieved using MS with (0.5mg/l) BA, (0.5mg/l) KN, (100mg/l) CH and (50mg/l) PVP. Root induction was possible using half MS with (5mg/l) BA and (0.5mg/l) KN. Root proliferation occurred when transferred to MS basal media. High frequency shoot multiplication and root induction were achieved in this species.

Ceropegia jainii

This is a rare plants collected from Goa. Successful micropropagation, multiple shoot production were achieved in this species using a shoot initiation medium containing MS and BAP (2mg/l). Multiplication and rooting were observed in MS with (0.5mg/l) BA and half with (1mg/l) respectively. Multiple shoot formation and *in vitro* tuberisation and *in vitro* flowering were observed. Multiple shoots at the rate of 6-8 per explant was achieved.

Uraria picta

This is a rare shrub used in Ayurvedic medical preparations. Both nodal segments and leaf discs were used as explants. Axiliary bud initiation was possible using MS (1962) medium with MS vitamins + KN (0.5mg/l), BA (0.1mg/l). Shoot multiplication was achieved using MS (1962) + MS vitamins + BA (0.25mg/l) + CM (20%). Rooting was observed under half MS medium. High rates of multiple shoot formation and rooting were observed.

Freria indica

This is a rare herbaceous plant where micropropagation was successfully carried out on a initiation medium of MS + BA (1.0mg/l) and rooting was achieved in half strength MS with 0.5mg/l IBA. We have successfully developed vegetative propagation methodology for this species which is being followed now.

Kampheria galanga

This herb is rare and is also an ornamental. Successful vegetative propagation using treatments with (0.5mg/l) KN and treatment with (0.2 mg/l) IBA gave encouraging results.

Hydrocotyl conferta

This is a herbaceous plant where successful micropropagation as well as vegetative propagation methodologies were proven to be successful. Micropropagation was achieved by using MS medium + BA (0.5 mg/l) for multiplication and rooted on about IBA (2.0 mg/l).

Syzigium travancoricum

Syzigium travancoricum is an endangered and endemic tree to Southern Western ghats. Mature nodal cuttings (binodal) and leaf were used as explant. Multiple shoot induction was observed on MS medium + BA (5.0mg/l) + AS (1.0 mg/l) + NAA (0.2 mg/l). Microshoots were transferred to 1/4 MS medium with low

cytokinins for elongation. The elongated shoots were rooted in 1/2 MS + 0.2 mg/l IBA.

Multiple shoot production

All the plants under investigation belong to the rare or threatened category. Hence there is an urgent need to multiply them in large numbers and arrange for transfer. Production of multiple shoots from single explants is a better way to achieve this objective. Hence we attempted to produce multiple shoots from all the species explants and were successful in achieving multiple shoot production.

Hardening of the micropropagated plants to facilitate field transfer

One of the essential prerequisites for field transfer of the micropropagated plants are that they need to be hardened before transfer so that they will be able to withstand the conditions in the field. Hardening is done in two phases. One in the controlled environmental chamber where 75% RH, 25°C temperature and 16/8 hrs photoperiod were maintained. The plants after rooting were transferred into small plastic pots (5 cms in diameter) with sterilised sand. About 1 0r 2 plants were potted and watered twice a day, once in the morning and once in the evening. The plants were maintained in the same way for about 3-5 weeks depending on the species. Once established well in this chamber they were then transferred to the mist chamber where there was no control over temperature, light but humidity is kept high with potting done in normal garden soil.

As we were planning field transfer immediately the plants were hardened for 4 - 7 weeks, again depending on the species under study and then were arranged for field transfer.

Field transfer of micropropagated endangered and rare plants

Field transfer of micropropagated plants was the immediate objective of this project followed by field evaluation of those plants. We have successfully transferred several species into the native habitat.

Species	Number of plants transferred
Ceropegia jainii	485
Piper Barberi	3560
Piper longum	2650
Freria indica	575
Kampheria galanga	785
Rauwolfia tetraphylla	560
Uraria picta	678
Hydrocotyl conferta	276
Syzygium travancoricum	2780
Rauwolfia micrantha	278
Tylophora indica	3750
Crotalaria longipes	270
Dianella ensiflora	275

Several of the protocols developed were also new and reproducible. Our interest in field transfer in phases has been mainly due to the following factors.

1. Many species require particular periods in the field to establish. Transferring over a certain period of time spread over a few months made us standardise the appropriate time for successful establishment of field transferred material.
2. Survival rate was better monitored since the chances for mass scale mortality is reduced.
3. Appropriate changes in hardening and timings were standardised avoiding large scale failures of field establishments.

Even in the process of field transfer we did not adopt a random approach. The transfer was carried out in designed plots where two kinds of transfers were carried out.

They are

1. Randomised plot design and
2. Species plots

Establishment, mortality and growth

In plot 1 several species were introduced randomly. This is to check the rate of survival of different species in a given soil condition and a management situations. In the 2 plot the transfer was done with each species specifically

planted and in isolation. This is to check any possible clues of inter-specific competition.

From our results we did not come across any difference in both the plots, suggesting the success of our transfer. But we may need a longer incubation time of atleast 1-2 years before we can conclude the success of our strategy to ascertain such any competitions.

Competition from neighbouring plants is offers the greatest single hazard faced by colonizing plants and the density-dependant mortality is another factor. Physical conditions such as wind, temperature fluctuation, nutrient availability and moisture regimes often decide on percentage establishment, mortality and growth of reintroduced plants.

We therefore carried out a Randomized block design (Fig. 1) and Species plot design (Fig. 2) to assess the above parameters. Comparison were also made with glasshover experiments. From the field transferred material which were observed for a 10 month period for parameters like mentioned above we found that the randomised plot design was superior to species plot design. Since most of the species field transferred are annuals there is a need for us to look at their phenological and reproductive capacities and differences for atleast two season. This will help us with better strategy. Planning for our future introductions.

Assessing the genetic fidelity of the micropropagated plants

Micropropagation is known to produce some variability within the plants. This kind of somaclonal variability is to be studied for the species to arrive at some conclusions regarding the genetic diversity that had occurred in the culture and their subsequent presence in the material that has been transferred into the field. A group of about 20 plants belonging to various stages of growth starting from the parent material, plants in shooting stage, rooted plants, plants in mist chamber that are fully grown and the plants transferred into the field will be assessed using Random Amplified Polymorphic DNA (RAPD) markers. Initial screening studies in species like *P. longum* have shown no variability in the micropropagated plants while those of other species like *U. picta* have shown some variability when screened suing several PCR primers.

Genetic diversity analysis carried out indicated some interesting variants in the propagated material especially in species like *Piper longum* and *Piper barberi.* Such variants are being continuously monitored now. We are not concluding any results on this front at the present time.

Participation and Training

Towards the end of the project it was felt that a "Withdrawal strategy" will be very useful and a must to our activities of propagation and transfer of endangered plants. This was primarily to ensure continuity of activities of conservation. As a part of this several local people in Gudalur Forest Reserve Area were trained in propagation techniques.
Construction of a low-cost hardening chamber and vegetative propagation methods were achieved for this purpose. Species of *Ceropegia jainii, Freria indica, Kampheria galanga, Dianella ensiflora, Hydrocotyl conferta* were propagated by vegetative means using our newly developed protocols. Participation of local forest officials and local people in these efforts enabled our field transferred material to day in safe hands and activities of conservation to continue.

Conclusions

Conservation of biodiversity encompasses genetic diversity of species, populations, richness of species in biological communities, processes whereby species interest with one another and with physical attributes within ecological systems.

Conserving biodiversity involves restoring, protecting, conserving or enhancing the variety of life in an area. This not only helps normal ecological functions but also adaptations and extinctions.

List of publications

Patil V and Jayanthi M 1997. Micropropagation studies in two species of *Rauwolfia*. Curr. Sci. Vol. 12, pp 961-965.

P. Balakrishna 1995 Plant tissue culture and commercialisation in sustainable development. Abstract at Second International Plant Tissue Culture Conference, Dhaka, Bangladesh. December 1995.

M Jayanthi 1996 Somatic embryogenesis and genetic variation studies in regenerants of Tylophora indica (Burm. F.) Merrill. Proceedings to National symposium on Current Trends in Plant Biochemistry and Biotechnology. Hisar. February 1996.

M Jayanthi, Ajith Anand, C Srinivasa Rao, V M Patil and P. Balakrishna 1996 *In vitro* propagation of some medicinal plants and wild relatives native to Western ghats. Abstract for the National Symposium on Plant Tissue Culture. G B Pant University, Pantnagar.

Ajith Anand 1996. Marker aided studies for screening genetic stability in microrpropagated plants of Piper species. Abstract for presentation at the National Seminar on Spieces Biotechnology, BIOSAAP, Calicut.

Ajith Anand, Srinivasa Rao and Balakrishna P 1997. *In vitro* propagation of *Syzigium travancoricum* Gamble, an endangered tree species. (Plant Cell Tissue and Organ Culture - in revision)

Ajith Anand, Srinivasa Rao, Latha R and Balakrishna P 1997. Micropropagation of *Uraria picta*, a medicinal plant through axillary bud culture and callus regeneration. In vitro (Accepted).

Jayanthi. M. Saving an Endangered plant. A case study on *Crotolaria longipes*.B.Ph.d Thesis submitted to University of Madras. 1997.

Plot Design

Fig 1. Randomised Plot design

A	B	C	F	A	G	M	J	I	K
D	M	G	I	C	B	J	F	E	A
G	H	E	F	D	B	A	C	I	K
M	I	K	L	A	B	D	E	F	M
E	F	H	J	C	A	A	B	I	

Fig 2. Species Plots

A	A	A	A	A	A	A	A	A	A
B	B	B	B	B	B	B	B	B	B
C	C	C	C	C	C	C	C	C	C
D	D	D	D	D	D	D	D	D	D
E	E	E	E	E	E	E	E	E	E

Legends to Figures

A) M. [illegible] hoot induction in *Piper barberi*

B) Rooting of micropropagated shoots in *Piper barberi*

C) Multiple shoot production in *Piper longum*

D) Rooting of micropropagaated shoots in *Piper longum*

E) Rooting of micropropagated shoots in *Ceropegia jainii*

F) Rooting of stem cuttins in *Frereria indica*

[illegible] *Dipcadi ensiflora*

H) Rooting of micropropagated shoots in *Kaempheria galanga*

I) Various stages of *in vitro* culture in *Syzigium travancoricum*

J) Hardened plants of *Rauvolfia tetraphylla*

K) Hardened plants of various species in the mist propagation facility

L) Field transferred micropropagated plants of *Uraria picta* and *tylophora asthamatica*

M) Field transferred plants of *Piper longum*

N) Micropropagated plants of *Syzigium travancoricum* in the field nursery

O) Field transferred plants of *Piper barberi*

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. : *Tinospora cordiflora*

2. Habit : Climber

3. Distribution : Plains of Peninsular India

4. Status : Common, but underthreat of exploitation

5. Relatives : *T. crispa, T. sinensis*

6. Important of the sp. : Whole plant is of medicinal value, anti-tumour property reported.

7. Area of collection : TBGRI, Trivandrum.

8. Micropropagation : A spp. recalcitrant to *in vitro* manipulation

Explant used : Leaf and axillary bud

Media used : MS and WPM

Initiation	- MS + 0.125mg/l TDZ + 0.25mg/l BA
Elongation	- WPM + 2 BAmg/l + 0.1 IBAmg/l
Rooting	- 1/2 MS + 0.25 IBAmg/l
Callus	- MS + 2.2-4,Dmg/l + 0.25 BAmg/l

Results achieved. : Shooting and rooting, no somatic embryogenesis and regeneration from callus

9. Status of Protocol : Reproducible.

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. : *Holostema ada-kodien*

2. Habit : Climber

3. Distribution : Plains of India

4. Status : Common

5. Important of the sp. : Medicinal value, Roots used for opthalmia, diabetes and eye treatment.

6. Area of collection : Western Ghats

7. Micropropagation :

Explant used : Nodal cuttings

Media used : MS Basal

a) Initiation : MS + 2 BAmg/l

b) Multiplication : MS + 1 BAmg/l + 0.1 IBAmg/l

c) Rooting : 1/2 MS Basal

Results achieved. : Multiple shoots and high percentage rooting

8. Status of Protocol : Reproducible

9. Remarks : Hairy root culture may be of significant use in multiplying root for pharmaceutical industry.

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. : *Kaempheria galanga*

2. Habit : Tuberous herb

3. Distribution : Plains of India

4. Status : Common

5. Relatives : *K. rotunda*

6. Important of the sp. : Medicinal, against cough, stimulant, carminative and diuretic.

7. Area of collection : KFRI, Peechi, Thrissur.

8. Micropropagation

Explant used : Rhizome

Media used : MS Basal

a) Initiation : MS + 1 BAmg/l

b) Multiplication : MS + 0.5 BAmg/l + 0.1mg/l Kn

c) Rooting : 1/2 MS Basal

Vegetative propagation through Rhizome

Results achieved. : Multiple shooting and rooting detected. Vegetative propagation may be of relevance for rural poor.

9. Status of Protocol : Reproducible

10. Remarks : The plant being of high medicinal value both vegetative and *in vitro* multiplication can be used for providing seedlings to economicaly backward people for cultivation

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. : *Aristolochia indica*

2. Habit : Climber

3. Distribution : Western Ghats and Plains

4. Status : Common

5. Important of the sp. : Medicinal value

6. Area of collection : Western Ghats

7. Micropropagation

Explant used : Axillary bud

Media used : MS Basal

a) Initiation : MS + 1 BAmg/l + 0.5 Knmg/l

b) Multiplication : MS + 0.5 BAmg/l + 0.1 Knmg/l

c) Rooting : 1/2 MS Basal + 0.2mg/lIBA

d) Multiplication rate : 10 ± 2 shoots on 3rd week.

Results achieved. : High multiplication and sucessful rooting and hardening.

8. Status of Protocol : Reproducible, Efficient.

9. Remarks : A plant of great relevance and pharmaceuticals industry. Studies related to isolation and purification of active principles is much needed.

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. : *Freria indica*

2. Habit : Shrub

3. Distribution : Endemic to Goa

4. Status : Rare

5. Area of collection : Goa

6.Micropropagation

Explant used : Shoot tip culture

Media used : MS Basal

a) Initiation : MS + 1 BAmg/l

b) Multiplication : 1-2 shoots per node.

c) Rooting : 1/2 MS + 0.1 IAAmg/l

Vegetative propagation : cuttings with pretreatment of 0.5 IAA about 5 to 10 minutes gave 100% response.

Results achieved. : Vegetative propagation is economically suited for multiplication.

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. : *Syzygium travancoricum*

2. Habit : Shola tree species

3. Distribution : Evergreen forest of Western Ghats

4. Status : Endangered

5. Relatives : *S. cumini, S. tamilnaduensis*

6. Area of collection : Gudalur

7. Micropropagation

Explant used : Shoot tip and leaf.

Media used : MS and WPM

a) Initiation : MS + 5 BA + 1 AS + 0.2 NAA

b) Elongation : 1/4 MS + 0.5 Casein Pentathonate

c) Rooting : 1/2 MS + 0.2 IBA

Results achieved. : Multiple shoot with an average of over 15 shoots per explant recorded. 100% roots with 75% rate of survivality in green house condition.

1. Name of the Plant sp. : *Syzygium cumini*

2. Habit : Tree

3. Distribution : Tropical areas.

4. Status : Common

5. Area of collection : Gudalur

6. Micropropagation

Explant used : Nodal segment

Media used : MS + WPM

a) Initiation : MS + 2mg/l BA+0.5mg/l AS+ 0.25mg/lNAA

b) Multiplication : MS + 1mg/l BA+ 0.5mg/l AS + 100 mg/l glutamine

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. : *Ceropegia jainii*

2. Habit :

3. Distribution : Endemic to Maharashtra.

4. Status : Rare

5. Relatives : *C. bulbosa*

6. Important of the sp. : Rare and habitat specific species

7. Area of collection : Goa

8. Micropropagation : Successful micropropagation by multiple shoot formation and somatic embryogenesis

Explant used : a) Nodal segments for multiple shoot formation
b) Shoot segments for somatic embryogenesis

Media used : Initiation - MS with 2mg/l of BA
Multiplication - MS with 0.5mg/l of BA
Rooting - 0.5 MS with 1mg/l of IBA

Results achieved. : Multiple shoot formation, Somatic embryogenesis, *in vitro* tuberisation and *in vitro* flowering.

9. Status of Protocol : Reproducible

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. : *Rauvolfia micrantha*

2. Habit : A large Shrub

3. Distribution : Nilgiris

4. Status : Rare

5. Relatives : *R. serpentina*

6. Important of the sp. : Alkaloids from the species is of medicinal importance

7. Area of collection : TBGRI, Trivandrum, Kerala

8. Micropropagation : Multiple shoot formation

Explant used : Nodal segments

Media used : Initiation - MS with 2mg/l of BA

Elongation - MS with 1mg/l of Ba and 0.05 mg/l of Adenine sulfate

Rooting - 0.5 MS with 1mg/l of IBA

Results achieved. : Multiple shoot formation and complete protocol for regeneration established. Plants are to be transferred into the field.

9. Status of Protocol : Reproducible.

10. Remarks : The plant is economically important due to the presence of alkalodis and is also a rare taxon.

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. : *Piper barberi*

2. Habit : Shrub

3. Distribution : South Western ghats

4. Status : Endangered

5. Relatives : *P. nigrum, P. atematum, P. bettle and P. longum*

6. Important of the sp. : Can be used for the improvement of cultivated Piper sp.

7. Area of collection : Shiruvani and adjoining areas of Western Ghat forests.

8. Micropropagation :

Explant used : Nodal cuttings (1.0-1.5 cm), axillary and apical meristem and leaf discs.

Media used :

Shoot Initiation - MS + BAP (1.0 mg/l) + KN (0.5 mg/l)

Shoot multiplication - MS with (0.5mg/l)BAP, (0.5mg/l)KN, (100mg/l) CH and (50 mg/l) PVP

Root induction - 0.5 MS + 0.5mg/lIIBA + 0.5mg/l kn

Results achieved. : High frequency shoot multiplication and root induction

9. Status of Protocol : Reproducible.

10. Remarks : A plant of high importance as it can be used to screen *Phytopthora* resistance in the cultivated *Piper* species.

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. : *Ceropegia bulbosa* var *lushii*

2. Habit : A fleshy twinning herb

3. Distribution : Through out India in dry localities and especially near the West coast

4. Status : Common

5. Relatives : *C. jainii,* a rare plant

6. Important of the sp. : The leaves and tubers are edible

7. Area of collection : Peechi

8. Micropropagation : Multiple shoot formation and somatic embryogenesis

Explant used : a) Nodal segments for axillary bud proliferation
b) Shoot segments for somatic embryogenesis

Media used :

Initiation - MS with 1mg/l of BA
Multiplication - MS with (0.5mg/l) of BA
Rooting - 0.5 MS with 6% Sucrose

Results achieved. : Multiple shoot formation and *in vitro* tuberisation

9. Status of Protocol : Reproducible.

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. : *Rauvolfia tetraphylla*

2. Habit : Shrub

3. Distribution : Found in most parts of India

4. Status : Vulnerable

5. Relatives : *R. serpentina*

6. Important of the sp. : Roots of this plant used as a substitute of R. *serpentina* Alkaloids like ajmaline, ajmalcine, reserpine, serpentine, tetraphyllicine etc. found

7. Area of collection : Western Ghat forests

8. Micropropagation :

Explant used : Nodal cuttings (1.0-1.5cm)

Media used : Shoot Initiation - MS + BAP (0.5-2.0 mg/l) or KN (0.5-2.0 mg/l)
Shoot multiplication - MS with BAP and KN alone and in combination with Adenine sulphate and also with IAA
Root induction - Half MS basal with 6% Sucrose

Results achieved. : Shoot Initiation media - MS + BAP (2 mg/l)
Shoot multiplication media - MS+BAP (0.5mg/l) + ADS (0.005mg/l)

9. Status of Protocol : Reproducible.

10. Remarks : Since it is a wild relative of Rauvolfia serpentian (which is threatened) steps should taken to exploit this plant and also to conserve it.

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. : *Uraria picta*

2. Habit : Shrub

3. Distribution : Along Himalaya and Southern Western ghats

4. Status : Rare

5. Relatives : -

6. Important of the sp. : Tribal medicine in Ayurvedic preparation

7. Area of collection : Coimbatore

8. Micropropagation :

Explant used : Nodal segments (1.0-1.5cm), Leaf discs

Media used :

Axillary bud Initiation - MS (1962)+MS Vitamins + Kn(0.5mg/l) + BA(1.0mg/l)

Shoot multiplication - MS (1962) + MS Vitamins + BA (0.25mg/l)+CM(20%)

Rooting - Half MS (1962) + MS Vitamins

Results achieved. : High rate of multiple shoot formation and rooting

9. Status of Protocol : Reproducible.

10. Remarks : The plant might be of economic importance due to its medicinal property. In light of no studies carried out on its metabolite composition, systematic approach is needed.

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. : *Piper longum*

2. Habit : Shrub

3. Distribution : Western ghats and Southern Peninsular India

4. Status : Vulnerable

5. Relatives : *P. nigrum, P. atematum, P. bettle and P. barberi*

6. Important of the sp. : A medicinal plant used for treatment of high fever, dysentry and cold. The fruits and roots are used as an important gradiant in indiginous medicine.

7. Area of collection : Shiruvani and adjoining areas of Western Ghat forests

8. Micropropagation :

Explant used : Nodal cuttings (1.0-1.5cm)

Media used :

Shoot Initiation	- MS + BAP (0.5-2.0 mg/l) + KN (0.5 mg/l)
Shoot multiplication	- MS with (0.5mg/l)BAP, (0.5mg/l)KN
Root induction	- Half MS basal

Results achieved. : High frequency shoot multiplication and root induction

9. Status of Protocol : Reproducible.

10. Remarks : RAPD analysis carried out in 20 randomly selected in vitro regenerated plants with nine primers.

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. : *Hemidesmus indicus*

2. Habit : Slender Shrub

3. Distribution : Upper Gangetic plain eastwards to Assam and throughout central and western and southern India

4. Status : Not threatened

5. Important of the sp. : Roots of this plant are medicinal. Used as a tonic alternative, demulcent, diaphoretic, diuretic and as blood purifier. Also used in nutritional disorders, syphilis, urinary disorders, rheumatism and skin ailments. Syrup from roots used as a flavouring agent.

6. Area of collection : Trivandrum

7. Micropropagation :

Explant used : Nodal cuttings (1.0-1.5cm)

Media used : Axillary bud Initiation - MS + BAP or KN (0.5-2mg/l)

Shoot multiplication - MS + BAP (0.5-2mg/l)

Rooting - Half MS + IBA (2mg/l)+6% sucrose

Results achieved. : Best Shoot induction media : MS + BAP (2mg/l)

Best Multiplication media : MS + BAP (0.5mg/l)

8. Status of Protocol : Reproducible.

9. Remarks : This plant, through not threatened is being exploited for its medicinal value and its's found listed in the priority list of FRLHT.

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. : *Myxopyrum serratulum*

2. Habit : A large climbing shrub

3. Distribution : Western ghats, Wynad, Anamalai and Travancore hills

4. Status : Endemic

5. Important of the sp. : Medicinal importance

6. Area of collection : TBGRI, Trivandrum, Kerala

7. Micropropagation : Axillary bud culture established, plants in green house ready for transfer to the field

Explant used : Nodal segment

Media used : Murashige and Skoog (1962) with 2mg/l of BA and roting was done on 0.5 MS with 6% Sucrose

Results achieved. : Axillary bud culture

8. Status of Protocol : Reproducible.

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. *Casearia rubescens*

2. Habit A large shrub

3. Distribution Western Ghats, in the forests of Coorg and hills of Travancore

4. Status Endemic

5. Relatives *C. esculenta* and *C.tomentosa*

6. Important of the sp. Fruits of C. tomentosa are used for making pickles.

7. Area of collection TBGRI, Trivandrum, Kerala

8. Micropropagation

Explant used : Nodal segments

Media used : Murashige and Skoog (1962)

a) Initiation - MS with 1mg/l BA + 0.1mg/l Kn

b) Multiplication - MS with 0.5 mg/l BA + 0.005 mg/l Adenine sulfate

c) Root induction - 0.5 MS with 0.5 mg/l IBA

Results achieved. : High rate of multiple shoot production and root induction

9. Status of Protocol Reproducible.

10. Remarks The relative species are of economic importance, it would be worthwhile to know if this species has similar properties.

Data Sheet For Plant Species Under in vitro Culture

1. Name of the Plant sp. : *Sesuvium portulacastrum*

2. Habit : Herb

3. Distribution : Restricted to mangrove areas

4. Status : Abundant in the mangrove areas

5. Important of the sp. : This is a salt accumulating type of plant which can withstand to high salinity levels and it is found in the areas which have rendered useless after salt production.
This species may be utlized for the reclamation of saline soils or soils which have become useless due to aquaculture practices for a long time as it can be grown both on the fresh water and high salinity conditions.

6. Area of collection : Pichavaram, Tamil Nadu

7. Micropropagation :

Explant used : Nodal cuttings (1.0 - 1.5cm)

Media used :

a) Shoot initiation and multiplication - Unique macro + MS micro and vitamins + 0.5 mg/l BA + 0.5 mg/l Kn + 100 mg/l proline

b) Rooting medium - Unique macro + MS micro and vitamins + 0.5 mg/l BA + 0.5

Results achieved. : Multiple shoot induction (5-6) and high frequency rooting (90%) was achieved using the above media.

8. Status of Protocol : Reproducible.

9. Remarks : Highly salt tolerant mangrove associate

Legends to Figures

A) Multiple shoot induction in *Piper barberi*

B) Rooting of micropropagated shoots in *Piper barberi*

C) Multiple shoot production in *Piper longum*

D) Rooting of micropropagaated shoots in *Piper longum*

E) Rooting of micropropagated shoots in *Ceropegia jainii*

F) Rooting of stem cuttins in *Frereria indica*

G) Multiple shoot production in *Dianella ensiflora*

H) Rooting of micropropagated shoots in *Kaempheria galanga*

I) Various stages of *in vitro* culture in *Syzigium travancoricum*

J) Hardened plants of *Rauvolfia tetraphylla*

K) Hardened plants of various species in the mist propagation facility

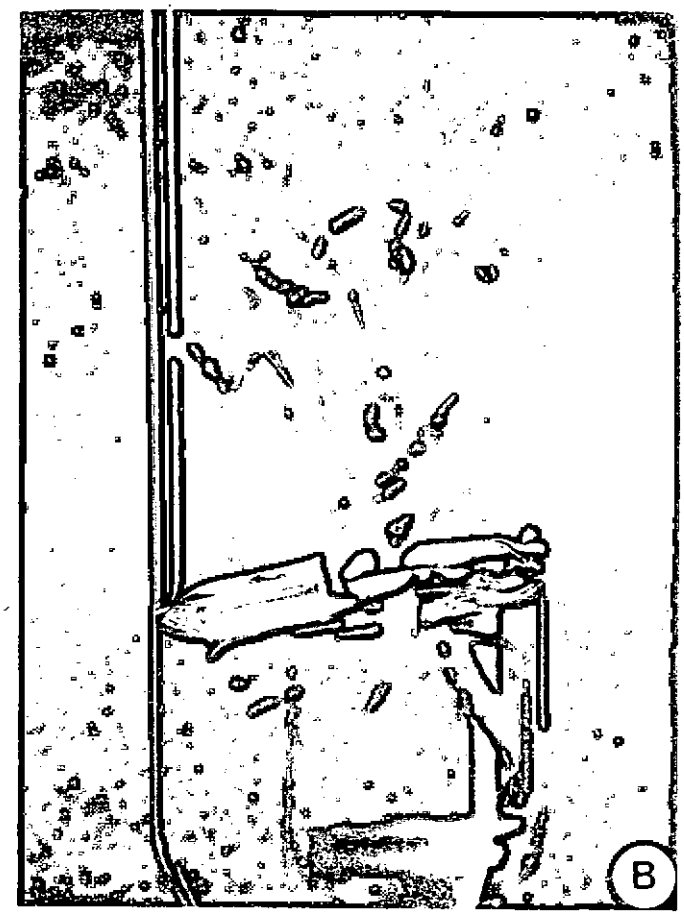
L) Field transferred micropropagated plants of *Uraria picta* and *tylophora asthamatica*

M) Field transferred plants of *Piper longum*

N) Micropropagated plants of *Syzigium travancoricum* in the field nursery

O) Field transferred plants of *Piper barberi*


A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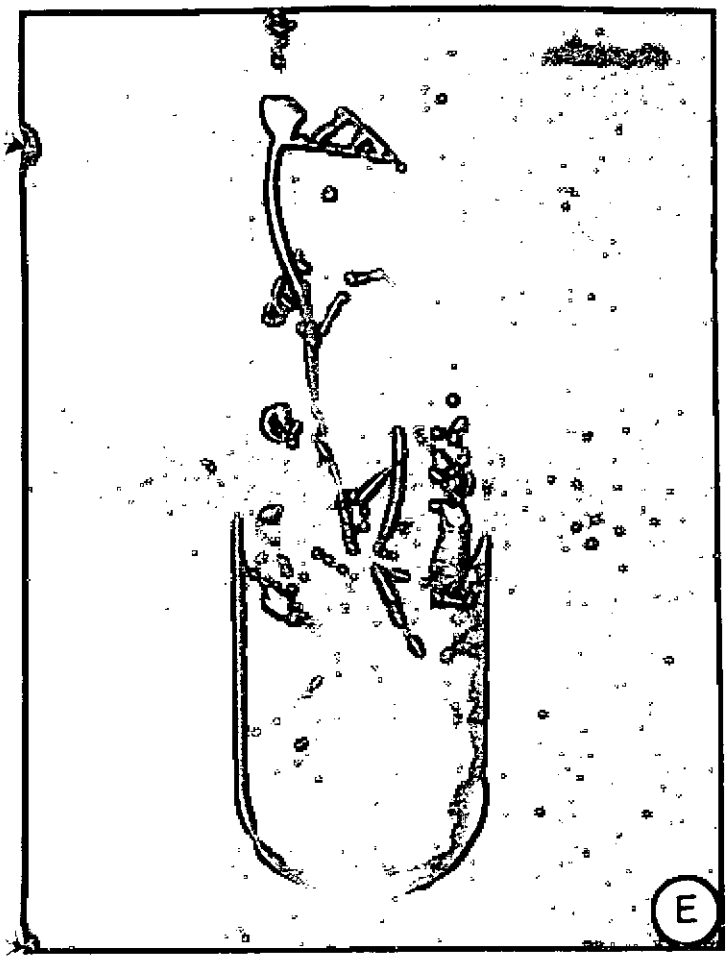
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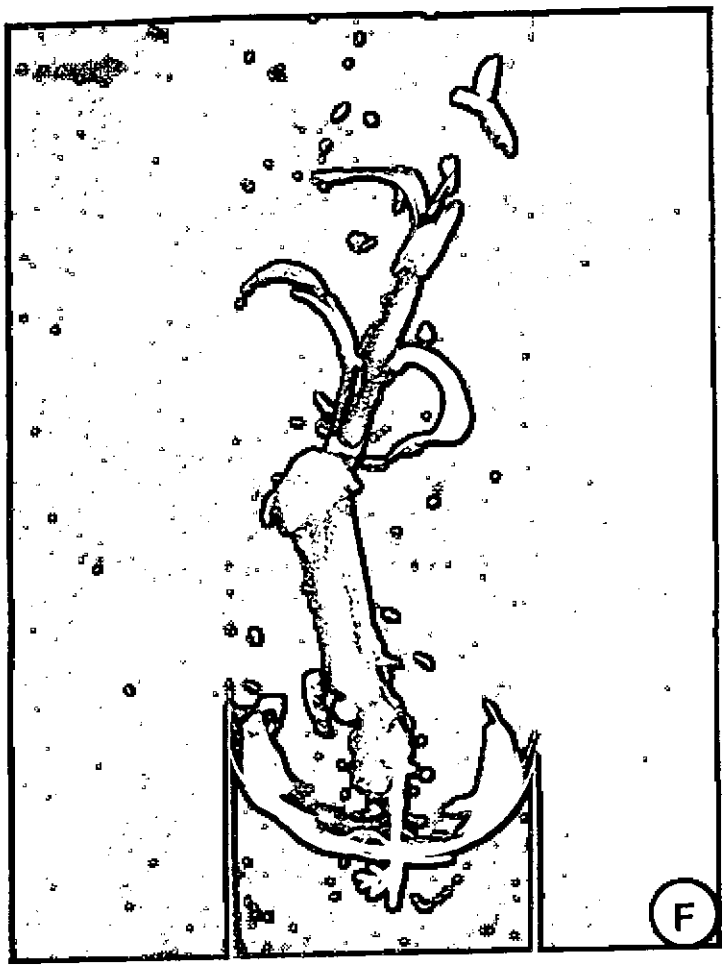
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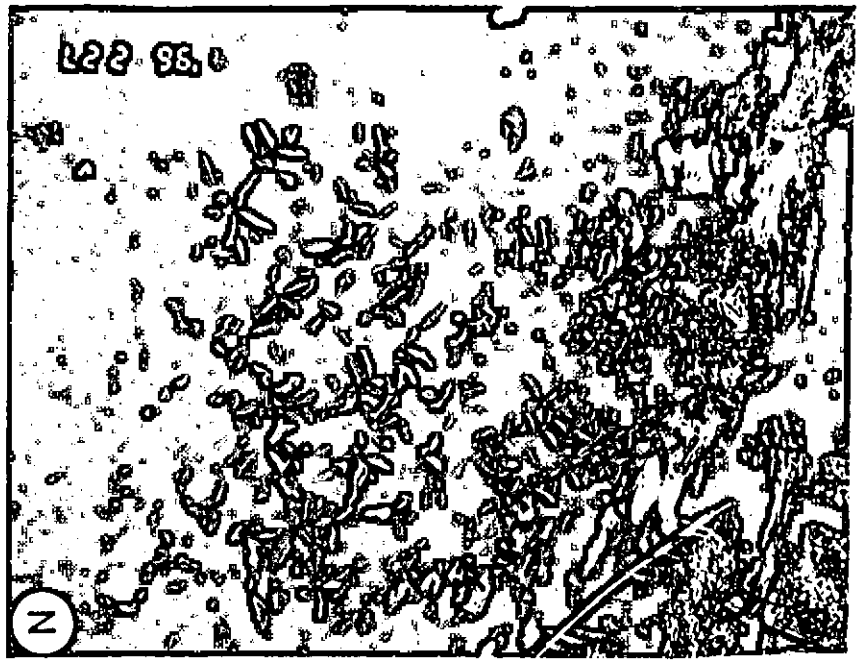
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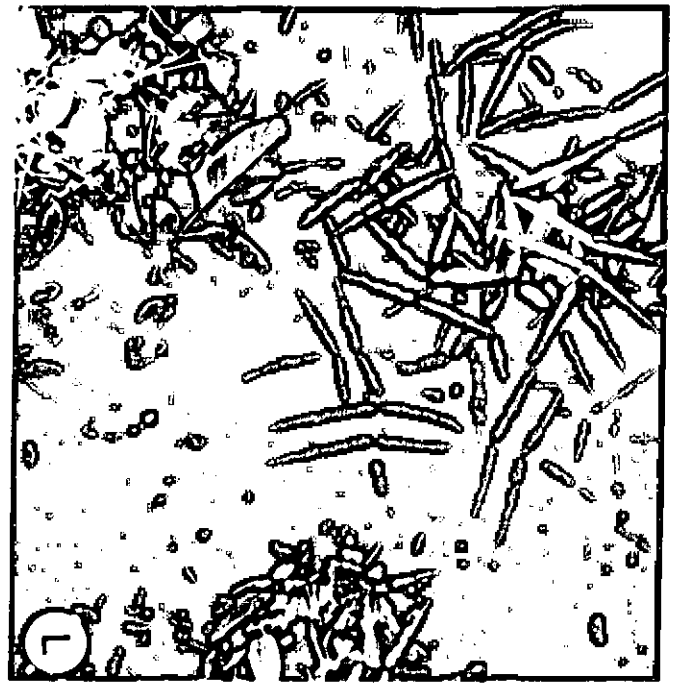
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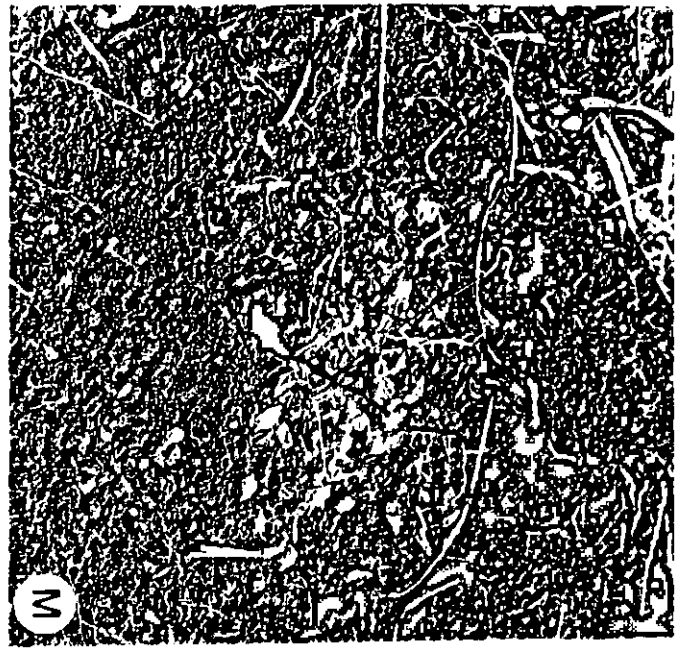
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MONITORING ECOSYSTEM HEALTH USING BIOINDICATORS

III. Monitoring Ecosystem Health Using Bioindicators

Introduction: The component on 'biomonitoring using bioindication technology' is complementary to the other two components, viz, 'micropropagation of endangered species' and 'use of molecular tools for measuring genetic diversity' which have been detailed in other two components. It should be realised that if conservation programmes are to be effective and placed on a secure scientific base, some ongoing and objective method of measuring this effectiveness is a prerequisite (Hawksworth, 1992), and since it is impractical to monitor the populations of all but microorganisms, it is necessary to identify particular organisms or groups of organisms that can serve as bioindicators of the health of the ecosystem under consideration. The complementary nature of the third component is thus obvious to the other components of the project.

Concept of Biological Monitoring: The environment is vastly bigger and more complicated than any man made machinery and the consequences of system failure are more disastrous, yet we commonly make major decisions about its management with scarcely and adequate indicators of its state, trends or response to our collective impact. The results, all too often, is unforeseen damage, diminished productivity, and a loss of what we now call 'sustainability' - *the ability to meet the needs of today without jeopardising the ability of future generations to meet their own needs.*

Monitoring is the process by which we keep the characteristics of the environment in view. It provides the essential data on how systems are changing and how fast. It provides the essential feed-back loops to management, so that we can adjust what we are doing and get the best out of the system. We need it, whether we are concerned to regulate pollution, manage fisheries, sustain soil fertility or look after nature reserves.

But what should be monitored, out of all bewildering complexity of nature? It is not possible to measure everything - choice is imperative. Very often that choice falls on physical attributes like temperature or chemical variables like the concentration of a key nutrient or of a significant pollutant. Such things are relatively easy to measure and the resulting Tables of data look impressive. However they tell us rather little about the response of ecosystems or species.

Biological monitoring starts at the end. Its logic rests on the fact that living organisms integrate the impact of many variables and that their biological efficiency, productivity or balance within the ecosystems they compose indicate the overall health of the system. Lichens growing on tree trunks are highly sensitive indicators of air pollution; the state of fresh waters can be judged from the faunas they support; the pattern of tropical vegetation can give eloquent expression to its history. And since we commonly manage the environment in order to sustain particular biological features, the direct surveillance of its

biological characteristics is likely to be the best way to establish whether the assumptions behind our management plans are valid.

There are no short cuts in the sphere of environment, and biological monitoring is not a miraculous cure at all. Its results can be as difficult to evaluate as any other scientific data about complex systems. But it is a tool we are using increasingly in today's world and has an important contribution to make in safeguarding tomorrow's environment.

Biological monitoring is important for several reasons as listed by Ian Spellerberg 1992.

- the world's living resources are being depleted all the time and impacts from developments are affecting the quality of those living resources. If sustainable development is to be a world objective then we need to monitor changes in those living resources as a basis for modelling strategies for sustainable development;

- biological and ecological monitoring has an important role in management of animals and plant (including microbes) populations for conservation. Without monitoring changes in the natural communities, of species, the effects of habitat loss, we have little on which to base good conservation practices;

- studies of land use and landscape change will come to rely more and more on good ecological monitoring techniques;

- the quality of our water, the air and the soil can be monitored through the use of indicator species and indicator communities, far more successfully than by chemical monitoring alone;

- there have been few long-term ecological studies and consequently very little known about natural long term processes of ecosystems. Biological monitoring programmes have an important role in our understanding of those processes and provide essential baseline data in studies of the effects of environmental impacts and

- biological monitoring has important applications in assessing methods of controlling pests and diseases of concern to agricultural and forestry.

Thus biological monitoring pertains to the use of biological variables starting from individual species, populations, community structure, ecosystem, physiological and biochemical aspects, ecosystem structure and dynamics such as food web, photosynthesis, behavioural physiology, nutrient cycling, etc. in monitoring the trend of the ecosystem changes. The conventional chemical and

physical method of monitoring alone are insufficient in clearly understanding the likely impact of the particular disturbance on the biological component and its function. Biological monitoring includes terminologies such as bioindicator, biomonitor, biosensor and bioprobe which are usually misconstrued with each other. Hawksworth (1992) has delineated the various terminologies that are encountered in biomonitoring and has given definitions of these. Organisms which are useful in monitoring the health of the ecosystem can fall under the categories **biomonitors, bioindicators** (early warning systems of changes) and **biosensors** (physiological changes).

To understand the extent of the damage of these disturbances may have on the biological components, which we strive to conserve, we need to study and understand the ecosystem structure, composition and dynamics through space and time. The data on the degree of disturbance or levels of pollutants alone may not reveal the subtle (but vital) changes that occur in the ecosystem structure or its processes. **The need for Biological monitoring studies is thus emphasised because, the systems that are affected are the biological systems ultimately.** Nevertheless, both chemical/physical methods and biological monitoring need to be integrated to understand the cause and effect and for any impact assessment studies. Many Western countries have initiated programmes on biological monitoring. Surprisingly, these studies hitherto are not popular in India and in other developing countries, some of which have world's richest flora and fauna.

