Studies on Pigeonpea Sterility Mosaic Disease: Isolation and Characterisation of the Causal Agent and Assessment of Genetic Variation within and between Populations of the Mite Vector, *Aceria cajani*

Thesis submitted to
Sri Venkateswara University
for award of the degree of
Doctor of Philosophy in Virology

By
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SEPTEMBER 1999

To
My Parents, Sisters and Naveen
Whose support and love has made it possible

And to
My Teachers, Drs P Sreenivasulu, DVR Reddy, AT Jones and Brian Fenton
Who provided invaluable source of strength and inspiration
and for their vision and life giving words

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CERTIFICATE

This is to certify that the thesis entitled ***Studies on Pigeonpea Sterility Mosaic Disease: Isolation and Characterisation of the Causal Agent and Assessment of Genetic Variation within and between Populations of the Mite Vector, Aceria cajani***, submitted by ***Mr P Lava Kumar***, for the award of degree of ***Doctor of Philosophy in Virology***, is a record of work done by him during the period 1996-99, under our supervision and that it has not previously formed the basis for the award of any degree or diploma or associate or fellowship or other similar title.

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22 September, 1999

DECLARATION

I declare that ***Studies on Pigeonpea Sterility Mosaic Disease: Isolation and Characterisation of the Causal Agent and Assessment of Genetic Variation within and between Populations of the Mite Vector, Aceria cajani***, submitted to the Department of Virology, Sri Venkateswara University, for the award of degree of ***Doctor of Philosophy in Virology***, is a record of work done by me during the period 1996-99, and that it has not previously formed the basis for the award of any degree or diploma or other similar title.

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ACKNOWLEDGEMENTS

It is with great pleasure and respects, I wish to express my gratitude and reverence to my research supervisors, Dr P Sreenivasulu, Professor and Head, Department of Virology, Sri Venkateswara University, Tirupati, and Dr DVR Reddy, Principal scientist, Virology, Crop Healthy Unit, GREP, ICRISAT, for inspiring guidance, constant encouragement, moral support and erudite discussions, which greatly contributed to successfully complete this project.

I would like to express my profound gratitude and heartfelt thanks to Dr AT Jones, Senior Principal Virologist and Head, and Dr Brian Fenton, Senior Scientist (Molecular Genetics), Unit of Soft Fruit and Perennial Crops, Scottish Crop Research Institute (SCRI), Dundee, Scotland, United Kingdom, for their able guidance, unstinted encouragement, critical analysis and care during my stay at SCRI.

I am grateful to acarologist's, Dr Don Griffiths, former Director, Central Scientific Laboratories (CSL), York, UK; and Dr GP Channabasavanna, University of Agricultural Sciences, GKVK, Bangalore, India, for spending their valuable time in aspects related to morphological studies of eriophyid mites.

I wish to record my special thanks to Dr MV Reddy, Senior Scientist, Lam farm, Acharya NG Ranga Agricultural University (ANGRAU), Guntur, India; Dr Jim Amrine Jr, University of West Virginia, USA; Dr GN Oldfield, University of California, Riverside, USA; Dr C Hirukl, University of Alberta, Canada; Dr S Gordon, Unit of SFPC, SCRI; and scientists and extension workers of Regional Agricultural Station, Nepalgunj, Nepal and Myanmar Agricultural Service, Yangon, Myanmar, for supplying mite samples.

I wish to express sincere thanks to Dr GP Martelli, Universita degli Studi, Bari, Italy and Dr Stanley Jensen, Department of Plant Pathology, University of Nebraska, Lincoln, USA, for supplying PoLV and HPV antisera, respectively.

I sincerely thank Gaynor Malloch and Wendy McGavin, Unit of SFPC; Claire McQuade, automated sequencing laboratory; George Duncan and Ian Roberts, Electron Microscopy Unit; Graham Cowen, Virology; Dr Frank Wright, Biomathematics and Statistics Scotland (BioSS); SCRI, Dundee, UK; Mr AK Murthy, Engineer, Electron Microscopy Unit, ICRISAT, India for their technical support and co-operation.

I am very grateful to my friends and colleagues, Phil Irving and Kiri Stanley, SCRI, UK; KLN Reddy, Thirumala Devi, Ranjana Bhattacharjee, P Delfose, Dr A Rahman, A Sudharshan Reddy and S Veera Reddy, ICRISAT; M Hema and Venkataramana, Sri Venkateswara University; Dr G Kiranmai, DRF, Hyderabad; Joseph Ajay, University of British Columbia, Canada; and G Srinivas and P Sreekanth, Tirupati, for their support and endearing help.

I acknowledge the generous help from support staff of Virology Laboratory in ICRISAT, and Department of Virology, Sri Venkateswara University, Tirupati.

I gratefully acknowledge the Department for International Development, UK, for providing the fellowship and funding for the research under the holdback grant R6407(H), awarded to ICRISAT and SCRI.

Pigeonpea, is a multipurpose legume crop cultivated mostly for its protein enriched seed. It provides dietary protein to an estimated 1.1 billion people around the world. India is the major producer contributing 80% of the global pigeonpea production. Pigeonpea sterility mosaic (SMD), a virus-like disease of unknown etiology, severely affects production with annual losses estimated 15 years ago of over US$ 70 million. In annual incidence, SMD is next to *Fusarium* wilt but, in losses in India, it is double that due to wilt. Intense efforts at ICRISAT and ICAR centers have resulted in the identification of several SMD resistant genotypes, but the resistance appears to be location specific and, more recently, is breaking down in certain locations. Previous studies indicated that diverse mechanisms govern SMD resistance in pigeonpea; genotypes are either resistant to the SMD pathogen, or to its mite vector (*Aceria cajani*), or to both agents. One hypothesis for the variability in resistance to SMD of different genotypes is the occurrence of different strains of the SMD agent, or of different *A. cajani* vector biotypes.

'Studies on Pigeonpea Sterility Mosaic Disease: Isolation and Characterisation of the Causal Agent and Assessment of Genetic Variation within and between Populations of the Mite Vector, Aceria cajani' is aimed mainly to isolate the agent causing SMD, and to understand the variation in *A. cajani* populations in SMD endemic regions. In a broader sense this study paves the way to understand the variation in resistance shown by several pigeonpea genotypes across the Indian subcontinent.

SMD was described nearly seven decades ago. Despite intense efforts, especially during the last fifteen years, the causal agent has remained elusive and enigmatic. However, the available evidence indicates that it is probably a virus. Recently, considerable progress has been achieved, especially in the characterisation of the agents involved in mite-transmitted viruses of dicotyledonous plants. For a few viruses, like blackcurrant reversion associated virus, characterisation occurred only after transferring them to herbaceous hosts, whilst for others, like peach mosaic virus, characterisation was achieved after virus purification directly from the natural host. This study to elucidate the causal virus of SMD utilised both of these approaches - mechanical transmission from SMD-affected plants to herbaceous hosts (Chapter 3.3) and direct purification of virus from infected plants (Chapter 3.4).

Eriophyid mites are the smallest arthropods. Accurate identification of these mites, particularly by morphological characters, is difficult because of their very similar basic body architecture and morphological features. Recently, using modern molecular biological techniques, a novel method based on analysis of ribosomal DNA, was developed for identification of these mites. Due to its accuracy and simplicity, this method was used to determine any diversity among *A. cajani* populations (Chapter 4).

This thesis is broadly divided into 6 chapters. chapters 1 and 2 contain the Introduction and Review of Literature, respectively; chapter 3 studies on the SMD-pathogen; chapter 4 analysis of *A. cajani* populations. Each of these chapters contains sub-sections covering Materials & Methods and Results. Chapter 5 contains the discussion. The overall Summary and Conclusions are presented in chapter 6, and the cited literature in the chapter 7. Chapter 8, the Appendix, contains details of the composition of buffers and reagents.

Outline

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List of Abbreviations and Symbols

A	Absorbance	K	Kilo
AGDD	Agar gel double diffusion	kbp	Kilobase pairs
ALP	Alkaline phosphatase	kDa	Kilodaltons
APS	Ammonium persulphate	l	litre(s)
BSA	Bovine serum albumin	LB	Luria-Bertani medium
bp	base pairs	LIV	Longevity *in vitro*
Cat.	Catalogue	M	Molar
cDNA	Complementary DNA	m	Meter
cm	Centimeter(s)	max	Maximum
cv	Cultivar	ME	Mercaptoethanol
Da	Dalton	min	Minute(s)
DAC- ELISA	Direct antigen coating-	mg	milligram(s)
	ELISA	ml	Millilitre(s)
DEP	Dilution end point	mm	Millimeter(s)
DIECA	Diethyldithiocarbamate	mM	Millimolar
DIG	Digoxygenin	mol. wt.	Molecular weight
DI	Defective interfering	MOPS	3-(N-morpholino)
dH_20	Distilled water		propanesulfonic acid
DMBs	Double membrane bodies	mRNA	Messenger RNA
DNA	Deoxyribonucleic acid	M_r	Molecular ratio
ds	double stranded	nm	Nanometer
DTT	Dithiothreitol	ng	Nanogram
EDTA	Ethylenediaminetetraacetic	No	Number(s)
	acid (disodium salt)	nts	Nucleotides
ELISA	Enzyme linked	NTP	Nucleotide tri-phosphate
	immunosorbent assay	OD	Optical density
EtOH	Ethanol	ORF	Open reading frame
ETS	External transcribed	PAGE	Polyacrylamide gel
	spacer(s)		electrophoresis
F	Forward	PBS	Phosphate buffered saline
g	Grams	PCR	Polymerase chain reaction
g	Gravitational force	PEG	Polyethylene glycol
h	Hours	pH	Hydrogen ion
ICTV	International committee of		concentration
	taxonomy of viruses	PTA	Phosphotungstic acid
IGS	Intergenic spacers	PNP	para-Nitrophenyl
IPTG	Isopropayl-β-		phosphate
	thiogaloctopyranoside	PVP	Polyvinyl pyrrolidone
ITS	Internal transcribed	rDNA	Ribosomal DNA
	spacer(s)	R	Reverse

RE	Restriction enzyme
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription-PCR
S	Svedberg unit
sat RNA	Satellite RNA
ss	Single stranded
SDS	Sodium dodecyl sulphate
sec	Seconds
SEM	Scanning electron microscopy
spp	Species
SM	Sterility mosaic
SMD	Sterility mosaic disease
TEM	Transmission electron microscopy
TEMED	N,N,N,N-tetra methylethylene diamine
Tris	Tris (hydroxymethyl) amino methane
U	Unit(s)
UA	Uranyl acetate
UV	Ultraviolet
V	Volts
v	Volume
var	Variety
VIDE	Virus identification and data exchange
W	Watts
x-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

List of Symbols

α	Alpha
β	Beta
°C	degree Celsius
/	Per
%	Per cent
µg	Microgram
µl	Microlitre
µM	Micromolar

One letter codes for nucleotides

A	Adenosine
C	Cytosine
G	Guanosine
T	Thymidine
U	Uracil

One letter symbols of amino acids

A	Alanine
R	Arginine
N	Asparagine
D	Aspartic acid
B	Asparagine
C	Cysteine
Q	Glutamine
E	Glutamicacid
Z	Glycine
H	Histidine
I	Isoleucine
L	Leucine
K	Lysine
M	Methionine
F	Phenylalanine
P	Proline
S	Serine
T	Threonine
W	Tryptophan
Y	Tyrosine
V	Valine

VIRUS ACRONYMS

AMV	Avian myeloblastosis retrovirus
BMV	Brome mosaic bromovirus
BSMV	Barley stripe mosaic hordeivirus
CLSV	Cucumber leaf spot tombusvirus
CMLV	Cherry mottle leaf closterovirus
CymRSV	Cymbidium ringspot tombusvirus
HPV	High Plains virus
LMV	Lettuce mosaic potyvirus
MMLV	Moloney murine leukaemia retrovirus
MSpV	Maize stripe tenuivirus
OYDV	Onion yellow dwarf virus
PATV	Pigeonpea associated tombusvirus
PMV	Peach mosaic closterovirus
PoLV	Pothos latent tombusvirus
PPSMV	Pigeonpea sterility mosaic virus
PVX	Potato X potexvirus
PVY	Potato Y potyvirus
RGMV	Ryegrass mosaic rymovirus
RGSV	Rice grassy stunt tenuivirus
RHBV	Rice hoja blanca tenuivirus
SMAV	Sterility mosaic associated virus
TBSV	Tomato bushy stunt tombusvirus
ToMV	Tomato mosaic tobamovirus
TMV	Tobacco mosaic tobamovirus
TRSV	Tobacco ringspot nepovirus
WSMV	Wheat streak mosaic rymovirus

Chapter 1

Introduction

INTRODUCTION

1.1. Background

Pigeonpea (*Cajanus cajan* (L.) Millsp), a drought-resistant pulse crop, provides vital protein for large populations of the poor in the tropics and sub-tropics of Asia, Africa and the Caribbean. Pigeonpea is especially important in small-scale farming in semi-arid regions. India accounts for nearly 85% of the world's pigeonpea production (Muller *et al.*, 1990). The simultaneous use of pigeonpea for food, fodder and fuel, its ability to ameliorate soils and its use as a hardy crop on marginal soils fitting into many intercropping situations, make pigeonpea a crop with a bright future. Many high yielding short duration cultivars can fit into many cropping systems including cereal based ones, thus contributing to sustainability of such important cereal crops as wheat and rice. It has also recently been shown to mobilise bound phosphorous and to make it available to subsequent crops. It provides much needed vital protein for large populations, especially in India. Thus it can contribute to adequate supplies of much needed protein for a balanced diet. However, production of pigeonpea in India and several other Asian countries is seriously affected by sterility mosaic disease (SMD), a virus-like disease of unknown etiology (Ghaneker *et al.*, 1992). Yield losses caused in most genotypes by SMD occurring early in the season can reach >90%, with an estimated annual loss of over $70 million in India alone during 1975-80 (Kannaiyan *et al.*, 1984). The causal agent of SMD is transmitted naturally by the eriophyid mite, *Aceria cajani* (Channabasavanna), and experimentally by grafting, but not by mechanical inoculation of sap. Despite several attempts in the past several years, the agent of SMD remained uncharacterised (Reddy *et al.*, 1994), preventing the development of sensitive techniques for its rapid and unambiguous detection in plants, which are essential to develop integrated management programmes for SMD. Despite many technical problems, work over several years in co-ordinated projects between International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India, and the Indian Council of Agricultural Research (ICAR), has produced new pigeonpea varieties with field resistance to SMD infection. The

resistance mechanism to SMD is not characterised, but previous studies indicated that diverse mechanisms govern SMD resistance in pigeonpea. Genotypes are either resistant to pathogen or to the vector or to both (Reddy and Nene, 1980; Muniyappa and Nangia, 1982; Sharma *et al.*, 1984; Saxena and Sharma, 1990; Reddy *et al.*, 1995). Although the resistant lines have performed well in field trials at ICRISAT, Patancheru and surrounding regions, their resistance elsewhere in India has been much less effective. This variability in resistance to SMD of the different pigeonpea genotypes is assumed likely to be due to the presence of either different *A. cajani* biotypes, or species of *Aceria* mites, or to the occurrence of different strains of the causal agent (Reddy *et al.*, 1998).

About 20 virus or virus-like agents are known to be transmitted by eriophyid mites (Oldfield and Proeseler, 1996). Most of the agents infecting perennial woody plants are unknown. Only a few mite-transmitted agents, mostly those infecting monocotyledonous plants have been characterised. Several attempts to characterise the causal agent of SMD have not been successful (Reddy *et al.*, 1994). However, a great deal of information has been obtained regarding the nature of the causal agent and its transmission. This data indicated that the causal agent is not a fungus, bacterium, phytoplasma or viroid and that the disease is not caused by mite toxaemia and was presumed to be caused by a virus (Ghanekar *et al.*, 1992). In recent years, using various approaches, considerable progress has been achieved, especially in the characterisation of the agents involved in mite-transmitted viruses of dicotyledonous plants. For a few viruses, like blackcurrant reversion associated virus (BRAV), characterisation could be achieved only after transferring them to herbaceous hosts (Lemmetty *et al.*, 1997). Whilst for others, like peach mosaic virus (PMV), characterisation was made after purification directly from the natural host (Gispert *et al.*, 1998). Application of such approaches may be usefully applied to characterise the causal agent of SMD.

Eriophyid mites are amongst the smallest arthropods. Accurate identification of these mites, particularly by morphological characters, is difficult because of their very similar basic body architecture and very similar morphological features. Several protein- and DNA-based molecular techniques have been developed to identify

physiological strains within pest species (Dowling *et al.*, 1996; Loxdale *et al.*, 1996; Murphy *et al.*, 1996; Sessions, 1996; Cavalli-Sforza, 1998; Loxdale and Lushai, 1998). However, the microscopic size, soft body, high host specificity and cryptic existence of eriophyid mites on host plants pose serious difficulties in manipulating them for experimental purpose and for the use of many of these techniques. Additionally, information on genetic variability within and between mite populations and the possible interchange of genes between them, has been difficult to study. Recently, a polymerase chain reaction (PCR)-based DNA analysis technique was developed for distinguishing morphologically closely related *Cecidophyopsis* mite species (Fenton *et al.*, 1995). This method is based on the finger printing of PCR amplified products of nuclear ribosomal RNA (rRNA) genes and associated spacer regions with restriction enzymes. This technique was found to be rapid and sensitive for the identification of different species of *Cecidophyopsis* mites. Therefore, the development and application of this technique seems ideal for determining if different species of *Aceria* or biotypes of *A. cajani* exist on pigeonpea in SMD endemic regions in India, Nepal and Myanmar. Furthermore, it should also provide the means to understand the degree of genetic variation and relationship within and between *A. cajani* populations and hence the basis of differences in resistance to SMD in pigeonpea growing regions of Asia.