Biological monitoring, thus requires, initially a knowledge of the ecosystem, its composition, (species, population) its structure (food web, population structure, biology, prey-predator relationship) and function (carbon fixation, energy flow, nutrient cycling etc.) In addition, the natural changes (deviation from the expected norm) that occur in the ecosystem structure and dynamics through space and time need to be understood.

Selection of Study areas for Biological monitoring: World Conservation Monitoring Centre (WCMC), UK has warned that unless urgent steps are undertaken biological diversity of immense economic and ecological value may be lost at several locations in India such as Western Ghats, Eastern Ghats and Western Himalayas. Botanical Survey of India (BSI) has also so far published three Red Data Books (1987, 1988, 1990). These 3 books contain a list of 814 threatened taxa of Indian flora. The Siruvani forests, in the Nilgiri Biosphere Reserve of Western Ghats, is relatively less disturbed, largely fall under the category of Southern Tropical Evergreen forest. These forests exhibit considerable diversity in structure and composition due to climatic, edaphic and altitudinal variations. Accessibility is limited in any of the hillocks due to steep slopes and thus areas remain more or less undisturbed (e.g. slopes of Vellingiri hills and Kunjiran hills). The following forest types are encountered in this region; Southern Tropical Evergreen forests, Southern Dry Mixed Deciduous,

Grasslands and Southern Thorn forest at the foot hills. This area has a major watershed which caters to the drinking water needs of Coimbatore City in addition to irrigation purposes of Palghat District. This forest also provides habitat for endangered animals like macaques and mountain squirrels in addition to various other animals and innumerable insect species.

In addition, mangrove ecosystem, which are unique inter tidal forests and considered as good as tropical rain forests in biological productivity is also taken up for this study. They have immense value in protecting coastal areas. But these ecosystems, in view of their conversion to arable lands or for various developmental activities are being doomed as waste lands, are considered endangered ecosystems or 'Hotspot' areas. The significance of conserving the mangroves of Pichavaram is mentioned elsewhere in this report.

Hence the study areas were primarily chosen from the mangrove ecosystem (Pichavaram) and the Nilgiri biosphere region of Western Ghats namely, Siruvani Hills.

Choice of organisms: There is no general rule as regards choice of organisms for monitoring or surveillance, monitoring has to be based on those organisms which are most likely to provide the most appropriate information for the questions being asked. Moreover monitoring the health of the ecosystem is a very general term in which the exact nature of the stress is not revealed.

Physical disturbance and change in environmental variables such as temperature or salinity will result in a change in the species composition of the biotic community. Such community changes can be used in monitoring current status of the environment and also in a predictive sense in relation to environmental assessments.

The EPA in the USA, UN Economic and Social council, the UNEP and other agencies have drawn up lists of indicators in the context of indicating environmental quality. The operation of these environmental indicator systems is, however, in its infancy. By way of contrast there has been much detailed research on the use of biological indicators for the detection of pollution and specific pollutants.

1) The stress for an ecosystem can be from physical clearing of the forest covers for developmental aspects.
2) The other stress can be from the various developmental (industrial or agricultural) activities.

The impact of the physical clearing can be best monitored using higher plants although micro-organisms can be of use in such monitoring programmes too. The other stresses in the form of toxic effluents or toxic gases can be monitored

by using the micro-organisms. Hawksworth (1992) has listed certain criteria for selection of the organisms useful as bioindicators. Although **microbes** in general, as the term suggests may not match all requirements to be indicators, there are **macro fungi, and lichens** which can be effectively used as biomonitors. Apart from studying individual microbial species or communities for bioindication, various **physiological and biochemical functions of these species/guilds** can also be used as **bioindicators** (biosensors).

As a whole, **micro-organisms certainly have a clear edge over other plants and animals to serve as early warning systems of changes (Bioindicators)** because of

- their ubiquitous distribution
- simpler thallus/cell structure
- shorter life cycle
- lack of specialised protective mechanisms
- high sensitivity to various disturbances
- important role in nutrient dynamics, energy flow and food web structure of all ecosystems, and
- their labile genetic make up which enables them to be exploited for the benefits of human race (bioremediation, bioaccumulation, detoxification) etc.

Review of Literature: Microbial indicators of soil health and sustainable productivity: Soil health and sustainability necessarily go hand in hand. The soil biomass that is present in a fertile soil roughly equals to the above ground biomass of plants supported by fertile soils in a pasture, or woodland or agro-ecosystem (Lee, 1991; Crossley *et al.*, 1992; Paoletti *et al.*, 1992). The possibility of using the biodiversity of soil organisms populations as a tool for monitoring soil health needs to be explored. Monitoring microbially mediated processes in soil as tools for bioindication of environmental impact is a developing concept (Paoletti *et al.*, 1991). Diverse microbiological studies have been attempted to assess deterioration or improvement in soil quality. Studies have been attempted at three levels: population level studies of the dynamics of species that are presumed to be important and or sensitive, community level studies of microbial community structure, such as species diversity and frequency of occurrence of species and ecosystem level studies of a range of soil processes (Visser and Parkinson, 1992). Total populations of bacteria and fungi in the soil are sensitive to and respond differently to soil management practices. Microbial biomass has been found to be a sensitive indicator of management induced changes in soil biological properties (Carter, 1986; Doran, 1988; Powlson et *al.*, 1987). Methods based on the physiology of micro-organisms such as respiration and enzyme activities may be a powerful tool for revealing small changes in the ecosystem (Ohtonen et *al., 1990;* Nannipieri et *al.*, 1994). Microbial diversity has also been used as indicators of agricultural ecosystems (Parkinson and Coleman, 1991). **Soil enzymes** are the biological catalysts of innumerable reactions in soils. Although some enzymes (e.g. dehydrogenase) are only found in viable cells

most soil enzymes can also exist as exoenzymes secreted by micro-organisms or as enzymes originating from microbial debris and plant residues that are stabilised in complexes of clay minerals and humic colloids (Ladd et *al.*, 1978; Dick, 1992). Since it is difficult to extract enzymes from soils, enzymes are studied indirectly by measuring their activity via assays done under in vitro conditions. It is difficult to relate activities in vitro to those occurring in situ. Nonetheless, soil enzyme activities provide insight into biochemical processes in soils and as early and sensitive indicators of management induced changes in soil fertility and stress. Oxido-reductases, transferases and hydrolyases have been the most studied as potential indicators of productivity, sustainability and pollution (Nannipieri et *al.*, 1994). Soil microbial biomass and soil enzymes have been used to measure direct and indirect microbial activity as an indicator of soil quality (Pnakhurst, 1994; Jordan, 1995; Turco, 1992) and indicators of disturbance of forests (Dhruva Kumar et al., 1992; Pohl and MalKomes, 1994; Kshattriya, 1991).

Cellulose decomposition rate, soil respiration rate, nitrification and activity of dehydrogenase (Zwolinaki, 1987) were found to be the most sensitive indicators of industrial pressure on the forest environment. Assessment of free living nitrogen fixation activity as a biological indicator of heavy metal toxicity in soil was done by Lorenz et *al.* (1992). Nitrification as an ecotoxicological indicator for agrochemical has also been attempted (Malkomes 1992).

Soils contaminated with heavy metals have been found to contain reduced levels of microbial biomass compared to soils receiving equivalent levels of nutrient from farm yard manure (Brookes and Mc Grath 1984; Chander and Brookes, 1991). An alternative strategy that can be used to detect the presence of heavy metals or pesticides residues in soil is to look for the development of microbial populations (bacteria and fungi) that are resistant to the contaminants (Summers, 1985; Redford, 1981; Silver and Mishra, !988). Nitrification has also been used as an indicator of heavy metal stress (Sharada and Sudha Nair, 1996; Mathan and Sudha Nair, 1996).

One of the major difficulties in developing biodiversity as an indicator *per se* is that only a limited number of soil organisms are well known taxonomically. An alternative approach could be to measure soil organisms biodiversity based on statistical analysis of taxonomic groupings of organisms randomly retrieved from soils or by genetic diversity analysis of DNA extracted from soils (Atlas et al., 1991). Using these approaches, they were able to show that the taxonomic and genetic diversities of microbial communities disturbed by chemical pollutants 245 T petroleum were lower in undisturbed reference communities. Functional diversity is also used as potential indicator (Zak, 1994).

High populations of beneficial soil organisms, soil organisms biodiversity, active microbial biomass, high levels of hydrolase and oxidoreductase enzymes are

among other good indicators of sustainable land use, while bioindicators of soil pollution include low microbial biomass, presence of microbial populations resistant to the toxicant.

Macrofungal Diversity as Monitors of Ecosystem Health: Macrofungi have all the characteristics to serve as early warning system of drastic changes in ecosystem health. Their position in the hierarchy of living organisms, as microbes, having most sensitivity to all kinds of stresses in addition to their conspicuous size enlist them as ideal candidates in biological monitoring. The following characteristics justify their selection/ candidature .

- Macrofungi are those which produce their reproductive structures (carpophores/fruit bodies) that are visible to naked eyes
- Their spores/mycelia are perennially present on litter /wood or sub-terraneousl only under conducive environmental conditions they form fruit bodies
- They are usually formed immediately after rain fall in tropical ecosystems. Within a year their production clearly indicates a successional pattern and some are also known to exhibit periodicity
- In tropical ecosystem the major roles can be diverse such as **litter decomposition** (a cumulative action with other microbes and microfauna) **wood decomposition-** white rot *(Fomes, Trametes)* and brown rot (Polyporus) **pathogenesis-bracket** fungi and **nutrient capture and mobilisation -** ectomycorrhizal fungi
- Some of them are edible and some are deadly poisonous, some have hallucinogenic effect and medicinal importance, hence many amateurs and local people show much interest in their study
- They have potential for varied biotechnological application
- Because of their integral association with nutrient dynamics and structure of the ecosystems their loss can sound death knell to any ecosystem. In this context they are considered ideal candidates for monitoring soil and ecosystem health, including forestry practices

Role of macrofungi in evolution and ecosystem function: The role of fungi in the evolution of land plants through mycorrhizal association has been emphasised by Hawksworth (1991). According to him, if rock breakdown involving fungi and establishment of mycorrhizas were crucial to the evolution of the land flora, without fungi there would be no lichens or bryophytes, no vascular plants, no dinosaurs to feed on them, and consequently no man. In spite of these facts, only scant attention has been accorded to the role of fungi in ecosystems.

Fungi are essential components of biocoenoses by their functions as decomposers of organic matter, parasites on other organisms and mutualistic symbionts in mycorrhizas. There are many wood rotting and litter decomposing fungi in an ecosystem with 'redundant' function. This redundancy imparts resilience in the

event of a loss of some species which acts as a buffer against any stress. In some cases fungi can be **'keystone species'**, the loss of which would lead to a major change in the ecosystems. The litter that accumulates on the forest floor must be degraded and these mineralization products must be effectively translocated and released to the macrophytes. Mycorrhizas play an important role in these processes and hence have carved a significant niche in the ecosystem. The reduction of mycorrhizal fungi may lead to an increasing instability of forest ecosystems, by increasing the water and nutrient stress in trees among other ill effects (Meyer, 1984).

Importance in biological monitoring: Fungi can be excellent bioindicators in view of their functions; niche differentiation and species diversity (Arnolds, 1991) especially, many species of ectomycorrhizal fungi are bioindicators of the degree and kind of air pollution (Fellner, 1988, 1989; Arnolds, 1990). A number of studies have been done on the response of ectomycorrhizas to air pollutants like SO_2 (acid rain), fluorides, ozone etc. (Ohtonen and Markkola, 1989; Joshi et al., 1991; Wallander and Nyland, 1992; Temmorshuizen, 1993). The number of fruit bodies may be a reliable index of number of mycorrhizas formed and that decrease in polluted region may be related to inability of mycorrhizal fungi to colonise (Schlechte, 1986; Termorshuizen and Schaffers, 1987; Jansen, 1991). Mycorrhizas can also be the indicators of many other pollutants like Cu, Pb, Cd, Zn and Ni (Gobl and Mutsch, 1985; Koomen and Mc Grath, 1990; Rhuling and Soderstrom, 1990). Apart from these, they can also act as the accumulators of heavy metals (Heyser and Donner, 1990). Accumulation of tannin from a pulp mill, in the cortical and pericycle cells, intracellular hyphae in the cortical cells of mycorrhizas has also been reported (Holopainen, 1990).

There is ample evidence that mycorrhizal fungi play a vital role in the nutrient dynamics of the heath land, forest and grass land ecosystems of the world. However, limited literature is available on biomonitoring aspects, hence their role as bioindicators of environmental changes has to be understood with reference to tropical, especially Asian conditions. A number of such studies have been successfully done in the Western Europe, but of the three types of mycorrhizas (ecto, endo and ericoid), the ectomycorrhizas will be most useful from the biomonitoring point of view, since they produce conspicuous fruit bodies generally above the ground.

Lichens as Biomonitors- Distribution and Ecosystem Function: Lichens are widely distributed from intertidal levels up to mountain peaks and from polar regions to the tropics because of their symbiotic nature. There are about 13,500 lichen species reported world wide and nearly 2000 species occur in India (Awasthi 1988, 1991). They form stable communities **notable for their** longevity and persistence. The lichen communities tend to remain metabolically active for a greater period of time, if the environment is left unchanged. Generally lichens are light-loving and very slow growing. They are adapted to survive in dry

habitats often withstanding the prolonged drought and extremes of temperature, and they tend to dominate habitats in which competition from other plants is minimal. Lichens are vital components of the ecosystems since they are an integral part in nutrient cycling and food webs. They fix enormous amount of carbon during the conversion of silicates to oxals during the weathering process of rocks by them. Huge amounts of carbon is locked up in the lichen thalli in the form of secondary metabolites. Lichens with cyanobacterial photobionts fix atmospheric nitrogen and enrich the nitrogen content in the ecosystem where they live. They are also responsible in the cycling of various minerals. Lichen colonies provide food and shelter to various micro invertebrates, thus becoming an integral part of the lower food web, and also used by various insects, birds, mammals including man for varied purposes. Thus lichens occupy a key position in various ecosystem functions.

The importance of lichens in biomonitoring: Lichens, unlike flowering plants possess no roots or other assimilation and vascular systems. They also lack a protective cuticle and an epidermis. Lichens are poikilohydrous in nature, which make them more vulnerable to the various environmental factors. The other major environmental factors like degree of illumination, humidity of the environment, age of the substratum, degree of corrugation of bark/rock which determine the moisture retaining capacity of the substratum, degree of rate of sloughing of bark, continuity and age of forest cover in a particular site, inclination of the surface, aspect and pH of the substratum determine the establishment of lichen communities. On the basis of various field observations on lichens, it is proved that, **within a single climatically uniform region each particular substrate has eventually a characteristic and often remarkably uniform lichen vegetation under the influence of similar environmental factors.** This phenomenon led to the formation of various lichen phytosociological federations (James et al. 1977). So each lichen federation is unique to a particular vegetation type with similar environmental factors. However a lichen federation tends to change if any one of the environmental factor is modified. So the changes in the lichen communities can be used as an indicator of ecological continuity/disturbance of the forest site studied. The above said characters of lichen communities allow, any data collection on lichens from different geographical zones all through the year irrespective of seasonal and climate changes. Many studies both ex *situ* and *in situ* have already successfully utilised lichens as their experimental samples(Hawksworth 1994). The conspicuous nature of lichens is also an added advantage for an easy identification in the field with a help of a 10 x hand lens. Hence lichens are widely used in Europe and in North America to indicate ecosystem health by the general public, school children and foresters.

Lichens as air pollution indicators: Lichens are widely used to assess air quality (esp. sulphur di oxide, fluoride, particulate matter, radioactive materials and other toxic substances). The first observation on lichens and air pollution started

at an early stage. In 1886, Nylander recorded the species of lichens present in the Jardins du Luxembourg in Paris. After thirty years when he re-examined the lichen flora of this area he found that all the lichens had disappeared. At that time Nylander attributed the loss of lichens was due to air pollution. This was proved accurate after a century that lichens were returning to colonise these trees following a fall in air pollution levels. The bioindicator value of lichens, demonstrated by Nylander created interest among researchers, to develop both qualitative and quantitative pollution assessment methodologies using lichens. So far around 1800 scientific papers are available in this subject along with 120 papers being catalogued each year (Hawksworth 1994).

Lichens as monitors of ecosystem continuity and disturbance: The lichen diversity and its distribution pattern are used as good indicators in assessing the ecological continuity and disturbance of forest habitat enabling interpretation of the site history and management. A number of lichen epiphytes are found in mature, primary forests with a long history of ecological continuity, possessing an unusually high number of faithful species especially belonging to the lichen families Lobariaceae, Pannariaceae, Collemataceae and Peltigeraceae (James et al. 1977). This phenomenon is widely used to assess ecological continuity in UK, continental Europe, North America, Tasmania and some countries in South East Asia (Rose 1976, 1988, Wolsley, 1991.).

On the other hand increased 'opportunistic' lichen diversity at a local or regional level often results as a consequence of habitat disturbance, fire-induced successional vegetation, for example is often a much richer habitat for lichens that have evolved climax vegetation (Galloway, 1992,) This phenomenon is also observed in various habitats like mangroves of Brazil, (Marcelli, 1991) and lower montane rain forests of Ecuador (Arvidsson, 1991).

Relevance to present study: These are some of the background justifications for selecting lichens, macro fungi and microbial (bacterial and fungal guilds) functions to monitor the health of the ecosystem. In spite of these facts, the major draw back one faces while selecting microbes for biological monitoring is the limited knowledge, literature and on going studies on these groups with reference to our ecosystem. "Studies on the microbes generally pertain to the biotechnological applications. A **basic inventory of the microbial diversity in the natural ecosystem is totally lacking in our country. It may be noted that this study using the microbial diversity for monitoring the ecosystem health is a pioneering work in our country.**

In this study stress has been given on the soil biological criteria (micorbial diversity and the microbially mediated processes monitored through functional guilds of both fungi and bacteria) and lichen distribution pattern in the Pichavaram mangrove ecosystem. While in the second study site at the Siruvani Hills in the Nilgiri Biosphere Reserve forests, stress was laid on lichen and

macromycetes occurrence and distribution pattern for monitoring studies. A preliminary analysis of the soil biological criteria in the lines carried out at Pichavaram was initiated to confirm the findings of that site. Consequently, we have initiated a fresh inventory of the areas under study for these groups. For the soil microbes with reference to bacteria both phenotypic and genotypic characters have been used to group them. Wherever identification was not possible the concept of OTU (Operational Taxonomic Units) has been employed for the convenience of sampling and monitoring procedures. In the case of macromycetes, their production is dependent on the total rainfall and its pattern and they have innate quality of successional pattern, seasonality and periodicity. Since a preliminary understanding of the trend in macrofungal fruiting is a prerequisite before formulating a sampling procedure, a seasonal survey has been done. The methodology, results and discussion of the work carried out is detailed in the following sections of the report.

Monitoring marine and soil health through microbial (including Lichens) community structure and function at the Mangrove Ecosystem of Pichavaram-Study site 1:

The mangrove ecosystems are very peculiar formations that develop best as far as structure and productivity go, along sheltered tropical low lying coastal, estuarine, deltaic areas where brackish water results from the mixing of sea water land runoff. Humidity should preferably be high and tidal fluctuations should be medium. All dead organic matter is quickly attacked by bacteria and fungi, although the tannins have an inhibitory effect on this process. These are the same bacteria and fungi that are active in the mineralisation part of the nutrient cycle. Complex associations of proteolytic and cellulolytic fungi and bacteria are associated in a complex manner. Iron and sulphur compounds are attacked by oxidising and reducing bacteria that occur every where in typical mangroves because of the ubiquitous presence of iron and sulphur derived from pyrites. In brief both fungi and bacteria are not only present every where, but they are abundant and varied lot in the mangroves each going tirelessly about its business of breaking down large molecules building up their own proteins and thus becoming and endless source of food for scavengers detritus feeders and flier feeders.

Thus the mangrove ecosystem offers a number of unique ecological niches having a strange combination of ecological factors yet to all of them, some species or other have become adapted, but extent of the genetic variation among strains is still anybody's guess. This is not, however, the only domain where the micro-organisms of the mangroves are poorly known. All aspects of fungi, bacteria, are vastly unknown. Even a mere list of species and their abundance is not available, equally poorly known are the causes behind the presence and

abundance of species in relation to environmental factors, in other words ecology of micro-organisms of the mangroves is still an open field.

Thus in this component of the project attention has been given to the microbial aspect of this system to know and understand the microbial flora (including the bacteria, fungi and the lichens). The methodologies have been worked out and optimised by us to suit the aim of the study. Modifications have been carried out in the culturing techniques, media used, sampling has been standardised as detailed out in the following sections.

Bacterial Guilds as tools to monitor ecosystem health: Among a variable pool of micro-organisms, bacteria and phyto plankton comprise the two largest microbial components of this ecosystem. About equal in biomass the bacteria contain about 95.5% of the bioactive surface area. The bacteria are considered critical to the estuarine environment as decomposers of organic matter, as transformers of organic substrates into inorganic compounds and as agents influencing the physico-chemical properties of these shallow coastal system. The heterotrophic forms are the largest group of bacteria occurring in the estuaries. Classical energy flow diagrams for estuaries depict heterotrophic bacteria as the principal processors of organic matter, responsible for the breakdown and mineralisation of a substantial fraction of this material. Especially detritus-based systems, such as mangroves, salt marshes and sea grasses, decomposition of organic material in sediments is essentially a microbially mediated process.

In this study the phenotypic diversity was compared with the genotypic diversity of the heterotrophic bacterial population occurring in the inter tidal region of the mangrove. A major purpose of the this study was to characterise the microbial communities, quantitatively and qualitatively as a first step to understand the diversity. The microbial diversity (both physiological and genetic) associated with the sites were utilised as biological indices of the environmental. The vegetation pattern in the three zones chosen presented an opportunity to examine selected properties of the established microflora. The functional guilds especially the nitrifiers, ammonifiers, denitirifers, phosphate soulbilisers, sulphur and iron bacteria were also monitored (alternative months) for a year to cover the entire seasonal variation.

Since the microbes primarily are responsible for the degradation and detoxification of many environmental contaminants changes in the composition or activity of microbial communities might have immediate or lasting effects on ecosystem and functioning. To assess the threshold limits of the various functional groups and the community struture, soil microcosms were treated with heavy metals.The effect of these on the total hetetrotrophic populations and on the functional guilds especially with reference to nitrogen cycle was studied to develop ecotoxicologial indicators. The rich microbial diversity of this region was also screened for novel properties.

The other important component is to integrate biological monitoring with aerial (satellite) monitoring methods. Data from other monitoring agencies needs to be integrated before formulating a biomonitoring method. This aspect although is not within the scope of this project, is however very essential to develop a successful monitoring programme. It may be noted this lacuna in this project has been duly filled and included in the project submitted to Department of Biotechnology, in 1996. as a continuation of this one. Finally biological monitoring cannot be economical and successful unless the local people are trained in the use of various organisms in monitoring. In this regard a training programme on the "Application of bioindication technology to the conservation of biodiversity" for senior level people was conducted in April 1992 by MSSRF. In addition to this, various other training programmes for NGOs and local people, farmers school children and college students have been conducted.

Materials and Methods: Site Characterisation: The Pichavaram mangroves are situated in the east coast of India, 250km south of Madras city. It lies between 11 23' to 11 30'N latitude and 79 45'E to 79 50' E longitude (Fig.1). These mangroves are limited to the north and south by Vellar and Coleroon rivers and are criss crossed by large number of channels and creeks and covers an area of approximate 1100 hectares (Kannan and Vasantha, 1992). The fresh water source for this mangroves are the Khansahaib channel, Coleroon and Vellar rivers. However the diversion of fresh water to various irrigation purposes **reduces** the fresh water flow in to the mangroves resulting in an increased salinity causing threat to this ecosystem.

Sample collection and bacterial enumeration: From each study zone, Neduodum, Periaguda and Kudianthittu selected on the basis of the density of the vegetation, (Krishnamurthy *et al* 1981), six sets of soil samples were randomly collected using a soil core sampler. Samples were transferred to sterile containers, stored with ice and taken up for bacterial enumeration.

Enumeration of Microbial population: Microbial numbers were determined by the pour plate method (Wollum, 1982) using appropriate aliquots of the sample at appropriate dilutions. All media used were from HiMedia and chemicals were of the highest quality. Sterilisation was carried out in a horizontal autoclave at 121C (151bs) for 15 mins.

Total heterotrophic Count: Soil sample (10g) was added to sterile blank of water and kept in a shaker (lhr/120rpm) for uniform mixing. Appropriate aliquots of sample were plated on Zobell agar. Triplicates were incubated for 2 weeks and were recorded. All colonies were numbered sequentially and subcultured. After sub culturing twice on Zobell agar to ensure purity and viability they were taken for further analysis. They were maintained on marine agar slants at 4C and subcultured monthly. Some strains lost viability during testing (less than 10% from each zone) and were discarded.

Nitirifers : For the enumeration of nitrifying bacteria the nitrifying agar media was used (Subha Rao,1986) gms/l ammonium sulphate 1gm, dipotassium hydrogen phosphate 0.1gm, sodium chloride 0.2gms, magnesium sulphate 0.5gms, ferrous sulphate trace, agar 15gms. Excess of sterilised calcium carbonate was added after sterilisation. The sample was plated out according the above mentioned procedure and incubated. At the end of incubation the total number of colony forming units which appeared on the plates were counted and inoculated into tubes containing nitrifying broth (5ml). After 10 days of incubation 1.2ml of sulphanilic acid (8gms of sulphanilic acid in 11 of 0.5N acetic acid) and 1ml of naphthalamine (gms/l naphthalamine 5.0ml in 11 of 0.5N acetic acid) were added to each tube containing the cells. The development of pink colour indicates positive result.

Ammonifiers: ammonifying agar media (gms/l, potassium dihydrogen phosphate 3gms, potassium chloride 0.2gms, magnesium sulphate 0.02gms, ferrous sulphate 0.01gm peptone 10 gms, agar 15gms) was used to enumerate ammonifiers following the procedure as mentioned earlier. At the end of the incubation the total number of colony forming units (CFU) which appeared on the plate were counted and inoculated into tubes containing ammonifying broth (5 ml) After 10 days of incubation, production of ammonia was tested by using Nesssler's reagent. Reddish orange colour indicates positive result.

Phosphate solubilisers: Pikovskaya's medium was taken and 5gms of soil was added and left for enrichment for 3 days. At the end of the incubation the sample was taken for enumeration on phosphate agar (gms/l glucose 10gms, tricalcium phosphate 5gms, ammonium sulphate 0.5gms, potassium chloride 0.01 gm, magnesium sulphate 0.1gm, ferrous sulphate trace, yeast extract 0.5gms, agar 15gms). The sample was plated out according to the above mentioned procedure and incubated. At the end of incubation the total number of colony forming units (CFU) which appeared on the plates with clearing zones were counted.

Sulphur bacteria: sulphur bacteria was subjected to enrichment in Thiosulphate broth according to the procedure mentioned above. The sample was plated on Thiosulphate agar (gms/l, sodium thiosulphate 5gms, dipotassium hydrogen phosphate 0.1 gm, sodium bicarbonate 0.2 gms, ammonium chloride 0.1gm, agar 20 gms and an excess of calcium carbonate after sterilisation). At the end of incubation the total number of colony forming units (CFU) which appeared on the plates were counted.

Iron bacteria: This was also subjected to enrichment according to the procedure mentioned above. The sample was plated on iron agar medium (gms/l, ammonium sulphate 0.5gms, sodium nitrate 0.5 gms, magnesium sulphate, 0.5gms, dipotassium hydrogen phosphate 0.5gms, calcium chloride 0.2 gms, ferric ammonium citrate 10 gms, agar 15gms) and incubated. At the end of

incubation the total number of colony forming units which appeared on the plate were counted.

*** For all the media used for the isolation of the functional guild specific isolates the respective isolation media was prepared in 50% estuarine water from the respective sites to maintain the saline conditions.**

Characterisation of isolates: Approximately 98 phenotypic characters were determined for each strain (Table 1).

Morphology: Colonial morphology was examined according to the criteria described by Colwell and Weibe. The characters scored for each strain included the presence of creamy-white, yellow, and orange pigments, fluorescent, transparent, opaque, matt and shiny colonies, round colonies, entire, serrated, rhizoidal and irregular edges, and spreading, flat and wrinkled colonies. The presence of fluorescein and pyocyanin were recorded after 7 days, under ultraviolet light, using the media described. Motility and micromorphology were determined microscopically from wet mounts and the presence of pleomorphism, curved rods, round-ended cells, chains, filaments and granular cytoplasm were noted. In addition, the length to width ratio of rods was measured and recorded as 2:1, 4-5:1, or 5:2 using a calibrated micrometer eye piece. Cultures (14d depending on growth rate) from marine agar slants were examined for cell shape, size and motility (wet mounts), spores, refractive granules of poly-b-hydroxybutyrate (Stanier et al 1966) and capsules, (India ink stain - phase contrast microscopy), gram reaction, acid fastness. Cultures were also examined for colony morphology and size, and for production of diffusable and non diffusable pigments. Fluorescent pigment formation on marine agar containing 0.15% (w/v) glycerol was assessed daily with uv light (260nm). Following 10 to 15 min. adaptation of the observer to the dark, bioluminescence was tested in the dark daily for 10 days.

Physiological and biochemical tests: Cultures were tested for the distribution of growth, oxygen relations were determined from stab cultures in marine agar butts. Indole production and ammonia production (Nesseler's reagent) were determined from 10d cultures. Cultures (2-4d) on marine agar were tested for catalase (with 3% H_2O_2) and cytochrome oxidase production allowing 1 min. for the blue colour to develop, Methyl red and Voges-Proskauer tests were done in MR-VP broth. Nitrate and nitrite reduction were tested in nitrate broth. Nitrite was detected with napthylamine-sulphanilic acid reagent. Acid production from D-ribose, D-fructose, cellobiose, lactose, sucrose or D-mannitol (all 1% w/v) was detected in MOF medium. Oxidation/fermentation tests were done in MOF medium containing 1%(w/v) D-glucose. Gas production from glucose was detected with inverted Durham tubes in the liquid medium used for determining growth distribution, supplemented with 1%(w/v) D-glucose. Substrates were filter sterilised. Lipase activity was tested in marine agar

containing 0.01%(w/v) $CaCl_2$ and 1%(w/v) Tween 20 or Tween 80. Starch hydrolysis was tested by flooding plate cultures (7d) on marine agar containing 0.5%(w/v) potato starch with Lugol's iodine. Gelatin hydrolysis was tested by flooding cultures (7 to 10d) on marine agar containing 10%(w/v) gelatin with acid $HgCl_2$. Casein hydrolysis was tested on marine agar overlaid with a double layer of 10%(w/v) skim milk agar. For the last three tests, clear zones around colonies were recorded as positive.

Nutritional Tests: Substrates were filter sterilised. These were mixed with the sterilised basal medium just before pouring to give a final concentrations of 0.1%(w/v). A total of 12 substrates were tested.

DNA isolation and RAPD analysis: For genomic DNA isolation, cells were harvested from 1ml culture. The pellet was suspended in 400ul of extraction buffer (10mM Tris- HCl, 10mM EDTA and SDS 10%, pH 8.0). The cell suspension was incubated at 100 C for 10 min. and centrifuged at 10,000 rpm for 10 min. The DNA in the supernatant was precipitated with 2.5 volume of ethhanol and 1/10 volume of 3.0M Sodium acetate (pH 5.2). The DNA pellet was washed in 70% ethanol and dissolved in TE buffer.

The PCR condition were optimised for Perkin- Elmer DNA thermal cycler 480 with genomic DNA of isolates as templates (Williams *et al* 1990). Each 25ul of reaction mixture contained 2.5ul of 10x assy buffer (100mM Tris -HCl, pH 8.3, 0.5M KCl, 0.1% gelatin), 2mM Mg Cl_2, 100uM dNTPs, 15ng primer, 40-50ng template DNA and 0.5 units *Taq* DNA polymerase (USB), overlaid with equal volume of mineral oil. The amplification conditions comprised of 40 cycles with 1 min. at 94 C, followed by 1 min. at 40 C and 2 min. at 72 C. The reaction was further incubated at 72 C for 15 min. for primer extention and stored at 4 C. The amplified products were analysed by electophoresis in 1.4% agarose gels in 1x TAE buffer. Three decamer universal primers OPD7,OPD6 and OPA3 (Operon Technology Inc. CA, USA) were used for amplification.

Data processing and analysis: Data were coded in binary form. Clusters of strains with similarities greater than 75% were designated as taxonomic groupings.

Results and Discussion Pichavaram: Most recent work on the pelagic marine organisms lumps together all of the prokaryotes that lack photosynthetic pigments into a black box called the 'heterotrophic bacteria'. Such lumping has been necessary because it has not been feasible to determine what kinds of bacteria make up the communities. However, ignorance of species and their distribution hinders to account for many ecological interactions. As an initial step to understand the distribution of the microflora,sampling was done in the three zones at the intertidal region for over a period of one year. Isolation was done with special reference to their functional roles using selective media. The

analysis was spread over this period to essentially study if there is any difference in the occurrence of microflora. The recovery of populations associated with the functional guilds like nitrifies, ammonifiers and dentirfiers were comparatively low when compared with the heterotrophic populations (Plate 1a and Fig.1). All these strains are being maintained in the culture collection.

To understand the distribution and composition of the heterotrophic population in this area 313 isolates were picked out from the plates and well defined colonies were picked out for further analysis. Less than 10% of the strains were lost from each site during the course of the study. Presumptive identification of the strains were done using Bergey's manual of systematic bacteriology and other keys. The recovery from Neduvodam and Periaguda was comperable which in the Kudianthittu area it was low. This trend was observed right through the year. Majority of the bacterial isolates from the samples were gram negative. The general characteristics revealed by the isolates is detailed as follows: Pigmented isolates were 33%. A high proportion of such isolates could be due to the fact that the orange and the yellow pigmentation is an adaptive protection mechanism of microorganisms once exposed to intense sunlight during summer. Catalase was produced by 64% and oxidase by 61%. 42% of the isolates were capable of reducing nitrate to nitrite, 55% were capable of fermentative metabolism of glucose, however, 39% showed no acid or base production. 55% isolates utilised glucose as a sole carbon source. Out of 67 isolates 55% of isolates were able to produce H_2S from cysteine. This was along the expected line as these isolates occupy, the inter tidal area which are marshy and is typically rich in H_2S and organic matter. A large number of anaerobic and aerobic bacteria in these regions help in the upward diffusion for the anoxic zone and its subsequent oxidation in the aerobic, surficial layer.

The dendrogram constructed on the basis of the phenotypic results revealed (Fig 2) inter group similarities ranging from 11 to 100% suggesting that significant diversity existed among the strains isolated from Pichavaram Mangrove ecosystem. The intra group similarities in the 8 groups formed at the level of 70% similarity - 96% in group 1 with 7 isolates, 72 - 90% in group 2 with 7 isolates, whereas rest of the groups formed two member clusters sharing the common node at the level ranging from 70 - 80%. Interestingly, considerable number of *Aeromonas* sp. were recovered from the mangrove ecosystem with the intra group similarities ranging from 77% to 96%.

Marinococcus sp. (formerly known as *Planococcus*), a gram +ve motile, yellow pigmented, cocci which was able to utilise all the carbohydrates tested and the other gram +ve yellow pigmented non motile isolates with inter group similarity of 66% along with *Marinococcus* identified as *Micrococcus* fell in one cluster at with *Aeromonas* at 66% similarity value. This was primarily due to the similarities in carbohydrate utilisation pattern. Many compounds, both organic

and inorganic, enter the aquatic environment resulting in the alteration of microbial communities, and it has been reported that Aeromonas is an indicator of organic load. In this study *Aeromonas* populations have been isolated in more numbers in Neduodum thereby suggesting that the chosen sites were rich in organic load due to the thick vegetation which in turn leads to a generation of litter. The next group of clusters formed at similarity level ranging from 90% to 62% included mainly of *Alteromonas* sp. followed by *Paracoccus* sp. and *Bacillus* sp. and a representative of Enterobacteriaceae. All theses strains were able to utilise all the carbohydrates tested (except arabinose) as a carbon source. They were non-motile and produced both catalase and oxidase. *Alteromonas* sp. were motile, pigmented and were able to degrade complex molecules such as starch, gelatin and tween compounds. *Bacillus* sp. were catalase positive able to degrade starch and tween but not gelatin and negative to Proskauer test. *Erwinia* sp. which was found to be non-motile, oxidase negative, not able to degrade starch and gelatin but could hydrolyse tween 40 and was identified as a representative of Enterobacteriaceae.

Flavobacterium could not utilise glucose, fructose, maltose, galactose and could utilise sucrose, glycerol and arabinose and positive in oxidase and catalase This isolate was clustered with *Erwinia* sp. at a similarity level of 62%. Surprisingly *Pseudomonas* sp. which are found to be a dominant genera in the marine environments was encountered in low proportion in the present study. Only two isolates was observed. This trend has also been reported in Beaufort sea bacteria, a tropical marine system which is quite different from the previously reported analysis from temperate marine environments (Tonsuo *et al* 1979). A facultative aerobic strain was identified as *Cytophage* due its light red pigmented colony, amylase and lipase positive cellulase and oxidase negative and penetration into agar medium. But in contrast to the previous report, this organism was notable to degrade gelatin. *Ocenosprillum* sp. with similarity value of 75%, oxidase positive and indole was not produced, nitrate reduced, not fermerntative and fails to attack starch, gelatin and inability to utilise carbohydrate, catalase positive. A gram negative pigmented isolate which was motile, formed hydrogen suphide from cystein, did not hydrolyse starch, but hydrolysed gelatin. It produced both catalse and oxidase and none of the carbohydrates were utilised. A group of isolates with intra group similarities of 74% to 54% were identified as *Photobacterium sp.* Another group with intra group similarities of 83% to 75% was identified as *Vibrio* sp. Such species have been reported in high proportion in some temperate marine environments (Cook and Goldman 1976, and Loveiace *et al* 1967)

Coryneform and *Flactobacillus* sp were also identified. Surprisingly, large number of *Acinetobacter* was identified with similarity value of 30% to the highest value of 100%. Invariably all these organisms were catalase positive and oxidase negative and highly unreactive to all the tests. There were many isolates which could not be identified bringing out the need to do more work and for

development of more data bases against which the results could be compared. All the characters were recorded onto data sheets and the culture are being maintained in glycerol stocks. The results of this portion of the study has been communicated to FEMS Microbial Ecology (Mathan et al).

An attempt was also made to assess the diversity in populations recovered from individual sites and compared between sites by applying the Shannon Weiner index. The diversity thus calculated was 0.938 for Neduvodum followed by 0.918 for Periaguda and 0.769 for Kudianthittu. In general, the species composition seems to be highly heterogeneous in the populations which were analysed in this study. The diversity index was found to be almost the same in the two near normal sites of Periaguda and Neduodum. Here the vegetation is quite dense and relatively undisturbed and the litter generated is also quite high. In Kudianthittu, where it is disturbed due to several anthropogenic pressures the vegetation is sparse hence, the diversity is comparatively low. **This suggested that such a study using the diversity index could be used as indices of environmental disturbances between sites.**

To assess the threshold limits of the functional groups, with special reference to guilds involved in the nitrogen cycle, soil microcosms from the different zones were treated with heavy metals. Cadmium, Chromium, Lead, Mercury, Copper and Zinc the potential hazards have been studied by creating *ex situ* microcosms. It was observed that the nitirifers were completely eliminated even at the lowest concentration of heavy metals. Mercury had the most striking effect followed by CU, Pb and Zn (Fig.3). This indicated that this particular guild could be used as a sensitive ecotoxicological indicator for heavy metal toxicity. The effect of the same heavy metals on the heterotrophic populations was also studied. It was observed that even as low as 25 ppm of Hg excepting for two kinds of bacteria all the rest were observed to grow even at 100ppm. One of them a halotelarant isolate could be used as an indicator of mercury toxicity as it was very obviously present in large numbers especially when the concentration of mercury increased (Plate 1b). In control conditions this one was found to occur only in very low numbers. For the other heavy metals although the diversity did decrease the percentage of decrease was comparatively low (Fig.3) There was an observed 80% reduction in all three sites from 25 ppm when the soil microcosms were treated with mercury. This trend was observed till 100ppm. When Copper was added 40% reduction was observed in Kudianthittu region and @10% in Neduodum and Periaguda. For lead not much of change was observed upto 400%. In Zinc 40% reduction was observed in Kudianthitu region while 20% was observed in Neduodum and Periaguda. When a comparison was done between sites the threshold limit of the microbial flora was found to be the same for both Periaguda and Neduodum while the Kudianthittu sites it was low. Since the diversity was found to be comparatively higher in these two sites it could be explained that a higher number of bacteria

got selected when compared to Kudianthittu where in the control conditions itself it is low.

RAPD Analysis: Molecular tools are being used to unravel the microbial diversity currently. In the present study RAPD analysis was performed successfully on the isolates from the three different zones Periguda, Neduodum and Kudianthittu of Pichavaram mangrove ecosystem using 3 random primers. The DNA profiles generated for each site and primer is consensus of three replicates. Typically 38 to 48 major DNA bands and several less intense bands were obtained when the PCR products were size fractionated on agarose gels (Plate 2) All the isolates were subjected to replicate analysis (to facilitate cross gel comparisons and assess reproducibility) and always resulted in the same patterns of DNA bands. No two isolates showed identical similar RAPD fingerprints indicating that the isolates were different from each other and that diversity was more. Dendrograms were constructed for the three individual populations separately. When the similarity level of 70% was applied here as in the phenotypic analysis, it was observed that the eight groups which were formed in the clusters in the phenotypic got scattered completely except for A1 and A2 which fell in the same cluster. The other observation was that C4 and C5 which fell in different clusters in phenotypic analysis fell in the same cluster in the RAPD clustering at a similarity level of 60%). Tonso *et al* 1995 used RAPD for the rapid identification of similar but not identical (i.e. closely related) isolates in their study. Further, their study suggests that identical or highly related RAPD pattern always had similarity indices above 85%. Similarity below this value indicates unrelated RAPD patterns and thus unrelated or distantly related organisms. Since none of the groupings showed a similarity level of more than 60% (excepting for two C4 and C5) in the RAPD analysis, it can be concluded that populations analysed were highly heterogeneous as revealed in the phenotypic analysis.

The isolates were also screened for novel properties. Strains which showed good enzyme production (proteolytic, amylolytic and cellulolytic) both in alkaline and acidic conditions, DNase producers, salt tolerance etc.,. Plate3 showing (a) phosphates solubilisation bacteria, (b) Dnase producers and (c) alkaline amylolytic isolate is enclosed. The characteristics of the isolates are recorded in data sheets which are being kept for compiling into data bases.