1.2. Objectives

Successful breeding and development of durable host-plant resistance to plant pathogens and pests depends on an understanding of pathogens and pests and variation within their populations. Isolation and characterisation of the causal agent of SMD, the development of diagnostic tools for its detection in plants, and an understanding of the bio-diverisity amongst *Aceria cajani*, the vector of the SMD pathogen, are therefore vital to develop pigeonpea cultivars with durable resistance to SMD. The objectives of the present study are:

I. To isolate and characterise the causal agent of SMD and develop diagnostic tools for its detection.

II. To determine the biodiversity of *Aceria cajani* populations in Indian-subcontinent.

1.3. Work plan

To achieve the above objectives the work programme is given below.

1. Develop protocols for the isolation of the causal agent of SMD.
2. Characterise the causal agent of SMD.
3. Develop diagnostic tools for SMD agent detection in the plants.
4. Collect of mites from pigeonpea from different locations in India, Myanmar and Nepal.
5. Develop and standardise PCR-based protocols utilising nuclear ribosomal DNA (rDNA) as a marker system for the mites.
6. Develop protocols for the extraction, amplification and analysis of *A. cajani* rDNA.
7. Clone and sequence the amplified product and compare the sequence with known sequences of other eriophyid mites to determine phylogenetic relationships.
8. Design primers for the specific detection of *A. cajani* mites.
9. Study the *A. cajani* mite morphology by scanning electron microscopy.
10. Assess the phylogenetic relationships of eriophyid mites using rDNA regions.

Chapter 2

Review of Literature

2.1. The Pigeonpea Crop

Pigeonpea, grown commercially, is an important food legume in India, Eastern Africa, particularly Kenya, Uganda, Malawi and Tanzania, and in the Caribbean region. It is grown to a lesser extent in many other tropical countries of Asia and South America (Van Der Maesen *et al.*, 1985). Pigeonpea is commonly grown as an annual, inter-cropped with cereals, fibre crops and other legumes. It is also grown as a perennial, particularly in hedges. World-wide the crop is cultivated on about 3.4 million hectares with an annual production of 2.7 million tonnes (Nene and Sheila, 1990). Pigeonpea has a high productivity including nitrogen fixation (98-280 kg/ha) and net primary productivity (2-12 t/ha) compared to many other legumes (Duke, 1985). It is mainly cultivated for its seed which contains nearly 30% protein and provides a vital protein diet for estimated 1.1 billion people around the world. Among legumes, it ranks fifth in area and fourth in production after beans, peas and chickpeas, but it is used in more diverse ways than other pulse crops (van der Maesen, 1995).

2.2. Diseases of Pigeonpea

More than 210 pathogens (Nene *et al.*, 1996) and 200 species of insect pests (Lateef and Reed, 1990; Shanower *et al.*, 1999) have been reported on pigeonpea. But only a few of them are economically important and common over large areas (Kannaiyan *et al.*, 1984; Reddy *et al.*, 1998). The most widespread and most important diseases of pigeonpea are sterility mosaic, *Fusarium* wilt, *Phytophthora* blight, *Macrophomina* root rot and stem canker, and *Alternaria* blight in the Indian sub-continent; witches broom in the Caribbean and Central America; wilt and *Cercospora* leaf spots in eastern Africa (Vakili and Maramorosch, 1974; Mehan and Reddy, 1990; Nene *et al.*, 1996). Because of the wide-spread occurrence and high economic losses caused, extensive research has been done on sterility mosaic, *Fusarium* wilt and *Phytophthora* blight.

2.3. Virus and Virus-like Diseases of Pigeonpea

Natural infection of pigeonpea with 15 viruses, 3 virus-like diseases and 1 viroid has been reported (Nene et al., 1996; Brunt et al., 1996a; Reddy et al., 1998; see Table 1). Information regarding the distribution, disease epidemiology and economic losses caused by these agents are meagre. Pigeonpea is susceptible to 23 of 49 viruses tested by experimental inoculation (Brunt et al., 1996b; Table 2). Most of these viruses cause mosaic, stunting and proliferation of vegetative growth.

Sterility mosaic is the most important virus-like disease and mung bean yellow mosaic begomovirus and cowpea mosaic comovirus caused diseases are of minor importance. Yellow mosaic of pigeonpea, caused by mungbean yellow mosaic begomovirus, is reported from the Indian sub-continent and the Caribbean (Reddy et al., 1990; Mandal et al., 1998). This virus causes bright yellow patches alternating with green patches on the leaf lamina. It is vectored by the whitefly *Bemisia tabaci* Genn, and is serologically unrelated to *Rhynchosia* begomovirus reported from Puerto Rico (Y. L. Nene, unpublished data). This disease is rarely severe but its incidence is higher in late-sown crop, sometimes resulting in an yield loss of about 40% (Mishra and Gurha, 1980; Beniwal et al., 1983). Infection of cowpea mosaic comovirus was reported from the Kenya and Caribbean (Reddy et al., 1998), but no information is available regarding its effects on plant growth and yield loss.

2.4. Sterility Mosaic Disease

SMD is the most important disease of pigeonpea in Indian subcontinent. The disease was first more than 65 years ago reported from Pusa, Bihar State, India, (Mitra, 1931). Alam (1933) first described SMD in detail, and Capoor (1952) established the infectious nature of the disease by graft transmission. Seth (1962) showed that under natural conditions the SMD pathogen is transmitted by the eriophyid mite vector *A. cajani*. The disease is confined to Asia, in addition to India, it has been reported from Bangladesh, Nepal and Thailand (Nene and Sheila, 1990); Myanmar (Su, 1931); and Sri Lanka (Newton and Peiris, 1953).

2.4.1. SMD symptoms and yield losses

SMD is characterised by a bushy and pale green appearance of plants, excessive vegetative growth, stunting, reduction in leaf size, leaf distortion, mosaic and mottling of leaves and the complete or partial cessation of reproductive structures (Reddy et al., 1990). Symptoms are often masked with the growth of the plant, but when ratooned

Table 1
Reported natural infection of pigeonpea with virus and virus-like agents

Virus	Distribution
Alfalfa mosaic alfamovirus	New Zealand
Arhar mosaic virus[2]	India
Clitoria yellow vein tymovirus	Kenya
Cowpea mosaic comovirus	El Salvador, Kenya, Puerto Rico, Trinidad and Tobago
Foliar vein yellowing (Rhabdo)virus[2]	USA
Horsegram yellow mosaic begomovirus	India
Mosaic[3]	Kenya and Zambia
Mung bean yellow mosaic begomovirus	India, Jamaica, Nepal, the Philippines, Puerto-Rico, Sri Lanka
Pigeonpea mosaic mottle (Viroid)	India
Rhynchosia mosaic begomovirus	Puerto Rico
Ring spot	Zambia
Sterility mosaic[3]	India, Bangladesh, Myanmar, Nepal, Sri-Lanka, Thailand
Tobacco mosaic tobamovirus	India
Tobacco streak ilarvirus	USA
Tomato black ring nepovirus	Kenya
Urd bean leaf crinkle virus[2]	India

[1] Nene *et al.*, 1996
[2] Unassigned virus
[3] Virus-like diseases of unknown etiology

Table 2
Susceptibility of pigeonpea to experimental inoculation of various plant viruses[1]

Susceptible to	Insusceptible to
Bean common mosaic potyvirus	Bean southern mosaic sobemovirus
Bean golden mosaic begomovirus	Bhendi yellow vein mosaic begomovirus
Bean yellow mosaic potyvirus	Broad bean wilt fabavirus
Clitoria yellow vein tymovirus	Cassava Indian mosaic begomovirus
Cowpea chlorotic mottle bromovirus	Cassia ringspot virus
Cowpea mild mottle (?) carlavirus	Cassia yellow blotch bromovirus
Cowpea mosaic comovirus	Chickpea chlorotic dwarf (?) mastrevirus
Cowpea mottle (?) carmovirus	Chickpea distortion mosaic potyvirus
Cowpea severe mosaic comovirus	Clitoria mosaic (?) potexvirus
Glycine mosaic comovirus	Clitoria yellow vein tymovirus
Horsegram yellow mosaic begomovirus	Crotalaria spectabilis yellow mosaic (?) potexvirus
Kennedya yellow mosaic tymovirus	Eggplant severe mottle (?) potyvirus
Lucerne Australian latent nepovirus	Groundnut eyespot potyvirus
Melilotus mosaic (?) potyvirus	Hop mosaic carlavirus
Mung bean yellow mosaic begomovirus	Maracuja mosaic (?) tobamovirus
Okra mosaic tymovirus	Marigold mottle potyvirus
Pigeonpea proliferation (?) rhabdovirus	Nerine X potexvirus
Pigeonpea sterility mosaic (?) virus	Pea streak carlavirus
Quail pea mosaic comovirus	Peanut chlorotic streak caulimovirus
Rhynchosia mosaic begomovirus	Peanut green mosaic potyvirus
Sunnhemp mosaic tobamovirus	Peanut mottle potyvirus
Swordbean distortion mosaic potyvirus	Potato V potyvirus
Urd bean leaf crinkle virus	Soybean chlorotic mottle caulimovirus
	Soybean crinkle leaf (?) begomovirus
	Soybean mosaic potyvirus
	Tephrosia symptomless (?) carmovirus
	Turnip mosaic potyvirus