Conclusion: This exercise of analysing the microbial diversity as part of soil biological criteria, has generated a lot of data on the type of microflora present here. The study having been spread for over a period of three years has given us an opportunity to analyse the differences over the period of time. In the last three years not much of difference has been observed. Thereby revealing that this parameter of microbial diversity and functional guilds could be used as an indicator on its own as indicators of disturbance and heavy metal toxicity. More than that an insight into the characteristics of the isolates of this region with

reference to their functional roles have been identified. RAPD, which has been used in this study, is useful for detailed characterisation and separation of strains within a single species, the data generated from this and the phenotypic analysis was limited preventing us from drawing taxonomic conclusions not only to species level but also at the generic level from the studies, thereby highlighting the need for data base against which a comparison can be made. However, we feel that these characteristics would provide us opportunities to explore further the genotypic delimitation of isolates belonging to same species or similar functional ability. Overall we have shown that microbial diversity and microbial functions have been used successfully to assess the health of this particular ecosystem.

Monitoring using Fungal Guilds - Celluolytic Guild: Nutrient dynamics is of 'open' type with regard to mangroves, since the mangrove litter is partly washed away into the ocean and may not be recycled to the plants. The organisation of the food web suggests that micro-organisms degrading the structural material of the mangrove leaves support a population of detritus consumers including invertebrate species and fish (Odum and Heald, 1975). The dead leaves, wood and bark which accumulate as litter, are disintegrated and decomposed by the cumulative action of microinvertebrates, bacteria and fungi. Microbes solubilize these complex phytopolymers into assimilable forms. The microbes usually accomplish this breakdown as 'guilds' or groups (group of species involved in similar function). Hence one may come across a number of different species involved in a function like cellulose breakdown. This phenomenon is described as 'boot strapping' by Hawksworth (1991). These guilds impart certain amount of resilience for the vital ecosystem function in the event of a stress. In this case, the community structure is altered, but not the function depending upon the magnitude of the stress. Hence these fungi as functional groups (guilds) are integral components of the mangrove soil, and they can serve as **'early warning systems'** of drastic change in the soil or marine health.

A seasonal survey to understand the microbial flora occurring in the selected areas of the mangrove ecosystem was taken up. A general idea of fungi involved in the degradation of cellulose component of the litter was studied from the litter samples. The involvement of these fungi isolated was confirmed by their ability to degrade CMC at experimental conditions. This study revealed that these fungi are not 'chance contaminants' but actively involved in the decomposition process. Since most of these fungi are readily encountered under cultural conditions, they have significant value from the biomonitoring point also. The same guild was subjected to various stresses that may have potential effect on mangrove ecosystem for e.g., Hg++, various other heavy metals and endosulfan, a pesticide used in paddy cultivation, were tested for their effect on these guilds because they have been black listed as potential threat from the marine ecosystem (BOBP, 1994).

Methodology: The entire mangrove ecosystem was divided into 3 areas based on the vegetation density. Periaguda area has maximum vegetation and least disturbed, Neduodum area is moderately disturbed, and Kudianthittu a maximum disturbed area. Samples were collected in triplicates from each area and sampling was done during summer, monsoon and post monsoon periods.

Litter samples, in the process of decomposition from the tidal submergence region only were collected in sterile bags and transported immediately to the laboratories and stored in the refrigerators. The samples were lightly blended and 0.59 of these samples were suspended in sterile water (25ml) with glass beads, and homogenised in an orbital shaker for 30 min. Aliquots of this suspension (0.5ml) was spread on PDA-Rose Bengal Agar medium. Samples from Periaguda and Neduodum were diluted 100 and 10 times respectively. The visible count of number of colonies after 48h incubation at room temperature was taken. The total fungal colonies and colonies of individual species were counted and tabulated (Table 1). The individual species were isolated, maintained in pure culture and used for studies involving response to stresses.

CMCase Assay: The ability to degrade CMC by the isolates was studied by growing them in CD media amended with CMC (carboxy methyl cellulose) as carbon source. Spore suspension (1 ml) of each isolate was inoculated in the specified media and incubated at 25+ 1° C. for 48 h. After incubation, culture filtrates were assayed for CMCase activity as shown in Table 2 (Chan and Au, 1989).

Effect of Various Stresses on the Cellulolytic Guild: Studies regarding the community's response to some stresses mentioned above were done by treating the fresh litter with stresses and plating them. The response of these communities under brief and chronic exposures of mercury toxicity (the source may be from the chlor-alkali plants in the coastal areas) was studied. Homogenised litter sample (500mg) were suspended in 25 ml water containing Hg++ (at different concentrations 0, 5, 10 and 25 ppm) and plated 2hrs after exposure (brief exposure) and after 7, 14 days of incubation (chronic exposure). Total number of colonies and individual species were counted at the end of the incubation period (Table 3). In addition, 'endosulfan' a pesticide widely used for agriculture purposes on the banks of Cauvery and tributaries was also studied for its effect on these communities by adopting the same procedure detailed earlier but at various concentrations (0, 0.01, 0.025 and 0.05%w/v). Results are presented in the Table 4 and discussed.

Results and Discussion: Among the mangrove areas, the relatively undisturbed area - Periaguda and partly disturbed Neduodum have almost all the mangrove species present in the entire Pichavaram mangrove forest. Hence their litter composition is different from that of the litter from Kudianthittu area which has only stunted *A. marina* and *Rhizophora* species restricted only to the fringes.

Table 1 gives the details of the number of fungal species, their percent occurrence through three different seasons. There are about 14 different species of fungi generally found to occur on the litter from intertidal zones (Table 2). The fungal flora is almost similar in Periaguda and Neduodum regions dominated by *Aspergillus niger* followed by other *Aspergillus, Cheatomium* and *Penicillium* species. In contrast, Kudianthittu area has only *Penicillium* and *Trichoderma* species. The total number of colonies are abundant in the case of Periaguda followed by Neduodum regions, whereas it is least in the case of Kudianthittu.

Changes in the seasons with regard to the fungal flora have very little impact on this fungal community. This may be due to the fact that a consistent collection and sampling procedure confining to decomposing litter only was observed every time. Repeated and ready isolation of 14 species only proves that these fungi dominate other mycoflora under marine conditions. As per Hyde (1990) there were as many as 40 fungal species involved in degradation of mangroves litter at intertidal level. Most of them belong to Ascomycetes. A number of other fungi belonging to ascomycetes, which require special isolation procedures have been described by many other workers also (Fell and Master, 1984; Subramaniam, 1986; Jones and Hyde, 1990). But from the points of biomonitoring, this fungal guild involved in the break down of cellulose and readily culturable nature in terms of their biomass and functional role would be far better than the entire fungal biodiversity, most of which require complicated isolation procedures.

CMCase Assay: Litter that falls on the mangrove forest floor is composed of 50% cellulose. The degradation of this polymer is achieved by colonisation of some fungi capable of utilising cellulose for their growth. However, microbial degradation of certain mangrove species have been studied only from the submerged samples (Cundell *et al., 1979).* The individual capacity to degrade cellulose based on CMC utilisation under *in vitro* conditions is represented in the Table 2 . All the fungi isolated (14 species) except 2 species have differential capacity to degrade CMC hence prove their role in the decomposition of cellulose component of the litter once again underlining the fact that they are not chance contaminants but actively take part in cellulose degradation. As Hawksworth 1991 described the redundant nature of these fungi in terms of cellulose decomposition, revealed at microbial level that the Nature has provided 'functional redundancy' imparting a certain amount of resilience to these important ecosystem functions in the event of a stress. Similarly, this 'guild' operating as one functional group provides back up for cellulose degradation under stressed conditions. Since this process is an essential aspect of nutrient recycling, this guild of fungal species can be used as biomonitors of various pollutants from marine or inter tidal zones.

What we observe from the experiment, under Hg++ toxicity is that the 'guild' (fungal community) structure is altered. A shift from the more dominating A. *niger* to A. *terreus* in Periaguda regions is apparent. Uninterrupted cellulose decomposition is implicit in this observation although at community structure level, a major revamp has occurred. Kudianthittu having a different mycoflora, exhibited considerable reduction in fungal biomass at high Hg++ toxicity.

In vitro studies with endosulfan (pesticide) the fungal guilds response was erratic. A consistent behaviour could not be obtained whenever the litter samples were treated with different concentrations of endosulfan (Table 4). This result only suggests that fungi may not be ideal candidates for monitoring the toxic levels of endosulfan.

The results of treatment with other heavy metals have also been very inconsistent reminding the fact that the fungi may not be sensitive to the toxic levels of other heavy metal like Cr, Cu, Pb, Zn, etc. However, the observation with Hg++ toxicity can be a useful indication for monitoring Hg++ pollution.

Conclusion

From these studies it is understood that the alteration in the fungal community structure - namely replacement of A. *niger* colonies by A. *terreus* species under 25 ppm Hg++ toxicity as an indication to mercury stress. Differences in the fungal community structure between Periaguda and Kudianthittu can be indicative of forest disturbance. A. *niger is* usually dominant in the litter of dense mangroves, whereas *Penicillium* and *Trichoderma* are prevalent in the degraded mangrove areas and hence can indicate degraded mangrove areas.

Lichen Distribution pattern in Pichavaram - a study to indicate Forest Disturbance in the mangroves of South India: In the present study the lichen diversity and its distribution pattern are used to assess the ecological continuity of the forest sites selected. Since this study is an initiative to use lichens, a detailed checklist of the lichen elements present in the study site is a prerequisite. Hence intensive collection of lichens were made at the sampling areas within Pichavaram mangroves (November 1992 - March 1993; in 24 field days) through regular field trips.

Methodology: Lichen samples were collected, after recording the field characters, cleaned and preserved in the form of lichen herbarium (MSSRF), at the Microbiology laboratory, M.S. Swaminathan Research Foundation, Chennai for future reference. Data from external morphology, anatomy, reproductive structures and chemistry were used in identification. Morphology was analysed by using a 10x lens in the field and a Zeiss trinocular dissection zoom microscope in the laboratory. For studying the internal morphology, lichens were sectioned in various planes by hand and observed under Nikon Optiphot

Compound microscope. Photobiont, asci, paraphyses and ascospores were examined on teased out preparations after soaking in water. Chemical spot tests, such as K, C, KC and P tests were also carried out.

The topography and vegetation structure in the Mangroves varies from that of Tropical Rain Forest ecosystem. The lichen diversity within Pichavaram mangroves is very low, and, lichen species belonging to a typical Lobarion community (indicator of forest ecosystem continuity), is also absent in this location. So it is felt that the disturbed forest site within Pichavaram, can be identified using the presence of increased opportunistic lichen species. Hence the diversity and the distribution pattern of Pichavaram mangrove lichens were quantified using a methodology described below. The lichen distribution pattern of Siruvani hills were studied through floristic surveys. The sampling procedures used for macromycetes and bacteria are not adopted to sample lichens because of the epiphytic/epilithic occurrence and distribution of lichens.

Selection of sampling sites and sampling procedure.

The four sampling areas (viz.: Sanikuttai vaikal, Neduodum, Periaguda and Keeriguda) were selected mainly on the basis of the six vegetation formations reported by Krishnamurthy *et al.* (1981). Out of these six vegetation formations, Formation No II, III and V contain mangrove vegetation and Formation No 1, IV and VI contain halophytic herbaceous species which do not support the growth of lichens. So the sampling area Sanikuttai vaikal lies in Vegetation Formation No II, Periaguda and Keeriguda in Vegetation Formation No III and Neduodum in Vegetation Formation No V. Since Vegetation Formation No III represents the core mangrove area two sampling areas were selected from this formation. Lichen colonies occurred only on *Excoecaria agallocha, Rhizophora apiculata, R. mucronata* and *R. lamarkii.* It was also found that lichen community structure is almost similar in all the species of *Rhizophora.* Hence *R. apiculata* (chosen as a model tree from *Rhizophora* members) and *E. agallocha* were selected for sampling. Moreover for convenient handling of the data, an *E. agallocha* and *R. apiculata* sample was collectively called as a 'Tree' and divided in to tree zones as mentioned below.

Tree Zone 1: *E. agallocha* trunk
Tree Zone 2: *E agallocha* canopy
Tree Zone 3: *R. apiculata* stilt roots
Tree Zone 4: *R. apiculata* trunk
Tree Zone 5: *R. apiculata* canopy.

Further as the *Rhizophora* trees form fringes along the margin of the water bodies, data was collected from both the waterward and landward parts of the sampling trees. In order to get an uniform sample size a 30 cm scale was used as a standard, placed on the sampling area of the tree and the lichen colonies in

contact with the scale was counted and recorded. A total number of 450 samples were counted from each sampling area of Pichavaram mangroves as mentioned below:

Total No of Trees	15
No of Tree Zones	5
No of aspects	2
No of samples/zone	3
Thus total No. of samples	450
Total sites studied	4
Total No samples counted	1800

There are only ten species of lichens present in the study site. However for monitoring purpose species belonging to the same genus are considered as single generic units and thus six generic units were derived. Finally all the data collected by the above method were Tabulated and interpreted.

Results and discussion: Lichens are host specific and the number of lichen species occurring in mangrove stands depends on the number of host tree species (Marcelli, 1995). This is true in the case of the lichen diversity of Pichavaram mangrove also. There are ten lichen species present in the Pichavaram mangrove area out of which 7 of them are crustose, 2 foliose and one fruticose (Table1). All these lichens are corticolous. Some of the interesting groups like foliicolous, saxicolous and gelatinous lichens are absent in the study locality. Even though all the lichens in this area are corticolous they are restricted to *E. agallocha* and species of *Rhizophora.* The lichen species distribution on *E. agallocha* and *R. apiculata is* presented in Table2. It is evident from the Table that *R. apiculata* trees bear more lichen colonies than *E. agallocha.* The number of colonies of *Roccella, Pyrenula, Dirinaria,* and *Lecanora* occurring on *R. apiculata* are remarkably higher than the number of lichen colonies occurring on *E. agallocha.* Among those lichen colonies occurring on *R. apiculata* trees, colonies of *Roccella* dominate and followed by colonies of *Pyrenula, Dirinaria* and *Lecanora.* Lichen species belonging to *Graphis* and *Buellia* do not show any distinct preference for either of these two trees. There are no lichen colonies seen on *Avicennia marina* and *A. officinalis.* This may be due to the nature of bark (papery - peeling off nature) of these trees. Stems of *Bruguiera* and *Lumnitzera* are not thick enough to bear lichens, although lichens are reported to occur on these trees elsewhere (Marchelli, 1991, Stevens 1991). Moreover this region is totally devoid of any rocky outcrops and thus no saxicolous maritime lichen communities are found. As mangrove leaves are short lived, foliicolous lichens are also absent.

Awasthi (1981) described the fertile specimens of *Roccella* collected from Pichavaram as *R. belangiriana* Awas. However inspite of repeated searches we are not able to relocate this species in Pichavaram now. The distribution pattern of lichen colonies within the five different Tree Zones were presented in Table 3. Here it is clear that the Tree Zone III (R. *apiculata* stilt roots) possesses more

lichen colonies when compared to the other Tree Zones. The difference is insignificant between Tree Zone I and II in terms of lichen colony number. Tree Zone 4 contains more lichen colonies than Tree Zone 5. Colonies of *Roccella* occur more on R. *apiculata* stilt root (Tz 3) than any other Tree Zones mentioned. However *Dirinaria* occurs more on R. *apiculata* trunk (TZ 4).

The *R. apiculata* trees with its stilt roots, trunk and canopy provide more space for lichen colonisation than *E. agallocha*.. This is evident from the occurrence of more lichen colonies on the stilt root (TZ 3) and trunk (TZ 4) of *R. apiculata*. The lichen colony distribution pattern on waterward and landward side of trees is given in Table 4. It is evident from this Table that generally more lichen colonies occur on the water facing side of the trees when compared to the land facing side of the trees. *Roccella, Dirinaria* and *Graphis* occur more on water facing side of the trees whereas colonies of *Buellia* occur more on land facing side of the trees. However colonies of *Pyrenula* and *Lecanora* do not show any preference. Likewise the spatial distribution of *R. apiculata* trees along the inter tidal zone enables them to trap more moisture and light which facilitates more lichen colonisation where as *E. agallocha* trees are distributed more towards the land. It is clear that the colonies of *Roccella, Dirinaria* and *Graphis* occur mostly on *R. apiculata* and towards water facing side of these trees. However colonies of *Buellia* shows preference towards land facing side of the trees. The distribution pattern of lichen colonies within four sampling areas is given in Table 5. It is evident from this Table that more lichen colonies occur in Neduodum and Sanikuttai vaikal followed by Periaguda and Keeriguda. The increased colony number in Neduodum and Sanikuttai vaikkal is mainly due to the increased occurrence of *Roccella* and partially due to *Dirinana*. However in Periaguda the observed increase in the lichen colony number is due to *Pyrenula* colonies. There is an observed increase in the lichen colony number in the Neduodum followed by Sanikuttai vaikal, Periaguda and Keerguda. Neduodum (Vegetation Formation IV) and Sanikuttai vaikal (Vegetation Formation II) are situated near the human settlements whereas Periaguda and Keeriguda (Vegetation Formation III) are situated in the central region of the Pichavaram Mangroves. Since Neduodum and Sanikuttai vaikal are near the human settlements, the forest in these areas are very much exposed to fuel and fodder extraction, which in turn causes a lot of forest disturbance, where as the forests in the Periaguda and Keeriguda region remain relatively undisturbed. Hence the forests in Neduodum and Sanikuttai vaikal are much exposed and thus there is an increased availability of moisture from the sea breeze and light which favours abundant lichen colonisation and growth of the moisture loving colonies of *Roccella* and *Dirinaria*. The forests in Periaguda and Keeriguda which are not very much exposed show less number of these lichens. However these forests in Periaguda and Keeriguda possess more *Pyrenula* colonies. Thus it is clear that an increase in the number *Roccella* and *Dirinaria* colonies on *Rhizophora* trees indicates forest disturbance whereas presence of more *Pyrenula* colonies on the

same host along with a lesser number of *Roccella* and *Dirinaria* colonies indicates relatively undisturbed forest areas within Pichavaram mangroves. The data collected in this study form a baseline for future studies to monitor the forest disturbance as well as air quality of this region in the future.

Monitoring Ecosytem Health through Microbial diversity of Siruvani Hills Of Nilgiri Biosphere Reserves (NBR), Western Ghats: Study Site II.

In this study site monitoring the ecosystem health was initiated by using the same techniques used for the previous site in the soil biological criteria. Preliminary studies were initiated to test the conclusions arrived in that area. In addition to lichens which were used in the previous study area macromycete diversity was also used for monitoring ecosystem health. The following section details the methodology, results and discussion of the work carried out in this area.

Soil Biological Criteria: The isolation procedures detailed in the earlier section was used in this site except that estuarine water was not used in the media. For the isolation of the heterotrophic population appropriate aliquots placed on nutrient agar medium (gms/l peptone 15gms, beef extract 3gms, sodium chloride 5gms, agar 15gms and pH adjusted to 7.4+_ 0.2). All the plates were incubated at 37C for 2 to 3 days. At the end of the incubation total number of colony forming units (CFU) which appeared on the plates were counted and expressed in log 10 cfu/gm (dry wt.) soil.

Effect of heavy Metal on Total Heterotrophic Count: To study the effect of heavy metals on the total heterotrophic count populations half strength nutrient agar was prepared and sterilised in an autoclave at 121C (151bs) for 15min. The heavy metals were UV sterilised. Lead acetate, Zinc chloride and Copper sulphate were added to the sterilised medium just before pouring to minimise the possible interactions between the media components and heavy metal. For the enumeration of heterotrophic count earlier mentioned procedure was employed and the plates were incubated.

Effect of heavy metal on the Nitrogen Cycle: To study the effect of the above mentioned heavy metals on the ammonifiers, nitrifier and denitrifier, aliquots as soil suspensions treated as mentioned above were plated on the respective media as mentioned earlier and incubated.

Antibiotic Resistance among heavy metal tolerants: Resistant isolates which appeared after treatment with heavy, metal were subjected to antibiotic sensitivity test. From the pure culture a loop full of colony was suspended aspectically into sterile water (500ul) in an Eppendoff tube. Appropriate aliquot (100ul) from this was transferred onto the nutrient agar plate and a lawn was

created using a L rod. Antibiotic discs were placed aspectically. All the plates were incubated for 24 to 48 hrs and the zone of inhibition was measured.

Enzyme assays: ***Amylase assay***:(Hofmann E., 1963, Kelly W.D. and Rodriguez Kabana R., 1975). Soil samples (5gms), were taken in an Erlenmeyer's flask (150ml) . Toluene (1.5ml) was added to this. The mixture was allowed to stand for 15min. To this distilled water (l0ml) and soluble starch (5ml of 2%[w/v]) was added. The flasks were then stoppered and placed in an incubator. Control consisting of soil without soluble starch was run concurrently. After 24 hrs. of incubation (at 37C., 120rpm) the flasks were opened, distilled water (15ml) was added, the contents were mixed and the suspension centrifuged to produce a clear supernatant, and made upto 100ml. The reducing sugar content of the supernatant was determined by Nelson's method.

Cellulase assay: (Pnacholy S.K and Rice E.L., 1973) Soil samples (5gms) were placed in Erlenmeyer flask (150ml) and toluene (0.5ml) added. The contents were mixed and allowed to stand for 15min. Acetate buffer (l0ml; pH 5.9) was added followed by carboxymethyl cellulose (l0ml of 1% w/v). The flask was then incubated for 24hrs at 30C control consisting of soil without CMC was run concurrently. At the end of this period, distilled water (50ml) was added and centrifuged to produce a clear supernatant. Volume was made to 100ml. The reducing sugar content was then determined by the Nelson's method.

Invertase assay: (Hofman E. and Seegerer A., 1951 and Ross D.J., 1965a. Soil samples (5gms) were placed in an Erlenmeyer's flask (150 ml). Toluene (1.5ml) was added to this and allowed to stand for 15mins. Acetate buffer (20ml 0.5M; pH 5.5) and sucrose (20ml of 5%w/v) were added to this and incubated for 24hrs. Control consisting of soil sample without sucrose was run concurrently. After incubation, distilled water (50ml) was added centrifuged to produce a clear supernatant. The supernatant was made upto 100ml distilled water. The reducing sugar content was then determined by Nelson's method.

Nelson's method: Appropriate aliquots were taken from the pooled samples and the volume made upto 2ml with distilled water. Alkaline copper tartarate [A - dissolve anhydrous sodium carbonate (2.5gms), sodium bicarbonate (2gms) potassium sodium tartrate (2.5gms) and anhydrous sodium sulphate (20gms) in water (80ml) and make upto 100ml; B - dissolve cupric sulphate (15gms) in a small volume of distilled water. Add one drop of sulphuric acid and make upto 100ml; Mix 4ml of B and 96 ml of A just before use.],(lml) was added to each of the test tubes containing the aliquots. The tubes were heated in a boiling water bath for 15min and they were allowed to cool. Arsenic molybdate reagent [A - dissolve ammonium molybdate (2.5gms) in distilled water (45ml), add sulphuric acid (2.1ml) and mix well; add disodium hydrogen arsenate (0.3gms) dissolved in water (25ml). Mix A and B and incubate at 370C for 24hrs]. (lml) was added to each of the test tube. After the colour developed, the volume was made upto

10ml with distilled water and the optical densities were recorded using a spectrometer at 620 nm. Water (2ml) was used as blank and given same treatment as the samples. Glucose was used as a standard stock contained 100mg/100ml. Appropriate dilutions were made from the stock to give 20, 40, 60, 80 and 100 ug/ml. A standard curve was prepared giving the same treatment as mentioned above.

Dehydrogenase assay: (Casida et *al* 1964; Kiss S. and Boaru M., 1965; Kiss et *al*, 1969). Soil samples (5gms) were put in test tubes and calcium carbonate (50mg) were added to it. The tubes were then saturated with 2ml solutions of 2,3,5 - triphenyl tetrazolium chloride (1%w/v). The contents were mixed thoroughly, the tubes were tightly sealed with plastic caps and were then incubated at 32C for 24hrs. Following incubation, methanol (25ml) were added to each tube and the contents were stirred. The resulting slurry was washed into a Buchner funnel (Whatman No.3 paper), extracted with successive aliquots of methanol and the filtrate was made to 100ml . The absorption of triphenylformazan in the filtrate was determined spectrophotometrically at 485nm, using methanol as the reference blank. Concentration were determined from a standard curve of triphenyl formazan in methanol.

Results and Discussion: As mentioned earlier the Siruvani Hills forms a major component of the Nilgiri Biosphere in the Western Ghats which is one of the hot spot areas of biodiversity. Monitoring of such an ecosystem becomes crucial for conservation studies. Although this area is vast we chose only three sites within this area for the preliminary survey.

The surface soil plays a crucial role and the loss of this through human or natural disturbance could be detrimental to the functioning of any ecosystem (Burke et al, 1989). An active soil microflora is necessary for the biogeochemical cycling of nutrients. The microbial activity of soils could be measured by several assays to obtain a complete picture of the microbial metabolic activity in soil (Nannipieri et al 1990). Methods based on the physiology of microorganisms such as respiration and enzyme activities may be more powerful for revealing small changes in the ecosystem. Methods of this kind are urgently needed as a basis for evaluating the long term cumulative effects on ecosystems (Ohtonen et al 1993). The data (form the preliminary survey) of the microbial populations recovered from these sites is summarised in the Table 2. The total heterotrophic counts were comparable in the Kodivelliamman and Kovai fall soils, while the counts obtained from the soil samples from the teak soils were low. A comparison of the heterotrophic counts among the soil samples taken in October, December and February showed no significant differences and the same trend was observed in all the three samplings. But a comparative study between sites revealed that a low count was observed in the managed forest (Teak and rose wood plantation) soil which could be attributed to the management practices. Although sampling was done in alternative months over a period of six months

the climate was predominanatly rainy and the samples were also collected at the same altitudinal level. An attempt made in this study to compare the functional diversity in the three different sites has shown that the colony counts for the ammonifiers, nitrifiers and phosphorous solubilisers were comparable in the moist deciduous soil except for the sulphur and the iron which were low. Whereas in the teak forest soils the counts of the of the total heterotrophs and all functional groups were low (Table 2).

The diversity among the physiological activities was vast. In the managed forests the physiological diversity was comparitively quite low when compared to other two sites which had a good representation of various populations, except for the cellulolytic ones which could be because of the function being carried out by the actinomycetes and the fungi, thus pointing to some kind of pressure (possibly the management practices) operating in the teak forest soils. Among the isolates in the Kovai fall region @ 86% of the population showed amylolytic property, while @63% had the ability to hydrolyse lipids. Proteolytic activity was observed in @50% of the isolates and 10% showed cellulolytic properties. In Kodivelliamman soil isolates the amylolytic population was only 19% while @55% showed proteolytic nature. Lipolytic property was observed in @36% and the lowest population present was cellulolytic. In the isolates from the teak forest soils the amylolytic populations were dominant with 30% while proteolytic, lipolytic and cellulolytic were found in low numbers.

As a rule, the most common ecosystems, without any special physicochemical or nutritional characteristic, usually carry low numbers of culturable microorganisms but a high diversity of species. At the opposite extreme, ecosystems submitted to extreme physical or chemical conditions can exert a strong pressure for selection and therefore bacterial diversity is low. That the diversity and population numbers were good in Kovaifalls and Kodiveliamman region show that these two sites because of the inaccessibility, the anthropogenic factors have not operated, while in that of the managed forest (teak forest). The low diversity indicated that a selection has taken place due to forestry maintenance practices followed over the last 50 years or so.

The use of soil enzyme activities as early and sensitive indicators of management induced changes in soil fertility and stress is accepted since they are a direct measure of the enzymatic activity of proliferating micro-organisms. Oxidoreductases, transferases and hydrolases have been the most studied as potential indicators of productivity, sustainability and pollution. As most of the organic material in forest soil is of plant origin, soil cellulase and other hydrolytic enzymes could be used as a tool to measure activity (Lahdesmaki P. and Piispanen R., 1988). Hovland (1981) assumes cellulase activity to be a more effective indicator of decomposition than basal respiration, however Nordgren *et al* (1986) found the cfu's of bacteria capable of degrading cellulose to be even more sensitive indicators of heavy metal pollution. Enzyme activities were also

measured. In this study however, low count of populations possessing cellulolytic property was observed. Cellulases was generally found to be low. Since in the forest lots of leaf litter is created and a study by us (earlier study) has shown that fungal population is very good they may be the predominant cellulase producers. Since the samples were also collected in the rainy season conditions were more favourable for fungal population. Dehydrogenases could also be used as general indicator of microbial activity because the assay is very quick and shows a significant positive correlation with the number of micro-organisms in soils and this has been widely used to measure the catabolic activity in the soils (Skujins J.J. 1967). In this study correlation could be established with the enzyme dehydrogenase and microbial count. The dehydrogenases enzyme activity was found to be the highest in the kodivellamman forest soils with the activity in the kovai falls soils followed a close second proportioned to the microbial diversity thereby indicating not much of disturbance is there. On the other hand in the teak forest the microbial diversity is low and so is the dehydrogenase activity.

In general the inverstase activity seemed to be very good and was once again the lowest in the teak forest. Amylases were the highest in the kovai falls region while it was comparatively low at the kodivellamman forest soils. Invertases and amylases generally show similar trends. The type of vegetation's and thus the organic matter play a role in the activity of the enzyme. As is seen in the table and the descriptions of the sites the organic content and the vegetation cover in the two moist deciduous forests are greater while on the other hand in the teak forest soils the organic content is low and the vegetation is basically teak and rose wood. This could be the reason for low microbial activity in the latter than the former two soils. It has been reported that high concentration of various soil enzymes are found in less disturbed sites than the more disturbed ones (Dhruva Kumar *et al*, 1992).

The supply of mineralised C,N,P and S from soil organic matter, the decomposition of plant and animal residues and the maintenance of soil structure are all dependant upon the correct functioning of the soil microbial ecosystem (De Haan *et al* 1989). Therefore it is important to determine and predict the adverse effects of heavy metals and other pollutants on soil micro-organisms (Flemming C.A. and Trevors J.T., 1989 and Baath E., 1989). Heavy metal introduced into the soil persist and accumulate and there is concern about the increasing concentration of heavy metals in soils. It is agreed that accumulated heavy metal affects soil processes, soil microbial biomass and microbial activities (Brookes *et al*, 1984, Brookes *et al*, 1986b, Reddy *et al*, 1987 and Reddy *et al*, 1989). Although many reports concerning the enumeration and identification of micro-organisms and the distribution and concentration of heavy metals in marine and estuarine sediments have been worked on (Grieg R.A. and McGrath R.A., 1977, Walker J.D and Colwell R.R., 1975, Sylvester A.J. and Ware G.C., 1977), not much has been worked on native soils of the forest.

Pioneering work on the effects of metal toxicity on soil microbial populations used plate count techniques, which are highly selective for certain groups of micro-organisms (Olson B.H and Thornton I., 1982, Duxbury *et. al* 1983). In present study effect of heavy metals on the heterotrophic populations was assessed by treating soil micro-organisms with various concentrations of metals like, copper, zinc, lead and plated.

The effect of copper on the heterotrophic populations had a drastic impact. Even at 50ppm as copper, 3/4 of the diversity was wiped out (Table 3). At 100pm there was no further drastic cut in the OTU's but no growth was observed at concentrations more than 100ppm. Although there was significant difference in the OTU's, those present were represented in large numbers. When the effect of copper between sites were studied the microbial populations present in the teak forest soil Was found to be more affected. The tolerance towards lead seemed to be more when compared to copper and zinc. Although there was a drastic reduction in diversity assessed by OTU's at 250 ppm itself, growth was observed to 750 ppm with the members present in quite large numbers even at 750 ppm. While at 1000ppm, KF soil samples had growth, no growth was observed in the other two sites.

The effect of zinc on the bacterial populations showed a drastic cut in the diversity as assessed by OTU's at 250 ppm. Growth was observed even at 500 ppm with t he OTU's dropping even further. Those present were represented in good numbers. At 750 ppm there was only one type of bacterium present in the kovai falls soils. Growth was completely inhibited in the other two soils at this concentration.

Although there was a reduction in the diversity as measured by applying operational taxonomic units (OTU's), it was observed that the ones which occurred were found in large numbers, thereby indicating that the treatment of the soil micro-organisms gave rise to a selection process as well as that to the adaptation of the species. All disturbances result in lower taxonomic and genetic diversities of microbial communities, microbial populations within disturbed communities demonstrated capabilities for enriched survival under a variety of conditions, demonstrating capacity for generalised adaptations to ecological disturbances. This trend was observed for all the three treatments . In a review article on the effect of heavy metals on soil on microbial processes and populations- emphasis is placed on temperate forest soils- the relative toxicity of the metals on a (mg/gm soil basis) decrease was observed to be in the order Cu> Zn>Pb(Baath E., 1989)

In another study by Ohya *et al* on the effects of zinc on the soil micro flora by assessing the influences on biological and chemical turnover changes in the soil micro flora were followed by counting the microbes and measuring the contributions - viable bacterial number decreases noticeably within the first 24

hrs of the incubation. During the course of subsequent incubation, however, this number increases and a selection for zinc tolerance was suggested. In a study on the influence of heavy metals on soil microbial activities (Hattori H., 1992) copper, lead and zinc, the effect exerted by cadmium, copper was highly significant while that of lead was the least significant. This is the trend observed in this study too.

Heavy metal accumulation in sites results in resistance building in cells. A great number of studies have tried to establish a relationship between heavy metal resistance and antibiotic resistance in hospital and polluted environments (Nakahara *et al*, 1977b, Smith D.H., 1967). Nevertheless more studies were carried with strains isolated from natural environments, so among forest soils where there has been no selection, not much has been done. In one analysis of isolates for tolerance to mercury in agricultural soils with no known mercurial input resistance was observed (Redford *et al* 1971). In such settings resistant microbes may be very rare but they might come into much greater quantitative prominence after industrial and agricultural pollution. (Silver S. and Misra T.K., 1988)

In this study antibiotic resistance was found in many isolates of copper tolerant ones. In most of them multiple resistance was observed. This phenomenon was observed more in the isolates from the Kovai Falls region while in Teak forest soils it was low. Antibiotic resistance to zinc and lead were found to be comparatively low. So many isolates were found to carry multiple resistance to antibiotics in native soils was itself quite surprising. Heavy metal resistance may select plasmids which will determine not only the resistance to these agents but also the resistance to antibiotics, (Pohl et al, 1977). When the isolates of the major group Pseudomonas were taken for plasmid analysis we were not successful in obtaining them. The only probable reason could be since the soil samples were treated ones, selection of plasmid could not have occurred.

Biochemical assays of the isolates resistant to various metals showed that all the isolates which grew in the presence of zinc were gram negative. While for lead it was found that about 60% of the isolates were gram negative the rest were gram positive. The isolate tolerant to copper isolated from KV soil alone was found to be positive and the rest were gram negative. Predominantly most of the isolates tolerant to these three metals fell in the negative category. Other biochemical assays showed that most of the isolates fell into three major groups, Pseudomonas being dominant among them. Among the resistant strains that the negative strains were found to be more, could be because the gram negative rods have more moisture containing interiors which protect them from adverse action. This trend was also observed by Doleman P. (1979) for lead.

In general nitrogen cycling processes (Kabata Pendias A. and Pendias H., 1984) and especially nitrogen fixation has been shown to be sensitive to small

concentration of heavy metals in soil. In this study the heavy metals completely wiped out denitrifiers and nitrifiers to a great extent. While the ammonifiers were present in good numbers. The reason for this could be that the medium used to enumerate them was a rich medium containing enough peptone to the extent that the population was able to recover from the stress. This trend was also observed in free living heterotrophic bacteria which was considerably reduced in Russian soils contaminated with copper, zinc, or lead. Zinc at various concentrations (Letunova *et al*, 1985 and Skujins *et al*, 1986) found potential heterotrophic nitrogen fixation and was sensitive to additions of copper and chromium salts. Strong sensitivity of heterotrophic N_2 fixation activity to metals was also found in soils to which metal contaminated sewage sludges has been applied (Brookes *et al* 1984, Skujins *et al* 1986, Martensson A. M. and Witter E. 1990). Such findings indicate that free living N_2 fixing bacteria may be suitable as biological indicators of heavy metal toxicity.

Conclusion: The result suggest that the abundance and diversity along with the activity of micro-organisms increase in undisturbed soils and that methods based on the assessment of microbial diversity and activity could be used as an effective tool to monitor the ecosystem functioning.

Monitoring ecosystem health through macromycete diversity of Siruvani hills of Nilgiri Biosphere Reserve (NBR), Western Ghats: Present study: Arnold (1996) has said studying macromycete diversity is difficult in a chosen area since macromycete exhibit seasonality, sucessional patterns, periodicity in addition to certain species exhibiting hypogeal habitat. In this particular project, the main thrust is being given to unravel the potential of macromycetes diversity in biological monitoring rather than to the inventorying of the entire macromycete diversity. Consequently it was felt, for the purpose of monitoring, an apparent diversity of species involved in litter wood decomposition the occurrence of certain dominant species can be ideal and sufficient. Hence, this present study does not emphasise the need for identification up to their species level and special detection or isolation procedures which may handicap easy monitoring methods. More over checklisting entire macromycete faces another major hurdle namely paucity of experts studying their taxonomy and classification. This is the reason for employing the concept of OTU (Operational Taxonomic Units) since identification is not possible upto species level, presently and that too within short span of the project. More over these studies are ultimately aimed to propagate the findings in simplified packages so that the target groups in training such as school children or local people can handle these packages easily without any prior expertise. Consequently this study does not give much importance to the check listing of diversity *in situ* but to apparent diversity having significance in ecosystem maintenance and structure.

As in Pichavaram mangroves this study area does not face any apparent extraneous stress from air or soil. The most obvious effect is from deforestation

and various afforestation programmes. This results in many degraded areas mixed with plantation forests. Hence an attempt has been made to compare the macromycete flora of a complete man-made forest, a moderately disturbed forest and a relatively undisturbed forest type. Although some species are consistently associated with specific disturbed conditions this result can be used as a baseline information for monitoring the health In the event of other extraneous stresses like, S02, F, or pesticide pollution. Studies involving *in situ* (controlled settings) and ex *situ* (in the laboratory) response of fungal species to some stresses will be taken up if the support for this project is continued. More over these findings will be simplified so that special packages for the use of nonspecalists and local people will be prepared. This is a very important step for the effective implementation of biological monitoring.

Methodology: Sites and Collection: Three sites viz., undisturbed, partially disturbed and man-made forests were selected (disturbance refers to clearing of forest area). Collection of data regarding the colour and other morphological details were done on the spot. Fruit bodies were collected, dried and preserved for further identification. Since we did not have field station and laboratory, preservation of deliquescent fruitbodies could not be done. We do not have an artificial drier to dry the samples immediately after collection. Culturing of selected species proved futile since no inoculation chamber was available in the near by area. Nevertheless, data regarding morphology have been recorded on data sheets and enough photographs and slide films of live materials have been taken.

Sampling studies for spatial and temporal distribution of macromycetes: Since macromycete fruiting is highly coinciding with rainfalls, an initial survey to understand the fruiting pattern through monsoon, winter and summer seasons was initiated. In this regard, regular field visits at intervals of 40-50 days (12 trips) have been undertaken. During initial few trips we were also training the local youths from the Irula tribe who live with in the biosphere area and whom we usually take along with us in our forays as trackers. These youths have been occasionally collecting certain macromycete species which they cook and relish. They have some local name for them and have fairly good knowledge about their occurrence. This familiarity came in handy for us in our work and we only had to train them to record the date, time, and area of collection, ways of collecting them, make rough sketches of them record certain observable features such as colour, pileus morphology, collared or not, rhizomatous or not etc.. They have faithfully recorded some of the for collected specimens along with meterological details.

Search for macromycete occurrence was usually taken up following rains. Equal man hours was given to each of the selected area during all the field trips. Initial 6 trips were spent in understanding the fruiting patterns in the selected areas. To start with 10 x 10m quadrates were put in the selected areas to study the

distribution pattern. But it has been now realised that this size is not sufficient and longer belt transect of 10 x 500m dimension should be ideal for sampling. This quadrat size will be followed in the future sampling studies. Field characters such as the colour and size of the pileus, stipe and lamellae, presence or absence of annulus, rhizomorph etc., were noted from the fresh specimens. The colour terminology of Komerup and Wanscher (1967) was used excepting the deliquoscent and short lived. The specimens were dried for further study.

Results and Discussion: Production of macromycete fruit bodies is highly influenced by rainfall patterns and not merely by the quantum of rainfall alone. If the total rainfall is equally distributed into intermittent showers throughout a monsoon period the quantity fruiting and to some extent diversity tend to increase.

From our present study starting from July, August of 1995 (S W monsoon), we have the observations Agaricals include soil, dung, wood, litter inhabiting gilled fungi (Table 5). The genera encountered are given in Table . As many as 56 species belonging to 19 genera occurred totally throughout the year. Of these 40% of them occurred on soil and are mostly encountered in the initial showers of the monsoon. The remaining percentage of Agaricales which occurred on the fallen wood and litter are encountered during the later phase of the monsoon rains. About 10-20% of the initially occurring soil Agarics are encountered throughout the season where as the rest showed a declining trend and replacement by other soil agarics depicting a clear successional pattern. In the peak of the rainy season the small agarics belonging to Marasmiales with their minute fruit bodies extensively covered the litter and small twigs (Plate 1). A similar trend was noticed even during the south west monsoon. Percentage of soil agarics (Plate 2 & 3) generally declined during winter season and by summer none was present. Some wood decomposing agarics were present in the end of the south west monsoon and by summer they were absent. Soil agarics become predominant with the commencement of the monsoon rains because these fungi must be having enough mycelial biomass already in the soil which under favourable conditions start fruiting. Those that have been occurring on wood or litter have to generate enough biomass when their substrata become moist, from the resting spores etc., and then commence their fruiting. Similarly the break down of substrata like litter and wood is not simple and mediated through the action of many fungi. There are some times a group of different fungi i.e. Marasmiales and xylariales involved in the litter decomposition. Even among these groups there are certain species which actively take part in decomposition of a particular component of the litter followed by the other. In addition, the day/night temperatures vary considerably between SW and NE monsoon seasons. All these conditions result in a clear successional pattern in the fruiting of macromycetes.

Consequently a standardisation of fruiting patterns represented by occurrence, succession, periodicity was necessary, before one begins studying of macromycete distribution. This was the reason to spend many field days during monsoon rains to observe and understand the habitat, occurrence and distribution trends of macromycetes so that appropriate sampling methodology can be worked out that is suitable for all periods of fruiting. With regard to the spatial distribution of macromycetes, as the vegetational pattern changes the macromycete flora also show some change i.e. the production of ectomycorrhizal fruit bodies may depend on the distribution of their host in a forest area. Similarly the degree of litter accumulation and quantity of fallen branches must be influencing fungi occurring on them. So within a area one has to standardise the size of the quadrat before using them studying for distribution pattern.