[1]Brunt *et al.*, 1996a and 1996b.

the new plant growth shows clear symptoms. Some pigeonpea genotypes like ICP 2376, show chlorotic ringspots and plants look as healthy. At some geographic locations like in Bihar and Nepal SMD infection results in reduction in internodes, shortening of the branches and leaves sometimes become filiform (Reddy *et al.*, 1998). The variation in symptom expression by some genotypes and at certain locations are attributed to the possible involvement of various strains of the causal agent (Reddy *et al.*, 1998). Three types of symptoms were recorded by screening different pigeonpea germplasm for resistance to SMD at ICRISAT, Patancheru (Reddy and Nene, 1979): 1. severe mosaic and sterility; 2. ring spots and no sterility; 3. mild mosaic with partial sterility. A susceptible genotype infected at an early stage of crop growth usually shows near complete sterility with a yield loss up to 95%. Susceptibility of the plant decreases with its age at infection and the yield loss varies with the cultivar. Disease incidence is highest in ratoond and perennial pigeonpea. Estimated annual losses due to SMD in India are about 205,000 tons valued at $70 million in 1984 (Kannaiyan *et al.*, 1984).

2.4.2. Physiological and biochemical studies

Although causal agent of SMD is not known, some aspects related to the physiological and biochemical changes in SMD-affected and healthy plants were studied. Decrease in pigment and total carbohydrate content, and an increase in chlorophyllase activity in SMD-affected pigeonpea leaves were reported (Narayanaswamy and Ramakrishnan, 1965a). Increase in respiration of diseased plants was shown to accompanied by general reduction in organic acid content, but accumulation of citric acid and succinic acid was noted in the stem and root (Narayanaswamy and Ramakrishnana, 1966). Calcium, potassium, sodium and magnesium contents were found to be less, and total nitrogen was found to be high in diseased than in healthy plants (Nambiar and Ramakrishnan, 1969a, 1969b).

2.4.3. The causal agent of SMD

Since the description of SMD, continuous efforts have been made to identify the causal agent in several laboratories. All such attempts were unsuccessful and the causal agent of SMD remains unidentified. Nevertheless, the quest for the isolation of the SMD pathogen has resulted in valuable information regarding the nature of the causal agent and its transmission. Convincing evidence was provided to show that a fungus, bacterium, phytoplasma or viroid was not involved in the disease and that it was not caused by mite toxaemia (Ghanekar *et al.*, 1992). The disease was predicted

to be caused by a virus. Approaches for the isolation of a possible virus using several protocols, various buffers, organic solvents and centrifugation methods were not successful in isolating any agent (Reddy *et al.*, 1994). Although the analysis of single and double stranded RNAs from SMD-affected and healthy plants revealed the consistent association of two dsRNAs of about 9.1 and 5.6 kbp in SMD-affected plants, these were later found to be derived from a virus associated with the powdery mildew (*Oidiopsis taurica*) fungus, which was found to prefer SMD-affected plants (W.R. Sacks and D.V.R. Reddy, unpublished information). Electron microscopic studies at ICRISAT, Patancheru, found no virus-like particles or inclusion bodies in the crude sap extracts, or in ultra-thin sections of diseased tissues or pellets of concentrated leaf extracts (A.K. Murthy, personal communication). Recently, the presence of nuclear inclusions in phloem cells of mid-vein sections of SM-infected leaves stained with Azure-A was reported by Singh and Rathi (1996). On this basis the authors concluded that the agent is probably a RNA containing virus.

2.4.4. Transmission of the causal agent of SMD

The agent of SMD is transmitted in nature by the eriophyid mite *A. cajani* and experimentally by grafting (Reddy *et al.*, 1989). Capoor (1952), first reported the transmission of the SMD agent by grafting and by sap inoculation. Subsequently it was shown that the SMD agent neither is sap transmissible nor pollen, seed or soil transmitted (Kandaswamy and Ramakrishnan, 1960; Narayanaswamy and Ramakrishnan, 1965b; Nene, 1972; Ghanekar *et al.*, 1992; Reddy *et al.*, 1994).

Three methods are being used for experimental transmission of SMD to healthy pigeonpea plants. The 'leaf-stapling' technique described by Nene and Reddy (1976a) is used to inoculate plants by mites both in the field and in the glass house. This technique, involves stapling of a portion of SMD infected pigeonpea leaves onto healthy pigeonpea seedlings. Mites from the stapled leaf migrates to the fresh leaf and transmits the pathogen. This technique was shown to facilitate inoculation at primary leaf stage and to rapidly express disease symptoms. The 'Infector-hedge' technique was used for large scale field-inoculations (Nene *et al.*, 1981). This consists of a hedge of pigeonpea plants infested with mites either by the leaf-stapling technique or spreading infected twigs on 10-days old plants at the upwind border of the field. The mites and the SMD causal agent multiply on these hedge row plants and serve as mite and inoculum source for disease spread through wind onto test material sown downwind. Perennial pigeonpea are often used to maintain the inoculum. This

technique was further modified to produce the 'spread-row' inoculation technique, where, instead of single hedge several rows of infected plants are established throughout the field to achieve more uniform disease spread (Nene *et al.*, 1981). These methods have successfully been used to identify sources of resistance to SMD.

2.4.5. The mite vector *Aceria cajani*

Like other eriophyids, *A. cajani* is highly host specific. It is restricted to pigeonpea and some of its wild relatives, *Cajanus scarabaeoides* and *C. cajanifolia* (Sheila *et al.*, 1988). They inhabit areas between thick filamentous trichomes covering the lower surface of young leaves. The eggs of these mites measure about 30x40 µm. At room temperature eggs hatch in 3-5 days and the adult emerges from the final nymphal stage about a week later (Oldfield *et al.*, 1981). They feed on the lower surface of the leaf with short cheliceral stylets. The short stylets (~2.03 µm) of these mites allow penetration of epidermal cells (Sheila *et al.*, 1988). Mites feeding cause no obvious damage to pigeonpea. Their dispersal and spread in nature is passive and depend on wind currents (Reddy *et al.*, 1989). *A. cajani* population density is greater on SMD plants than on healthy plants. They are distributed on all stages of the leaves with their numbers more on young and mature leaves. On healthy plants, mite populations are less and more concentrated towards the petiole end of young leaves (Dhar and Rathore, 1994). Several studies have shown that *A. cajani* populations on pigeonpea are almost uniform throughout the year in cooler parts of India, whereas in semi-arid zones, high temperatures decrease mite populations (Reddy and Raju, 1993b; ICRISAT Ann. Rep., 1995; Lakshmikantha *et al.*, 1997). Incidence and spread of SMD depends on vector population in the field. It was found that 5 mites/cm^2 of leaf area is correlated with SMD spread in the field and less than 1 mite/leaf results in very mild SMD incidence (Dhar *et al.*, 1998).

2.4.6. The causal agent-mite relationship

Studies by using SMD causal agent-free *A. cajani* population have established that mite toxaemia is not responsible and an agent transmitted by the mites is involved in the etiology of SMD (Ghanaker *et al.*, 1992). A single mite can transmit the agent and that all stages of the mites are equally efficient in transmitting, but transovarial transmission is not observed (Janarthanan *et al.*, 1972; Reddy *et al.*, 1989). The acquisition access period is 5-10 min and the inoculation access period is 30 min. It is not clear whether the transmission is of a semi-persistent or persistent type. Agent-carrying mites can retain their ability to transmit throughout their life span provided

they continue to feed on a susceptible host. This does not eliminate the possibility of reacquisition of the agent from such plants.

2.4.7. Host range of the causal agent of SMD

In the absence of any diagnostic test, information regarding the host range of the SMD pathogen is based entirely on symptom expression on mite inoculated plants. SMD was known to infect only pigeonpea. Recently, based on symptoms and presence of mites, *Cajanus scarabaeoides, C. platycarpus* and *C. cajanifolia,* wild relatives of pigeonpea, were reported as an alternative hosts (Reddy *et al.*, 1993a: 1998). However, in these plants disease incidence is rare and mite multiplication is limited.

2.4.8. SMD spread in nature

The information relating to SMD cycle in nature is limited. The pathogen is not seed-borne. The pathogen is most likely to be spread by mites only (Reddy *et al.*, 1989; Ghanekar *et al.*, 1992). Mites surviving on alternative host, such as *C. scarabaeoides,* and on off-season pigeonpea on field borders, volunteer and ratooned plants, those grown in kitchen gardens, serve as main sources for disease spread (Reddy *et al.*, 1988; 1990; 1993a). The survival of SMD inoculum in areas where there are no volunteer pigeonpea plants is not known (Reddy *et al.*, 1989).

2.4.9. Symptom variation in different pigeonpea genotypes

Alam (1933) was the first to report the existence of resistance in pigeonpea to SMD. Screening for the sources of resistance was initiated at ICRISAT in 1975 under the programme "ICAR-ICRISAT Uniform Trial for Pigeonpea Sterility Mosaic Resistance" and 'All India Co-ordinated Pulses Improvement Project' (Nene and Reddy, 1976b; Nene *et al.*, 1989; Amin *et al.*, 1993). Following screening of the world collection of pigeonpea varieties at ICRISAT, genotypes immune to the disease were identified. Of nearly 15,000 germplasm accessions screened, 326 resistant lines showed no overt symptoms and 97 lines showed only ring spot symptoms but no sterility (Nene *et al.*, 1989; Amin *et al.*, 1993). Scoring was based on visual symptoms. A number of genotypes were shown to possess field resistance to SMD but this was later found to be largely location specific. The resistance mechanism is not characterised. Sharma *et al.* (1984) reported that SMD susceptibility is dominant over tolerance, and that

tolerance is dominant over the resistance. Inheritance of resistance to SMD appears to be complicated and determined by multiple allelic series (Saxena and Sharma, 1990).

Observations at ICRISAT indicated that some resistant lines did not permit multiplication of the mite vector. Leaf anatomy was studied in relation to the resistance to *A. cajani*. In several resistant lines the leaf cuticle and epidermal cell wall was 50-100% thicker than in susceptible lines (Prameela *et al.*, 1990; Reddy *et al.*, 1995). The cuticle thickness in resistant lines was 3.03-3.79 µm and in susceptible lines 1.52-2.22 µm. The average stylet length of *A. cajani* is 2.03 µm, which is less than the cuticle size in the majority of resistant lines. Although mites can pierce the cuticle, the stylets may not reach the underlying cells to feed and consequently cannot transmit the agent. But some lines (like ICP 8136) support the continued multiplication of mites, but are resistant to the SMD pathogen. This indicates the occurrence of different resistance mechanisms to SMD in pigeonpea.

Evaluation of 16 pigeonpea genotypes for resistance to SMD at ICRISAT, Patancheru, and 9 different locations in India, revealed large variation in disease incidence and symptom expression (Reddy *et al.*, 1993c). Furthermore, it was also found that some germplasm lines resistant at ICRISAT centre were susceptible at the other locations. Further studies in 51 field and pot tests, using 7 different pigeonpea genotypes (ICP 2376, 7035, 8862, 8863, 1097, 10984 and 11146; see Table 3) in different seasons, and in tests at different locations in India, identified the occurrence of 5 different variants of SMD (Reddy *et al.*, 1991; Reddy *et al.*, 1993c). Differences were also found in the strains of SMD causal agent prevalent at ICRISAT, Patancheru, India and Nepalganj, Nepal (Chaurasia, 1993). The differential reaction of germplasm lines to SMD over the different locations suggests the possible occurrence of different strains of the causal agent.

Assessment of SMD-variants based on symptoms alone is complicated by the fact that symptoms are governed by many biotic and abiotic factors. In recent studies, host reaction to SMD was shown to be governed by more than one gene and was assumed to be "strain specific" (Srinivas *et al.*, 1997a and 1997b). Pigeonpea is a cross-pollinated crop. In addition to environmental factors, genotypic variability induced as a result of cross-pollination are also likely to play an important role in symptomatology. Thus, variability in the pathogen, the mite vector, the plant genotype and the environment, and mixed infection with other pathogens, may all contribute to variability in symptom expression.

Table 3
Differential reaction of pigeonpea genotypes to sterility mosaic pathogen in India*

Pigeonepa Genotypes	Host reaction to SMD variants				
	1	2	3	4	5
ICP 2376	R	T	S	S	S
ICP 7035	R	R	R	R	S
ICP 8862	R	R	R	R	S
ICP 8863	S	S	S	S	S
ICP 10976	R	T	R	R	S
ICP 10984	R	R	R	S	R
ICP 11146	R	R	R	S	S
Location	Gwalior	Badanpur Patancheru	Coimbatore Kumargunj Pudukottai	Bangalore Dholi	Kanpur

R - Resistant (no symptoms); T - Tolerant (only ring spots, no sterility); S - Susceptible (mosaic and sterility)

*The places were mapped in India map given in Appendix 8.5.

2.5. Characterisation and Identification of Viruses

Several diseases caused by plant viruses have been reported and apparently new ones continued to be reported (Brunt *et al.*, 1996a). When an unknown virus disease is being investigated, it has to be determined is the virus(es) concerned is identifiable with the reported virus. Correct identification of the virus causing disease in the field is essential, symptoms usually won't allow positive identification. Studies on the virus and virus nucleic acids offer enough information for proper characterisation and identification of virus. The guidelines for characterisation and identification of an unknown virus have been reviewed. (Matthews, 1991; Murphy *et al.*, 1995; Mayo and Horzinek, 1998; van Regenmortel *et al.*, 1999). Plant viruses are classified on the basis of their particle morphology (virion shape, size, presence or absence of peplomers and envelope, capsid structure and capsid symmetry), physicochemical and physical properties (virion molecular weight, buoyant density, sedimentation coefficient, stability of the particles in pH, buffers, temperature, solvents, detergents, cations and to irradiation), genome [type of nucleic acid (DNAor RNA), size in kpb, strandedness (single or double), configuration (linear or circular), sense (positive or negative or ambisense), number and size of segments, nucleotide sequence, presence of 5' terminal cap structure, 5' covalently linked protein and presence or absence of 3' poly A tract], protein [number and size, functional activities of structural proteins and non-structural proteins, details of special functional activities of proteins (trascriptase, RT, hemagglutinin, neuraminidase and fusion activities], carbohydrates and lipids (content and characters, post transcriptional modifications), antigenic properties (serological relationships), biological properties (natural host range, mode of transmission in nature, vector relationships, geographic distribution, pathogenicity, association with disease, tissue tropism, pathology and histopathology). Plant viruses are classified into 12 families, 60 genera out of which 23 are floating genera (not assigned to any family) with more than 1000 virus species and strains (see Figure 1; van Regenmortel *et al.*, 1999).

2.5. 1. Strains of the Causal Agent

Occurrence of virus variants (strains) that differ in several properties is well documented (for example, Magome *et al.*, 1997; Yang and Mirkov, 1997; Kiranmai *et al.*, 1997; Druka and Hull., 1998). Understanding variation in a virus causing a disease is important because strains vary in the severity of disease they cause and can mutate to break crop plant resistance to a virus in the field (for example, Hamamoto *et*

al., 1997; Revers *et al.*, 1997a; 1997b). A range of criteria based on structural (genome and amino acid composition, shape and size) serological (presence or absence, and degree of serological relationship between viruses determined using polyclonal or monoclonal antibodies) and biological (disease symptoms, host range, methods of transmission and cross-protection) properties is being used to differentiating strains (for details, see Matthews, 1991). However, there is no defined criteria to consider certain viruses as species and others as strains of the one species (van Regenmortel *et al.*, 1997; Mayo and Pringle, 1998). The kind of criteria to be used depends on the purpose of the study. In the absence of any knowledge on the causative agent of SMD and suitable diagnostic assays to detect the pathogen, it is difficult to characterise strains of SMD, and its effects on symptom expression and host resistance.