In accordance with temporal successional pattern, it was observed that out of 56 species of agarics 40% occur on soil immediately after the initial rainfall followed by those occurring on other substrata. The diversity of agaricals generally tends to be high in the undisturbed areas during the initial SW monsoon rains where as in the end of SW monsoon and during winter the agarics diversity of partially disturbed areas equals that of the undisturbed area. Excepting for initial showers the man made forest (MMF) do not show any agarics occurring in this area. The reason for the increase in the diversity in the partially disturbed area can be attributed to the resurgence of litter and wood inhabiting Agarics only after a lag period from the beginning of the monsoon rains.

The other predominant group Gasteromycetes include genera like, *Cyathus Dictyophora, Scleroderma, Geastrum, Lycoperdon, Phallus, Rhizopogon* and *Pisolithus* (Plate 4). These fungi produce fruit bodies during the end of the south west monsoon and during north east monsoon. Some species like *Geastrum* and *Cyathus* occur on litter and on twigs respectively. Most of the other are soil inhabitants. *Pisolithus is* an ectomycorrhizal fungus occurring with eucalyptus.majority of the species belonging to OTU Gasteromycetes occur in the last stage of the SW and during NE monsoon rains. Changes in climatic condition such as fall in day temperature or any other factor may be responsible for their production during this period. They are normally encountered in the road sides or in the partially disturbed areas (Table 6). *Geastrum, Cyathus* species occur on the litter and twigs and species like *Dictyophora Phallus, Rhizopogin. Scleroderma, Lycoperdom, Pisolithus* etc. occur on soil especially in the disturbed areas. This occurrence remains unexplainable although this phenomenon can be a good indicator of forest disturbance.

The OTU *xylariae* includes 4 species of *Xylaria* (Plate 5). Two species of *Clavariadulphus, Daldinia* sp., *Nectria sp., Peziza sp.* and *Clavulinopsis* sp., etc. Some species of *Xylaria* become predominant in the middle of monsoon rains and occur on the litter and wood along with the agarics. They have significant role in

decomposition of litter. The other species belonging to this group are normally found during the north east monsoon. Their number declines from the end of the NE monsoon rains. This species belonging to *Xylaria* profusely occur on the littertwigs during the initial monsoon rains. They, along with the agaricales (Marasmiales) must be involved in the litter decomposition actively. The other xylariae members belonging to *Clavariadelphus, Clavulinopsis, Daldinia, Nectria* occur in partially disturbed areas, since some of them occur on wood which may require microbial action in succession. Species of *Peziza* and *Daldinia* occur even during winter. They slowly get dried and vanish in the peak summer.

The OTU polyporales includes brackets, polypores, hydnoids etc. The genera encountered are given in the Table 7 . Excepting for the leathery, perennial polyporales, like *Ganoderma, Polyporus* etc. most of the polypores and bracket fungi like *Polystictus* (Plate 6) were formed during the end of the north east monsoon season and they remained till the end of the winter season. Most of the bracket fungi occurred in the fallen logs and not on the soil signifying their important role in nutrient recycling. As many as 10 species are commonly encountered (Table 2). The leatherly fruit bodies of perennial polypores occur on trees even in the undisturbed forests. Where as the annuals like brackets and other polypores are formed during the end of the SW monsoon rains on dead logs and are active even till the end of winter season. Their diversity seem to be more in the partially disturbed areas which can be explained by the presence of more fallen logs and dead trees in the partially disturbed areas.

Boletes are rarely occurring compared to the gilled macrofungi. There are two species encountered on dead logs during later phase of south west monsoon rains.

Conclusions:

These findings have resulted from our 70 days of field work totally from 12 trips. It may be premature to arrive at any major conclusions since these sort of studies must be done atleast for three consecutive years to amve at any meaningful conclusion. Nevertheless, based on the above observations it may be recommened that the following characteristics of certain species having some **indication values.**

1) The occurrence of large numbers of tiny Marasmiales and Xylaria species on litter during the initial rains is a striking phenomenon. This occurrence can serve as an indication of healthy soil conditions free of any stresses whether it is undisturbed or partially disturbed area.

2) The constant occurrence of the species belonging to the OTU Gasteromycetes especially that of *Geastrum, Cyathus, Lycoperdon* and *Pisolithus* in the partially disturbed areas only is another important aspect.

This phenomenon can be used to monitor the disturbance in the core regions and as well as to monitor the general health of the soil environment.

3) Occurrence of maximum diversity of macromycetes including agaricals, Polypores, xylariae (Woodlitter decomposing) and Gasteromycetes in an area can be an indication of partially disturbed forest condition. This indication can be useful in identifying areas of high pristinity and the extent of forestry practices that have existed earlier.

These observations can also used as baseline information regarding the apparent diversity of macromycetes against which effects of various stresses on this diversity can be monitored. As we already mentioned earlier changes in the macromycete diversity may have significant effect on the ecosystem as a whole. We have had a lesson from the Tsuga forest of W. Germany where the total destruction of the forest was preceded by a decline in the mycorrhizal fungi. A general macromycete diversity as we have reported can be helpful in monitoring the general health of the Siruvani Hills forest (both disturbed and undisturbed). Hence macrofungi can be considered as suitable "surrogates" for the overall health of the ecosystem. But as Hawksworth (1990) states that "**the implicatiion of lichen loss may be considerable for other organisms and for certain environment but the extend can not be estimated with any confidence because of lack of baseline surveys and ecological research**", these studies must be conducted for three to five years before any such recommendations can be made.

We have also prepared a macromycete calendar (Table 9) for this area and plan to prepare a field guide for macromycetes in local languages. This calendar and field guide will have all the details as mentioned in the Table and hence can be user-friendly even for non specialists.

Future plans:

If this project gets the continued support from DBT, will have the following objective to complete the study and in preparing a suitable biological monitoring method for Siruvani Hills.

1) Detailed sampling studies using long belt transects (10x500m) to understand their spatial and temporal distribution patterns with reference to the vegetational density of the Siruvani Hills.

2) Identification and culturing of species having important ecosystem function such as litterlwood decomposition and simultaneously study their potential for biotechnological applications.

3) Testing the response of certain fungal guilds species to stresses like acid rain, flouride, pesticides etc. under controlled settings in the natural environments.

4) Similar studies under laboratory conditions.

5) Preparation of simplified techniques from these findings. Preparation of various visual training modules packages organizing training programmes for school children and local people.

6) Conduct slightly higher level training for pollution Control board personnel, Forest personnel research scholars and others involved in the Conservation of Biodiversity.

Problems faced and Recommendations:

First and foremost thing to enable our future studies will be to establish a field station with minor laboratory facilities, research scholars and a vehicle to go to forest areas. We had the problem of timing field studies with the rainy seasons. Most of the collected specimens perished before they could the processed and dried since we did not have driers and a place to process them. Attempts of culturing them proved futile since we did not have inoculation chamber facility. All these problems could be solved by establishing a field station cum laboratory near the proximity of the study area. Since we have to do our studies in the areas infested with elephants, leeches and other wild animals we must have our own transport.

A MOU with the forest department PCB (state) and other monitoring agencies and like minded research institute Overseas/National so as to exchange data and to organise a coordinated research approach. The areas under study must be freely accessible. In this regard clear permission from the forest departments must obtained to enter collect data and conduct research experiments in the study areas.

Ecological continuity status of various forest types in Siruvani hills - a study using lichen community as indicators

The current research on the lichen diversity and its distribution pattern, aims in identifying the ecological continuity and forest disturbance of various sites/Land Scape Element types within Siruvani hills. These forests were not surveyed earlier for its lichen diversity and hence the present study forms the initiative to document the lichen wealth of this area. The various vegetation types within Siruvani hills (See Details about study site in the general introduction) were surveyed (Table 6) through regular field trips, for its lichen

diversity. Almost equal time was spent in surveying in all the vegetation type studied.

Lichens as Biomonitors of Siruvani Hills: Methodology: As a first step a detailed check - list of lichens of Siruvani hills was prepared. As a second step the lichen phytosociological federations were identified by studying the substrate preferences of lichen colonies (viz. bark - base, trunk and the canopy in case of trees; rocks, soil, leaves of various plants, wood, moss). The canopy lichens were recorded by analysing the fallen twigs and in some cases climbing up to the canopy. Lichens were classified in to 3 major groups with regard to exposure to sun (viz. fully exposed - 1, moderately exposed - 2 and fully shaded - 3), humidity, age of the substratum (by noting the GBH of the tree), bark / rock surface nature, inclination of surface, aspect were also recorded.

Observation and Result: The above observations on Siruvani hill lichens recognises atleast 9 lichen phytosociological federations within the study site. These lichen federations also tend to change when there are changes in the key factors determining lichen development in a particular ecosystem. The lichen federations recognised along with their characters in Siruvani hills is given in Table 7.

Discussion: State of Dry deciduous forest in Siruvani hills: This forest type occur in two localities 1. Filter house area, 2. Kovai falls area (Table 7).

The common lichen federations identified in these LSE type are Graphidion, Leprarion in Filter house area and Lecanorion in Kovai falls. The Graphidion, Leprarion prefers shade and moisture loving lichen federations. The forest type near filter house being in a protected area is not disturbed much and provide an ideal environment for the development of Graphidion and Leprarion federation. However the Kovai falls area is much affected due to its vicinity to human settlements and ecotourism. One can observe more open areas in this LSE type and thus Lecanorion federation (a federation dominate in well lit areas) dominate. Thus it is understood that the Kovai falls area is disturbed.

Status of Tropical Wet Ever Green forest in Siruvani hills(Table16)**Thannerpallam Area:** In this area one can distinguish three lichen federations (viz Myriotremion, Physodion and Laborion). The Myriotremion federation prefers to grow in moderately lited areas and occupies the substrates almost near the road sides and foot paths. However in well lit areas due to tree fall the lichen federation Physodion dominates. The interior of this forest type shows typical Laborion as an indication of an old forest.

Muthikulam lower tank - on west bank of Siruvani reservoir: (Table16). This forest type possess two lichen federations (viz. Trichotherion, Laborion with *Dictyonema, Pannaria, Phyllopsora* and *Leptogium*). The lichen federation

Trichotherion is composed of moderately light, moisture loving Parmelioid Taxa. The forest - Grass land ecotone provides a favourable condition for this federation to grow. The interior of this forest type possess Laborion but with that of a different species composition *(Dictyonema, Pannaria, Phyllopsora and Leptogium)*. The change in the species composition in the Laborion federation in this site indicate the changes in this forest type earlier. The interior though resembles a near normal site, during the British period timber was extracted from this area ultimately affected the lichen vegetation due to clearing. However after the departure of the British this area is closed for any further forest clearings. This facilitated re colonisation of lichen flora on the remaining forest trees, a reason responsible for an atypical Laborion federation.

Muthikulam and Singapara area: (Table 7) In both the areas the road and path side trees and rocks possess the Myriotremion and Usneion lichen federations. These federations replace the Laborion in moderately lit area. The Soil layers exposed due to excavations are dominated by the sun loving Cladonion. However these lichens are absent in the forest floor due to the coverage of humus. A typical Laborion federation can be located in the interior places of this forest type indicating the ecological continuity of this site.

Pulmedu: (Table 7). This area possess two types of land scape elements (viz. a dominant grass land in the elevated region and STW Ever Green forests in the east and west facing slopes. The grass land contain small rocks on which saxicolous light loving lichens like *Buellia sp, Caloplaca saxicola, Diploschistis scruposes, Parmelia cinerascens, P. saccatiloba* and P. *xanthia* occur. These lichens are well established over their substratum. The colony size of these lichens ranges from 5 - 30 cm, proving their long presence in this site. The soil is well covered by the grasses and the terricolous lichen communities are absent. The forests in the adjoining slopes possess typical Lobarian communities with *Lobaria japonica, Parmelia wallichiana, Pertusaria amara, Ramalina pacifca* etc., denoteing the long ecological continuity of this site.

Man made forests : Man made forests occur in the *foot* hills and in certain west facing slopes within the study site. These forests are monoculture of either *Tectona grandis* or *Dalbergia sisso.* The lichen *Bacidia sp. is* only recorded from these forests.

Conclusion: The above study is the first of its kind to use the lichen diversity and its phytosociological federations in Sinuvani hills. As a result the hither to unstudied localities were surveyed for its lichen wealth. The observation on the lichen federations, though through floristic surveys clearly indicate the intricate relationship of lichen communities with their respective ecosystems. The changes within the lichen communities were observed whenever the structure of the vegetation type get modified, is used here to determine the ecological continuity of the site studied. The changes in the lichen community structure

occurring in the Southern Tropical Rain forest type at Muthikulam lower tank, clearly indicate the changes occurred in this site five decades ago. When the lichen diversity is compared with similar forest type surrounding this area, the overall lichen diversity is similar. However the key species belonging to Lobarion like *Lobaria japonica, Sticta sylvatica* and S. *limbata* are replaced by members of *Dictyonema, Pannaria, Phyllopsora* and *Leptogium* proving the sensitiveness of lichens to the forest disturbance occurred earlier.

Lichen phytosociological studies to monitor ecosystem continuity has received no attention in India so far as compared with Europian countries. The present study is mainly based upon the lichen floristic observations. However more details on lichen phytosociology is required to identify forest continuity in a more precise manner. This can be possible only through various ecological sampling methodologies to quantify the distribution pattern of these symbiotic epiphytes, which will be carried out as a future work. The vegetation types within Siruvani hills is characterised by an interesting and varied flora, particularly regarding the lichens. Records of exceptional floristic interests include the first reports of *Dictyonema sericeum* - a basidio lichen, *Clathroponina olivacia* to India and saxicolous *Ponina interstes* to Indian mainland.

Significant Results

Soil Biological Criteria :

- Ecotoxicological indicator for heavy metal contamination has been identified and tested in the two study sites
- @ 313 isolates have been grouped according to their phenotypic characters
- RAPD analysis of 90 species has been completed and grouped according to their profile
- Novel strains such as alkaline amylase, protease, lipase, cellulase, heavy metal resistance strains and DNase producers have been identified by biochemical assays
- Purification of alkaline amylase is under way
- Intensive screening of other enzymes is under way for applied purposes
- All cultures are being catalouged and maintained in the culture collection at the Micorbiology Lab, MSSRF.

Macromycetes diversity:

- A survey of seasonal production of macromycete fruit bodies over a period of one year has been carried out
- About 150 species have been collected so far
- Indicators for healthy forest soil conditions were litter decomposition is very active, has been identified and members of marasmiales and xylariales
- Gastromycetes members can serve as indicators of human interference's (forest disturbances)
- Further study is on to develop a suitable sampling methodology to understand their distribution pattern over time and space

Lichen diversity:

- About 1500 specimens collected and 150 species have been identified
- Environmental factors affecting lichen vegetation were recorded
- Lichen phyto-sociological association identified with reference to near-normal, semi-disturbed and disturbed sites
- All lichen species collected is being catalouged and maintained as herbaria in the culture collection at the Micorbiology Lab, MSSRF.

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Legends

Soil Biological Criteria :

Mycology/Macromycetes

Fig 1 **Distribution of various bacterial guilds in three different zones of Pichavaram**

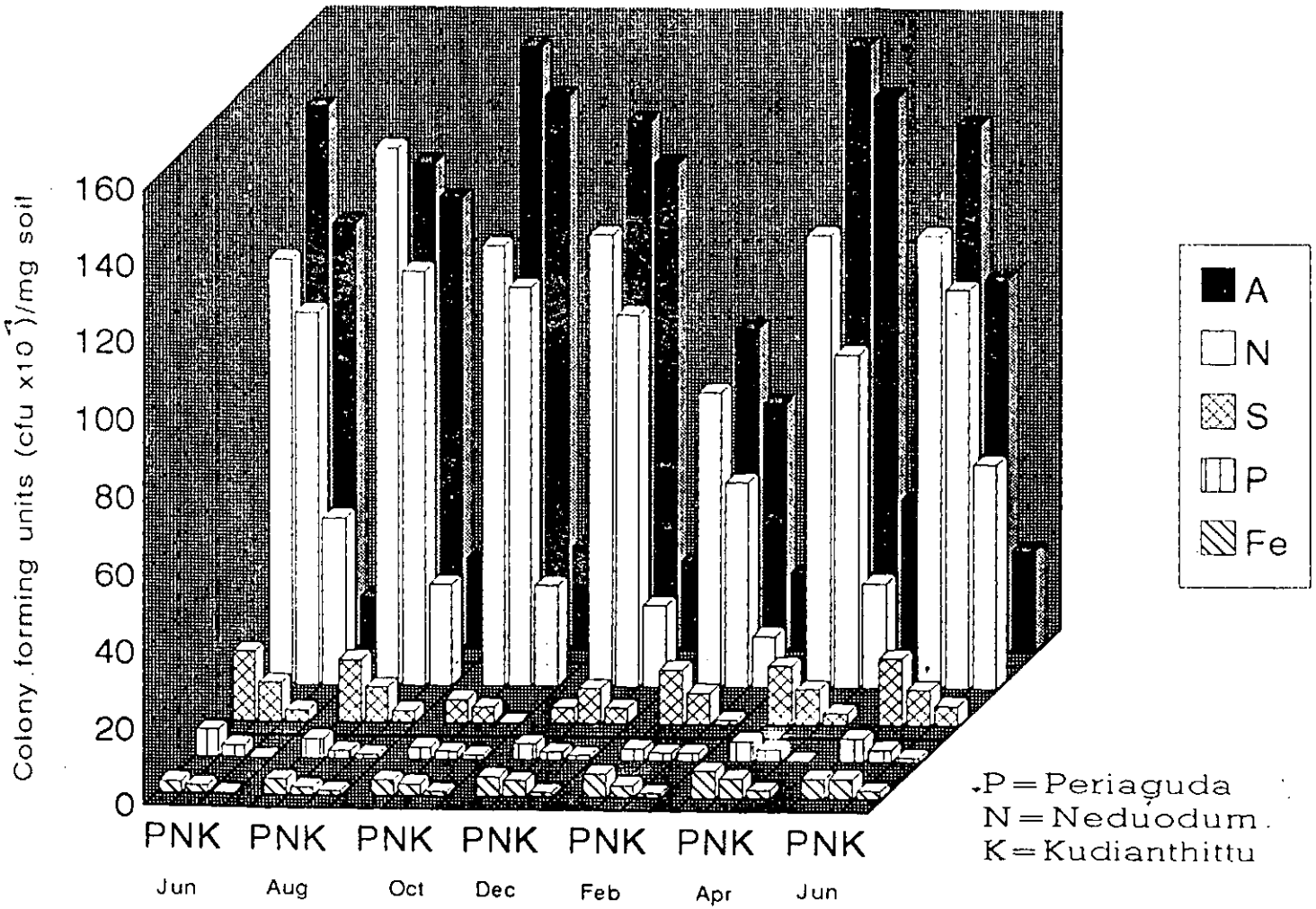
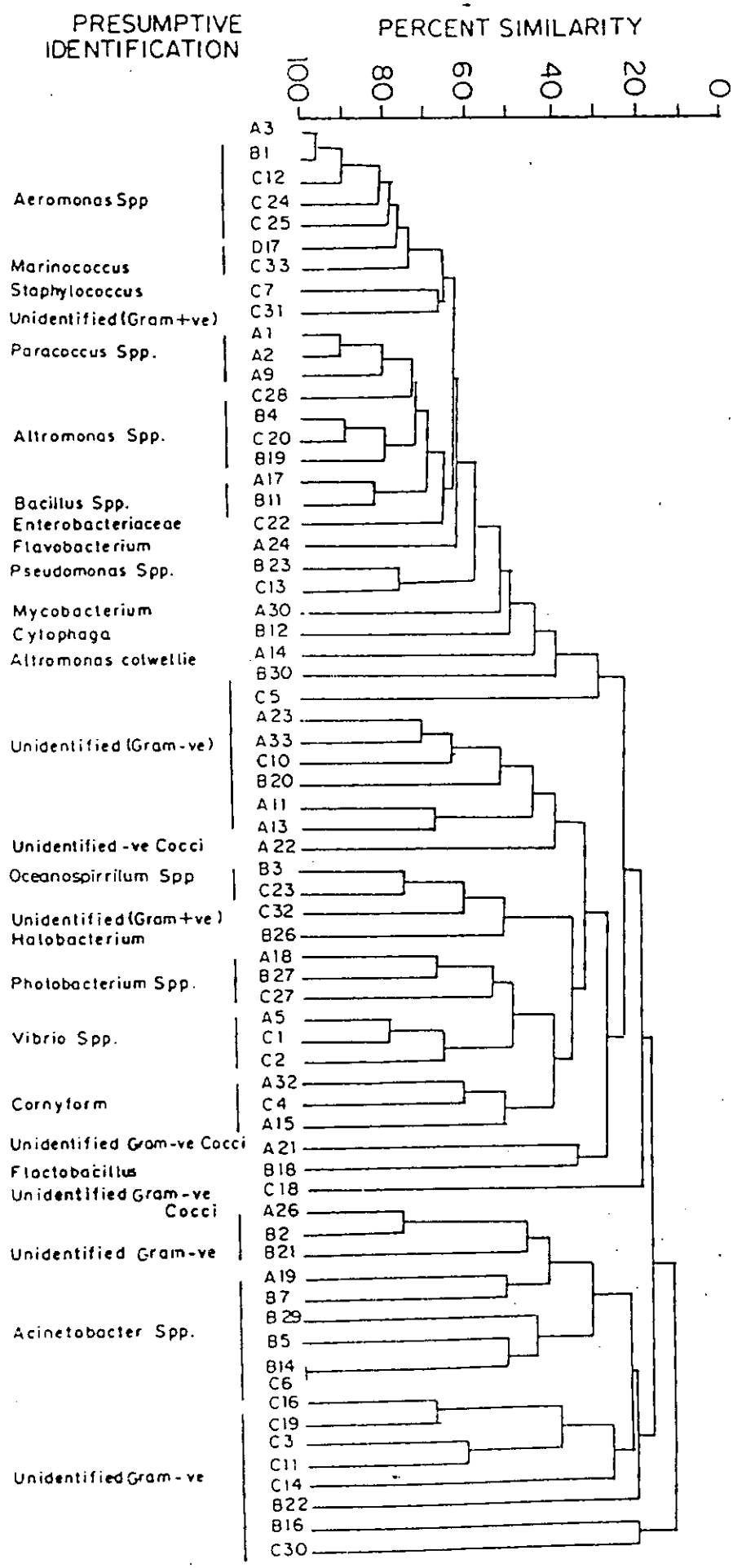


Figure 1. A simplified dendrogram of bacterial isolates (OTUs), based on phenotypic characters, from Pichavaram Mangrove Ecosystem



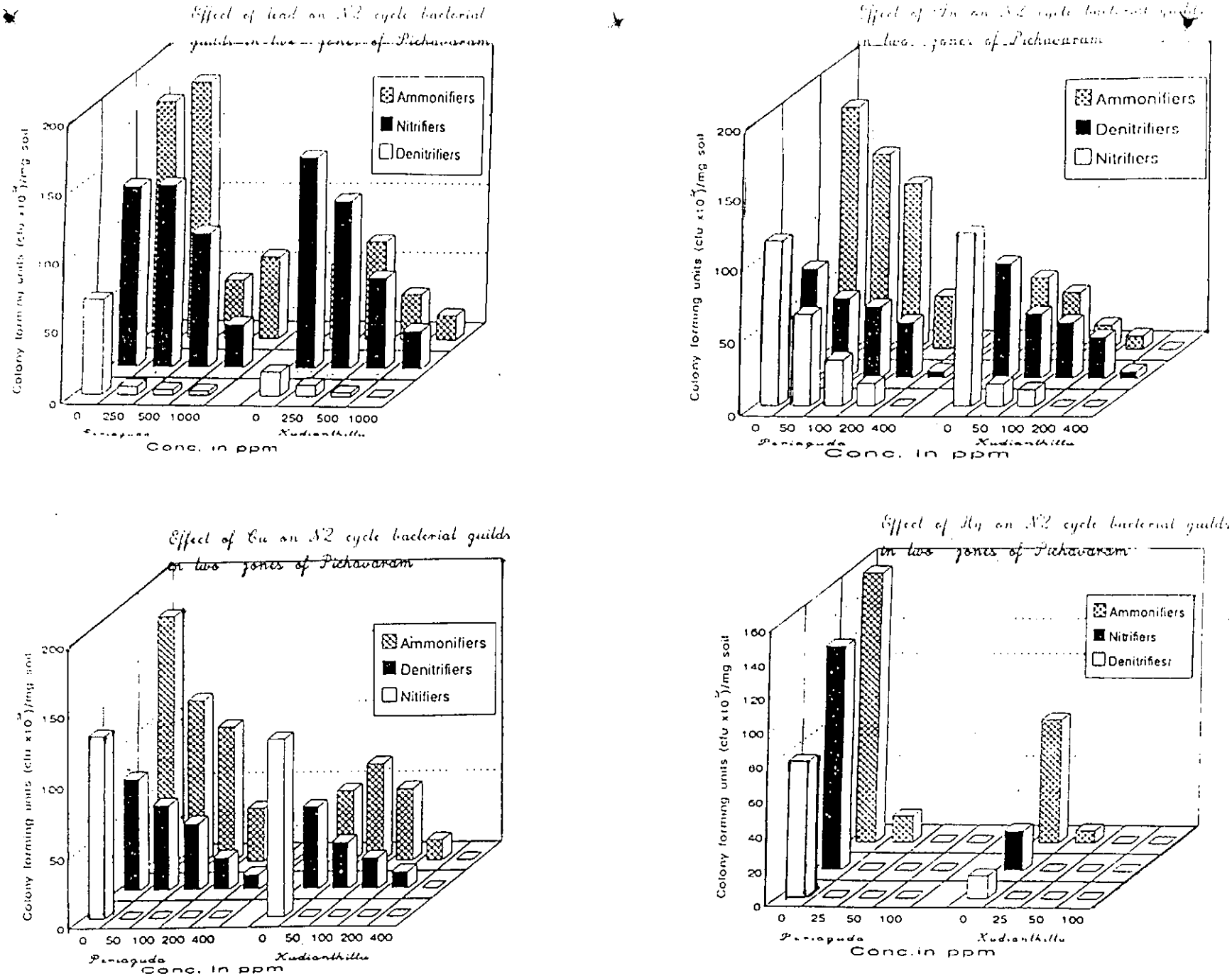


Fig 3 Effect of heavy metals on the bacterial guilds in the different zones of Pichavaram (a) lead, (b) zinc, (c) copper and (d) mercury

Table 1 Characters coded for analysis

Colonial Morphology [30]

elevation	surface	form	edge	colour	pigments	growth
flat	matt	punctiform	crenate	white	non-diffusible	no growth
raised	shiny	circular	smooth	off-white	diffusible	ring
convex	waxy	rhizoid	irregular	light yellow		pellicle
pulvinate	opaque	irregular		yellow		even
umbonate	glossy	scalloped		orange		slight turbitity
		mucoid		pink		moderate turbitity
		transparent		brown		heavy turbitity
		translucent				

Growth in liquid media [7]

Micro-Morphology [17]

cell form	cell end	arrangment	motility	endospore	staing reaction
rods	rounded	singles	motile	present	gram positive
coccus	tapering	pairs	non-motile	absent	gram negative
branched		chain			gram variable
filaments		cluster			
filament					
[branched]					

Biochemical characters [44]

Glucose metabolism	Hydrolysis of	Acid from	Utilisation of	Miscellaneous
fermentative	caesin	glucose	glucose	catalase
oxidative	starch	fructose	fructose	oxidase
alkaline reactin	gelatin	sucrose	sucrose	urase
no reaction	tween 20	galactose	galactose	DNase
	tween 40	mannitol	mannitol	ONPG
		arabinose	arabinose	nitrate reduction
		glycerol	glycerol	ammonification
		xylose	xylose	indole production
		insitol	insitol	citrate utilization
		maltose	maltose	methyl-red
		starch	starch	voges-prokauer
		sorbitol	sorbitol	H_2S production

Numbers in parenthesis indicate number of characters analysed

TABLE 2 ENUMERATION OF MICROBIAL POPULATIONS* IN THREE DIFFERENT SITES

Sites	THC	Ammonifiers	Nitrifiers	Sulphur	Iron	Phosphorous
KF	5.21	5.35	5.30	2.74	2.33	2.36
KV	5.28	4.83	4.82	2.65	2.49	2.41
TF	3.53	3.39	2.20	1.36	1.51	1.12

* - $\log_{10}$ Cfu gm^{-1} soil

KF - KOVAI FALLS FOREST

KV - KODIVELLI AMMAN FOREST

TF - TEAK FOREST

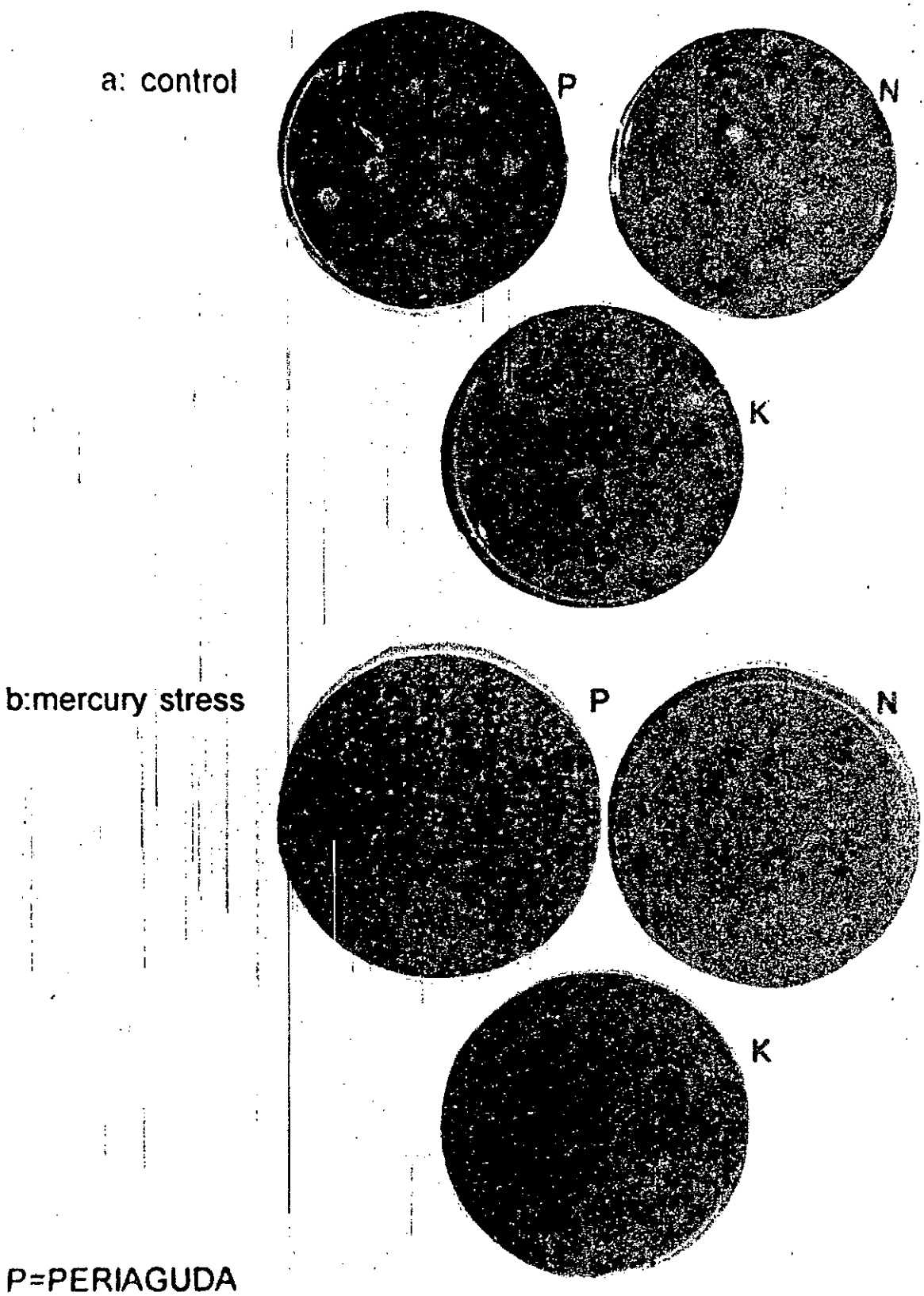
THC - Total heterotrophic count

Table 3 Effect of Heavy Metals on the Nitrogen guild of the Siruvani hills

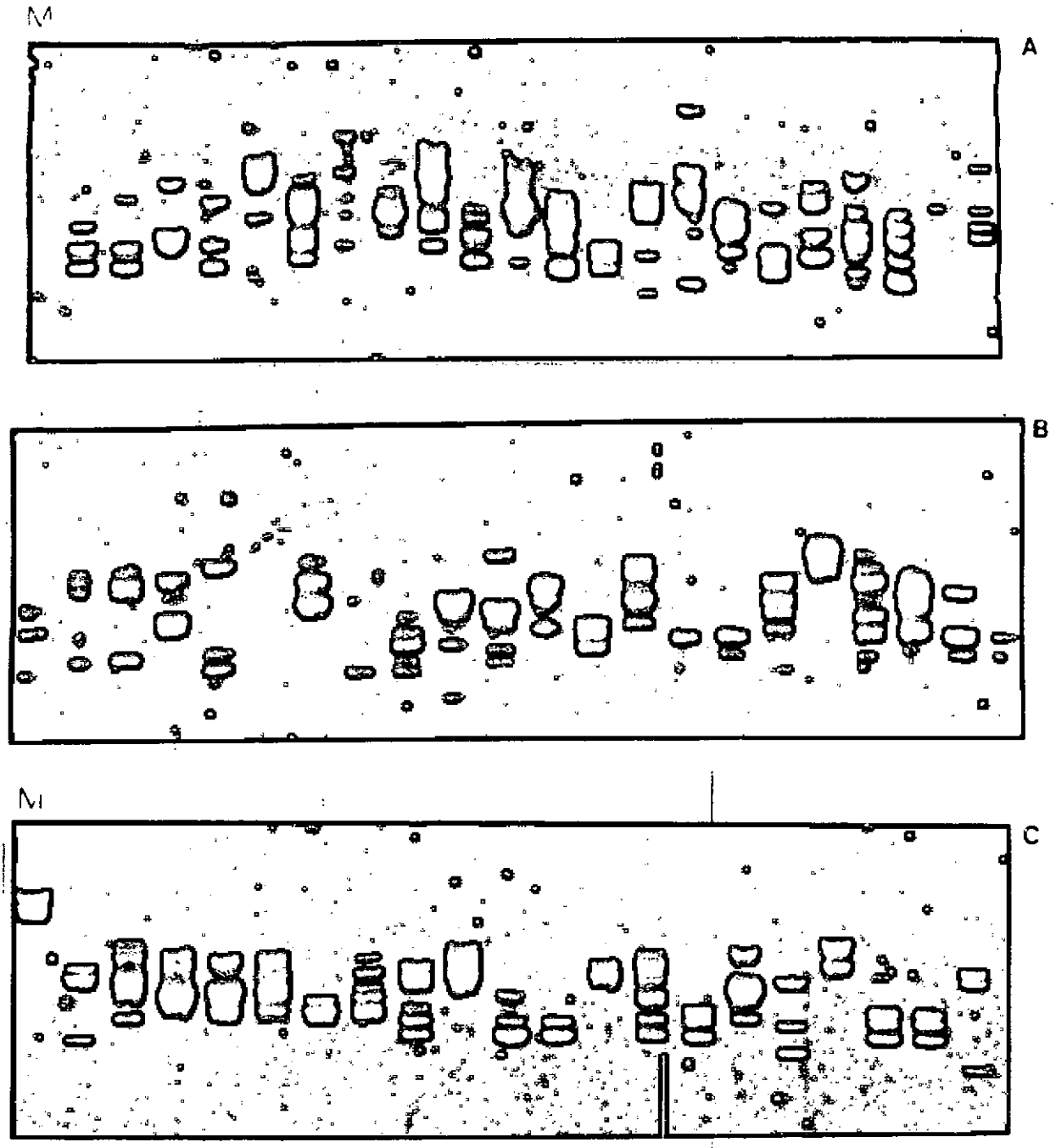
Functional Group	Sites	Concentration of Heavy Metal in ppm			
		Hg 25	Cu 100	Zn 750	Pb 750
Ammonifiers	KF	-	++	++	++
	KV	-	++	++	++
	TF	-	++	+	+
Nitrifiers	KF	-	-	-	+
	KV	-	-	-	-
	TF	-	-	-	-
Denitrifiers	KF	-	-	-	-
	KV	-	-	-	-
	TF	-	-	-	-

KF - Kovai Falls Forest
KV - Kodivelli Amman Forest
TF - Teak Forest

TOTAL HETERORTROPHIC POPULATIONS IN THE THREE DIFFERENT ZONES IN PICHAVARAM MANGROVE ECOSYSTEM

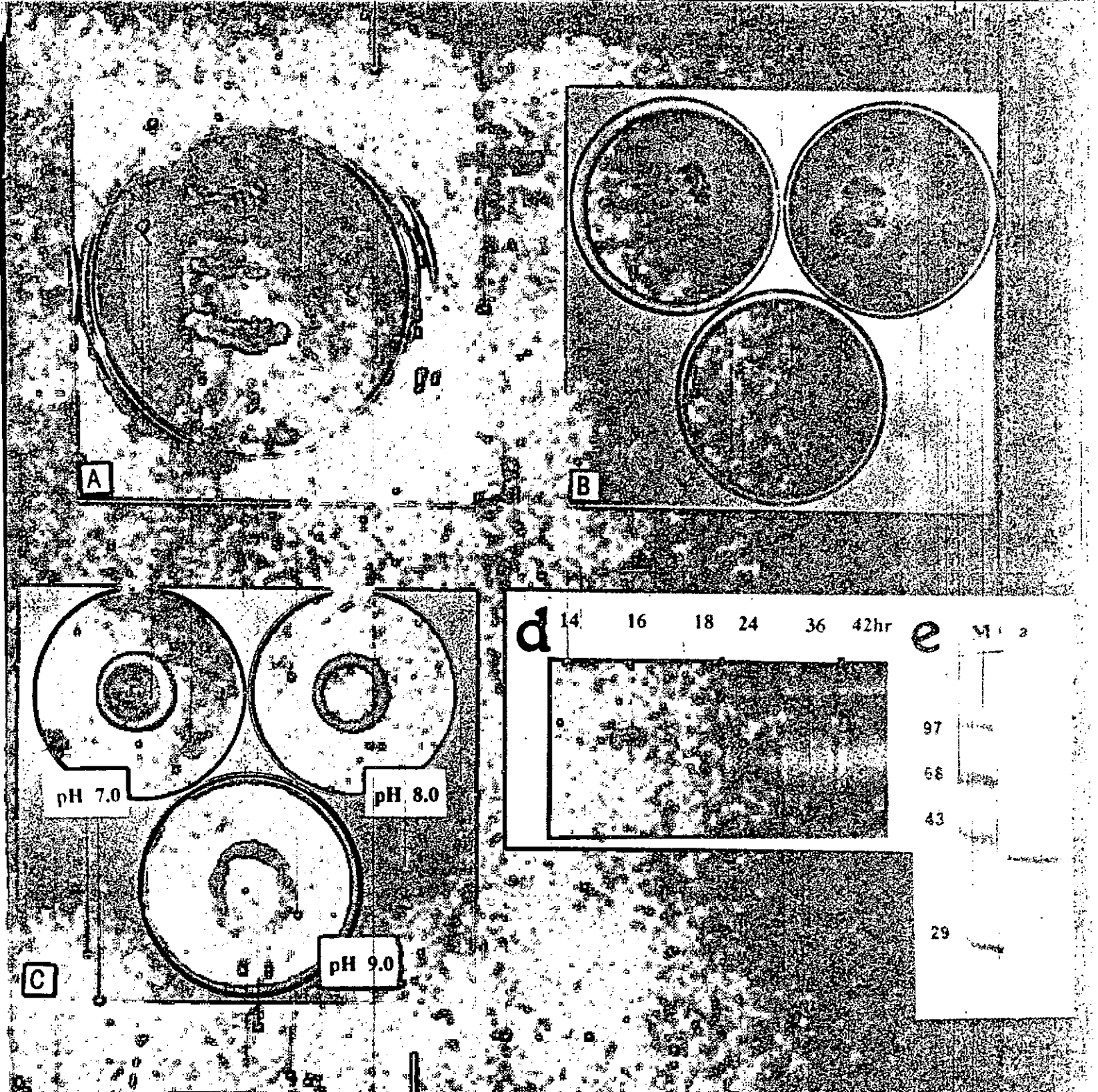


P=PERIAGUDA
N=NADUVODUM
K=KUDIANTHITTU



Profile of 64 isolates **from Pichavaram** Mangrove Ecosystem using Random Primer OPA-03

M=Marker [DNA: Hind III Digest]; A- Periaguda, B- Kudianthittu and C- Neduodum



a. Isolates showing Phosphate solubilising activity

b. Isolates showing DNase actvity

c. Production of Alkaline amylase by *Altromonas* sp. at different pH.

d. Changes in isozymes pattern of alkaline amylase by culture time

e. Lane a - SDS-PAGE of the alkaline amylase visualised by Coomassie brilliant blue staining.

lane M, Molecular marker.