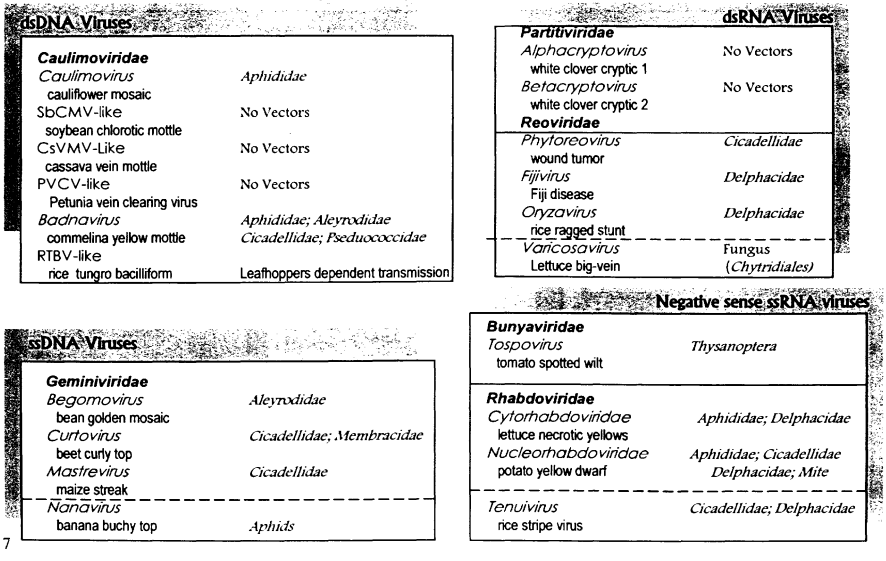
2.6. Invertebrate Vectors of Plant Viruses

Nematoda and *Arthropoda* are the two invertebrate phyla that contain vectors of plant viruses (Figure 1). The most important group of vectors numerically is in the insect order *Homoptera*. Although viruses transmitted by other vectors of *Arthropoda* are not as numerous as those found in *Homoptera*, they are very important viruses economically. Invertebrate vectors associated with the transmission of members of various genera of plant viruses are given in Figure 1. Virus genera and information regarding vectors were collected from the ICTV 7th report (van Regenmortel *et al.*, 1999), "Virus Identification Data Exchange (VIDE) Database" (Brunt *et al.*, 1996a) and Gray and Banerjee (1999). Note that, in this study, virus classification and nomenclature as given in the ICTV 6th report (Murphy *et al.*, 1995) and subsequent revisions as presented in Mayo and Pringle (1998) and Mayo and Horzinek (1998) was followed.

2.7. The Eriophyid Mites

Arachnida families, *Eriophyoidea* and *Tetranychidea* feed on plants and differ in many other respects from the members of the same class and phyla (Lindquist, 1996). Eriophyide mites are the smallest of all arthropods and measure about 150-250 µm. These are invisible to the naked eye and can be seen with the aid of a microscope. The mites have short life cycle of about 2 weeks that includes egg, two nymphal and adult stages (Manson and Oldfield, 1996). Unlike other mites, they possess only two

Figure 1
Principal vector species of plant virus families and genera



dsDNA Viruses

Caulimoviridae	
Caulimovirus	*Aphididae*
cauliflower mosaic	
SbCMV-like	No Vectors
soybean chlorotic mottle	
CsVMV-Like	No Vectors
cassava vein mottle	
PVCV-like	No Vectors
Petunia vein clearing virus	
Badnavirus	*Aphididae; Aleyrodidae*
commelina yellow mottle	*Cicadellidae; Pseudococcidae*
RTBV-like	
rice tungro bacilliform	Leafhoppers dependent transmission

dsRNA Viruses

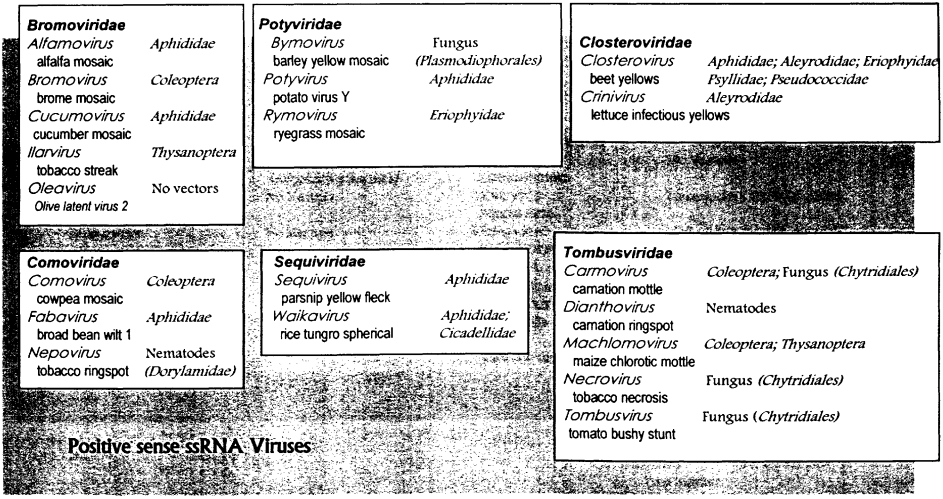
Partitiviridae	
Alphacryptovirus	No Vectors
white clover cryptic 1	
Betacryptovirus	No Vectors
white clover cryptic 2	
Reoviridae	
Phytoreovirus	*Cicadellidae*
wound tumor	
Fijivirus	*Delphacidae*
Fiji disease	
Oryzavirus	*Delphacidae*
rice ragged stunt	
Varicosavirus	Fungus
Lettuce big-vein	(*Chytridiales*)

ssDNA Viruses

Geminiviridae	
Begomovirus	*Aleyrodidae*
bean golden mosaic	
Curtovirus	*Cicadellidae; Membracidae*
beet curly top	
Mastrevirus	*Cicadellidae*
maize streak	
Nanavirus	
banana buchy top	*Aphids*

Negative sense ssRNA viruses

Bunyaviridae	
Tospovirus	*Thysanoptera*
tomato spotted wilt	
Rhabdoviridae	
Cytorhabdoviridae	*Aphididae; Delphacidae*
lettuce necrotic yellows	
Nucleorhabdoviridae	*Aphididae; Cicadellidae*
potato yellow dwarf	*Delphacidae; Mite*
Tenuivirus	*Cicadellidae; Delphacidae*
rice stripe virus	


Bromoviridae
Alfamovirus Aphididae
alfalfa mosaic
Bromovirus Coleoptera
brome mosaic
Cucumovirus Aphididae
cucumber mosaic
Ilarvirus Thysanoptera
tobacco streak
Oleavirus No vectors
Olive latent virus 2
Potyviridae
Bymovirus Fungus
barley yellow mosaic (Plasmodiophorales)
Potyvirus Aphididae
potato virus Y
Rymovirus Eriophyidae
ryegrass mosaic
Closteroviridae
Closterovirus Aphididae; Aleyrodidae; Eriophyidae
beet yellows Psyllidae; Pseudococcidae
Crinivirus Aleyrodidae
lettuce infectious yellows
Comoviridae
Comovirus Coleoptera
cowpea mosaic
Fabavirus Aphididae
broad bean wilt 1
Nepovirus Nematodes
tobacco ringspot (Dorylamidae)
Sequiviridae
Sequivirus Aphididae
parsnip yellow fleck
Waikavirus Aphididae;
rice tungro spherical Cicadellidae
Tombusviridae
Carmovirus Coleoptera; Fungus (Chytridiales)
carnation mottle
Dianthovirus Nematodes
carnation ringspot
Machlomovirus Coleoptera; Thysanoptera
maize chlorotic mottle
Necrovirus Fungus (Chytridiales)
tobacco necrosis
Tombusvirus Fungus (Chytridiales)
tomato bushy stunt
Positive sense ssRNA Viruses

Benyvirus	Fungus
beat necrotic yellow vein	*(Plasmodiophorales)*
Capillovirus	No Vectors
apple stem grooving	
Carlavirus	*Aphididae; Aleyrodidae*
carnation latent	
Enamovirus	*Aphididae*
pea enation mosaic	
Furovirus	Fungus
soil-borne wheat mosaic	*(Plasmodiophorales)*
Hordeivirus	No Vectors
barley stripe mosaic	
Idaeovirus	No Vectors
raspberry bushy dwarf	
Luteovirus	*Aphididae*
barley yellow dwarf	
Marafivirus	*Cicadellidae*
maize rayado fino	
Ourmiavirus	*No Vectors*
Ourmia melon virus	
Pecluvirus	*Fungus*
peanut clump	*(Plasmodiophorales)*
Pomovirus	Fungus
potato mop-top	(Plasmodiophorales)
Potexvirus	*No Vectors*
Potato virus X	
Sobemovirus	*Aphididae; Cicadellidae;*
Southern bean mosaic	*Coleoptera*
Tobamovirus	No Vectors
tobacco mosaic	
Tobravirus	Nematodes (*Trichodoridae*)
tobacco rattle	
Trichovirus	*Aphididae; Pseudococcidae*
apple chlorotic leaf spot	
Tymovirus	*Coleoptera*
turnip yellow mosaic	
Umbravirus	*Aphididae*
carrot mottle	
Vitivirus	*Pseudococcidae*
grapevine virus A	

Positive sense ssRNA Viruses (unassigned groups)