Table 1. Mycoflora of Mangrove Litter (Inter tidal) Pichavaram

Season	Area	Percent occurrence of fungal species												Total no. of colonies
		1	2	3	4	5	6	7	8	9	10	11	12	
	P	10	-	<5	74	5	-	-	-	-	5	-	-	4050
Summer	N	5	<5	10	64	7	<5	<5	<5	-	6	5	-	1250
	K	<5	-	-	15	9	-	-	-	<5	10	6	57	371
	P	15	5	5	70	<5	-	-	-	-	<5	-	-	9266
Monsoon	N	5	<5	<5	60	8	10	<5	-	-	<5	<5	10	789
	K	5	<5	10	<5	20	<5	-	<5	5	10	20	25	351
Post	P	<5	<5	-	49	<5	<5	<5	-	<5	40	<5	-	2266
Monsoon	N	-	-	-	56	<5	-	-	-	<5	26	7	5	700
	K	-	-	-	-	25	<5	-	<5	<5	13	-	55	208

P - Periaguda; N - Nedu odum; K - Kudianthittu

1 - *Aspergillus flavus* 2 - *A. fumigatus* 3 - *A. nidulans* 4 - A. niger
5 - *A. terreus* 6 - *Chaetomium globosum* 7 - *Chaetomium sp.* 8 - *Curvularia lunata*
9 - *Fusarium sp.* 10 - *Pencillium citrinum* 11 - *P. oxalicum* 12 - *Trichoderma viride*

Table 2. Fungal species, per cent occurrence and their capacity for cellulase production based on CMC utilization

Fungal species	% occurrence	Activity @
Aspergillus flavus	8%	0.193
A. fumigatus	<5%	0.613
A. nidulans	<5%	0.653
A. niger	50%	1.000
A. terreus	9%	0.908
Chaetomium globosum	8%	0.259
Chaetomium sp.	<5%	0.389
Curvularia lunata	<5%	0.231
Fusarium sp.	<5%	0.410
Pencillium citrinum	10%	0.532
Pencillium oxalicum	<5%	0.840
Trichoderma viridie	10%	0.614
Unidentified sp 1	<5%	0.219
Unidentified sp 2	<5%	0.028

@ - OD ml^{-1} culture filtrate

Table 3. Effect of HgCl2 on mycoflora

Study area	Treatment	No. of colonies	% reduction
	Control	272	-
P1	5ppm	121	65.6
	10ppm	153	43.8
	25ppm	84	69.2
	Control	177	-
P2	5ppm	125	29.4
	10ppm	84	52.6
	25ppm	28	84.2
	Control	100	-
N1	5ppm	74	26
	10ppm	56	44
	25ppm	11	89
	Control	272	-
N2	5ppm	211	22.5
	10ppm	104	61.8
	25ppm	19	93.1
	Control	148	-
K1	5ppm	85	42.6
	10ppm	39	73.7
	25ppm	3	-
	Control	21	-
K2	5ppm	19	9.6
	10ppm	16	23.9
	25ppm	4	81

Table 4. Effect on Endosulfan on mycoflora

Study area	Treatment	No. of colonies	% reduction
	Control	545	-
P1	0.01	504	8
	0.05	373	31.6
	0.10	452	17.1
	Control	680	-
P2	0.01	554	19
	0.05	437	36
	0.10	416	39
	Control	273	-
N1	0.01	262	4.1
	0.05	258	5.5
	0.10	183	33
	Control	636	-
N2	0.01	565	11.2
	0.05	398	37.5
	0.10	478	25
	Control	626	-
K1	0.01	465	27.2
	0.05	513	19.1
	0.10	406	35.2
	Control	253	-
K2	0.01	171	33.5
	0.05	242	4.4
	0.10	165	34.8

Table 5 Occurrence of macrofungal taxa through different seasons at Siruvani Hills

Season	Site	Agarics				Gastero mycetes	Polypores	Xylariae		Boletes
		S	W	L	D	S	W	L	W	W
S. west monsoon Jun-Sep	Und	24	2	1	-	-	1	1	1	-
	Pd	15	3	2	1	1	2	1	1	1
	MMF	2	-	1	-	-	-	-	-	-
	Total	**41**	**5**	**4**	**1**	**1**	**3**	**2**	**2**	**1**
N.east monsoon Oct-Nov	Und	8	6	-	-	-	2	1	2	-
	Pd	9	7	2	2	8	6	2	4	2
	MMF	2	-	1	-	-	-	1	-	-
	Total	**19**	**13**	**3**	**2**	**8**	**8**	**4**	**6**	**2**
winter Dec-Feb	Und	2	1	-	-	-	2	-	-	-
	Pd	4	6	-	3	1	8	-	2	-
	MMF	-	-	-	-	-	-	-	-	-
	Total	**6**	**7**	**-**	**3**	**1**	**10**	**-**	**2**	**-**
summer Mar-May	Und	-	-	-	-	-	1	-	-	-
	Pd	-	-	-	-	-	3	-	1	-
	MMF	-	-	-	-	-	-	-	-	-
	Total	**-**	**-**	**-**	**-**	**-**	**4**	**-**	**1**	**-**

Und-undisturbed; Pd-partially disturbed (due to human interference);

MMF- manmade forest; S-soil; L-litter; W-wood;

Table 6 Macromycete calendar and field guide for Siruvani Hills*
(under preparation)

Season	Species	Identification features	When to look for	Where to look for
SW monsoon	Marasmiales Xylariales	Small brownish Thread like	during rain fall	on litter
SW monsoon	Soil Agarics (see list)	mushrooms	immediately after rain fall	widely distributed
Sep-Oct	Gasteromycetes	stink horns, earth star, puff balls, bird's nest	shortly after showers	only in the disturbed areas on soil/ litter
Winter	Wood decomposing	bracket fungi (leathery)	almost through out the year	disturbed areas (mainly)

* in local language with illustrative charts and photographs for easy identification

Identification features

colour **size** **habit** **usage** **odour** **substrata**

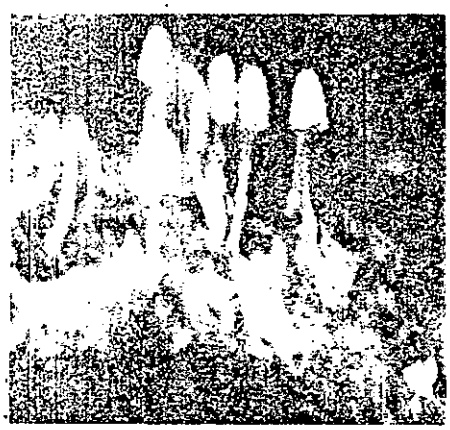
cap stalk cap stalk solitary groups poisonous edible medicinal

Table 7 Different genera of macromycetes, grouped under five Operational Taxonomic Units (OTU) occurring in various regions of Siruvani Hills

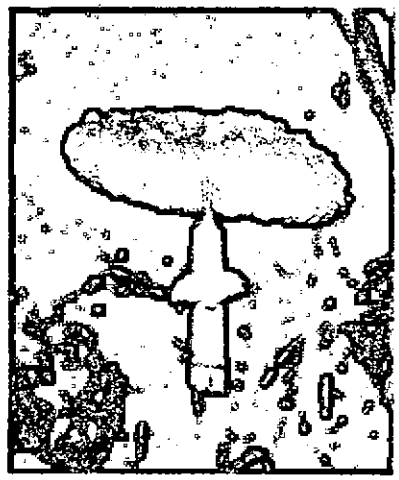
Agarics	Gasteromycetes	Polypores	xyalriae	Boletes
Agaricus (s)	*Cyathus* (bird's nest)	*Ganoderma*	*Clavariadelphus*	*2 unidentified species*
Agrocybe (l & s)	*Dictyophora* (stink horn)	*Fomes*	*Clavulinopsis*	
Amanita (s)	*Geastrum* (earth star)	*Hydnum*	*Daldinia*	
Collybia (l & s)	*Lycoperdon* (puff ball)	*Polyporus*	*Nectria*	
Conocybe (l & d)	*Phallus* (stink horn)	*Polystictus*	*Peziza*	
Coprinus (d & s)	*Pisolithus*	*Schizophyllum*		
Gymnopilus (w)	*Rhizopogon*	*unidentified* (jelly)		
Inocybe (s)	*Scleroderma*			
Lepiota (s)	*unidentified* (globose)			
Macrolepiota (s)				
Marasmius (l & w)				
Melanotus (w)				
Mycena (l & s)				
Panaeolus (d)				
Phaeomarasmius (w)				
Pleurotus (w)				
Psathyrella (l & s)				
Psilocybe (s & d)				
Termitomyces (s)				

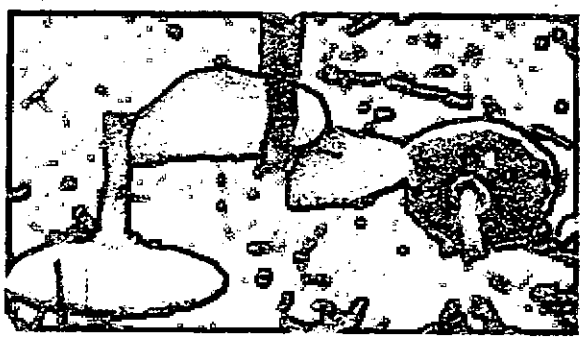
s-soil; l-litter; d-dung; w-wood

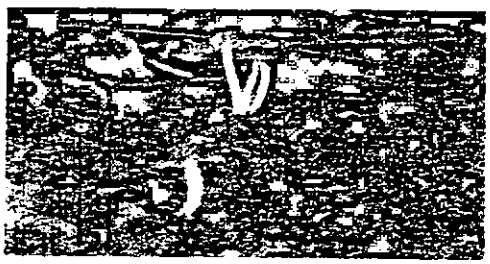
OTU Agaricales on wood - Siruvani Hills



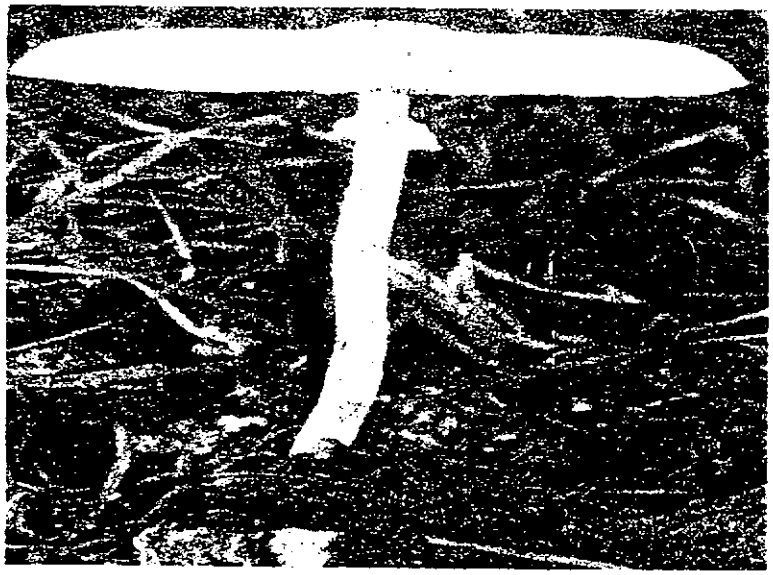
OTU Agaricales on soil- Siruvani Hills

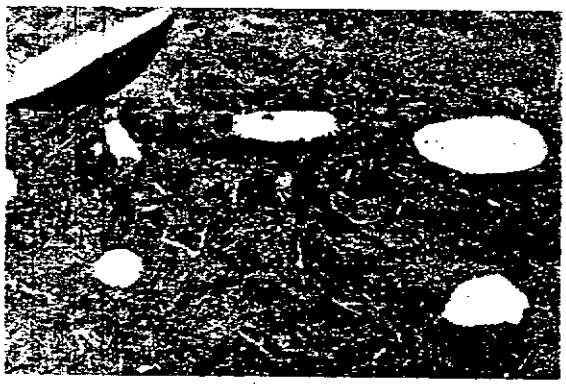




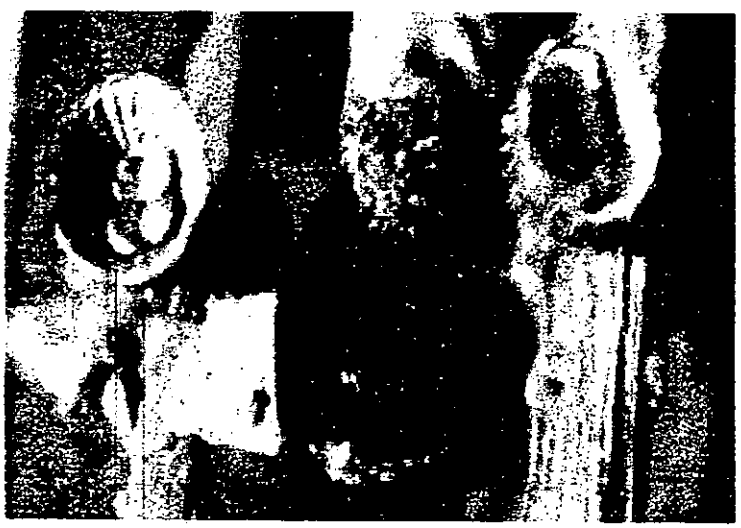


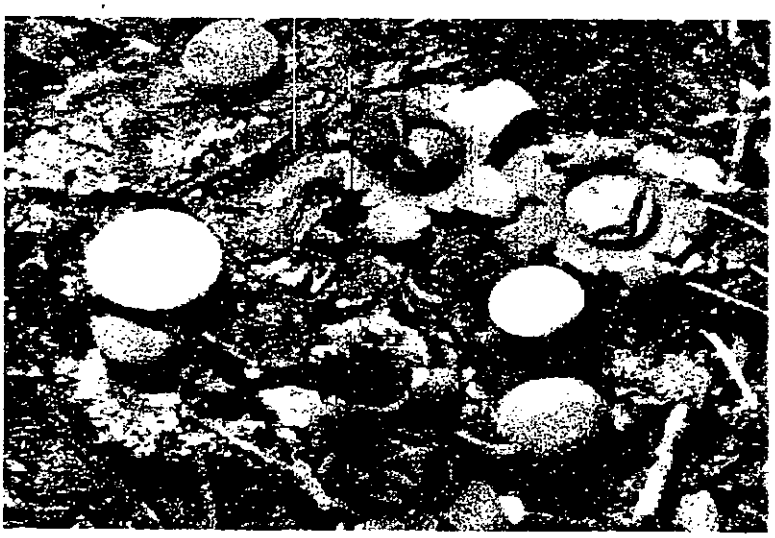
OTU Agaricales on dung - Siruvani Hills



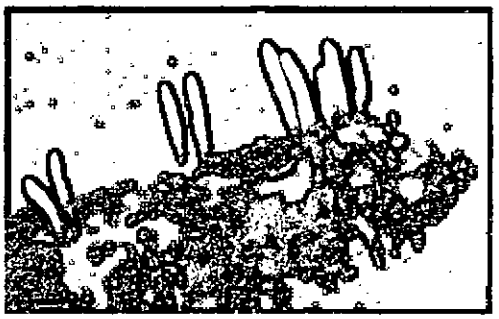


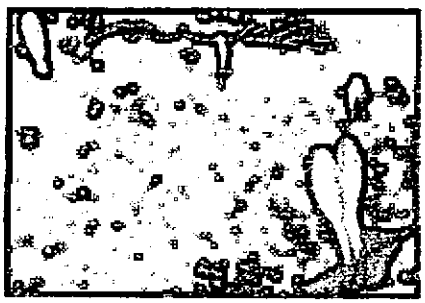
OTU Gasteromycetes - Siruvani Hills

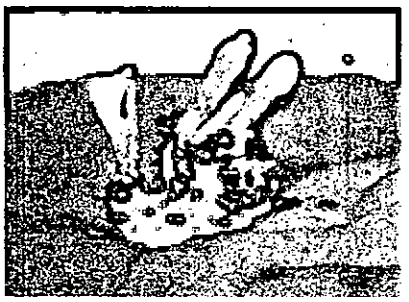


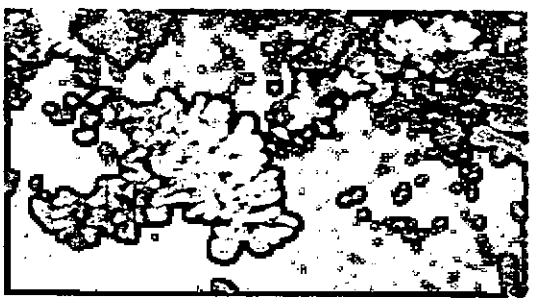


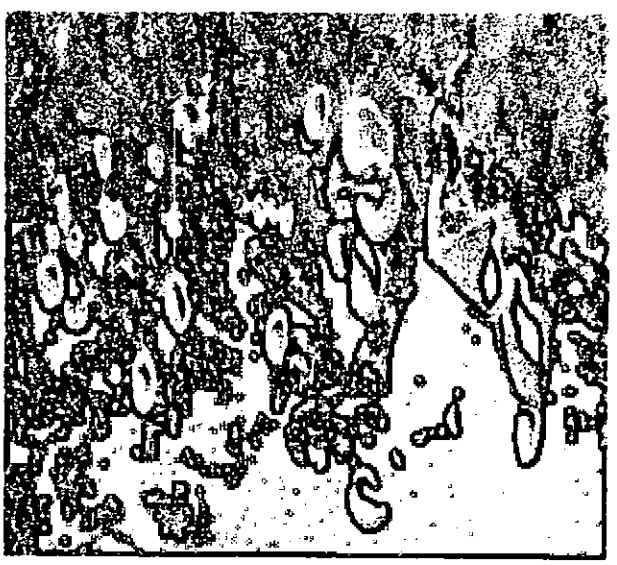
OTU Xyalriales- Siruvani Hills

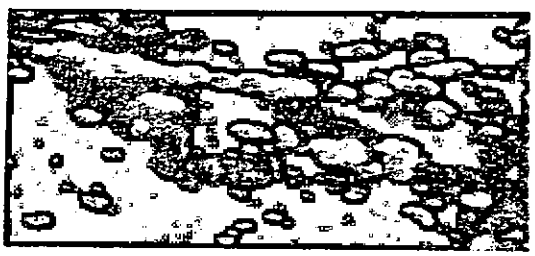




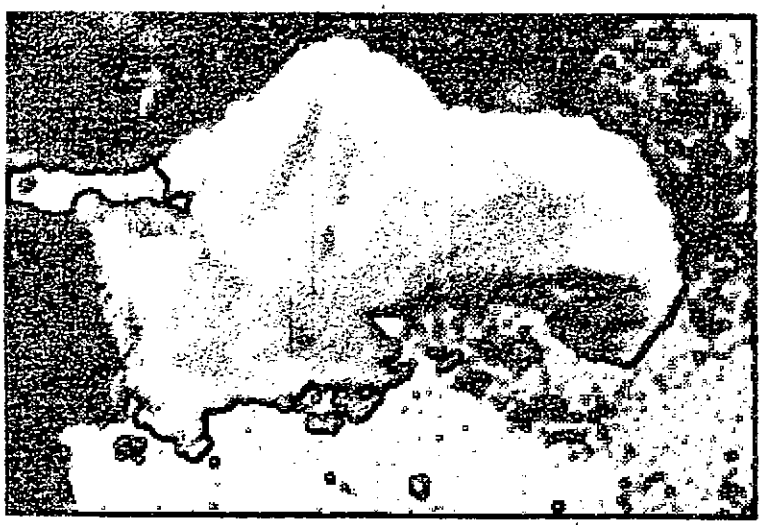


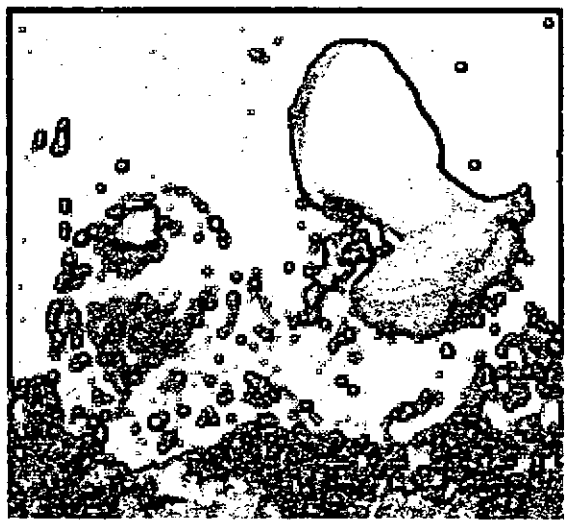






Wood-decomposing macro fungi - Siruvani Hills





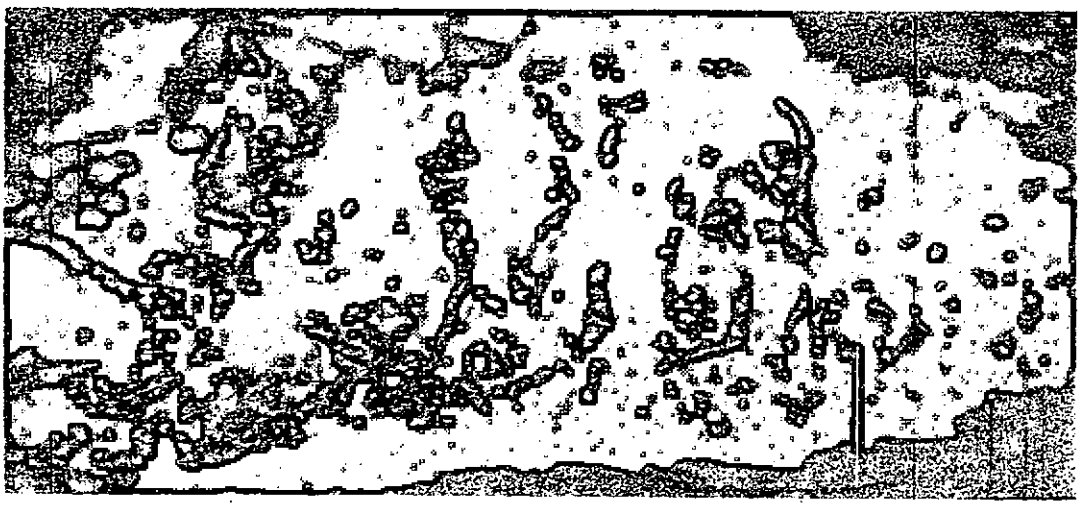


Table 1 Occurrence of Lichen species on the Mangrove Trees of Pichavaram

	MANGROVE TREES					
	Avicennia marina	*Avicennia officinalis*	*Excocaria aggalocha*	*Rhizophora apiculata*	*Rhizophora lamarkii*	*Rhizophora mucranata*
CRUSTOSE LICHENS						
Buellia montana	-	-	+	+	+	+
Buellia sp.	-	-	+	+	+	+
Graphis scripta	-	-	+	+	+	+
Graphis sp.	-	-	+	-	-	-
Lecanora sp	-	-	+	+	+	+
Pyrenula sp 1	-	-	+	+	+	+
Pyrenula sp 2	-	-	+	+	+	+
FOLIOSE LICHENS						
Dirinaria confluens	-	-	+	+	+	+
Dirinaria consimilis	-	-	+	+	+	+
FRUTICOSE LICHENS						
Roccella montagnei	-	-	+	+	+	+

Table 2 Lichen Colony distribution on *E. aggalocha* and *R. apiculata* trees in Pichavaram mangroves

Tree	Number of Colonies of induvidual lichen generic units					
	Roccella	*Dirinaria*	*Graphis*	*Buellia*	*Pyrenula*	*Lecanora*
E. aggalocha	17	476	339	221	729	315
R. apiculata	3997	2513	579	437	2528	1185
Total	4014	2989	918	658	3257	1500

Table 3 Lichen colony distribution on different Tree Zones

Tree Zones	Number of Colonies of induvidual lichen generic units					
	Roccella	*Dirinaria*	*Graphis*	*Buellia*	*Pyrenula*	*Lecanora*
1	6	208	176	134	482	160
2	11	268	163	87	247	155
3	1513	789	271	224	998	633
4	1384	1026	147	163	1024	349
5	1100	698	161	50	506	203

Table 4 Lichen colony distribution on Water facing and Land facing side of the Trees

Aspect	Number of Colonies of induvidual lichen generic units					
	Roccella	*Dirinaria*	*Graphis*	*Buellia*	*Pyrenula*	*Lecanora*
Water facing side	2326	1781	525	207	1643	708
Land facing side	1688	1208	393	451	1641	792

Table 5 Lichen Colony distribution among four mangrove areas within Pichavaram

Area	Number of Colonies of induvidual lichen generic units						Total
	Roccella	*Dirinaria*	*Graphis*	*Buellia*	*Pyrenula*	*Lecanora*	
Keeriguda	239	274	42	42	346	262	1205
Neduodem	1934	1668	333	101	601	67	4704
Periaguda	58	500	325	286	1381	671	3221
S. vaikkal	1783	547	218	229	929	500	4206

Table 6 Vegetation types studied within Siruvani hills

Vegetation type	Site	Altitude in meters
Dry deciduous	Forest above Filter house	450-475
Disturbed dry deciduous	Kovai Falls	450-475
Mixed ever green with dry ever green elements	Thannerpallam area	700
Semi ever green - wet ever green	Kungiranmalai base	1000
Grass land -ever green forest	Muthikulam lower tank,	800-900
	Pulmedu	900-1000
Wet ever green	Muthikulam forest area	900
Wet ever green	Singapara forest area	900
Man made forests		
Teak plantation	Vellapathi and Seengapathi	400-450
Rose wood plantation	Mylone bungalow area	950

Table 7. Phytosociological classification of the lichen communities recognised in the Siruvani forests

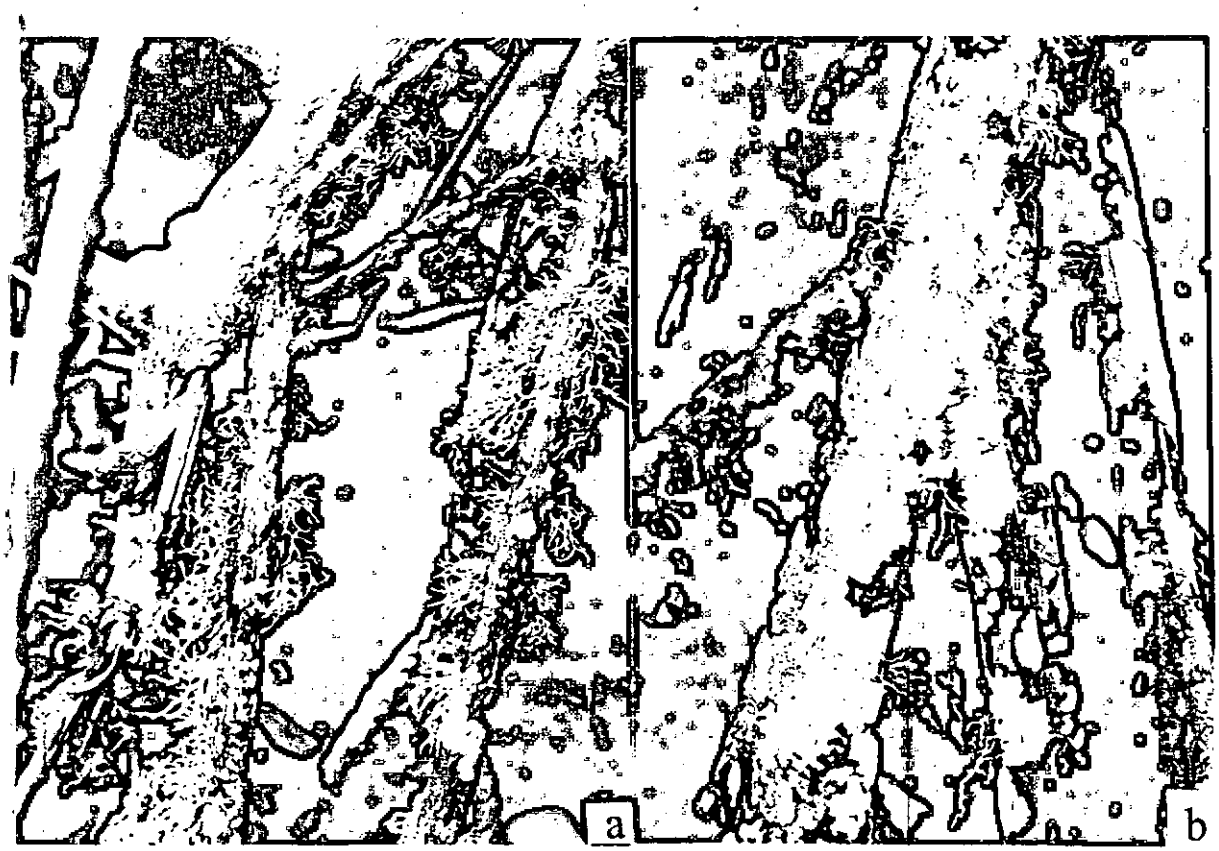
Federation	Unions	Characters
Graphidion	*Graphis scripta* (C)*. *G. longeramea* (S), *Pertusaria pertusa* (C), *Pyrenula nitida* (C)	Shade loving lichens, Found on smooth bark and rock surfaces. Crustose species dominate
Lecanorion	*Lecanora allophana* and associates	Replaces Grphidion in well-lit areas
Leprarion	*Crysothrix candelaris* (C) *C. chlorina* (C,T)	Moderate shade loving and on rough bark and soils. Leprose species dominate.
Myriotremion	*Myriotrema desquamasence* (C), *Porina mastoidea* (C), *P. internigrans* (C), *Pertusaria amara* (C).	Moderate shade loving lichens , humidity loving, on smooth bark, crustose dominate, Replace Laborion when light increases
Physodion	*Physcia aipolia* (L), *P. tribacoides* (L), *Parmelia austroindica* (C)	Sunny habitats, Mostly lignicolous - Foliose
Laborion	*Sticta sylvatica* (M), *Collema auriformi* (C), *Leptogium dendiculatum* (C), *L. cyanescens* (C), *Heterodermia pseudospeciosa* (C), *H. comosa* (C), *Pannaria stylosa* (S), *Phyllopsora parvifolia* (C), *Dictyonema ligulatum* (S, C)	shade - moisture loving lichens. Mostly on moss covered bark, Foliose dominate. Known as Old forest indicator species
Usneion	*Usnea rubicunda* (C), *U. picta* (C), *Ramalina pacifica* (C)	Shade - moderately lited - moisture loving lichens, Rough barked trees - trunks and canopy
Cladonion	*Cladonia coniocrea* (T), *C. ramulosa* (T), *Usnea sp.* (S).	sunny habitats - mostly terricolous, Lichens with two fold characters dominate
Trichoterion	*Parmelia reticulata* (C), *P. saccatiloba* (C), *P. tinctorum* (C), *P. santi-angelii* (C)	moderately sunny habitats, on rough barked trees - base, trunk and canopy, foliose lichens dominate, mostly on forest - grassland ecotone.

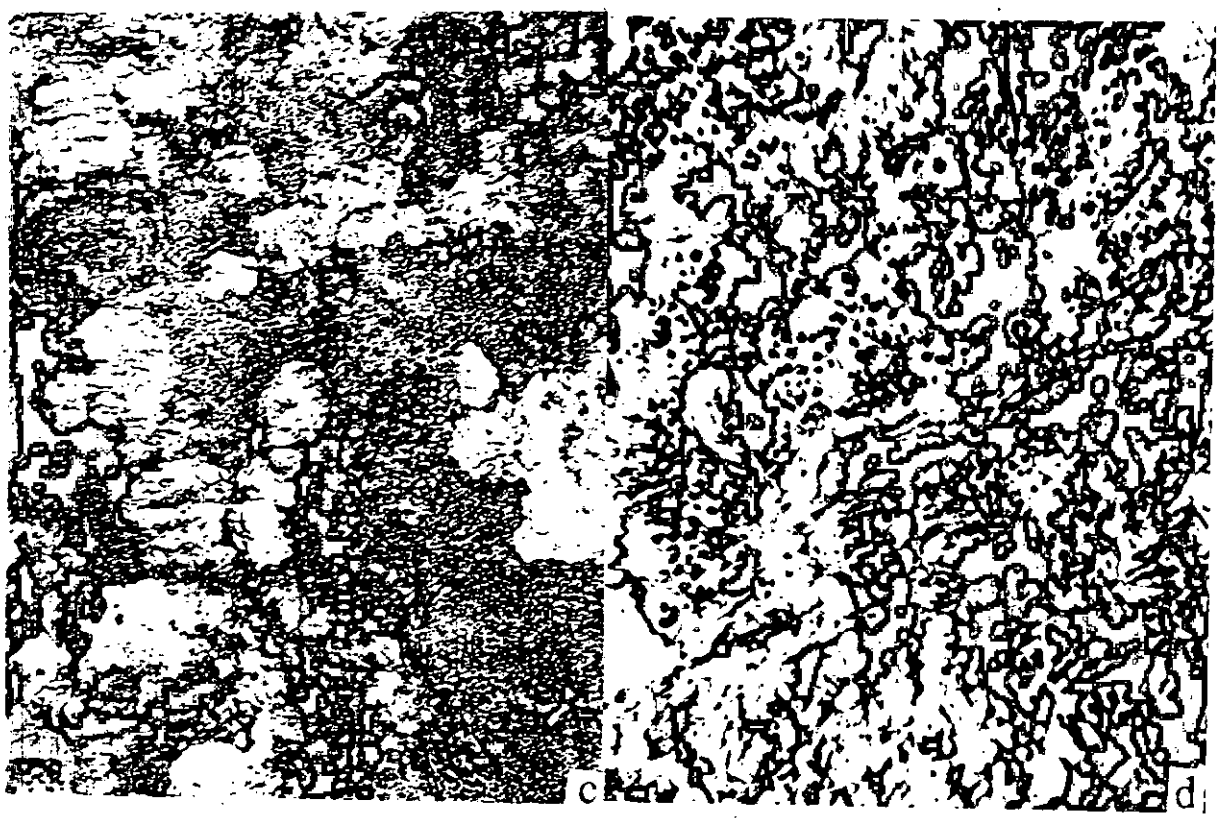
*C-Corticolous, S- Saxicolous, T- Terricolous, L- lignicolous, M- Musicolous

The lichen federations observed in the study site are specific to the vegetation type mentioned and listed in Table 8.

Table 8 Lichen associations found in various forest types with in Siruvani hills

Collection locality	Forest type	Lichen federation identified
Vegetation type near and above Siruvani filter house	Dry deciduous	Graphidion, Leprarion
Vegetation type near Kovai falls	Dry deciduous	Lecanorion
Tannerpallam area	S. Tropical Mixed Wet Ever Green (Mixed)	
	Road side	Myriotremion, Physodion Laborion
	Interior	
Muthikulam lower tank (On the west bank of Siruvani reservoir)	S. T. W. Ever Green On the forest - grassland ecotone.	Trichoterion
	Interior forests	Laborion with Dictyonema, Pannaria, Phyllopsora and Leptogium
Muthikulam (Near 20 check post area)	S. T.W. Ever Green on both the side of the Path.	Myriotremion & Usneion, on soil - Cladonion
	Interior forest	Typical Laborion
Singapara Area	S.T.W. Ever Green forest	as above.
Pulmadu	Grass land - STW Ever Green forest in the valley with long ecological continuity	Typical Lobarian


a
b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MOLECULAR TOOLS AS AN AID TO CONSERVATION OF MANGROVE GENETIC RESOURCES

IV. Molecular Tools as an Aid to Conservation of Mangrove Genetic Resources

Coastal ecosystem, that suffers from the twin problems of both low productivity and uncertain yield, is an important part of the natural base of our country. Since these regions form vital bridges between the terrestrial and aquatic ecosystems, their preservation is essential to maintain the ecological balance and biodiversity. Despite their ecological and economic significance, the effects of current resource use practices evident both in the inland and coastal areas have rendered various forms of stresses on the coastal ecosystem. Increasing soil erosion and water pollution caused by intensive farm practices in the inland areas that get transported through the river and canal systems are quite adversely affecting the coastal systems. Moreover, the sea water intrusion and the attendant soil and water quality problems caused by the ground water depletion have already started threatening the very sustainability of agricultural systems in the Saurashtra region of Gujarat and Tanjavur region of TamilNadu. At the same time, it is anticipated that by the year 2000, the human population living within 60km of the shoreline will grow to over 50 per cent. Many of the world's poor are crowded in coastal area and coastal resources are vital for their livelihood security. Above all, the prospect of sea level rise, expected to be in the order of 8-29 cms due to global warming by 2025, makes the need to take concrete steps to ensure the sustainable management of the coastal ecosystem all the more urgent.

The coastal ecosystems are characterised with various physical, climatic and anthropogenic pressures. The most productive vegetation in the coastal regions are a group of specialised tree species i.e the mangroves, growing in the inter tidal and estuarine areas and are subject to high physical stress conditions such as high salinity and water-logging. It is one of the most productive ecosystems of the world. It supports various micro-organisms and other invertebrate species. They are the primary nursery ground for a number of commercial shrimp, crab and fish species. The total fish catch in the mangrove swamps account for about 68 per cent of total world production. The mangrove vegetation help in stabilising shoreline and provide protection from tidal bores, ocean currents and storm surges. In addition, these plant species possess valuable characters for potential use in developing novel genetic material for adaptation to the ever fluctuating climatic extremes of the coastal zone.

Mangroves are very specialised forest species of tropical and subtropical coastal regions of the world bordering the sheltered seacoasts and estuaries. They are valuable natural resources with high productivity, hence, often over-exploited for various purposes. The magnitude of threat to this ecosystem is on the rise as more and more mangrove areas throughout the world are being used for agriculture, wood resources and forest products. The increasing dependency of

Table 1. Traditional usage of some important mangrove species

Species	Products	Usage
Acanthus ilicifolius	Leaf Extract	Relieve rheumatism
Avicennia alba	Seeds, bark, bark-extract	Cure of small-pox
	Sap from bark	Contraceptive
	Fruits	Human consumption
A. marina	Young leaves	Human consumption, cattle feed;
	Roots	Cure for minor fish stings;
	Bark	Astringent
	Wood	Firewood
A. officinalis	Seeds	Curing ulcers
Aegiceros corniculatum	Seeds, bark	Fish poison
Bruguiera gymnorrhiza	Fruits	Medicine for eye ailment
	Hypocotyl	Vegetable, seasoning raw fish
	Wood	Fire wood
	Bark	Preparation of adhesive
B. sexangula	Tender leaves, hypocotyl	Consumed as vegetables
Ceriops tagal	Wood	Fuel wood
	Bark	Curing malaria, Preparation of adhesive
Excoecaria agallocha	Sap	Fish poison and cure for fish sting
		Cure for tooth-ache, ulcers
Heretiera fomes	Wood	Timber for canoes and house building
Kandelia candel	Bark	Cure for diabetes
Lumnitzera racemosa	Leaves	Cure for health problem in infants
Nypa fruiticans	Leaves	Thatching material
	Fruits	Human consumption
	Sap	Alcohol
Rhizophora mucronata	Prop- and slit roots	Firewood
	bark	Mosquito repellant
	Fruits	Human consumption
		Cure of diabetes
R. apiculata	Wood	Fire wood
Sonneratia alba	Wood	Fire wood and timber
	Fruits	Human consumption
	Pneumatophores	Fishing floats, Preparation of scent
Xylocarpus granatum	Bark	Cure for diarrhoea, Astringent
	Wood	Fire wood, Charcoal
	Seed ointment	Cure of insect bite

the local population on the mangrove species for various products and usage (Tables 1 and 2) further threatens the sustainability of the system. Constant environmental and anthropogenic pressures on the ecosystem have led to a rapid decline of many natural populations of different species. This is evident

from the fact that once widely prevalent mangrove genus *Rhizophora* is now at the verge of extinction (Blasco,1975).

India with a coastline of about 7,500 km represents 8 per cent of the world mangrove areas (Status Report, 1987). However, in terms of species composition, it is represented by 60 species belonging to 42 genera and 29 families which are predominantly distributed along the eastern coast of India that accounts for about 82% of Indian Mangroves. An alarming rate of depletion in the mangrove areas is being seriously felt in India (It is reported that there has been about 25% reduction in the mangrove forest cover along the Indian region during the last 25 years or so).

Table 2: Medicinal value of some important mangroves as used by the tribals in Andaman and Nicober Islands

Species	Plant part	Medicinal properties
Acanthus ilicifolius	Leaves	Treating rheumatism and neuralgia
Acrostichum aureum	Pounded rhizome	Used as de-obstruent
Barringtonia racemosa	Fruits Kernels	Cure of cough, asthma, diarrhoea.Cure of jaundice
Caesalpinia bonduc	Leaf paste Seeds	Cure for swollen testicles. Cure of jaundice
Calophyllum inophyllum	Seed oil	Used for rheumatism, skin diseases and leprosy
Ceriops tagal	Leaves	Cure for malaria
Heretiera littoralis	Seeds	Used for diarrhoea and dysentry
Hibiscus tiliaceus	Roots Leaves	Possesses febrifuge, operative, emollient, laxative properties Used to cure pimples
Pongamia pinnata	Bark, Crushed seeds	Cure for malaria, skin diseases, rheumatic joints etc
Scaevola taccada	Leaves	Used as febrifuge, cure of headache, cough
Terminalia catappa	Leaves	Curing scabies, cutaneous diseases and skin diseases
Thespesia populnea	Leaves	Curing stomach troubles
Xylocarpus	Bark	Cure for dysentry

Given the ecological and economic importance of the mangrove vegetation, and the considerable threat both in terms of increasing population pressures in the coastal areas and the anticipated climatic changes in the region to which they are exposed, concerted and well visualised action plan has to be undertaken for the conservation, restoration, exploitation for important genetic and other useful traits and rational use of this unique genetic resource.

Despite the economic and ecological significance of the mangroves, experimental studies in this group of plant species have almost been neglected for long time. Available reports on mangroves are highly fragmentary and no worthwhile information is available to account for species identity, relationship and

evolution among the mangrove species. Based on the available information it is not possible to partition the observed variation into environmental and heritable components. The phenotype is known to be the result of intricate interaction of the genotype and the environment. The highly fluctuating environmental conditions in the coastal region, where the mangroves grow, influence the phenotypic variation within and between the species to a large extent. Hence, species identification and delimitation based on some quantitative characters and phenotypic markers have resulted in the confusing taxonomic status of the mangrove species. It was in this context, molecular markers based on the analysis of genetic material (DNA/RNA) or gene products (proteins/isoenzymes) were considered as the best bet for studies related to species identification, assessment of genetic diversity, elucidating species relationship and depicting phylogentic trends within and between the mangrove species of Indian region.

Unlike morphological markers, molecular markers are stable and are not prone to environmental influences and do precisely portray the genetic relationship between plant groups and hence are widely used in genetic resource characterisation and conservation. There are several marker systems available now, like the Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), fingerprinting etc. that have proved to be of invaluable assistance in molecular genetics and in applications of plant breeding, phylogenetic analysis and marker aided selection of traits. It is hoped that the advances in the field of molecular biology will contribute immensely to the genetic improvement of major crop and non-crop species thereby, ensuring better productivity to meet the challenges of the ever growing population.

Use of molecular markers in the recent years has made it possible to precisely :

- fingerprint the genotype
- verify the F1 hybrids
- estimate genetic distance and heterotic groups
- select best individuals for breeding
- break undesirable genes of interest and recover reccurent genotypes
- undertake genetic analysis of QTL
- clone genes of economic importance

With the advancements in the field of molecular biology, a number of techniques have emerged and have been commonly used for a number of applications in genetic characterisation and improvement. Table 3 details out some of the examples where molecular markers have been used successfully for a variety of purpose. A detailed description on some of the commonly used marker systems in a number of plant species are given below.

Restriction Fragment Length Polymorphism (RFLP)

DNA markers generated by RFLP are the result of differences in the sequences of nucleotides in the DNA of two individuals. RFLPs can either be due to loss of a restriction site or an addition of a new one which primarily occurs through base changes, deletions, additions or re-arrangements in the DNA sequence. Thus polymorphism revealed by digesting (any) genomic DNA with a restriction enzyme is the result of a sequence difference within the restriction site of that enzyme.