Family	
Genus	*Vector species*
Type member	

Key for identification

pairs of legs and their dispersal in nature is by wind currents. They occur widely on ferns, conifers and flowering plants throughout the world. Most of the mite species are quite specific for the host plant on which they feed, usually being confined to one plant genus, or at most the members of a single family (Oldfield, 1996a). Most of the plant species they inhabit are perennials. They cannot survive for long periods away from the host. They feed on succulent parts of the plant such as buds and young leaves, and feed by puncturing plant cells and sucking the contents. The short cheliceral stylets (about 20 μm) of eriophyid mites limit feeding to epidermal cells only, and the structure of the mouth parts allows penetration only to about 5 μm (Orlob, 1966). Some mite feeding produces no observable effect on the host plant, but with other species either feeding or associated toxins may cause different types of symptoms including malformed growth. For example, red kernel disease in wheat by *A. tosichella*; big buds of balckcurrants by *Cecidophyopsis ribis*. (Keifer *et al.*, 1982; Oldfield, 1996b; Westphal and Manson, 1996). Viruses or other agents transmitted by mites may also induce various types of symptoms on host plants. They are distinguished from toxaemias or other feeding effects of mites which are restricted to close proximity to the mite feeding sites. The pathogens transmitted by mites induce systemic symptoms even after the vector is eliminated, unlike toxaemias which are not systemic.

2.7.1. The eriophyid mite vectors

Eriophyid mites are vectors for about a dozen important plant viruses and several other pathogenic agents of unknown etiology. (Hiruki, 1992; Maramorosch, 1994; Oldfield and Proeseler, 1996; Table 4). The relationship between eriophyid vector and transmitted agent is highly specific. Plant pathogens transmitted by eriophyid mites are not known to be transmitted by members of any other taxa or usually by more than one species of eriophyid. Exceptions are *Abacarus hystrix* Nalepa, *Aceria tulipae* Keifer, and *A. tosichella*, that are reported as vectors for two or more plant pathogens (see Table 4).

Due to constant association of mites with the diseased plants, often it is difficult to determine whether the disease is due to the mite feeding or some pathogenic agent. More than 70 years ago blackcurrant reversion disease agent was first recognised to be associated with mites (Amos *et al.*, 1927). Since then little progress has been made in understanding the specifics of the transmission mechanism of mite-borne agents. The best understood mite-pathogen relationship is that of wheat

Table 4
Disease agents vectored by eriophyid mites

Disease agent	Vector	Pathogen	Sap Transmission	Natural Hosts	Distribution	Reference
Dicotyledons						
Blackcurrant reversion	*Cecidophyopsis ribis*	Nepovirus	-	*Ribes nigrum*	Europe, New Zealand	Jones, 1994 Lemmetty *et al.*, 1997
Cherry mottle leaf	*Eriophyes inaequalis*	Closterovirus	+	*Prunus emarginata*	Canada, USA, Europe	James and Mukerji, 1993; James, 1994
Fig mosaic	*Aceria ficus*	Unknown	-	Figs	World-wide	Oldfield and Proeseler, 1996
Peach mosaic	*Eriophyes insidiosus*	Closterovirus	+	*Prunus* spp.	Southwestern USA, Mexico	Gispert *et al.*, 1998 James and Howell, 1998
Pigeonpea sterility mosaic	*Aceria cajani*	Unknown	-	Pigeonpea	South and East Asia	Ghanekar *et al.*, 1992
Prunus latent mosaic	*Vasates fockeu*	Rymovirus(?)	-	*Prunus* spp.	North America	Hiruki, 1992
Rose rosette	*Phyllocoptes fructiphilus*	Unknown	-	*Rosa* spp	North America	Epstein and Hill, 1994
Monocotyledons						
Agropyron mosaic	*Abacarus hystrix*	Rymovirus	+	*Agropyron repens*, *Triticum aestivum*	Eurasia, Canada, Finland USA	Langenberg and Robertson, 1996a
Garlic mosaic (WSMV infection of garlic)	*Aceria tulipae*	Rymovirus	+	Garlic	Indian sub-continent	Ahmed and Benigno, 1985
High plains disease	*Aceria tosichella*	HPV	-	Maize	USA	Jensen *et al.*, 1996
Hordeum mosaic	Not well classified mites	Rymovirus	+	*Hordium* spp	Canada	Langenberg and Robertson, 1996b
Oat necrotic mottle	Not well classified mites	Rymovirus	+	*Avena sativa*	Canada	Gill, 1976
Onion mite-borne latent virus	*Aceria tulipae*	Rymovirus (?)	+	*Allium* spp.	Europe	van Dijk *et al.*, 1991.
Ryegrass mosaic	*Abacarus hystrix*	Rymovirus	+	*Lolium* spp. *Dactylis glomerata*	Eurasia, Australia North America	Plumb, 1996
Shallot mite-borne latent virus	*Aceria tulipae*	Rymovirus (?)	+	*Allium* spp.	Europe and Asia	van Dijk *et al.*, 1991
Spartina mottle	Not well classified mites	Rymovirus	-	*Spartina* spp.	United Kingdom	Jones, 1980
Wheat spot mosaic (Wheat spot chlorosis)	*Aceria tulipae*	Unknown	-	*Triticum aestivum*	Canada, USA	Slykhuis, 1956 Nault and Styer, 1970
Wheat spotting	*Aceria McKenziei* *Eriophyes tritici*	Unknown	-	Wheat	North America	Maramorosch, 1994
Wheat streak mosaic	*Aceria tulipae*	Rymovirus	+	Wheat, Oats, Barley, corn, wild grasses	World-wide	Styer and Nault, 1996 Oldfield and Proeseler, 1996

'+' Sap transmissible; '-' Not sap transmissible; Note: All agents are transmitted by grafting

streak mosaic rymovirus (WSMV) and its vector *A. tosichella*. Studies indicate that WSMV is not transovarially transmitted. Immature stages as well as adults can transmit (Slykhuis, 1955). However, adults cannot acquire the virus (Orlob, 1966). WSMV persists in the midgut for at least 5 days (Slykhuis, 1955; del Rosario and Sill. 1965; Paliwal and Slykhuis, 1967; Stein-Margolina *et al.*, 1969; Sinha and Paliwal, 1976). WSMV was suggested to circulate through various body tissues and inoculated into plants via the saliva. This is based on detection of WSMV particles in midgut, and occasionally in the haemocoel and salivary glands of vector mites (Paliwal, 1980). However, virus transmission by regurgitation was not ruled out. Some studies indicate the possible involvement of receptors which determine vector specificity. For example, *A. tosichella* can acquire but not transmit barley stripe mosaic hordeivirus (BSMV). *A. hystrix*, the vector of ryegrass mosaic rymovirus (RGMV), can multiply on WSMV infected wheat, without serving as a vector (Paliwal, 1980). Specific studies relating to other mite-transmitted agents are limited. Studies on other mite-transmitted diseases like peach mosaic (Wilson *et al.*, 1955); wheat spot mosaic (Slykhuis, 1956); RGMV (Mulligan, 1960); fig mosaic (Proeseler, 1972); and SMD (Reddy *et al.*, 1989) suggest that mite-borne pathogens are not transmitted through eggs. In general, virus persists in the vector for at least 2-5 days. Acquisition access period varies from 15 min to 16 h, inoculation access period is 15 min and in most cases single mites can transmit the disease agents.

2.7.2. The nature of mite-transmitted disease causal agents

The nature of most of the disease causal agents transmitted by mites infecting monocotyledons are known, but a few of those that infect dicotyledons. All the well characterised mite-transmitted agents are shown to be viruses (Table 4). In a few instances, the association of bodies with double membrane (DMBs) were recognised. However, the precise role of these bodies in disease etiology is not understood. There are no reports of eriophyid mites transmitting other plant pathogens such as bacteria, fungi, phytoplasmas, spiroplasmas and viroids.

All mite-transmitted viruses have long flexuous-rod shaped particles and belong to the genus *Rymovirus* and *Closterovirus* (Table 4). Recently, a virus with properties similar to nepoviruses was shown to be closely associated with blackcurrant reversion disease and was transmitted by mites (Lemetty *et al.*, 1997; Latvala *et al.*, 1997).

Under natural conditions, virus usually spreads to only hosts which are compatible to the mite vector. For example, under natural conditions *A. tosichella* is known to transmit WSMV to wheat, corn, garlic and several wild grass species (Connin, 1956; Sill and del Rosario, 1959; Ahmed and Benigno, 1985); peach mosaic virus transmitted by *Eriophyes insidiosus* infects several *Prunus* spp. on which vector can multiply (Creamer *et al.*, 1994). Many of the viruses infecting monocotyledonous, and cherry mottle leaf closterovirus and peach mosaic closterovirus infecting dicotyledonous plants are readily sap transmissible. Such mite-borne sap transmissible viruses have a wide experimental herbaceous host range. None of the mite-transmitted disease causal agents are transmitted through seed or pollen but appears to be present in all parts of the host tissue, and are not localised to any particular host tissue like *Luteoviruses*. Most mite-borne viruses within a virus genus are usually related serologically (Creamer *et al.*, 1994; Van Dijk, 1996). For example; WSMV, RGMV, agropyron mosaic and oat necrotic mottle; cherry mottle and peach mosaic; onion mite-borne latent virus and shallot mite-borne latent viruses are serologically related.