Using restriction enzyme nucleases, DNA molecules can be cloned, this has allowed major portions of the plant genome to be assayed for genetic markers. In order to detect polymorphism at the nucleic acid level, genomic DNA from two genetically distinct individuals are digested with a restriction enzyme and the DNA is separated by gel electrophoresis. By Southern blotting using the standard procedure (Sambrook et al. 1989) the DNA from the gel is transferred to nylon membrane for probing subsequently using specific or non-specific DNA probes.

Following transfer of the DNA to nylon membrane by Southern blotting, a labelled (either with radioactive or non-radioactively labelled dATP) DNA clone (probe) was hybridized to the membrane. Polymorphism can result due to differences in the distribution of the restriction sites in the two genotypes. The DNA sequences that hybridise to a particular probe constitute discrete chromosomal loci. Alleles can be differentiated with the variation in the restriction sites. Accordingly, restriction fragments are well suited as genetic markers.

The production of probes is crucial for RFLP and in particular single-copy DNA sequences are required for mapping. In most higher plants the nuclear genome consists of a large proportion of repetitive DNA sequences. Often these repeats are interspersed with unique single-copy sequences that render the isolation of low-copy DNA sequences more difficult. The proportion of repeated DNA and the extent to which it is interspersed with single-copy DNA is generally a function of the overall DNA content of the organism. Compared with other organisms plants have a wider range of DNA content, more DNA per nucleus and complex genomic make up. The large genome size of certain crops makes it difficult to isolate clones consisting entirely of single-copy DNA.

RFLP analysis has been used for a variety of purposes. Since restriction sites are actual samples of nucleotide sequences, the variation for the presence of sites has been used to estimate genetic divergence of individuals. Variation in the restriction site does not reflect a functional difference in the sequence analysed.

Random Amplified Polymorphic DNA (RAPD)

RAPDs uses short oligonucleotides (generally 10-mer) sequences to identify the hololologous region in the target DNA. Based on the principles of polymerase chain reaction (PCR, Williams et al 1989), it amplifies million number of copies of a fragment and the electrophoretic migration and resolution of the fragments account for the similarity/differences between individual species.

RAPD is a considerably recent technique, popular and widely used for its many advantages over the RFLP's and has virtually been used for all those purpose the RFLP were being used. The other advantage of this technique has been the requirement of low quality and quantity of the template DNA. They are very cost and time-effective when compared to the RFLP's, where the chemicals and enzymes used are more and more-over there is always a hazard of working with radio-active chemicals. Another limitation of the RFLP being the level of polymorphism detected itself. In-general, inter-specific variation is high although the variation detected in between the cultivars of the species can be very low; thereby the chances of finding a useful polymorphism can be increased by using enzymes which cut more frequently or by using greater variety of enzymes. All these difficulties are minimised by the use of RAPD's which are dominant markers. However, they suffer from difficulties with regards to the reproducibility.

DNA Fingerprinting

Eukaryotic genomes contain a substantial amount of repeated DNA sequences. Repeated DNA are organised in long arrays of tandem repeats or dispersed throughout the genome. A number of studies on repeated DNA sequences have been done on both animals and plants, and a common feature is that both the abundance and sequence of the repeat units change during the course of evolution. Some tandemly repeated sequences have been mapped to the telomeric and centromeric regions of the chromosomes thereby suggesting that the repeated sequences do play a role in evolution and speciation. Genome specific and species specific repeated sequences have provided very useful phylogenetic tools to study evolutionary relationships among species as also analysing the genomes of hybrids and amphiploids. Both the dispersed and tandemly repeated sequences have been used to identify the species/genotypes/ strains.

Individual specific fingerprinting, mostly in case of human beings, has been obtained by employing tandem repeat sequences. A number of plant species have also been fingerprinted using mini-or macro-satellite probes. Another approach of DNA fingerprinting have been using artificial probes of simple repeated sequences such as (GACA)4, (GATA)4 etc. These studies have yielded

variable DNA fragment patterns in several plant species. Among other potential uses the mini-satellite probing pattern have been useful in identification of genotypes, parentage analysis and assessment of genetic relatedness.

Table 3: Use of molecular markers (some specific examples)

Species	Marker system used	Utility	Reference
Oryza sativa	RFLP and RAPD	Studies on genetic diversity and compatibility	Zheng et al. 1994. Fukuoka et al. 1992.
	RAPD	Marker aided selection for gall midge resistance gene Gm4t.	Nair et al. 1996.
	DNA fingerprinting	Identification of rice genotypes.	
		Phylogeneic distribution and genetic mapping of (GGC)n.	Ramakrishna et al.1995.
	DNA amplification fingerprinting		Zhao et al. 1993.
Festuca arundinacea	RFLP	Genetic variability	Xu *et al.* 1994..
Rosaceae	DNA fingerprinting	Genetic variability in apple cultivars	Nybom et al. '90.
Rosa sp	RAPD	Phylogenetic relationship	Millan *et al.* 1996.
Pisum sp	RAPD	Phylogenetic analysis	Hoey *et al.* 1996.
Brassica oleracia.	RAPD	Cultivar identification	Hu *et al* 1991.
Cauliflorous vegetables	RAPD& RFLP	Identification of breeding lines	Nishio *et al.* 1994.
Daendranthema grandiflora	RFLP & RAPD	Genetic variability	Wolff et al. 1994.
Rauvolfia sp	RAPD	Identification for strictosidine synthase	Bracher et al 1992.
Brassica oleracea	RAPD	characterisation of genetic identities and relationships	Kresovich et al. 1992
Brassica rapa	DNA fingerprinting	Linkage maps and VNTR analysis	Rogstad 1994.
Solanum sp	RAPD	Genetic variation of androgenic monoploids	Singsit et al. 1993.
Populus sp	RFLP & RAPD	Genetic variability studies	Liu et al. 1993.
Pauloumia taiwaniana	RFLP & RAPD	Establishing hybrid origin	Wang et al. 1994.
Brassica sp	RAPD	Genetic diversity and heterosis.	Jain et al. 1994.
Maize and tomato	RFLP	Construction of genetic linkage maps.	Kresovich et al 92; Helenjaris et al.86.
Phaseolus vulgaris	M13 DNA finger-printing	Evolution and domestication	Sonnante et al 1994.
Triticum sp	repeat sequences	phylogeny of wheat B and D genomes.	Dvorak et al 1990.
Plantago sp	DNA fingerprinting	population and species variation.	Wolff et al. 1994.

Hordeum sp	Microsatellite	Allele variation and mapping. Cultivar selection	Becker et al. 1995. Saghai Maroof et al. 1994
Lycopersicum esculentum	microsatellites	identification of highly polymorphic DNA regions for cultivar	Vosman et al 1992.
	RAPD	identification. Cultivar Improvement	Klein-Lankhorst et al. 1991
Mangifera indica	DNA fingerprinting	Identification and genetic analysis	Adato et al. 1995.
Cicer arietinum	DNA fingerprinting	genetic diversity and genome mapping Interspecific and intraspecific polymorphism	Sharma et al.1995. Weising et al 1992.
Carica sp	DNA fingerprinting	Identification and genetic analysis.	Sharon et al. 1992.
Glycine max	DNA amplification fingerprinting	Genome mapping.	Prabhu et al.1994. Rangwen et al 95.
Cynodon sp	DNA amplification fingerprinting.	Genetic relationships	Caetano-Anolles et al. 1995
Phaseolus vulgaris	RFLP	Species relationship	Stockton et al 1994.
Medicago sativa	RAPD	Genetic improvement	Echt et al 1992.
Persea americana (avocado)	DNA fingerprinting	Cultivar identification	Lavi et al.1991.

The polymorphism from this repetitive DNA results from the variable number of tandem repeats (VNTR) of a core sequence. Minisatellite regions contain a core sequence (Nakamura et al., 1985), from 16-64 bp long and were first used to detect multiple band patterns that are highly polymorphic in humans and thus are used for fingerprints (Jeffereys et al., 1985). These band patterns are generated by the hybridisation of the probe to the genomic DNA digested with a restriction enzyme. The simple-sequence repeat regions consist of 2-6 bp sequences (Weising et al., 1991) and are a major source of genomic variation (Tautz et al., 1986). The abundance of the simple-sequence repeats in the plant genome and the ease with which they are applied make this the best choice for DNA fingerprints. The abundance and the amount of information derived from markers and also the ease with which they can be identified, make them an ideal marker system for plant genetic linkages and physical mapping, population studies and varietal identification.

DNA Amplification Fingerprinting (DAF)

DNA amplification fingerprinting uses very short arbitrary primers (> 5nt in length) for the amplification of discrete portions of a genome and produce

characteristic fingerprints. Profiles can be tailored in the number of monomorphic and polymorphic products, for example template digestion prior to the amplification can dramatically enhance the detection of polymorphic DNA. Polymorphism from single banding patterns are useful as genetic markers while more complex and informative patterns are suited for DNA fingerprinting. This can be useful in studying closely related organisms, in fingerprinting, systemic and phylogenetic studies, generate sequence tagged markers for diagnosis of mutationally defined genomic regions and find markers linked to phenotype-defined genetic loci in positional cloning.

Protein profiles - Protein and isoenzyme patterns have been used for a number of applications

Isozymes were originally defined by Markert and Moller (1959) as different variants of the same enzyme, having identical or similar functions, and present in the same individual. Their importance in understanding gene action in development and differentiation has been exploited for many years. They are of immense use to make precise quantitative estimates of genetic variability based upon one parameter of the genetic structure of the primary products of the genes themselves. This technique of extracting and analysing the electric mobility of soluble proteins, particularly enzymes, has a wide range of applications ranging from population genetics, evolutionary process studies, and the phylogenetic relationships of the populations. These make possible comparisons between individuals and populations on the basis of several gene loci, rather than one or two. Moreover they follow typical mendelian segregation patterns by which progenies of organisms can be analysed. Isozymes provide valuable information with respect to hybridisation and gene duplication, including polyploidy.

The Present Study :

Particular emphasis was placed on the mangrove species primarily because of their significant ecologically economic importance.

The objectives of this subcomponent of the project were to use molecular markers for :

a) analysis of intraspecific genetic polymorphism in selected species
b) establishing genomic relationships
c) depicting evolutionary and phylogenetic trends and
d) developing probes for RFLP through construction of genomic libraries in endemic mangrove species.

Materials and methods

Species under investigation

As already mentioned, mangroves are the most diverse group of species with preferential adaptation to varying climatic and edaphic conditions. Therefore, the composition of species and their relative abundance vary to a large extent in a given population. They are wide-spread all over the coastline of the Indian region. The populations for the present study were collected from both the Eastern and Western coast of India (Table 4) . Samples of individual species were collected depending on their occurrence. A detailed account of the geographical location and other physical characters are given in Table 6. In the present investigation a detailed analysis of all the mangrove species in the Pichavaram mangrove forest were undertaken. In addition, some of the associated species and parasitic ones on the mangrove species were taken into account. Due to the diversity in species composition, a brief description is provided here for the analysed species with respect to their taxonomic positions. In Table 5, a detailed description on the number of genera and species within the families to which the investigated species belong both in India and world-wide are given.

Table 4: Name of the populations analysed

Eastern Coast	Western Coast
Bhitarakanika	Goa
Koringa	Bombay
Pichavaram	Ratnagiri
Muthupet	Calicut
Adyar	Vikroli
Ennore	Thane
Pondicherry	Ganapathiphule
Gulf of Mannar	
Karaikal	
Porto Novo	

Rhizophoraceae: It is a small pantropical family of about 16 genera of tree and shruby species belonging to the order Rhizophorales (Tobe and Raven 1983). Within the family a number of tribes are recognised. Of them, Rhizophoreae Blume (or 'mangrove Rhizophoraceae) is universally recognised as a natural and discrete taxon, and it includes four exclusive mangrove genera *Rhizophora, Kandelia, Bruguiera, Ceriops.* The uniformly viviparous condition is particularly distinctive. In all the species of the genus the reported chromosome numbers are 2n=36 (Sidhu 1968). In India, *Rhizophora* is virtually exterminated by over exploitation (Blasco 1977), since they do not coppice and can not regenerate in the absence of seedling parent. *Rhizophora mucronata, R. apiculata, R. x lamarckii,*

Kandelia candel, Bruguiera cylindrica, B. gymnorhiza, Ceriops decandra and *C. tagal* are the species included in the present study.

Table 5 : Distribution of species of mangrove family

Family	World wide		In India		Species studied	Reported status
	No. of genera	No. of species	No. of Genera	No. of Species		
Rhizophoraceae	16	120	8	16	*Rhizophora mucronata(7/2)*	Mangrove
					R. apiculata	
					R. x lamarkii	
					Kandelia candel(1/1)	Mangrove
					Bruguiera cylindrica(6/5)	Mangrove
					B. gymnorrhiza	
					B. sexangula	
					B. parviflora	
					Ceriops decandra(2/2)	Mangrove
					C. tagal	
Sterculiaceae	60	700	24	80	*Heritiera fomes(35/5)*	Mangrove
Meliaceae	50	1400	35	5	*Xylocarpus granatum(3/3)*	Mangrove
Avicenniaceae	1	8	1	3	*Avicennia marina(8/3)*	Mangrove
					A. officinalis, A. alba	
Myrcinaceae	35	1000	11	117	*Aegiceras corniculatum(2/1)*	Mangrove
Combretaceae	20	600	6	32	*Lumnitzera racemosa (2/1)*	Mangrove
Euphorbiaceae	300	5000	72	466	*Excoecaria agallocha(40/8)*	Mangrove associate
Acanthaceae	250	2500	81	424	*Acanthus ilicifolius(50/6)*	Mangrove associate
Chenapodiaceae	102	1400	18	46	*Suaeda maritima(110/4)*	Salt marsh plant
					Salicornia brachiata	Salt marsh plant
Aizoaceae	130	1200	10	18	*Sesuvium portulacastrum (8/1)*	Satl marsh plant
Palmae	217	2500	56	142	*Nypa fruticans(1/1)*	Mangrove
Poaceae	620	10000	246	1175	*Porteracia coarctata(1/1)*	Salt marsh plant
Pandaceae	3	700	2	40	*Pandanus ascicularis (600/37)*	Coastal plant
Loranthaceae	36	1300			*Dedrophthoe falcata(30/11)*	Parasite
					Viscum orientale(60/14)	Parasite
Verbenaceae					*Clerodendron inerme(...)*	coastal plant

Avicenniaceae: *Avicenniaceae* is a pantropical monogeneric family with the exclusive mangrove genus, *Avicennia* L. The genus includes eight species world wide. Though, the genus was initially included in the family Verbenaceae, its segregation as a separate family Avicenniaceae is now generally accepted. However, phytochemical evidences do not favour the inclusion of the taxa belonging to *Avicennia* to a separate family (Reddy et al. 1993). All the three species of the genus *Avicennia* occurring in India viz. *A. marina, A. officinalis, A. alba* were analysed.

Euphorbiaceae: *Excoecaria agallocha* is the only species of the genus found in the mangrove habitats and have been included in the present study. . Among the 5000 odd species of this family, *Excoecaria agallocha* is generally considered as a mangrove associate with landward habitation with low salinity. However, this species was observed well inside the mangrove forest along with *Avicennia marina* which is reported to be a highly saline tolerant mangrove species (Chapman 1977a).

Myrsinaceae: In the present study only one species *Aegiceras corniculatum* was analysed. Myrsinaceae is a large family with above 30 genera which includes an exclusive mangrove genus *Aegiceras*. Only two species of this genus have been reported from mangrove communities in the Asian tropics. This genus is distinctive within the family by virtue of its elongated capsular, dehiscent fruit and elongated seed without an endosperm. Inclusion of this genus in a separate 'family' Aegiceraceae (Candole 1844) was not favoured as these features largely relate to its unusual fruit and seed biology which in turn may be related to its habitat (Tomlinson 1986).

Combretaceae : This family contains two genera, *Leguncularia* and *Lumnitzera* which are typical constituents of mangrove communities. *Lumnitzera racemosa,* is identified by its white petals and exerted stamens. The species has restricted distribution in Asian region and have been investigated in the present study.

Acanthaceae : *Acanthus* is the only genus of *Acanthaceae* that has representatives in mangrove ecosystems. Among the 30 species of *Acanthus,* 3 species are reported to occur as mangrove associate in India. (van Steenis 1937). Very often they have been treated as a single variable species with no prominent differences in vegetative features. *Acanthus ilicifolius* is generally identified by spiny leaves and attractive inflorescences. This species has been included in the present study. This species is one of the most wide-spread mangrove species that could be observed in all types of mangrove habitation.

Sterculiaceae: This family represents a number of commercially important species of which three species of the genus *Heritiera* are found in mangrove ecosystem. *H. fomes* is a tree species widely distributed in Ganges and

Brahmaputra Delta and the Sundarbans. The other species of this genus are found in tropical rain forests. *H. fomes* has been included in the present study.

Meliaceae: It is a large tropical family of 50 genera and over 100 species commercially important for high quality timber. Two of the three species of *Xylocarpus* occur in the landward mangrove communities. *X. granatum* (analysed species) is a tree species and its mature fruit is the size of a melon and weighs approximately 2 to 3 kg.

Chenopodiaceae *:* Among different species *Suaeda maritima and Salicornia brachiata* were included in the present investigation. Members of this group include many herbaceous or semi- woody halophytes which are often found in mangrove areas in places of habitat disturbances as a result of human interference. Members of *Chenopodiaceae, Salosa, Atriplex, Sueda* and *Salicornia* are considered as characteristic tropical salt marshes and their presence in mangrove wetlands stands reflects marginal disturbance. The Pichavaram Mangrove forest from which *S. maritima* and *S. brachiata* were collected for the present study, represents a disturbed forest, *S. maritima* was observed to a great extent. However, *S. brachiata* was observed only in the landward side where salinity was comparatively less.

Table 6 : Geographic location and physical characteristics of the study sites

Name of the study site	Latitude & Longitude	Rainfall (mm)	Salinity (ppt)	Soil pH	Dominant soil type
Gulf of Mannar	8° 47'N 79°14'E	900	7.0-17.0	6.0-7.5	Sandy
Muthupet (M)	10° 46' N 79° 52' E	1280	6.0-18.0	5.9-7.3	Fine sand and clay
Pichavaram (P)	11° 27' N 79° 47' E	1300	3.0-27.0	6.2-8.0	Fine sandy-clay
Ennore (E)	13° 30' N 80° 15'E	1200	5.3-13.4	6.2-7.7	Sandy-silt
Coringa (Co)	16° 30'N 82° 20'E	1150	5.0-30.0	7.5-8.4	Clayey-silt
Bhitarkanika (B)	20° 40' N 86° 52' E	1125	2.0-21.0	6.7-7.8	Fine silt/ clay
Calicut (C)	11° 55'N 75° 81'E	1380	6.0-18.0	6.2-7.4	Sandy and clayey soil
Goa (G)	17° 08'N 73° 52'E	890	2.0-35.0	6.8-7.7	Lateritic rock and clay
Ratnagiri (R)	17° 08'N 73° 19'E	946	10.0-24.0	5.2-7.6	Lateritic rock with gray soil
Bombay (Bo)	19° 25'N 73° 05'E	700	6.0-15.0	7.7-8.3	Sandy-clay
Karaikal	10°78'N 79° 50' E	1300	12-21	6.1-7.5	Clayey, dry and compact
Pondicherry	12° 08' N 79° 52' E	1250	10-25	5.8-7.6	Fine sand
Adyar	13° 00' N 80° 15' E	1250	6.1-19.5	6.6-7.3	Fine sand and silt
Thane	19° 21' N 73° 06' E	720	7.0-16.0	6.8-7.9	Sandy, clay
Ganapathiphule	17° 06' N 73° 16' E	950	9.0-18.0	5.4-7.8	Lateritic rock with clay
Portonovo	11° 30' N 79° 49' E	1290	6.0-21.0	6.1-7.9	Fine sandy-clay
Vikroli	19° 18' N 73° 04' E	820	6.2-19.9	5.8-7.6	Sandy, clay

Aizoaceae: The family includes about 130 genus world wide with about 10 genera found in India. 18 of the 2000 odd species are reported from India. This family is being represented by *Sesuvium portulacastrum* in the present study.

Pandanaceae: Among about 40 species of two genera belonging to this family, *Pandanus fascicularis* has been included in the present study.

Gramineae: *Porteracia coarctata* (= *Oryza coarctata*) is the only wild relative of rice and has inherited tolerance to salinity and water submergence. This species is widely distributed along the coastal areas of India and have been included in the present study.

Palmae *(Nypa fruticans* Wurmb*) Nypa* is the oldest mangrove with geological age of 69 million years (the end of Cretaceous age). It has been listed as one of the main woody species of mangrove, but has not been reported from Cauvery delta mangroves. It is a monotypic genus belonging to a separate family Nypaceae, however, recent taxonomy logically considers this genus as a member of Palmae (Arecaceae) with independent line of specialisation, as numerous distinctive features of mangroves (like incipient viviparity) have no relation to Palmae. Relationship with Pandanaceae has been suggested on the basis of superficial similarities of the fruiting head, but the basic morphology of the two forms are quite dissimilar (Tomlinson 1986).

Loranthaceae: Loranthaceae is the only family which represents the parasites associated with mangroves. The parasitic species can be described as mistletoes and seem indiscriminate in their choice of host (Tomlinson 1986). However, we have found *Dendrophthoe falcata* only on *Rhizophora* and another parasite *Viscum orietale* only on *Exocoecaria agallocha* (not reported elsewhere). These species were also included in the present analysis.

METHODS

Cytological studies

Mitotic chromosome numbers were determined from the root tips. Actively growing root tips, either from the germinated seeds or from the plants growing in plastic bags, were pre-treated in water at 4C for 24 hr and fixed in 1:3 acetic acid and absolute alcohol for a minimum period of 24 hr. Fixed root tips were washed in water, hydrolysed for 30 min in 5N HCl at RT and stained in Fuelgen stain (pH 2.2) for 1 hr. Meristematic zones were squashed in 1 % acetocarmine and observations were made from temporary mounts.

DNA isolation

Leaf materials were collected from matured individual plants from six ecogeographically distinct zones of the Indian coast. Twigs containing young leaves were dipped in 0.05% charcoal water and transported to the laboratory under ice. The young leaves were collected, frozen under liquid nitrogen and preserved at -80C for DNA isolation. In certain cases, leaves were collected from the planted propagules in the mist chamber at the Foundation. Mangrove leaves in general contain substantial amount of latex, phenolics, secondary metabolites and mucilages. Modifications in the protocol were made so as to obtain quality DNA from these species. Leaves were ground under liquid nitrogen in a buffer containing 20mM Tris HCl (pH 7.5), 250 mM NaCl, 25mM EDTA, 0.5% w/v Triton X. The homogenate were passed through four layers of cheese cloth and the filtrates (after centrifugation) were incubated with 2% CTAB (Saghai-Maroof et al 1984) with altered salt concentration at RT for 2h or overnight at 4C. Equal volume of Chloroform and Isoamyl alcohol (24:1) was subsequently added on, vortexed and centrifuged at 10,000 rpm for 10 min. The aqueous phase was extracted with 2/3rd volume of isopropanol. The precipitated DNA was recovered by centrifugation at 5,000 rpm for 10 min. The pellets were air dried, washed in 70 per cent ethanol and dissolved in appropriate volume of TE (pH 8.0). The purification of DNA was carried out through RNase treatment and subsequent phenol:chloroform treatment. DNA was recovered through ethanol precipitation and dissolved in TE.

RAPD analysis

Amplification reaction (final volume 25ul) contained 10-50ng genomic DNA, 240uM each of dATP, dGTP, dCTP and dTTP (Pharmacia), 30ng of primer (Operon), 10x Taq Polymerase buffer and 1u Taq DNA polymerase (USB) and 2.5 mM $MgCl_2$. Each reaction mixture was overlayered with 25ul of mineral oil (Sigma). DNA amplification was performed in a DNA thermal cycler (Perkin Elmer Ceteus) programmed for: 1st cycle of 3.5 min at 94 C, 1 min at 40 C and 2 min at 72 C followed by 44 cycles of 1min at 94 C, 1 min at 40 C and 2 min at 72 C. An additional cycle of 15 min at 72 C was used for final extension. Amplification products were analysed by electrophoresis on 1.5% agarose gel in 1x TBE buffer. Minor modifications were made in the amplification conditions for individual species only with respect to the amount of template DNA. Other conditions were kept identical.

RFLP analysis

DNA digestion and blotting: Six micrograms of genomic DNA were digested at 37C with different restriction enzymes (*Eco RI , Eco RV, Hind III, Pst I, Taq I, Sau3A*) etc. The fragments were separated by electrophoresis in 0.8% agarose

gels in 1x TBE (45 mM Trisborate and 1mM EDTA). The fractionated DNA from the gels were blotted onto Hybond N^+ (Amersham) membrane by Southern transfer (Southern 1975).

RFLP detection: Probe labelling was done either using non-radioactive fluorescein 11-dUTP or radioactive ^{32}P-dCTP. Probe hybridization and detection for non-radioactive method was done according to manufacturers' instruction (ECL Random Prime Labelling and Detection Systems, Ver. II, Amersham) and for radioactive method the procedure described in Sambrook et al 1989 was followed.

Genomic library construction

For use in RFLP studies, genomic clones were prepared following two approaches. The first was through PCR amplifications. Prominent amplified fragments of varying sizes revealed through individual RAPD (10 mer) primers were eluted from the gel. These gel purified fragments were further amplified using the same primer. The amplified and eluted fragments were used as probes for RFLP analysis.

The second approach was to develop specific probes by constructing genomic libraries. As a first step in genomic library construction, DNA samples from different species were digested to completion either with *Pst* I or *Eco* RI enzyme. The digested DNA samples were electrophoresed on a 0.8% agarose gel and fragments of 0.5 to 2.0 kb were eluted from the gel. In certain cases the digested DNA samples were directly used for ligation with the vector. After extracting with phenol and chloroform the digested DNA was ligated into *Pst* I or *Eco* RI digested and phosphatased *pUC* 18 or *pUC* 19 plasmid vectors. The ligation was done overnight at 8 C. Transformation using the ligated mix was done using DH5 alpha *E. coli* strain by standard procedure.

Data analysis - Both RFLP and RAPD assay were taken into consideration. Results of the RAPD assay represented a consensus of atleast two replicates. Only reproducible bands were considered for comparison. Pairwise comparison of amplification products and RFLP loci was done using Nei's similarity index (Nei and Li 1979). Cluster analysis were carried out using unweighed pair group mean average method (UPGMA, Sneath and Sokal 1973) and used to generate the dendrogram.

Methods of production of single-copy DNA probes

Random complementary DNA (cDNA) clones have been extensively used for RFLP analysis in plants (Landry and Michelmore, 1987). cDNA clones are derived from gene transcripts and are a good source of single-copy clones

(Tanksley and Pichersky, 1987). The mRNA population consists of both less and more abundant mRNAs, however, the high abundancy of a specific mRNA is very often correlated with a particular phase in the growth period or during stress.

Random genomic clones on the contrary, are likely to contain repeated DNA sequences, making them undesirable for RFLP mapping. However using specific methylation-sensitive enzymes single-copy clones can be produced by the following methods:

a) Colony hybridization with ^{32}p-labelled total genomic DNA is one method of selecting single-copy genomic clones (Bernatzky and Tanksley, 1986).
b) A strategy for producing genomic libraries enriched for single-copy sequences is based on the use of methylation-sensitive restriction enzymes. Data from maize suggest that repeated DNA sequences are methylated to a greater degree than single-copy DNA, with the result that cloning with methylation-sensitive enzymes such as *Pst* I produces libraries enriched for single-copy sequences (Burr *et al.* 1988). Genomic libraries are also developed using *Eco* RI restriction enzyme, however, the percentage of single-copy clones generated are less than that of *Pst* I enzyme.

Competent Cell preparation

For transformation of *E.coli* (DH5 alpha) with the recombinant plasmid, high efficiency competent cells are needed which are prepared as follows:

a) Initiate an overnight culture of DH5 alpha strain (3ml).
b) Inoculate 100 ml of Luria broth with the 24h old culture and incubate at 37 C for 2 h or until the absorbance reaches 0.2-0.4 at 560nm.
c) Pellet the cells in cold by centrifuging at 8K for 5 min.
d) Suspend the cells in (100 ml) sterile 100mM $CaCl_2$ for 30 min in ice.
e) Centrifuge in cold and resuspend the pellet in 4 ml of 100mM $CaCl_2$.
f) Add 15% (final)sterile glycerol, aliquot in 100 ul volumes.
g) Until use store in -80 C.

Transformation of E.coli

a) To 100 ul volume of competent cells add 2-5 ul volumes (100ng) of ligated DNA and incubate in ice for 45 min.
b) Heat shock for 2 min at 42 C.
c) Add 500 ml of Luria broth (without ampicillin) and incubate for 1h at 37 C.
d) Plate 200ul volumes of broth onto amp+ LB agar plate with 20 ul of 100mM IPTG and 50 ul of 2% X-gal.
e) Incubate the plates at 37 C overnight.

f) Recombinant plasmids are selected by their white colour against non-recombinant blue colonies.

Construction of cDNA library

Construction of cDNA library was initiated for isolating stress-induced genes. The target species for this study was *A. marina*. Total RNA were isolated from fresh leaves of *A.marina* using the GITC-LiCl (Guanidine thiocyanate-Lithium chloride) method.

The standardised protocol for isolation of quality RNA from mangrove species is as follows:

a) Grind 3g of tissue under liquid nitrogen and suspend it in 20 ml of GETM buffer (4M GITC, 10mM EDTA, 50 mM Tris-HCl, pH 7.5 and 8% 2 B-mercaptoethanol)
b) Shake well and add 20 ml of chloroform-isoamyl alcohol (24:1), vortex briefly and centrifuge at 10,000 rpm for 10 min. at 4C).
c) Transfer the aqueous phase to a fresh tube and precipitate the total RNA with 1.4 volume of 6M LiCl at 4C for 15 h.
d) Centrifuge at 15,000 rpm. for 30 min. at 4C and dissolve the pellet in 10ml of TE buffer (10mM Tris-HCl and 1mM EDTA at pH 8.0) containing 0.3M NaCl.
e) Remove the proteins by extraction with equal volume of phenol folowed by phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform.
f) To the aqueous phase, add 2.5 volume of ice-cold ethanol and incubate at -20C for 30 min.
g) Centrifuge at 10,000 rpm. for 10 min. at 4 C and dissolve in RNase free water.
h) Check the quality and quantity of RNA by Spectrophotometer.

Using the above method quality RNA (OD-260/280= 2.0) was obtained. mRNA was extracted from this preparation using the standard Oligo-dT column chromatography which is done as given below:

Preparation of oligo dT column

All the solutions prepared for RNA isolations were prepared in sterile DEPC treated water.

a) Mix 0.5 to 1 gram of oligo dT cellulose in 0.1 N NaOH in a DEPC (Diethyl pyro-carbonate) treated beaker.
b) Pour the mix in a sterile glasswool plugged 3 cc syringe without air bubbles.
c) Wash the column with 3 volumes of DEPC (sterile) water.
d) Wash the column with high salt buffer (HSB- 0.5 M LiCl, 10 mM Tris.Cl (pH 7.5), 1 mM EDTA and 0.1% SDS prepared in DEPC treated sterile water) till the pH is 7.0 to 7.5 and the column is ready for loading or store in -20 C after

a series of washes (wash 5 times in 0.1 M NaOH followed by Sterile water wash until the pH is neutral. Finally wash in ethanol and store in -20 C for future use).

Loading of RNA

e) Before starting loading wash the column with 10 ml of 0.1M NaOH.
f) Wash the column with 10 ml of sterile water.
g) Wash with 10-20 ml of HSB. The pH of the flow through should be 7-7.5 .
h) Load the water dissolved RNA to the column.

Elution of mRNA

i) Heat the RNA sample by incubating at 68 C for 10 min.
j) Add 10 M LiCl (0.5 final) and 20 % SDS to the heated sample.
k) Pass the sample through the column and collect the flow through.
l) Heat the flow through again at 68 C for 10 min. and pass through the column.
m) Collect the flow through and heat for the third time in the same manner and save the eluate.
n) Wash the column with 20 ml of HSB and save the eluate.
o) Wash the column with 25 ml of MSB and save the eluate.
p) (Medium salt buffer (MSB) -0.15 M of LiCl, 10 mM of Tris. Cl (pH 7.5), 1 mM of EDTA and 0.1% SDS prepared in DEPC water).
q) Wash the column with 10 ml of elusion buffer or the low salt buffer (LSB) and collect the eluate which contains the poly A+RNA (mRNA).
r) (LSB- 2m M EDTA and 0.1% SDS prepared in DEPC water).
s) Precipitate the eluted mRNA in 3 M sodium acetate and ethanol overnight at -20C.
t) Centrifuge at 26K at for 90 min. Air dry the pellet and dissolve in water.
u) Absorbance at 260 and 280 with a ratio of 1.8 - 2.0 indicates pure mRNA.
v) Following the above method, pure (OD=2.0) mRNA was obtained and synthesis of first strand of cDNA is being initiated using the cDNA synthesis kit from BRL, U.S.A. Further steps of cloning are underway and cDNA will be constructed in lambda-gt11 phage or plasmid vector and expressed in *E. coli* or yeast.

Salt tolerance Studies

One year old plants of *Avicennia marina* are treated for varying durations at increasing concentrations of NaCl, using distilled water as negative control. Crude protein was extracted from the leaves by grinding with a buffer (containing 250mM Tris, 20mM EDTA, 0.4% SDS and 0.1% 2-mecaptoethanol) in a 1:3 ratio. The suspension was spun down at 10,000rpm for 10 minutes at room

temperature and the supernatant used for SDS PAGE analysis. Purification of the protein is yet to be standardised.

The identification of proteins/enzymes playing a central role in salt tolerance would be followed by their cloning for further analysis of their regulation and (if suitable) for gene transfer into important crops.

RESULTS

GENETIC DIVERSITY STUDIES:

Analysis of inter- and intra population genetic diversity was one of the major objectives of this project. A number of species have been selected for the present study. Polymorphism at intra-and Inter-population level were undertaken only in the species with wide range of habitats. In all 27 species, belonging to 20 different mangrove genera, including some of the epiphytes were analysed for establishing relationship among them. Detailed studies on the genetic polymorphism have been completed in 12 different species using 1-12 distinct populatons. Table 7 summarises the number of species, number of populations, number of plants analysed, number of primers used and percentage polymorphism in them. Detailed description is given for four mangrove species where substantial informations were obtained.

EXCOECARIA AGALLOCHA

Genus *Excoecaria* comprises about 35-40 species distributed from the tropical Africa and Asia to west-Pacific region, with only three species reported from the mangrove areas. *E.agallocha*, a characteristic and predominent association of the mangrove community and the type species of the genus, has a wide distribution range from east Africa, India to Hawaiian islands and some parts of Australia. This species primarily occur in the mangrove forests with occasional distribution in inland stations. Within the mangrove forests, this species most commonly occupies exposed beaches or sandy estuaries and is the second most wide-spread species seen after *Avicennia marina*. The species has also been recorded in disturbed sites to an elevation of about 400 Mts. Despite the widespread distribution, the plants show no obvious morphological adaptation and varietal distinction has not been reported. The genus is distinguished from closely related members of the family by a combination of characters including dioecious condition, axillary inflorescence, male flower with 2-3 stamens and absence of a caruncle from the seed.

The present experiments were designed to assess the nature and extent of genetic polymorphism at intra and inter population level in this wide spread mangrove species using molecular markers. A comparison was also made to

asses the level of polymorphism between male and female plants of the same location.

Table 7: Extent of Genetic Polymorphism based on RAPD profiles

Species	No. of Populations Analysed	No. of plants	No. of primers	No. of Amplification products	% Polymorphism
Acanthus ilicifolius	8	48	18	86	7.3
Bruguiera cylindrica	1	16	7	58	10.6
Ceriops decandra	1	15	6	66	11.6
Excoecaria agallocha	6	36	16	149	65.0
Lumnitzera racemosa	1	17	8	48	11.2
Rhizophora apiculata	1	20	12	67	11.1
Rhizophora mucronata	1	25	12	75	12.3
Avicennia marina	10	200	17	172	76.7
A. officinalis	1	20	16	115	32.3
A. alba	1	12	12	111	37.8
H. fomes	1	12	12	96	23.5
X. granatum	1	15	16	82	12.6
S. portulacastrum	1	10	12	46	9.6
S. maritima	1	15	10	74	12.6
Nypa fruitcans	1	10	10	112	14.3
S. bracheata	1	20	12	96	12.6

RAPD analysis

In the present study 42 genotypes from seven distinct populations were analysed for RAPD using 16 random decamer primers. Table 2 details out the total number of amplification products, their size range and the number of polymorphic products observed in each primer. The comparison was based on the presence or absence of a particular band. Differences in the intensity of the bands were ignored. In all, 149 amplification products were revealed across all the plants analysed, of which 111 products were polymorphic and only 37 were common to all the genotypes. The number of amplification products ranged from 7(OPA 09) to 13 (OPA 16), with an average of 9.93 fragments per primer.

The overall size range of the amplified products ranged from 291bp (OPA 08) to 3658 bp(OPA 16). Fig 1 shows the RAPD profiles in two randomly selected genotypes from each populations using 4 different primers(OPA18, OPD06, OPD11, and OPA12, Fig. 1a).

In the subsequent analysis, genotypes belonging to individual population were assessed for the extent of intra population polymorphism. Table 7 gives details of the total number of the polymorphic products observed in each population. While 72% of the amplification products were polymorphic across all the populations, the intra population polymorphism ranged from 20.40 to 30.64 in population 6 and population 1, respectively. The other populations had 29.56 (pop 2), 27.10 (pop 3), 26.47 (pop 7), 23.36 (pop 5), and 21.90 (pop 4) per cent polymorphic loci.

TABLE 7 : Total number of amplificationfragments , and their size range in all the genotypes analysed

Sl no	Primer	Amplification products			Size range
		mono morphic	poly morphic	Total	From -To
1	OPA 06	3	7	10	510 -2401
2	OPA 18	0	7	7	470 -1918
3	OPD 03	1	9	10	377 -2358
4	OPA 15	3	6	9	444 -2367
5	OPA 08	2	6	7	291 -2007
6	OPA 06	2	10	12	472 -3499
7	OPA 20	2	5	7	526 -1643
8	OPD 16	2	7	8	398 -2293
9	OPA 12	4	7	11	388 -3658
10	OPD 12	1	12	13	353 -3409
11	OPD 11	6	4	10	536 -2555
12	OPD 15	3	6	8	694 -3508
13	OPD 13	3	6	9	672 -1243
14	OPA 14	3	7	10	509 -3126
15	OPA 16	0	10	10	340 -2019
16	OPA 17	3	5	8	510 -2128
	Total	**38**	**111**	**149**	

RFLP Analysis

RFLP analysis was carried out at inter-population level. One genotype from each population was selected for the present analysis. Genomic DNA from each genotype was digested with four restriction enzymes (*Eco*R I, *Eco*R V, *Hind* III and *Bam*H I). They were probed with six different probes (5 from the genomic libraries from the mangrove species *E.agallocha, Acanthus ilicifolius* and *Avicennia marina* and Cox I probe from rice). The Total number of loci detected using these

probes were 68,of which 26 were common across all the analysed genotypes, thereby showing an overall polymorphism of 61.76%. Maximum polymorphism was encountered with Cox I probe where all the bands were polymorphic. Fig 1c shows the RFLP profiles of one genotype from each population digested with *Eco*R I and *Eco*R V, probed with the genomic clones from *Acanthus ilicifolius*, *Avicennia marina* and Cox I.

TABLE 8 : Intra-population polymorphism in the amplified products.
Figure in the paranthesis are the polymorphic ones in each population

Sl.No	Primer	Pop 1	Pop 2	Pop 3	Pop 4	Pop 5	Pop 6	Pop 7
1	OPA 06	9 (3)	7 (2)	7 (3)	8(3)	5 (1)	7 (1)	6 (2)
2	OPA 18	6 (2)	5 (1)	4 (1)	5 (1)	6 (1)	5 (2)	4 (2)
3	OPD03	7 (3)	7 (2)	7 (2)	6(2)	8 (2)	9 (2)	7 (2)
4	OPA 15	7 (4)	4 (3)	4 (2)	4 (3)	4 (1)	3 (0)	5 (2)
5	OPA 08	7 (2)	6 (2)	5 (2)	3 (0)	9 (2)	6 (2)	6 (3)
6	OPA 06	10 5)	10(2)	8 (2)	10(2)	8 (2)	4 (3)	7 (2)
7	OPA 20	7 (3)	5 (3)	5 (1)	4 (0)	6 (1)	5 (0)	6 (2)
8	OPD16	7 (0)	7 (0)	8 (1)	7 (0)	7 (0)	7 (0)	6 (1)
9	OPA12	8 (3)	10(2)	9 (1)	9 (1)	7 (2)	6 (1)	7 (3)
10	OPD12	11(2)	11(3)	11(3)	11(3)	5 (1)	9 (2)	10 (2)
11	OPD11	7 (1)	8 (2)	4 (2)	3 (1)	4 (2)	7 (1)	5 (1)
12	OPD15	10(3)	8 (2)	8 (0)	9 (1)	8 (1)	7 (1)	8 (0)
13	OPD13	5 (2)	5 (2)	6 (1)	5(1)	6 (1)	4 (1)	5 (1)
14	OPA 14	10(2)	9 (2)	9 (3)	8 (1)	9 (3)	9 (1)	8 (2)
15	OPA 16	7 (2)	7 (3)	7 (3)	7 (3)	9 (3)	7 (1)	8 (1)
16	OPA 17	6 (1)	6(3)	5 (2)	6 (1)	6 (2)	3 (2)	4 (1)
	Total	**124(38)**	**115(34)**	**107(29)**	**105(23)**	**107(25)**	**98(20)**	**102(27)**

Comparison between male and female individuals

E.agallocha is a dioecious species where the morphological differences are not obvious. Due to the high level of polymorphism encountered in the species, we selected four male and four female genotypes from four different populations and subjected them to analysis using 20 random decamer primers. The RAPD profiles of these genotypes using three random primers are depicted in Fig 1b. Differences between the male and the female genotypes in their RAPD profiles was evident both at inter and intra population levels. The level of polymorphism among the males (46 out of 98 amplification products) and the female (48 out of 96 amplification products) were of the same magnitude. The eight genotypes (4 male and 4 female) in all had 106 amplification products of which 42 were polymorphic across the genotypes. No sex-specific amplification product was observed in any of the primers used.

Two primer assay

It is reported in *Lycopercicum esculentum* and *Brassica juncea* (Klein-Lankhrost *et.al* 1991, Jain *et.al* 1994) that two primer assay generates additional amplified products between individuals. In the present study, we analysed two individuals with a combination of two primers (1:1) for ascertaining its usefulness in genetic polymorphism assay. In total 6 primers were used both individually and in combinations. The profiles obtained through the combined primers, shared the bands present for both the primers when used individually. No additional bands were observed.

Cluster analysis

Representative samples (one each) were selected from each population and were subjected to analysis for inter population divergence. Pair-wise comparisons were made and the similarity index were used for calculating the dissimilarity co-efficient (DC) using the unpaired group mean average method. Cluster analysis is based on 149 RAPDs and 68 RFLP of which 111 and 26, respectively were polymorphic. Fig 2 depicts the inter population relationship based on the dissimilarity coefficient. The population belonging to the eastern and western coast formed two distinct clusters separated by a DC of 3.78. The population of Bhitarakanika, although geographically distinct from the other three populations of the eastern coast shared the same cluster. However, it was distinct within the group separated by a DC of 3.09.

***Avicennia* sp.**

Avicennia L. is a pantropical, exclusive mangrove genus of eight species, occupying diverse mangrove habitats. The genus was initially included in the family Verbenaceae but its segregation as a separate family, Avicenniaceae, is now generally accepted. However, phytochemical evidences do not favour the inclusion of the taxa belonging to *Avicennia* to a separate family (Reddy *et al.* 1993). The genus is uniform in its gross morphology and anatomy. Although, there are often useful diagnostic characters seen in the field, they are rarely transmitted to the labels of herbarium specimens (Tomlinson 1986), making the species identification difficult.

In the Indian subcontinent, the genus is represented by *A. marina* (Forsk.) *Vierh.*, *A. officinalis* L. and *A. alba* Blume. Among the three species, *A. marina* has the broadest distribution not only in India, but also throughout the world both latitudinally and longitudinally. With decreasing temperature from equator, the number of mangrove species decreases: only *A. marina* (var. *resinifera*) remains as the most frost resistant species in Southern Australia and Nothern New Zealand (Chapman 1977). While there are about 35 species in North-eastern tip of Cape York Peninsula, it is reduced to a solitary species, *A. marina* at the southernmost coastline of mainland Australia. The wide range of distribution of *A. marina* itself

has been attributed to its wide range of salinity tolerance (Macnae 1966). *A. officinalis* is most eastward and distribution range of this species is from South India through Indo-Malaya to Papua Guinea and Eastern Australia. It is one of the most distinctive *Avicennia* species and is recognized by its large orange-yellow flowers. *A. alba* has a distribution range from India to Indochina. This species can readily be distinguished from other species by its characteristic lanceolate, acute leaves with white undersurface.

Within *A. marina,* several varieties have been recognized, but morphological distinction is never clear-cut and much of the segregation is geographic. *A. marina* var. *typica* Bakhuzen (= *A. marina* var. *marina* Moldenke) is reported to be of wide occurrence in India, while *A. marina* var. *acutissima* Staf & Moldenke is restricted to the vicinity of Bombay, India (Rao 1967). The other varieties reported elsewhere are *A. marina* var. *resinifera* (Western Pacific), *A. marina* var. *intermedia, A. marina* var. *rumphiana* (Malay Archipelago), *A. marina* var. *anomala* (Low Island, Port Douglas and Queensland) and *A. marina* var. *australis* (Australia). Varietal description has not been reported in *A. officinalis* and *A. alba.*

The primary objectives of the present study were to assess the nature and extent of intra-specific variation in the three species of *Avicennia,* and to establish inter-specific relationship between them using RAPD and RFLP markers, which shall be useful for genetic conservation and improvement.

Intra-population variation in *A. marina* as measured by proportion of polymorphic RAPDs was between 17.8 (Ennore) and 38.9 per cent (Muthupet). Clustering based on Euclidean distance showed two distinct clusters including varying number of genotypes in each population Table 10. One genotype from each cluster was selected at random (total 20 genotypes) for studying inter-population variation using RAPD and RFLP markers. Percentage of polymorphism as revealed by individual primers varied from zero (OPD04) to 100 per cent. In total 172 RAPDs were amplified, out of which 132 were polymorphic in at least one pairwise comparison. The inter-population RAPD profiles amplified by OPD01, OPD04 and $(GATA)_4$ are shown in Figure 6a. RFLP analysis of the same genotypes showed 66 per cent polymorphism. The dendrogram constructed based on 132 polymorphic RAPDs and 62 RFLPs is shown in Figure 7.

Intra-population variation in *A. officinalis* (Fig. 6b) and *A. alba* (Fig. 6c) as measured by the proportion of polymorphic RAPDs was 32.3 and 37.8 per cent respectively. Percentage of polymorphism as revealed by individual primers varied from zero (OPD04) to 67 per cent in *A. officinalis,* and 72 per cent in *A. alba..* Clustering based on Euclidean distance showed 10 genotypes of *A. officinalis,* and 8 genotypes of *A. alba* forming major clusters. One genotype from the major cluster of Pichavaram population of *A. marina* and *A. officinalis* and

Coringa population of *A. alba* was selected at random to study the inter-specific relationship between the three species. In total 184 RAPDs were amplified, out of which 109 were polymorphic. Hybridization with 24 enzyme-probe combinations showed 136 RFLP bands, out of which 84 were polymorphic. The inter-specific relationship among the three species based on the genetic distance as revealed by RAPDs and RFLPs is shown inFigure 7c.

Table 10: Details of RAPD and RFLP analyses in *Avicennia* species

Species/ Populations	RAPDs/ RFLPs		% Polymorphism
	Total	Polymorphic	
A. marina			
Gulf of Mannar	98	30	30.6
Muthupet	95	37	38.9
Pichavaram	92	29	31.5
Ennore	90	16	17.8
Coringa	93	26	28.0
Bhitarkanika	91	32	35.2
Calicut	85	17	20.0
Goa	93	29	31.2
Ratnagiri	88	20	22.7
Bombay	85	16	18.8
Average	**91**	**25.2**	**27.7**
A. marina			
Interpopulation	172	132	76.7
RAPD	94	62	66.0
RFLP			
A. officinalis			
Pichavaram	96	31	32.3
A. alba			
Coringa	111	42	37.8

Acanthus ilicifolius

Acanthus ilicifolius is a predominent mangrove species occupying all types of mangrove formations. The species in general occupy regions away from the sea coast and inhabit less saline conditions. In certain places, *A. ilicifolius* forms distinct monoculture in degraded and denuded places. This species has been collected from a large number locations.

The mitotic chromosome analysis was carried out for the samples collected from three populations (Pichavaram, Goa and Calicut) using root tip meristems cells. The cells were characterised by the presence of 48 chromosomes resolved into 24 homomorphic pairs. The complements were characterised by chromosomes with mostly submedian centromeres. No variation in chromosome number was observed either in the plants belonging to the same or to different population.Figure 8 shows the metaphase chromosome complement of a plant from Pichavaram and Calicut populations.

RAPD analysis

Forty-eight genotypes from eight geographically distinct populations of *A. ilicifolius* were selected for the present analysis.The samples were initially screened for intra-population variation using 15-18 random primers. The number of amplification products ranged from 69-78 for different populations (Table 11) and polymorphism was between 3.8 to 7.3 per cent. RAPD profiles of seven genotypes of the Pichavaram population obtained for five random primers are shown in Figure 3. The profiles were reproducible as can be seen from the comparisons between the lanes 8 to 14 and lanes 22 to 27, obtained through the use of a single primer in two replicates.

Based on the results from RAPD analysis of intra-population variations, one representative sample from each population was selected and the genomic DNA was amplified for 13 primers to account for inter-population variation. The number of amplification products observed ranged between 3 (OPA 06) and11 (OPA 19) with a size of 0.35 kb to 3.6 kb. A total of 73 amplification products were observed and of which 25 were polymorphic in atleast one pairwise comparison. Of the 13 primers tested, only two primers (OPA 03, OPA 06) revealed similar profiles for all the genotypes analysed representing all of the eight populations. Although for other primers, the RAPD profiles of these eight populations shared a number of common bands, population-specific profiles could be observed through the use of one/ two primers. Figure 4 depicts the RAPD profile of one sample, randomly selected, from each population. The profiles of each population are distinguishable.

Table 11: Intrapopulation polymorphism in *Acanthus ilicifolius*

Popula-tion	RAPD Analysis				RFLP Analysis		
	No of plants analysed	No of primers used	No. of amplification products	Per cent polymorph -ism	No. of plants analysed	Total no of RFLP fragments	Per cent polymor-phism
1	7	16	78	3.8	5	31	3.2
2	6	16	74	4.1	6	33	9.1
3	6	18	83	4.8	6	29	6.9
4	6	16	72	6.9	6	33	6.1
5	5	16	79	5.6	5	36	8.3
6	6	18	86	6.9	6	27	7.4
7	6	14	68	7.3	6	29	6.8
8	6	15	72	5.6	5	33	6.1

RFLP analysis

Forty five plants of *A.. ilicifolius* representing eight populations were analysed with RFLPs. The level of polymorphism both within and between the

populations was assessed using PCR generated fragments and clones with low copy sequences from the *Pst* genomic library as probes.

Genomic DNA of five to six genotypes from each population were digested with three enzymes and probed with two PCR generated and three *Pst* genomic probes. The size of probes used was 0.5 to 2.0 kb. Table 11 lists the total and polymorphic RFLP fragments observed for each population using 15 probe/enzyme combinations. The size of the bands ranged from 0.5 to 10 kb for *Eco* RI, 0.9 to 12 kb for *Eco* RV and 1.0 to 6.5 kb for *Hind* III. Figure 11 shows the intra-population RFLP pattern in five individuals from the Pichavaram (population 1) revealed through the use of two restriction enzymes and probed with a *Pst* genomic clone. The polymorphism in RFLP loci ranged narrowly from 3.2 (population 1) to 9.1 (population 2) per cent (Table 11).

As the level of polymorphism at intra-population level was negligible, we selected five populations (three from the western coast and two from the eastern coast) for RFLP analysis between populations. For each population one genotype was analysed. Here also, fifteen probe/enzyme combinations were used. They produced a total number of 96 RFLP fragments of which 44 were polymorphic across the populations in atleast one pair- wise comparison. The overall size of the fragments produced ranged from 0.8 to 7.0 kb for *Eco* RI, 0.3 to 9.0 kb in *Eco* RV and 0.35 to 8.0 kb for *Hind* III.

Cluster analysis

Pairwise comparisons were made for the RAPD profiles obtained through the use of thirteen random primers in the representative samples of all the eight populations. The five populations analysed from the eastern coast formed the main cluster separated at a DC (dissimilarity coefficient) of 0.38. In the subsequent analysis, two populations from the eastern coast and three from the western coast were surveyed for both RAPD and RFLP markers. The relationship between the five populations was anslysed using 69 RAPD and 96 RFLP fragments of which 22 and 44 fragments, respectively were polymorphic.The two eastern populations formed a distinct cluster separated at a DC of 0.28, and the three populations from the western coast were separated from this cluster at a DC of 0.36, 0.39 and 0.42 (Figure 5).

***Rhizophora* Sp.**

The species and hybrids of *Rhizophora* constitute the dominant component of mangrove ecosystems. They occur as the most seaward species and protect the mangrove wetland from tidal waves and storm surges. Litter fall from this component (885-978g dry wt/m^2/year) supports the microorganisms and the benthic fauna of the ecosystem by nutrient recycling (Rao, 1987). The extensive

root systems provide the breeding place for fishes and prawn, as well as the shelter for the juveniles. In all these aspects, role of the hybrid is more significant than the species as a result of its hybrid vigour.In India, the genus *Rhizophora* have already been reported to be at the verge of extinction (Blasco, 1977), among which, sterile hybrids are the most vulnerable. In the present study area, Pichavaram Mangrove Forest, Tamil Nadu, India, *Rhizophora* is represented by *R. apiculata* Blume, *R. mucronata* Lamk. and a co-occurring sterile hybrid. The hybrid blooms profusely but rarely produces viviparous propagules (Muniyandi and Natarajan, 1985; and the authors' personal observation). Production of synthetic hybrids by sexual hybridisation between the parental species and introduction into the original habitats is one strategy for conservation. However, reports on parentage based on morphological features for this hybrid were contradictory (Lakshmanan and Rajeswari, 1983; Muniyandi and Natarajan, 1985; and Kathiresan, 1995). Therefore, the present study, based on Random Amplified Polymorphic DNA (RAPD, Williams *et al.*, 1990) and Restriction Fragment Length Polymorphism (RFLP) markers was undertaken.

Table 12. Morphological features of the single trees of *R. apiculata*, *R. mucronata* and the hybrid

Character	*R. apiculata*	*R. mucronata*	The hybrid
Leaves	dark green, elliptic, mucron not present	light green, obovate, mucron is prominent	dark green, broad obovate, mucron present but not prominant
Peduncle	brown and short (<1cm)	light green and long (1.5-4cm)	green and intermediate (1-2.5cm)
Anthers	9-12 anthers equal in size	8 (4+4) equal in size	11-14 anthers unequal in size. 3-6 small anthers and 8-10 big anthers
Style	sessile	sessile	1.5-2.5mm
Pollen	fertile	fertile	sterile (98-100%)
Hypocotyl*	short, blunt tip	long, pointed tip	---

** We did not find any propagule in the hybrid tree used for the present study. However, a few propagules collected from other trees were short or intermediate between R apilculata and R mucronata, and the tip of the hypocotyl was blunt.*

The primers used, sequence of the primer, number of amplification products (RAPD bands) for each primer, polymorphism between the two species and similarity between the combined banding pattern of the two species and the banding pattern of the hybrid are given in Table 13. PCR amplification of genomic DNA for twenty-five primers produced 94, 105, 125 amplification products in *R. apiculata*, *R. mucronata* and the hybrid respectively. Polymorphism (proportion of polymorphic bands to the total number of bands) between the species for individual primers varied from 25 per cent (OPA16, OPD01 and OPD13) to 80 per cent (OPAF10 and OPAF13) with an average of 42 per cent.

The banding pattern for the primers OPA14 and OPD08 were monomorphic, and hence they were not useful in parentage analysis. Among the remaining 23 primers, every band observed in the hybrid was present either in *R. apiculata* or in *R. mucronata* or in both for 19 primers (eg., OPAF12, OPD13, OPA20, OPA15, OPA17, OPAF13). For the primers OPAF10, OPD03 and OPD15 one amplification product each observed in *R. mucronata* was not present in the hybrid (missing bands). Amplification by OPA08 produced a high molecular weight band in the hybrid which was absent in both the species (non-parental band).

Table 13: Identification of the parentage of a *Rhizophora* hybrid using RAPD markers

	No. of Amplification Products			
Primer	*R. apiculata*	*R. mucronata*	Hybrid	Similarity between the species and hybrid
OPA06	1	2	2	100
OPA08	3	2	4	75
OPA10	4	5	6	100
OPA14	1	1	1	100
OPA15	2	1	2	100
OPA16	4	3	4	100
OPA17	7	5	7	100
OPA20	3	6	6	100
OPD01	4	3	4	100
OPD03	4	6	5	83
OPD06	1	2	2	100
OPD07	2	3	3	100
OPD08	6	6	6	100
OPD13	3	4	4	100
OPD15	2	3	3	75
OPAF07	3	5	5	100
OPAF10	2	4	4	80
OPAF11	11	10	13	100
OPAF12	7	4	7	100
OPAF13	4	2	5	100
OPAF14	5	9	10	100
OPAF15	3	3	4	100
OPAF18	4	5	6	100
OPAR16	3	6	6	100
OPAR20	5	5	6	100
Total/ Average	94	105	127	96.5

The banding pattern of the two species were combined to simulate the expected hybrid banding pattern and compared with that of the actual hybrid to calculate percentage of similarity. The similarity for individual primers varied from 75 to 100 per cent with an average of 96.5 per cent. Restriction digestion of the total

genomic DNA with three enzymes followed by probing with mitochondrial genome specific probe (atp 6) exhibited 100 per cent polymorphism between the two species. The RFLP pattern of the hybrid showed 100 per cent similarity with that of *R. apiculata*, and none of the restriction fragment in *R. mucronata* was observed in the hybrid.

Genetic relationship among various mangrove genera

In a preliminary study of 20 mangrove species belonging to 9 genera a dendrogram was constructed based on RAPD markers The dendrogram showed that two species within a genus shared a common node and if there were more than two species in a genus they formed a cluster to the node with narrow difference in similarity (Parani *et al.* 1997, unpublished). Later, a detailed study on three of the 20 species namely *Avicennia marina, A. officinalis and A. alba* using RAPD and RFLP markers also revealed close inter-specific relationships with 63-84 % similarity between the species (Parani & al. 1997a). For the present study, therefore, one species from a genus was arbitrarily selected to learn about more number of mangrove and its associated genera. The genera represented in the present study included 11 true major mangroves, 3 true minor mangoves, 2 mangrove associates, 2 mangrove parasites, 3 terrestrial wild species and 1 cultivated species.

Genomic DNAs from these species were isolated using different protocols as described in materials and methods. Barring *Rhizophora* and *Sonneratia*, the DNA isolation protocols yielded consistently good amount of DNA suitable for PCR amplification and digestion with restriction enzymes. In case of *Rhizophora* and *Sonneratia*, DNA yield was very less compared to other species, and the co-precipitating mucilage made the DNA viscous. Although, it did not hinder PCR amplification, restriction digestion required more enzyme (10-15 units/µg DNA) and incubation overnight for complete digestion. Twenty primers with arbitrary sequences of ten nucleotides (random primers) and three microsatellites viz.(GATA)4, (GACA)4 and (GTG)6 were tested for amplification. The random primers had G + C content ranging from 50 to 100 per cent. Among the tested primers, nine random primers and (GATA)4 gave satisfactory amplification. The amplified primers had either 60 or 70 % G + C content except (GATA)4 which had 25% G + C content. The size of the amplified products ranged from 0.3 to 4.6kb. Amplification with these ten primers was carried out three times and only those amplification products which were consistently observed in two replications (consensus products) have been scored for further analysis. The primer sequences of the ten primers, and the number of DNA products are listed in Table 14. For RFLP analysis, genomic probes developed from the mangrove species *Acanthus ilicifolius, Avicennia marina, A. officinalis, Excoecaria agallocha, Bruguiera cylindrica* and *Rhizophora apiculata* were used. The membranes were washed at high stringency and exposed overnight. The probe code, size of the probes and their source is given in Table 14. Since polymorphism with different

enzymes for the same RFLP probe is not considered independant, for a set number of two enzymes 14 different probes were so as to have 14 independant enzyme-probe combinations for analysis. The probe and the number of loci detected are listed in Table 15.

The number of RAPD and RFLP loci detected per primer or enzyme-probe combination was much higher than that of estimated at intra- and inter-specific level in certain mangroves earlier (Parani *et al.* 1996, Parani *et al.* 1997a, Parani *et al.* 1997b, Lakshmi *et al* . 1997, Parani *et al.* 1997, unpublished). The number of RAPDs amplified by the primers varied greatly from 16 to 31. All the RAPDs generated by all the primers were polymorphic, except the two fragments produced by the primer OPD06 which were monomorphic across all the species. Each primer, except OPD06 produced species specific fingerprints which were diagnostic. The number of RFLPs detected by individual enzyme-probe combinations varied from 6 to 20. All the fragments detected by the probes were polymorphic, except the six fragments detected by three probes which were monomorphic. However, RFLPs detected by single enzyme-probe comination was not sufficient to get species specific pattern for all the species. RFLP profiles detected by hybridisation with the probes to the *Eco*RI digested DNA of the 22 species are shown in Fig. 8a.

Table 14: The number of loci scored among the 22 species against each primer and enzyme-probe combination.

	RAPD analysis		RFLP analysis			
			*Eco*R I blot		*Hind* III blot	
Primer	Sequence	No. of loci	Probe	No. of loci	Probe	No. of loci
OPA03	5'AGTCAGCCAC	24	ACP101	9	ACP201	11
OPA07	GAAACGGGTG	20	AMP105	18	AMP262	21
OPA11	CAATCGCCGT	18	EXP202	13	EXP102	17
OPA18	AGGTGACCGT	16	BCP012	16	BCP006	15
OPD04	TCTGGTGAGG	31	RAP002	15	RAP112	13
OPDO6	ACCTGAACGG	22	AOP252	14	AOP152	16
OPD08	GTGTGCCCCA	18	AME082	6	AME082	10
OPAR13	GGGTCGGCTT	21				
OPAF08	CTCTGCCTGA	19				
$(GATA)_4$	$(GATA)_3$	24				
	Total	213		91		103

Table 15: Name and source of the genomic clones used to probe the blots made from *Eco* RI and *Hind*III digested DNA

S. No.	*Eco*RI blot	*Hind*III blot	Source
1.	ACP 101 (2.0)*	ACP 201 (2.0)	*A. ilicifolius Pst*I/pUC18 library
2.	AMP 105 (1.8)	AMP 262 (1.8)	*A. marina Pst*I/pUC18 library
3.	EXP 202 (1.5)	EXP 102 (1.5)	*E. agallocha Pst*I/pUC18 library
4.	BCP 012 (1.8)	BCP 006 (1.8)	*B. cylindrica Pst*I/pUC19 library
5.	RAP 002 (3.2)	RAP 112 (3.2)	*R. apiculata Pst*I/pUC19 library
6.	AOP 252 (2.5)	AOP 152 (2.5)	*A. officinalis Pst*I/pUC18 library
7.	AME 082 (2.0)	AME 082 (2.0)	*A. marina Eco*RI/pUC18 library

* Figure given inside the bracket indicate the size of the insert in the respective clone

In total 213 RAPDs and 194 RFLPs were detected. The presence/absence data of RAPDs and RFLPs were analysed independently as well as after pooling the data from both, and three separate dendrograms were constructed. Comparison of the denrograms of independant analyses of RAPD and RFLP data with that of from the pooled data, although, revealed differences in the strength of relationship between certain groups, by and large, the pattern of clustering remained the same. Therefore, for the purpose of discussion, the dendrogram given in Fig. 8b which was constructed from the pooled data has been used. In the dendrogram clustering of 12 species representing 10 major mangrove genera, *Excoecaria* and *Acanthus* into one major group was observed. In that major group, *Bruguiera* and *Ceriops*, *Rhizophora* and *Kandelia*, *Avicennia* and *Aegiceras*, and *Excoecaria* and *Acanthus* shared common nodes. Another major cluster included *Nypa*, mangrove associates, parasites, *Pandanus* and *Lycopersicon*.

Studies on mechanism of salt tolerance:

Progressive salinization of irrigated land poses a threat to the future of agriculture. In contrast to halophytic plants living partially in sea water, crop plants are sensitive to high NaCl concentrations. Within salt sensitive species there is great diversity with respect to salt tolerance, suggesting that genetic engineering has the potential to improve salinity tolerance in crop species. A scrutiny of cellular processes involved in the adaptation of organisms to salinity suggest that halotolerant genes could respond to components of crucial defence

responses, i.e. ion transport, Ca^{2+}/Cl^- channels and sensory mechanoreceptors, or metabolic processes such as protein or osmolyte synthesis.

Two basic approaches have been used to identify salt tolerant genes :

a) characterisation of new and abundant proteins that occur during adaptation,

- salt treatment of plants at varying concentrations for varying durations
- crude protein extract analysis by SDS-PAGE
- Purification of proteins
- Amino acid analysis and antibody studies

b) looking for changes in specific genes coding for proteins logically involved in adaptation,

- Assay activity of peroxidases,catalases, superoxide dismutase (oxidative stress)
- plasma membrane and vacuolar H^+ATPasesa
- kinase/phosphatase activity- enzymes involved in signal transduction
- enzymes involved in osmolyte synthesis

An ideal system for the study of the mechanism of salt stress tolerance, is the coastal mangrove ecosystem where the salinity varies with the incoming tide and seasonal monsoons from 0 ppt to 38.0 ppt. The plant chosen for the present study, *Avicennia marina* is one of the most widespread core mangrove species along the Indian coast. It is known to tolerate salinity of 38ppt and more. Special physiological features as well as the presence of osmolytes in some of these species has been documented. A detailed analysis and characterisation of the proteins induced on salt stress in a temporal and tissue specific manner, will reveal vital information on salt stress management. Importantly, a better understanding of the relationship between tissue specific gene regulation and stress responsiveness will help configure our course towards future crops tolerant to tomorrow's saline and desiccated soils.

Genomic Library

Recombinant clones were selected on IPTG- X-gal plates and the plasmids were initially screened for the presence of inserts. This is done by quickly lysing the cells in high salt (1xSTE, Sambrook et al. 1989) buffer and extracting with phenol and chloroform. Recombinant plasmids were digested with either *Pst* I or *Eco* RI as the case may be for releasing the inserts. The size of the inserts was checked with control DNA size marker alongside in agarose gel. The size range of the inserts of the clones thus developed in different mangrove species (as mentioned in the text below) are given below in Table 16. Some of the genomic clones of *A. ilicifolius* appear in the Fig. 4c.

Table 16: Total number of high copy genomic clones and identified repeat positive clones.

Species	Cloning site	No.of clones	Insert size range (kb)	No.of repeat positive clones
Avicennia marina	*Pst* I	412	1.5 - 9.0	4
	Eco RI	60	1.2 - 3.5	2
A. officinalis	*Pst* I	475	0.8 - 9.0	6
	Eco RI	110	1.4 - 4.5	3
Acanthus ilicifolius	*Pst I*	320	0.5 - 4.5	5
	Eco RI	80	0.6 - 2.5	1
Excoecaria agallocha	*Pst* I	75	2.0 - 5.0	1
	Eco RI	22	1.5-2.5	
Lumnitzera racemosa	*Pst* I	25	1.0 - 4.5	-
	Eco RI	10	0.3 - 2.2	
Rhizophora apiculata	*Pst* I	78	1.2 - 5.1	-
Bruguiera cylindrica	*Pst* I	45	0.9 - 4.4	2
Ceriops decandra	*Pst* I	50	1.0 - 5.0	1

The clones developed both by PCR method and genomic libraries were used for radioactive and non-radioactive methods of probing in our RFLP studies. All the genomic clones in *pUC* plasmids are maintained as glycerol stocks at -70 C.

Genomic clones have been prepared in the following mangrove genera: *Acanthus ilicifolius, Avicennia marina, A. officinalis, Excoecaria agallocha, Rhizophora* spp., *Ceriops decandra, Bruguiera cylindrica* and *Lumnitzera racemosa.* So far, more than 1500 clones have been prepared with a maximum representation in *Avicennia* spp. and *Acanthus* sp. Genomic clones from the above mentioned genera are being accumulated with an ultimate objective of screening them for any gene(s) of interest, for example gene(s) for salinity tolerance.

Screening of the genomic clones for DNA-repeat sequences

Screening of the genomic clones is being carried out using microsatellites ie., DNA repeat sequences like $(GATA)_4$, $(GACA)_4$, $(GTG)_5$, $(CA)_8$, and $(AT)_8$. Few positive clones (upto 10) for each microsatellite have been obtained in *Avicennia* spp. and *Acanthus* sp. Characterization of these repeat positive clones by double stranded DNA sequencing is underway in order to synthesize species-specific flanking primers for intervening VNTR (variable number tandem repeat) loci.

DNA sequencing of the repeat positive clones

Few GTG and GATA positive clones have been sequenced using forward and reverse universal primers using Sequenase version II (Amersham). As the number of tandem repeats in them were not many, sequencing of more clones is under way for finding more number of tandem repeat regions for the synthesis of flanking primers which would facilitate studying of polymorphism more closely.

Preliminary screening for stress- induced enzyme genes

Colony hybridization of the genomic clones from few mangrove species is being carried out using stress-induced genes as the DNA probe. To begin with, osmotic-stress induced osmotin-like protein gene from tomato (a gift from Dr. Rosa Rao, University of Naples, Italy) and pathogen-induced thaumatin-like protein gene sequence from rice (a gift from Dr. S. Muthukrishnan, Kansas State University, U.S.A) were used as DNA probes. By Southern blotting of the digested genomic DNA and probing we confirmed the presence of sequences homologous to these two cDNA probes in the target species (*Avicennia marina*). Tentative positive clones obtained in our preliminary screening are being analysed.

Shot-gun cloning was carried out to identify osmotin and thaumatin-like gene sequences in the target species. Essentially in this approach, Southern hybridization was done for osmotin/thaumatin DNA probes and the homologous region on the blot which showed up as intense signal in the autoradiogram (2.0 to 2.5 kb) was used to elute the similar region on the twin gel loaded with the same DNA for use in constructing the library. The eluted fragments in turn served as markers for direct elution of the homologous regions without the need for Southern hybridization. Eluted and purified fragments were used to construct genomic library enriched for the homologous regions in the genomic DNA. Currently more such clones are being accumulated in order to be followed by conventional screening to locate true positive clones.

DISCUSSIONS

In general, mangroves are typical plant formations occupying inter-tidal and estuarine zones of the tropical and sub-tropical regions of the world. On account of their location in the coastal region, where resource use conflicts are serious, the mangroves are under severe threat of extinction. Given the rate at which the area under mangroves are declining [area under mangrove in India has come down to 356,500 ha in 1977 (Blasco 1977) from 681,976 ha in 1963 (Sidhu; 1963)], and the fact that many mangrove species are on the way to extinction due to human intervention, reduced precipitation and climate change, it is essential to

develop sound conservation strategy for any mangrove species. As a prelude to conservation, intra-specific genetic variation in different mangrove species occurring in India was studied using molecular markers. Such studies, among other things it was hoped, could help to select priority areas for conservation and provide vital information for development of genetic sampling and improvement (Waugh and Powell 1992).

Mangroves are recalcitrant plants where both physical and systemic constraints are obvious. Information on reproductive biology and population genetics of mangrove species, to a large extent, is lacking. Conventional genetic analysis is difficult in mangrove species and so far no detailed studies have been carried out in this unique group of plants. The available information on the genetic structure of mangrove species is scanty and largely restricted to some stray reports on chromosome number and isoenzymes (Kumar and Subramanian 1988, McMillan 1968). Based on the available information it is not possible to partition the existing variation in mangrove species into environmental and genetic components. As an aid to our ongoing conservation programme, we have initiated investigations to document both intra-site and inter-population polymorphism in various mangrove species using molecular markers, with an overall objective of selecting/identifying distinct genotypes for long term conservation.

RAPD markers provide a convenient and rapid assessment of the differences of the genetic composition of related individuals. The relative ease and greater convenience (Aitkin et al 1994) in the analysis of RAPD markers and their stable nature against environmental changes have been useful in the assessment of variation (Halwart et al 1991, Tao et al 1993, Aitkin et al 1994), fingerprinting (Caetano-Anolles et al 1991), cultivar/ species identification (Hu and Quiros 1991, Welsh et al 1991, Jain et al 1994, Parida and Raina 1994), assessment of stability in micropropagated plants (Rani et al 1994), quantitative phylogenetic comparison of genotypes (Halwart et al 1992) and genetic mapping (Williams et al 1990).

In the present study, different populations of *A. marina* revealed varying degrees of genetic polymorphism in their RAPD profiles. Polymorphism was highest in the Muthupet population (which represents pure stand of *A. marina* with conspicuous absence of other mangrove genera like *Rhizophora, Ceriops, Bruguiera* etc) followed by Bhitarkanika, Pichavaram, Goa, Gulf of Mannar and Coringa populations which are rich in terms of species diversity (Untawale 1985; Status Report 1987). The Ennore population exhibited lowest polymorphism of 17.8 per cent. This population represents back water mangrove comprising of patches of *A. marina*. Constant use of its foliage for fodder and extensive cattle grazing prevent seed set and reduces the chances of new genetic recombinations to establish. Added to it, the release of effluent from industrial units into the

habitat makes the soil no longer suitable for establishment of seedlings. These might have also resulted in directional selection and colonisation of genetically uniform genotypes. Calicut, Ratnagiri and Bombay populations represent the locations most affected by the conversion of mangrove areas for agriculture, mining and urbanization. This has drastically decreased the area under mangroves, and increased the anthropogenic pressure over the remaining parts of the ecosystem. The net loss of the genotypes and genetic erosion was reflected in reduced polymorphism in RAPDs. Even, the total number of RAPDs observed in these populations was less. The most striking observation was that the populations representing true mangrove formations display greater degree of variability in *A. marina*. In comparison, the percentage of polymorphism observed in the populations of *A. officinalis* and *A. alba* from Pichavaram and Coringa respectively was greater than the average percentage of polymorphism observed in *A. marina* (see Table 10).

The dendrograms constructed for individual populations of the three species showed two clusters each including varying number of individuals and no genotype formed OTUs (Operational Taxonomic Units). One genotype from each cluster of each population of *A. marina* was selected at random to study the variation at inter-population level. Polymorphism at inter-population level was more than intra-population variation indicating high degree of genetic divergence between the populations. This may be attributed to the diversity in the environmental and physical conditions of the geographical locations where these populations have evolved (see Table 6). Exclusion of Ennore, Calicut, Ratnagiri and Bombay populations from the data analysis reduced the percentage of polymorphism by 12.3 per cent (data not shown) which indicated that a considerable amount of polymorphism observed in this species is unique to these populations.

In the inter-population analysis, any RAPD or RFLP particular to a group of genotypes was not observed and the dendrogram did not show any distinct grouping among the genotypes. Therefore, though, molecular markers have been used to delineate varieties (Fukuoka *et al.* 1992) and to identify cultivars (Schnell *et al.* 1995), the present study does not indicate the existence of distinct entities in *A. marina*, atleast among the populations studied. This is in accordance with the observations based on morphological characters and distribution (Tomlinson 1986). Therefore, it seems inappropriate to recognise *A. marina* var. *acutissima*, the only variety other than *A. marina* var. *typica* reported in India as a distinct entity.

One genotype each of *A. marina* and *A. officinalis*, selected from the major cluster of Pichavaram population and *A. alba* from Coringa population was analysed to establish inter-specific relationship. Genetic distance as measured by similarity percentage in RAPDs and RFLPs was more between *A. marina* and *A. alba* than

between *A. marina* and *A. officinalis*. Among the three species *A. alba* and *A. officinalis* were closely related.

The present study showed that genetic polymorphism in *A. marina* as measured using molecular markers varied between populations and genetic divergence was significantly higher between the populations than within a population. These observations have indicated that :

1. The locations like Ennore where genetic erosion was of higher magnitude should be given priority for *in situ* conservation as these populations also contribute substantially to the overall genetic variation in the species

2. for the same reason, all the populations should be included in genetic sampling to represent the species in any *ex situ* conservation program, however, sample size may proportionately be reduced for populations like Ennore, Calicut etc., which show less polymorphism.

We assessed the nature and extent of genetic polymorphism in *E. agallocha*, a widely distributed mangrove species using markers generated through RAPD analysis. The present analysis demonstrates a high level of polymorphism between the samples analysed from six ecogeographically distinct populations to the extent of about 70% of amplification products being polymorphic. However, at intrapopulation level the variation encountered was significantly low ranging from 29.7 to 46.7 per cent, compared to the overall polymorphism in the species (Table 8).

Mangrove ecosystem is a complex and dynamic one. The uniqueness of this ecosystem is that the biota is constantly under physiological stress caused by fluctuating growing conditions. Despite such extremes, mangroves have successfully colonised by developing morphological, reproductive and physiological adaptations (Saenger 1982, Claugh et al 1982, Claugh 1994). The mangrove community is therefore not uniform structurally or functionally as number of environmental factors influence individual species differently (Kathiresan 1994). Secondly, the variability encountered in the mangrove species with respect to their morphological and genetic characteristics in a given environment is predominently determined by the basic characteristics of the species that gets colonised in a given region. The type, size and frequency of occurrence of a species is, therefore, a function of a particular mix of fluvial, tidal and wave energy found in a particular region and also the microclimatic conditions prevailing in that region. Different species could, therefore, colonise at a site depending on their adaptation and edaphic preferences. Differences in the climatic factors prevailing in each locations, therefore, would result in differential adaptibility specific to the sites and depending upon the genomic constitution of the species, it could show variable degree of differences. It is

therefore envisaged that the extent of polymorphism displayed by the mangrove species would be species-specific. This is supported by the extensive polymorphism encountered in the present case, compared to other mangrove species (eg. *Avicennia marina* and *Acanthus ilicifolius*) where both the inter and intra population differences are extremely low (Unpublished data). The present analysis, therefore, shows highly variable genetic constitution in *E. agallocha* as also the high degree of polymorphism encountered in the samples collected from different populations.

The other reason for variability in this species could well be attributed to the reproductive nature of the species. Since it is a dioecious species, predominent cross pollination between different plants is an inevitable consequence. The cross pollination between two genetically variable plants could well trigger the overall genetic polymorphism in the species. Based on the present analysis, it is logical that the polymorphism encountered between the populations is the result of the differences in microhabitats and climatic conditions of the location (Table 6) and the species' adaptation. And the intra-site polymorphism could well be ascribed to the outbreeding nature of the species. At the same time, absence of polymorphism between male and female plants inhabiting the same location with a number of primers points to the fact that the degree of differences in the species is independent of their sexual differences. In fact, the degree of polymorphism exhibited by male and/or female genotypes of any population was of different magnitude.

A large body of information is available depicting RAPD variability in both crop and non crop species. The extent of variation in RAPD loci varies from species to species. At present, we do not have any information on the extent of genetic variation in different mangrove species. However, in case of another dioecious species (*Populus deltoides*), to which a parallel can be drawn for the present study, RAPD markers has been useful in differentiation of the clones developed through controlled pollinations between selected male and female clones (Rani et al 1994), and the species in general exhibits a high degree of intraspecific polymorphism. However, no analysis have been carried out to account for the intraclone polymorphism in this species (Rani and Raina, Personal communication).

The present analysis also points out that two primer amplification is not useful in the investigated species in generating additional markers that contradicts the earlier reports (Jain et al 1994, Klein-Lankhorst et al 1991). However, studies on other mangrove species could tell us as to whether any generalisations can be made or not.

The present analysis of 48 genotypes of *A. ilicifolius* representing eight distinct populations of Indian coastline, reveals a low level of genetic differences

between the genotypes at the intra-population level. Although 4-6 per cent of the amplification products in these cases were polymorphic across the genotypes, the RFLP profiles were largely uniform between the individuals of a given population. At the inter-population level, the polymorphism in RAPD loci was about 28 per cent. This was the case with the RFLPs also. The observed low level of polymorphism at intra-population level could therefore broadly speak of the stable genetic constitution of the species. In case of chromosome analysis also, no numerical and/ or structural variations were observed.

Mangrove species are constantly subjected to physiological stress caused by fluctuating growing conditions. Despite such extremes, they have successfully colonised suitable areas by developing morphological, physiological and reproductive adaptations (Claugh *et al.* 1992, Clough 1982, Saenger 1982). Therefore, depending on the genetic architecture of these species and their edaphic preferences and adaptations, different species display varying degrees of polymorphism. Present observations in *A. ilicifolius* and our results in other mangrove species (Parani *et al.* 1995, Parida *et al.* 1995, Parani et al. 1997, Rajalakshmi et al. 1997, Lakshmi *et al.* unpublished data) do support this presumption. In fact, the extent of polymorphism in *Excoecaria agallocha,* a dioecious mangrove tree species, was of much higher magnitude; intra-population polymorphism ranging from 20.4 to 31.0 per cent while as much as 65 per cent of the amplification products were polymorphic at the inter-population level (Lakshmi *et al.* 1997).

A. ilicifolius is an opportunistic coloniser of suitable habitats (Claugh 1994) and shows widespread occurrence in all types of mangrove formations inhabiting swampy sites away from the sea coasts. The observed inter-population differences could be ascribed to the fluctuating microclimatic conditions in the locations these populations inhabit (Table 11). However, the overall polymorphism in this species is of lower magnitude. Therefore, collection of samples from geographically and physically distinct populations would be a better option for conserving the diverse gene pools of this species, than selection within a particular population. Based on our present observations, distinct genotypes (identified through molecular marker analysis) from each populations have been selected for conservation. An *in situ* Genetic Resources Centre has been established in the Pichavaram Mangrove Forest for assembling these distinct genotypes for long term conservation. This is a part of an anticipatory research programme designed for consolidation of genetic material capable of facing the altered growing conditions in the coastal regions that may arise in future from rise in the sea level due to global warming. In conclusion, our results demonstrate that molecular markers provide an effective tool to assess the existing genetic polymorphism in otherwise difficult mangrove species and to design the conservation strategy.

There are six species and three putative hybrids reported in *Rhizophora* (Tomlinson, 1986). *Rhizophora xlamarckii* Montr. is one of the putative hybrids between *R. apiculata* and *R. stylosa* Griff. found in New Caledonia, Papua Guinea and Queensland (Duke and Bunt, 1979, Banerjee, 1986, Tomlinson, 1986,). Initially, the *Rhizophora* hybrid found in the Pichavaram Mangrove Forest has been identified as *R.* x *lamarckii* (Lakshmanan and Rajeswari, 1983; Muniyandi and Natarajan, 1985) based on morphological features and co-occurence of the putative parents. However, parentage of this hybrid was disputed for the reasons that *R. stylosa* does not occur in the Pichavaram Mangrove Forest and morphological features of the *R. xlamarckii* described by Duke and Bunt (1979) differed with that of the hybrid present in this area (Kathiresan,1995). In this regard, the molecular markers can not only establish the parentage but also identify the maternal parent which is almost not possible with morphological markers.

Paternity analysis of natural hybrids using molecular markers involves establishment of polymorphism between the putative parental species and its application in hybrid identification (Wang *et al.*, 1994). In the present study, RAPD profile for individual primers exhibited upto 80 per cent polymorphism between *R. apiculata* and *R. mucronata*. Among the 25 primers used, 23 primers were readily scorable for hybrid identification. The banding pattern of the hybrid had an average similarity of 96.5 per cent with the combined banding pattern of *R. apiculata* and *R. mucronata* as against the expected similarity of 100 per cent in case of synthetic hybrids. Since it is not possible to take exactly the same parental genotypes of a natural hybrid, missing bands and non-parental bands may appear due to variations within the species and hybrid. Moreover, missing bands and non-parental bands were also observed in RAPD analysis of hybrids of known parental genotypes and could not readily explainable (Gejiao Wang *et al.*, 1994; Marshal and Marchand, 1994). Considering these facts, a deviation of 3.5 per cent from expected similarity is taken as insignificant, and *R. apiculata* and *R. mucronata* are proposed as the most probable parental species of the hybrid.

Since the organellar DNA is of maternal origin, RFLP for mitochondrial DNA could be applied for identification of the maternal parent. In RFLP analysis, polymorphism was observed between the two species, and the RFLP patterns of the hybrid for all the three enzyme-probe combinations were exactly similar to that of *R. apiculata*. This indicates that *R. apiculata* must be the maternal parent of the hybrid genotype under study.

From the results, we conclude that the *Rhizophora* hybrid found in the Pichavaram Mangrove Forest is a cross between *R. apiculata* and *R. mucronata*, and that the former was the female parent of the hybrid under study. Based on the results, hybridisation between the two species was carried out using *R.*

mucronata as the pollen parent, but the flowers dropped 48h after pollination. Studies on the nature and the extent of incompatibility are in progress. There are a number *Rhizophora* self-sown seedling in the Pichavaram Mangrove Forest, but the hybrids could not be identified at this stage based on morphological characters. Therefore, using the RAPD fingerprints of the *Rhizophora* species and the hybrid established in the present study as reference, the DNA isolated from the leaves using the same protocol described for the petals are being analysed to identify the hybrid genotypes for *in situ* conservation. RAPD analysis which is simple, fast and easy to perform in large number of samples was found to be more useful than other marker systems for this purpose.

Diversity in mangroves is a widely accepted phenomenon and that was also evidenced by the molecular data from different mangrove species accrued in the recent past. RAPD analysis revealed 34, 74 and 76% intra-specific polymorphism in *A. ilicifolius* (LAKSHMI *ET AL.* 1997), *E. agallocha* (LAKSHMI *et al.* unpublished) and *A. marina* (PARANI *ET AL.* 1997a) respectively. The present study has strengthened the molecular data as almost all the RAPD and RFLP loci observed were polymorphic at inter-specific or inter-generic level. Nevertheless, mangroves have similar physiognomy, physiological and structural similarities as a result of convergent evolution (YANNEY-EWUSIE 1980). The mangrove community has distinct commonness in its characteristics like tolerance to salinity and submergence, susceptibility to frost and low temperature etc. Therefore, despite rich diversity in its forms there must be a line of relatedness among the flora which is rather unexplored.

Exploring the phylogeny of mangroves by conventional cytological and morphological means seems very difficult given the level of taxonomic isolation between the families from which they have evolved. The RAPD and RFLP analyses which basically testify the degree of homology between any taxa irrespective of their taxonomic isolation through practically unlimited number of phenotypically neutral DNA based markers could be effectively employed to elucidate the inter-relationship at molecular level. In very many cases the RAPD and RFLP data have supported, complemented and helped to extrapolate the data from cytological and morphological observations. The present study, being the first of its kind in mangroves to the best of our knowledge, presents a dendrogram constructed from a data set of variation in 213 RAPD and 194 RFLP markers (characters) which portrays the inter-relationship among the mangroves and between the mangroves and certain terrestrial genera. The RAPD primers that we have used in the present study showed more discriminatory power of distinguishing the species than the enzyme-probe combinations used in RFLP analysis. This may be, because we have used probes from the same mangrove species and/or the number of enzyme-probe combinations tried was not sufficient to yield species specific RFLPs. It was also observed that RAPD analysis produce slightly higher estimates of similarities at inter-specific level

(Powel *et al.* 1996). We discuss the relationship based on the dendrogram which was constructed by pooling both RAPD and RFLP data.

Among the taxonomically diverse taxa ranging from *Rhizophora* to *Lycopersicon*, clustering of all the true mangroves except *Nypa* (Arecaceae) into one group indicated that the homology among the mangroves is more than that between the mangroves and other genera. This is further supported by the fact that *Avicennia* shared a node with another mangrove species *Aegiceras* but not with *Clerodendron* which belongs to Verbenaceae under which Avicennia was placed earlier. It could assume that the clades occupying the mangrove habitats may not have evolved from certain randomly chosen families. Rather, they might have had certain genetic homogeneity and specialisation as to become better adapted to the prevailed environmental condition in the mangrove ecosystem. It is, therefore, important to be aware of diversity of mangroves, and at the same time to recognise features (including those at molecular level) common to all mangroves. However, whether this homology existed in the ancestral terrestrial vascular species and predisposed them to evolve as mangroves and/or it is the result of convergent evolution is a matter of further investigation.

A close look at the clustering pattern gave many interesting features of association. At 60% similarity level, a cluster between *Ceriops* and *Bruguiera* was observed and it possesed the highest similarity (73%) among the mangroves. The other two members of the tribe Rhizophorae which is sometimes called as mangrove Rhizophoraceae, *Rhizophora* and *Kandelia* shared a node with 44% similarity and formed a cluster with *Ceriops* and *Bruguiera* at 28% similarity level. All these species are characterised by viviparity which is one of the most distinguishing features of the mangroves. They also belong to the salt excluding type of mangroves which are reported to have an ultrafiltration mechanism in the roots for excluding salt. Among the other true mangroves analysed in the present study, the genus *Xylocarpus* of Meliaceae represented by *X. granatum* was found to be closest to the *Rhizophora* complex. This *Rhizophora* - *Xylocarpus*-complex showed a close telationship with another cluster formed by *Aegiceras, Avicennia, Sonneratia, Heritiera, Excoecaria* and *Acanthus. Lumnitzera* of Combretaceae was found outside this cluster. This preliminary report on the degree of relatedness among the mangroves would be of much use in further studies, on phylogeny, systeamics and evolution.

Avicennia which was earlier placed in the family Verbenaceae shared a node with *Aegiceras* of family Myrsinaceae, another true mangrove rather than with *Clerodendron*, a member of Verbenaceae. This supports the separation of *Avicennia* species into a monotypic family Avicenniaceae based on phenotypic characters (Moldenke 1967), though, biochemical evidence did not favour the segregation (Reddy *et al.* 1993). While *Aegiceras* perpetuates through viviparous propagules, incipient viviparity in the seeds of *Avicennia* has been frequently reported. Both the species belong to the salt excreting type of mangroves which

have salt glands on the leaf surface for secreting the excess salt out of the plant system. Moorthy & Kathiresan (1997) also reported close relationship between *Aegiceras carniculatum* and *Avicennia* species in terms of photosynthetic efficiency.

Another node at 54% per cent similarity was shared by *Excoecaria* and *Acanthus* and is a part of the cluster formed by other true mangroves. It is to be noted from the dendrogram that these species are more closely related to the other mangroves than at least one true mangrove, *Lumnitzera*. *Acanthus* is the only genus in Acanthaceae which has representation in the mangrove communities. A. *ilicifolius* is widespread in almost all the mangrove formations in India. *E. agallocha* is distributed thoughout the Asian tropics and mangroves. One of our study sites, the Pichavaram Mangrove Forest (Tamil Nadu, India) is locally named after the vernacular name of this species, *'Thillai'* tree as *'Thillai Vanam'* meaning *'Thillai'* forest owing to its widespread distribution in the forest since long time. Physiological features of *E. agallocha* also showed its close relationship with other mangroves like *Avicennia, Bruguiera* and *Ceriops* (Moorthy & Kathiresan 1997). Therefore, based on the present study and observations made earlier (Tomlinson 1986, Seanger *et al.* 1983) these two species could be considered as true minor mangrove. With larger genome size (2n = 148), greater polymorphism in RAPD markers and cent per cent polymorphism with relatively conserved mitochondrial gene, cox 3 (Lakshmi *et al.* unpublished), *E. agallocha* possesses a naturally diverse resource base upon which forces of evolution could operate to select the genotypes most suitable for the mangrove ecosystem. However, this is probably not the case for *A. ilicifolius* because phenotypic features even at species level does not differ much as there has been a tendency to treat the three species of *Acanthus* described from mangrove habitats (Steenis 1937) as one single variable specis. Also, a detailed analysis of *A. ilicifolius* (2n = 48) showed only 34 and 45 per cent polymorphism for RAPD and RFLP markers respectively (Lakshmi *et al.* 1997).

The other major cluster consisted of mangrove associates, terrestrial genera and *Nypa*. In this cluster, *Porteresia coarctata* which was not considered as a mangrove before, shared a node with *Pandanus fascicularis* and both formed a cluster with *Nypa fruticans*, a true mangrove. This cluster of monocots was closely related to another cluster of three species. The mangrove associate *Suaeda maritima* of Chenopodiaceae shared a node with another genus of the same family, *Salicornia brachiata* and both were related to *Sesuvium portulacastrum*, a salt marsh plant recorded in many mangrove areas. *Viscum orientale* which was earlier placed under Loranthaceae did not share a node with *Denrophthoe falcata* of the same family, and thus, favoured its segregation into a monotypic family, Viscaceae based on morphologial traits. *Lycopersicon* which was included as an outgroup for dicots neither clustered with true mangroves nor shared a node with mangrove associates.

The present study by employing the molecular markers has helped to understand the relationship between the taxonomiclly diverse or 'unrelated' mangroves species. The information like *Xylocarpus* is closely related to Rhizophorae tribe could not be reliably obtained though other conventional methods of phylogenetic analysis. However, the molecuar markers showed rather distant relationship between mangrove associates and most of the true mangroves, as part of the mangrove community they do interact with them. They may share or compete for the same pollinators, share same predators or parasites so that as alternative host their influence may not be entirely negligible. As diversity is perceived as the outcome of ecological interactions, particularly competition within small habitat, the increasing competition between the mangroves and its associates as evidenced by their intrusion deep and wide in to the mangrove habitat, may ultimately lead to evolution of new species better adapted to the emerging ecological conditions. Close association of *E. agallocha* and *A. ilicifolius* (which are not popularly considered as true mangroves) with the other true mangroves observed in the present study seems to be a positive indication towards that end. Further elaborate study on phylogeny of mangroves using molecular markers shall help us to better understand the evolution of these specialised taxa. And that may ultimately give clues to understand the mystery of the evolutionary pathways by which the highly specialised adaptive syndrome of mangroves has been achieved.

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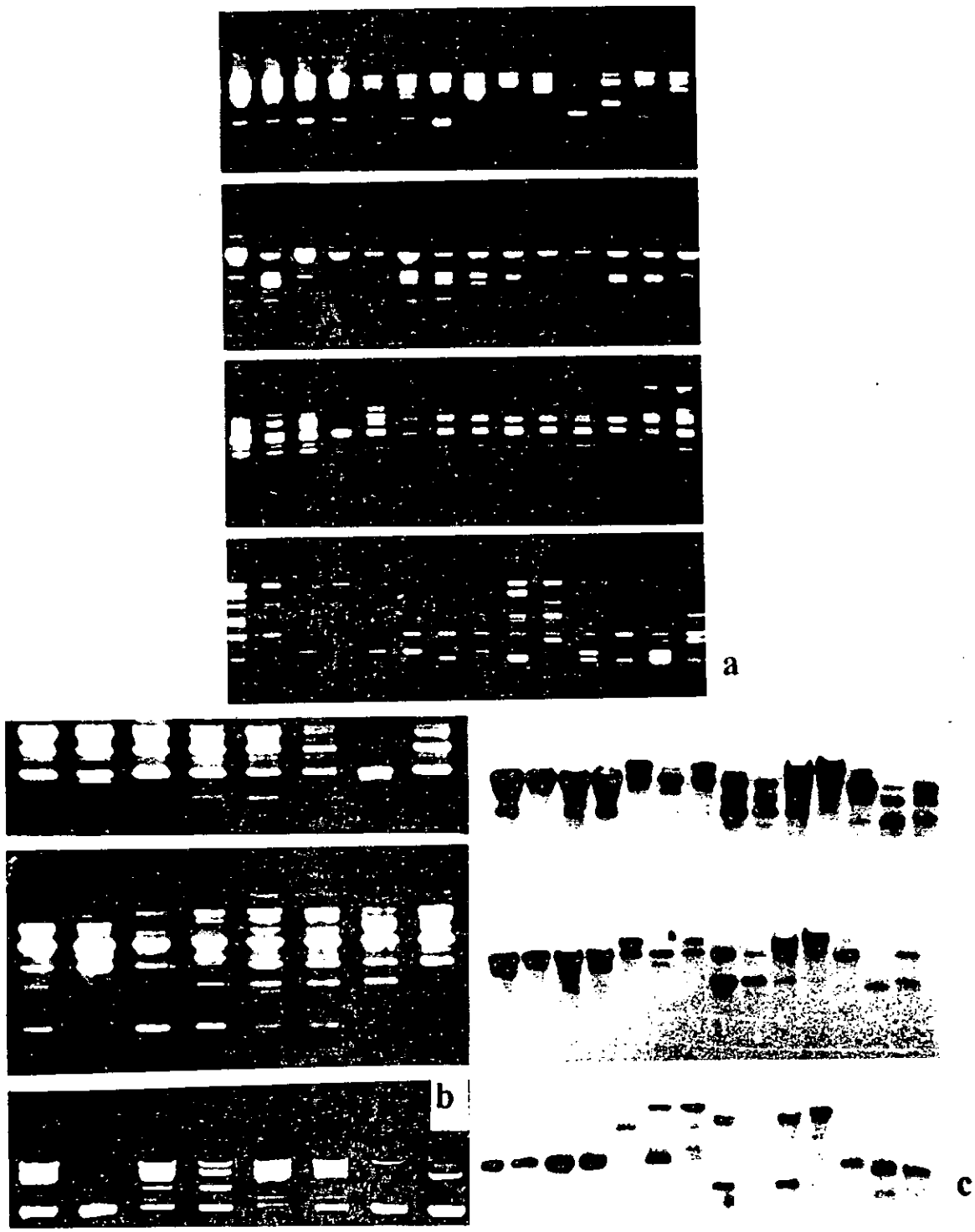
Excoecaria agallocha



a: RAPD profile in representative genotypes from seven populations
b. RAPD profile in male and female genotypes
c: Inter-population RFLP profile

Fig. 1

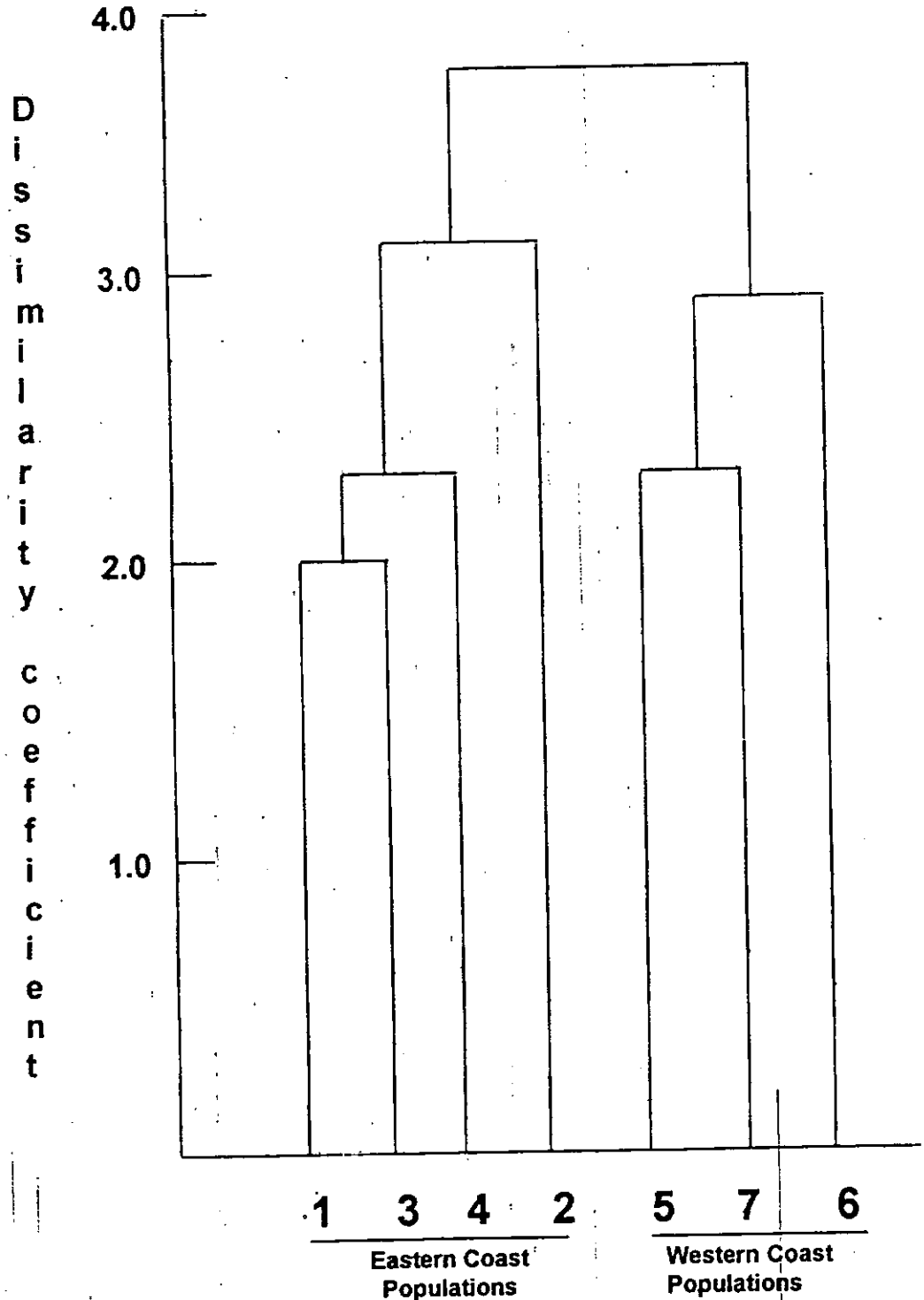

4.0
3.0
2.0
1.0
Dissimilarity coefficient
1 3 4 2
5 7 6
Eastern Coast Populations
Western Coast Populations

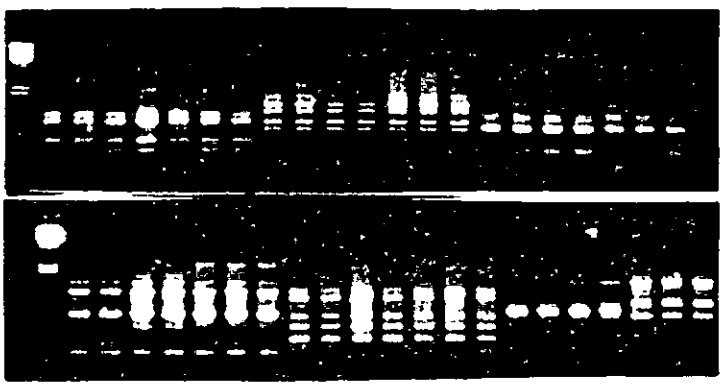
Fig. 2.

ACANTHUS ILICIFOLIUS

a

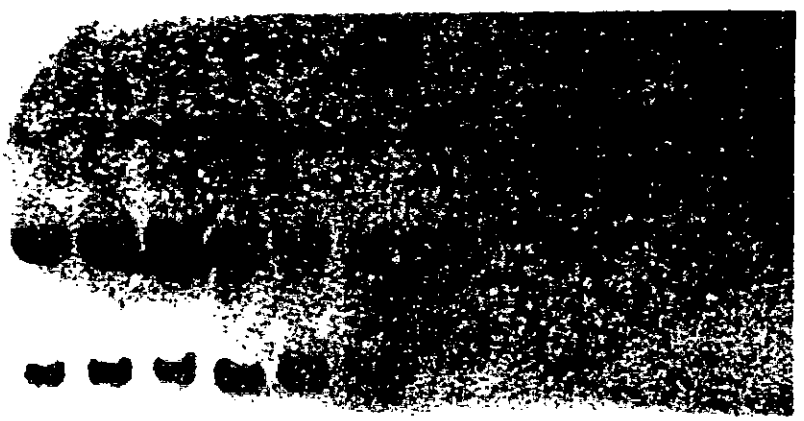
SOMATIC CHROMOSOMES

INTRA-POPULATION



b

RAPD analysis



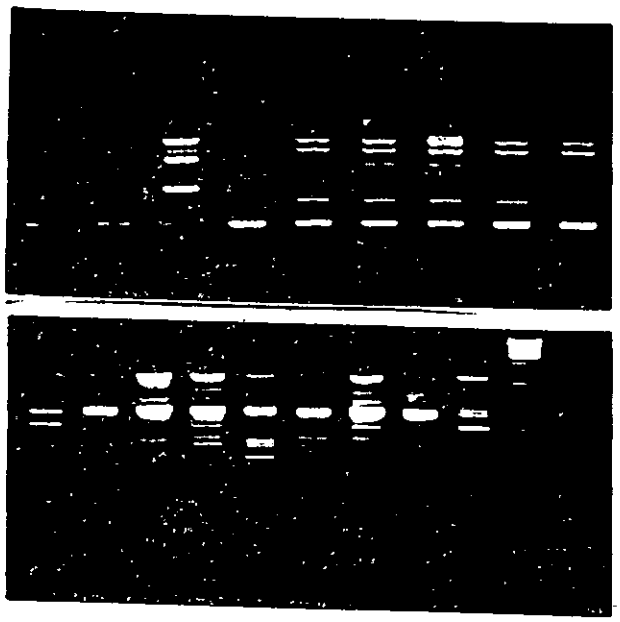
c

RFLP analysis

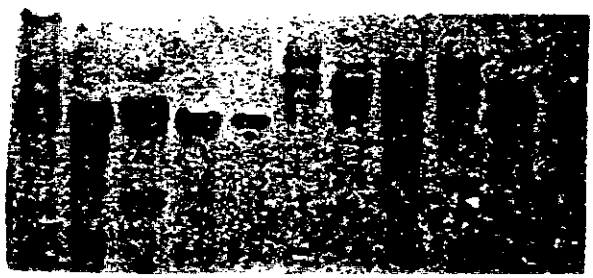
Fig. 3.

ACANTHUS ILICIFOLIUS

INTER POPULATION



RAPD analysis

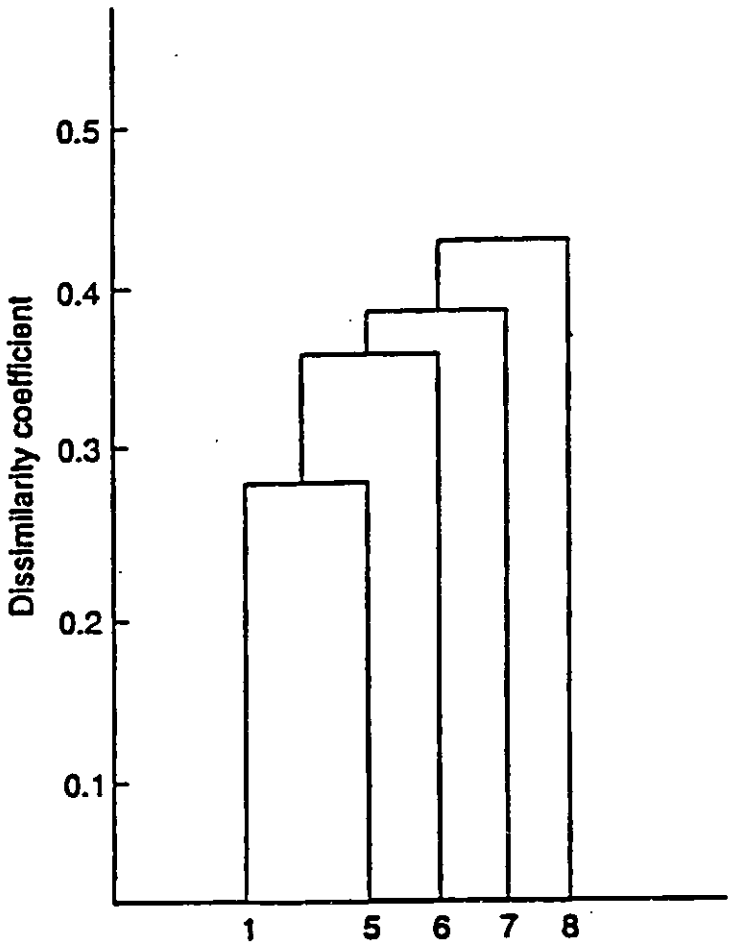


RFLP analysis

Genomic clones developed from mangrove species

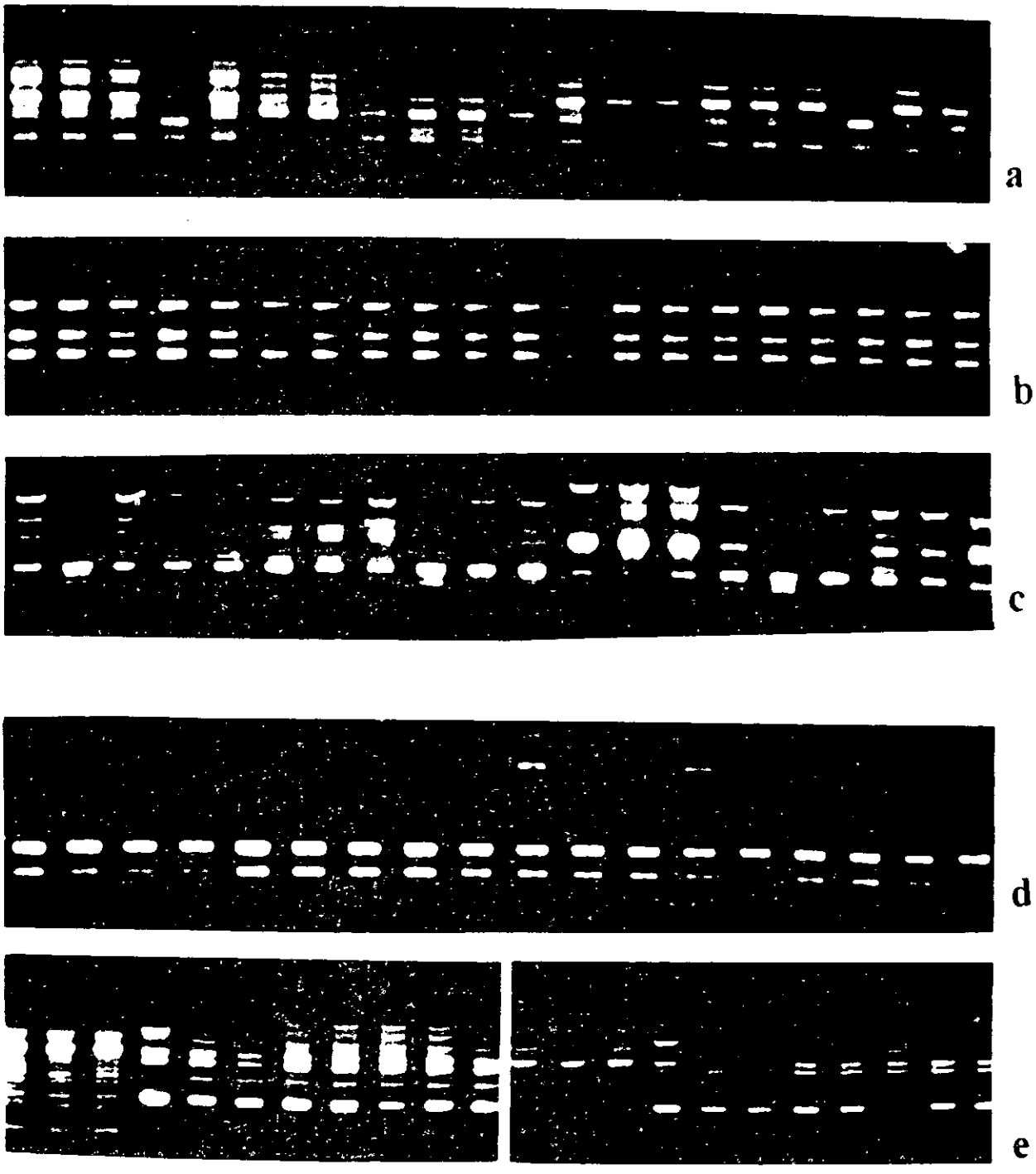
Fig. 4.

Fig. 5. Dendrogram depicting relationship between *Acanthus ilicifolius* populations collected from Eastern and Western Coast.



Avicennia Sp.

Fig. 6.



a-c: RAPD profile depicting inter-population variation in *A. marina*
d: RAPD profile depicting intra-population variation in *A. officinalis*
e. RAPD profile depicting intra-population variation in *A.alba*

Fig. 7. Dendrogram depicting inter- and inra-population and interspeifific relationship in *Avicennia* species

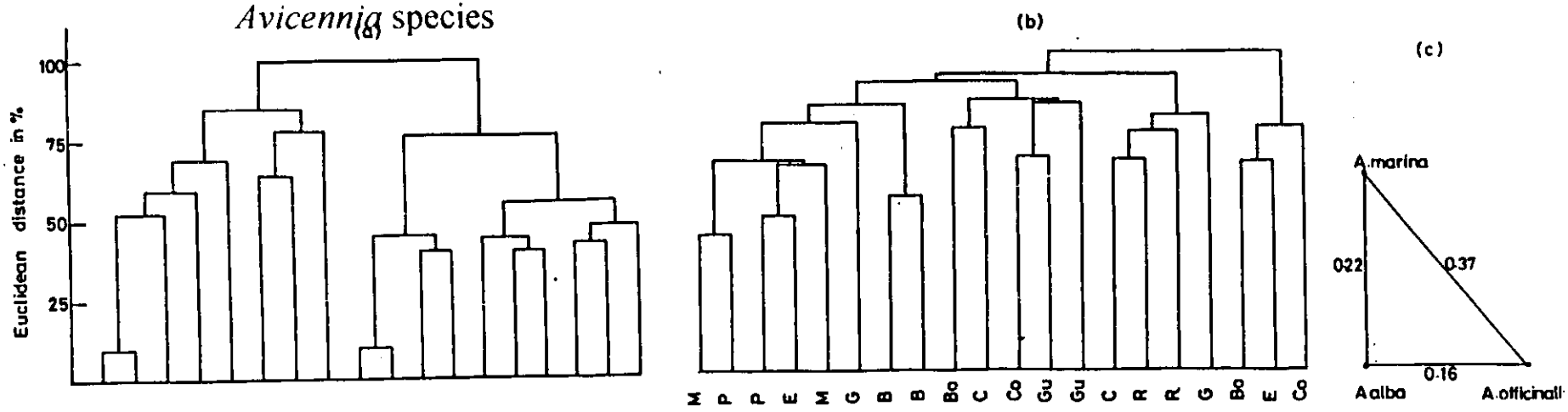
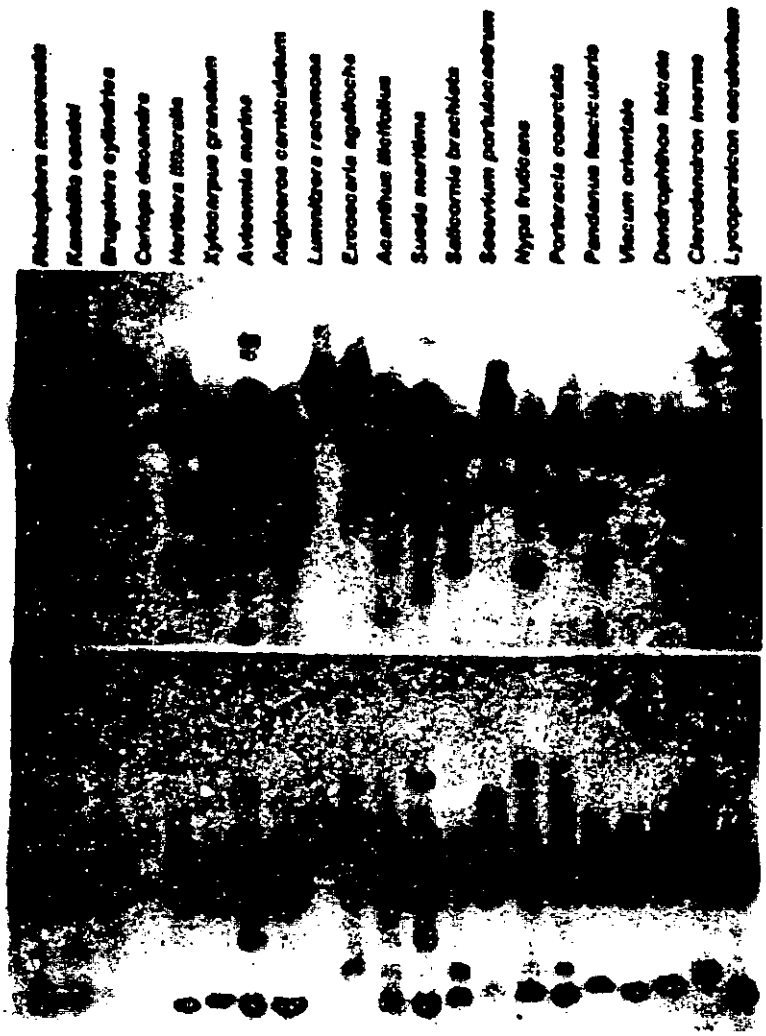
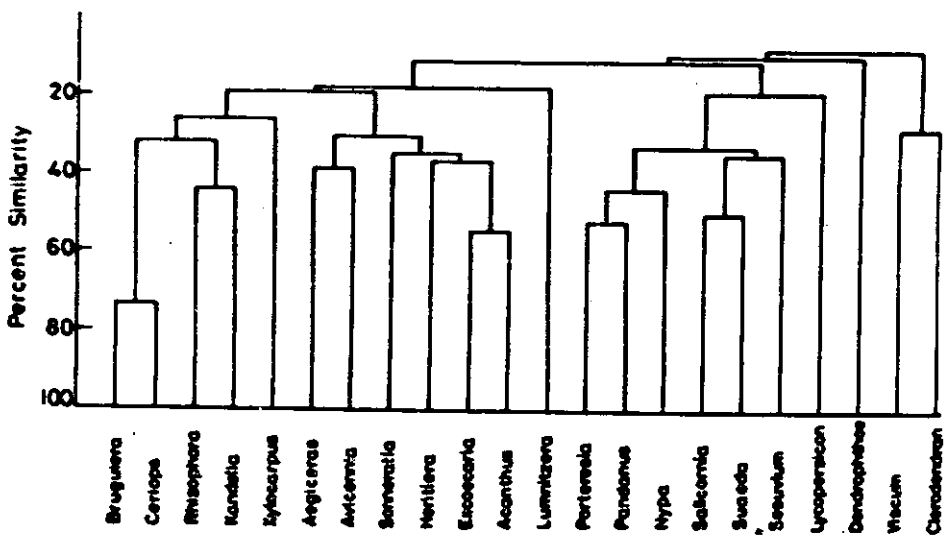


Fig. 8.



a



b

a. RFLP profile in 21 mangrove genera
b. Species relationship based on RAPD and RFLP profiles

Short-term Training Programme on "Yeast Genetic Manipulation and Molecular Markers"

A 2 week long training programme on "Yeast Genetic Manipulation and Molecular Markers" was conducted by the molecular biology group of our Foundation during November 27 to December 11, 1995. The training programme was designed for practicing scientists of various universities and institutions to give exposure and practical acquaintance with newer techniques in the field. Emphasis was given to the areas on few selected areas as evident from the course title itself and also to few important techniques of use in day-to day molecular biology research.

Yeast is a model eukaryotic system and can be profitably be employed to understand mechanisms of eukaryotic gene expression. However, expertise in handling and using this organism is limited in our country. Similarly, development and usage of DNA markers in plants have gained a great momentum currently and hence it has been decided to impart hand-on training in some of the state-of the-art technology in these fields especially yeast genetics, basic DNA techniques and molecular markers. By providing hands-on training the participants should be able to utilise these techniques most effectively and efficiently in their on-going research activities.

Faculty members of the course

The faculty members invited for the course were from both India and abroad.

Guest faculty
Prof. Deepak Bastia from the Department of Microbiology, Duke University Medical Centre, North Carolina, U.S.A.,
Prof. Bharat B. Chattoo, Biotechnology Centre, M.S. University, Baroda.
Dr. G.S.Khatri, Centre for Biochemical Technology, Mall Road, Delhi.
Dr. R.P. Ramanujam, Pharmacia Biotech Inc., Milwaukee, U.S.A.

Internal faculty
Dr. George Thomas, SPIC Science Foundation, Madras.
Dr. K.K. Narayanan, Scientist, SPIC Science Foundation, Madras.
Dr. C.S.Anuratha, Scientist, Molecular Biology, M.S.S.R.F.
Dr. P.Balakrishna, Scientist, Tissue culture, M.S.S.R.F.

Dr. Ajay Parida,Scientist, Molecular Biology, M.S.S.R.F.- Course Director

List of Participants

- Dr. P.S.Rajendra Babu, Research Officer, SPIC Pharma R&D Centre, Maraimalai Nagar, Tamil Nadu 603 209.
- Dr. J.S.Melo, Scientific Officer, Biotechnology Division, FIPLY, BARC, Trombay, Bombay 400 085.

- Rahul Sharma, Junior Research Fellow, Genetic Engineering Division, Centre for Biochemical Technology, Mall Road, New Delhi 110 007.
- Anuradha, Junior Research Fellow, Department of Botany, University of Calicut.
- Ashtekar, Senior Officer, R& D Division, FDC Ltd., SV Road, Jogeshwari, Bombay 400 102.
- N.Ravi, Research Assistant, Plant Biotechnology Division, Imstitute of Forest Genetics and Plant Breeding, Coimbatore 641 002.
- Dr. E.M. Muralidharan, Scientist, Division of Genetics, Kerala Forest Research Institute, Peechi, Trichur 680 653.
- V.Vanishree, Junior Research Fellow, Department of Biotechnology, Bharatidasan University, Trichy 620 024.
- Nirmal Babu, Scientist, NRC for Spices, Marikunnu, Calicut.
- Radhakrishna Pillai, Lecturer, Department of Life Sciences, University of Calicutt, Thenhipallam 673 635.
- Dr. S.Sandhya, Scientist, Environmental Biotechnology Division, Neeri, CSIR complex, Madras 600 113.
- Shaik Yazdani Basha, Senior Research Fellow, School of Biotechnology, Madurai-Kamaraj University, Madurai 625 021.
- K.Narashimhan, Scientist, Rubber Reseach Institute , Kottayam.
- Ajith Anand, reseaarch Fellow, MSSRF, Madras.
- N.Mathan, Research Fellow, MSSRF, Madras.
- Senthil Kumar, Research Fellow, SPIC Science Foundation, Madras.

Special Lectures Delivered

- Introduction to recombinant DNA technology.
- Molecular markers: Physical and high resolution mapping of plant genomes.
- Molecular markers: Documentation of genetic polymorphism in plants.
- Molecular markers: Application in plant breeding and crop improvement.
- Plant DNA isolation and purification & Random amplified polymorphic DNA (RAPD)
- Restriction fragment length polymorphism (RFLP): Principles and Methodology.
- Micro- and mini-satellite based marker systems.
- Pulsed -fieldgel electrophoresis (PFGE).
- Tools for DNA and RNA purification.
- Room temperature stable enzymes and nucleotides.
- Yeast as a model system for genetic studies.
- Enzyme purification methods.
- Population Genetics: Introduction.
- Population Genetics: Measuring variation.
- Current trends in understanding microbial diversity.
- Approach to sustainable human development.
- Conservation of biological diversity.
- An overview of enzyme purification, yeast genetics and transformation vectors.

- Genetics of DNA replication.
- DNA polymerases and replication progression
- Termination of DNA replication.
- Control of DNA replication.

Topics/ Techniques covered

The participants were provided hands-on training in some of the state-of the-art technology in the field of molecular biology. Apart from the special lecture schedules, the practicals also covered the principles and applications of the techniques used. They were also given training on the design of experiments, analysis and interpretation of data. The topics/ techniques covered during the programme were:

- DNA isolation methods.
- DNA purification and estimation methods
- Agarose gel electrophoresis.
- RAPD techniques.
- RFLP techniques (includes restriction enzyme digestion, electrophoresis, Southern blotting, labelling of DNA probes both radioactive and non radioactive, autoradiography etc.).
- Prepartion of DNA probes for RFLP by partial genomic library construction.
- Maintenance of bacterial cultures and clones, transformation of E.coli and selection of recombinants.
- Statistical analysis of data.
- Pulsed-field gel electrophoresis.
- Protein gels-SDS-PAGE, staining etc.
- Protein purification methods.
- Yeast culture transformation and selection of recombinants.

ANNEXURE

List of publications

Patil V and Jayanthi M 1997. Micropropagation studies in two species of *Rauwolfia*. Curr. Sci. Vol. 12, pp 961-965.

P. Balakrishna 1995 Plant tissue culture and commercialisation in sustainable development. Abstract at Second International Plant Tissue Culture Conference, Dhaka, Bangladesh. December 1995.

M Jayanthi 1996. Somatic embryogenesis and genetic variation studies in regenerants of Tylophora indica (Burm. F.) Merrill. Proceedings to National symposium on Current Trends in Plant Biochemistry and Biotechnology. Hisar. February 1996.

M Jayanthi, Ajith Anand, C Srinivasa Rao, V M Patil and P. Balakrishna 1996 *In vitro* propagation of some medicinal plants and wild relatives native to Western ghats. Abstract for the National Symposium on Plant Tissue Culture. G B Pant University, Pantnagar.

Ajith Anand 1996. Marker aided studies for screening genetic stability in microrpropagated plants of Piper species. Abstract for presentation at the National Seminar on Spieces Biotechnology, BIOSAAP, Calicut.

Ajith Anand, Srinivasa Rao and Balakrishna P 1997. *In vitro* propagation of *Syzigium travancoricum* Gamble, an endangered tree species. (Plant Cell Tissue and Organ Culture - in revision)

Ajith Anand, Srinivasa Rao, Latha R and Balakrishna P 1997. Micropropagation of *Uraria picta*, a medicinal plant through axillary bud culture and callus regeneration. In vitro (Accepted).

Jayanthi. M. Saving an Endangered plant. A case study on *Crotolaria longipes*.B.Ph.d Thesis submitted to University of Madras. 1997.

Training programmes organised /served as resource persons

1) Senior level training programme on The Application of bioindication technology to the conservation of Biodiversity April 1992.

2) Importance of Conservation and Monitoring of Mangrove ecosystems 1993-94 Training and demonstration for local school children at Pichavaram, Tamil Nadu, India.

3) Short term course on Environmental pollution: Causes and Remedies, University of Madras and Envriro Club, Madras November 1994.

4) On "Environmental Awareness" Workshop organised jointly with Rotary club of Madras Feb,1995.

5) Trainers' Training Progamme for the NGOs' on Biodiversity Conservation RHUSHA Vellore. Oct 1995.

6) Farmer's level training on "AgroBiodiversity Conservation and Integrated Pest Management" Sriperumbudur. July1996

7) Trainers' Training Progamme on Biodiversity Conservation. Kottakal, Kerala India. November 1996.

8) Farmer level training programme on Ecological Horticultural Techniques . Kattupakkam , Madras.

9) Environmental Monitoring : an Integrated Approach. in Biotechnology and Industry (College Level) Ethiraj Colege for Women, October 1996.

10) Trainer's Training Programme on Biodiversity Conservation for NGO's at KVK, Gandhigram, January 1997.

Papers Published/Communicated

- **Sudha Nair**. Biodiversity and Environmental Pollution. Paper presented at the National Symposium on Chemopollutants and Sustainable Ecosystems. Nov., 1995.

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