For some mite-transmitted agents of unknown etiology, DMBs were found in plant tissues, they have been detected in plants affected with rose rosette (Gergerich and Kim, 1983), fig mosaic (Plavsic and Milicic, 1980), wheat spot mosaic (Chen and Hiruki, 1990; Zaychuk, 1991; Hiruki *et al.*, 1994), High Plains virus (Ahn *et al.*, 1995). The nature of the DMBs is yet to be understood, but they are suspected as disease causal agents. The DMBs isolated from wheat spot mosaic diseased tissues are 100-200 nm in diameter and were found associated with endoplasmic reticulum (Chen and Hiruki, 1990). Molecular analysis of DMBs showed the presence of neither nucleic acid nor protein components. These data together with the relationship of the DMBs to the endoplasmic reticulum suggest that the DMBs are probably a host response to some mite or pathogen related factors (Chen and Hiruki, 1990).

Mites other than from the family Eriophyidae also reported as vectors of plant disease agents. Among these, Tenuipalpidae members, *Brevipalpus ovovatus and B. phoenicis* are linked with citrus leprosis transmission (Maramorosch, 1994). Coffee ringspot virus, an unassigned nucleorhabdovirus is reported as vectored by *B. phoenicis* (Chagas, 1996). Rice plants parasitised with *Steneotarsonemus spinki* Smiley (Tarsonemidae) have been reported to contain virus-like particles (Shikata *et al.*, 1984). However, it has not established whether the disease is due to mite feeding

or by the virus. Robertson and Carroll (1988) described a virus-like disease of barley transmitted by the spider mite *Petrobia latens* Muller. Reports of red spider mites (Tetranychidae), as vector of potato virus Y was not confirmed (Schulz, 1963; Orlob, 1968). However, it was shown that, these mites can acquire tobacco mosaic tobamovirus (TMV), potato virus X (PVX), tomato bushy stunt virus (TBSV), onion yellow dwarf virus (OYDV), brome mosaic bromovirus (BMV) and tobacco ringspot nepovirus (TRSV), which can reach high concentrations in the mites and their excretions are infectious (Orlob, 1968; Orlob and Takahashi, 1971). However, no infection with these viruses resulted from infestations of such mites.

2.8. Identification of Eriophyid Mites

Identification and classification of eriophyid mites are particularly difficult because their basic body architecture are very similar and homogenisation of useful morphological characters due to convergent evolution. The biological homogeneity displayed by this group suggests that they may have originated from a single primordial ancestor (Jeppson *et al.*, 1975). To date over 3,000 eriophyid species are described and about 300 new species await descriptions. This was estimated to account for about 10% of the world fauna, suggesting the existence of many unrecognised eriophyid species in natural and agricultural ecosystems (Amrine *et al.*, 1996).

Accurate identification of these mites is essential for the successful and economic application of control measures. For example, due to misidentification of *C. grossularia* as *C. ribis*, (a vector of the reversion disease agent), regulatory agencies enforced the destruction of all *Ribes* infested with the mites to avoid any possibility of introduction of 'reversion disease' into the USA (Amrine, 1993).

The microscopic size, soft body and cryptic existence of these mites on host plants pose considerable difficulties in handling and identifying them. Generally, the host specific nature and the type of damage caused are the most common features considered for mite identification (Keifer *et al.*, 1982). But this is complicated by recent evidences which suggest the cross-colonisation of mites to other host species and the existence of several uncharacterised mite species in nature (Fenton *et al.*, 1996; Amrine *et al.*, 1996). Because of confusion in the description of these mites in the literature and expertise required to distinguish the species, identification based on morphology is complicated (Keifer, 1975; Amrine, 1993; Amrine *et al.*, 1994; Conjin

et al., 1996). Consequently, validity of the suggested basis for mite biotypes, its speciation on different plant species has been the subject of conjecture. However, recent advances in molecular techniques have facilitated the study of the genetic composition of pests both at the population and individual level. These studies have been, and continue to be, crucial in the identification of individuals within a species as well as the differentiation of closely related species. Such information is vital for these mites both for the understanding of pest distribution *per se* and indirectly in relation to epidemiolgy.

The most important break thorough was made when modern molecular biological techniques were successfully used for the amplification of eriophyid mite rDNA by PCR. Subsequent analysis of amplified products revealed that rDNA ITS sequences can be used as molecular markers for mite identification (Fenton *et al.*, 1995 and 1997; Kumar *et al.*, 1999a). Application of such methods is useful to understand the genetic variation within and between populations of *A. cajani*.

2.8.1. Biological strains in eriophyid mites

Information regarding the occurrence of biological strains in eriophyid mites is meagre. Several studies, mostly based on host compatibility and virus vectoring ability, suggested the possible occurrence of mite biotypes (Frost and Ridland, 1996). Mites morphologically identical to and classified as *A. tulipae* are reported to be pests of plants in the family *Graminaeae* either because of feeding damage, such as kernel red streak disease in maize, or mainly because of the virus transmission, such as WSMV (Slykhuis, 1980; Keifer, 1982; van Dijk and van der Vlugt, 1994). Based on such characteristics biotypes in the population of *A. tulipae* (Slykhuis, 1955; Gibson, 1957; del Rosario and Sill, 1967; Schevchenko *et al.*, 1970), *A. hystrix* (Gibson, 1974), *Cecidophyopsis ribis* (Eisbein and Proeseler, 1969; Esterbrook, 1980; Csapo, 1992), and *A. tritici*, *A. hystrix*, *Aculodes mckenziei* and *Aculodes dubius* (Sukhareva, 1981) were suggested to occur.

2.9. Molecular Techniques for Biological Strain Differentiation

Every population have some degree of variation which reflects in genetic composition, the few exceptions are usually where single clones colonise new areas (for example Corell *et al.*, 1992). Various techniques based on biological (mating behaviour, host adaptability etc., examples: Navajas *et al.*, 1994; Dahal *et al.*, 1997; Jennings *et al.*, 1997; Sunnucks *et al.*, 1997), physical (based on morphology,

examples: Jones et al., 1993; Amrine et al., 1994) and biochemical (protein and nucleic acid based techniques, for review see Avise, 1994; Loxdale and Lushai, 1998; Cavalli-Sforza, 1998) characteristics are used to identify strains within the populations. Selection of suitable technique depends on the type of questions being asked and the organism under scrutiny. Because of its effectiveness and reliability, application of nucleic acid based techniques become popular for the species identification, to investigate the degree of variation (heterogeneity), relationships between pest populations and the basis of biotypic differences.

2.9.1. DNA based techniques

There are many advantages of DNA based techniques in the studies of population genetics, evolutionary ecology and systematics: 1. the genotype rather than phenotype is assayed; 2. based on the problem one or more appropriate sequences can be selected on the basis of evolutionary rate of mode of inheritance; 3. procedures for DNA based assays are simple; 4. DNA can be prepared from small amounts of tissue.

To use DNA sequences for any specific application, it is essential to know how these sequences are varied and how they are organised. This knowledge helps in choosing sequences appropriate for systematics and indicate fitting techniques for analysis.

2.9.2. Ribosomal DNA and PCR

The polymerase chain reaction (PCR) has revolutionised the isolation and study of sequenced parts of the genome (Saiki et al., 1988). Multi-gene families provide one of the most popular targets, as they provide multiple templates and have partially conserved sequences which are ideal for primer designing (Long and Dawid, 1980; Hills and Dixon, 1991). One of these families encodes ribosomal RNA (rRNA) which is present in all cellular life forms. It has been analysed at the structural level in a large number of multicellular eukaryotes (Hills and Dixon, 1991). The primary transcription unit of rRNA consists of 18S, 5.8S and 28S genes and additional regions like an external transcribed spacers (ETS) and two internal transcribed spacers (ITS-1 and ITS-2). All these sub-units together known as ribosomal DNA (rDNA). Multiple copies of rDNA transcription units are separated by intergenic spacers (IGS). The gene arrangement of rDNA primary transcription unit is as follows; ETS-18S-ITS1-5.8S-ITS2-28S-IGS-18S-ITS1-5.8S-ITS2-28S-IGS; see Figure 2). Analysis of this region has found widespread use in theoretical studies such as phylogeny and applied use in species-

Arrangement of Ribosomal RNA Genes

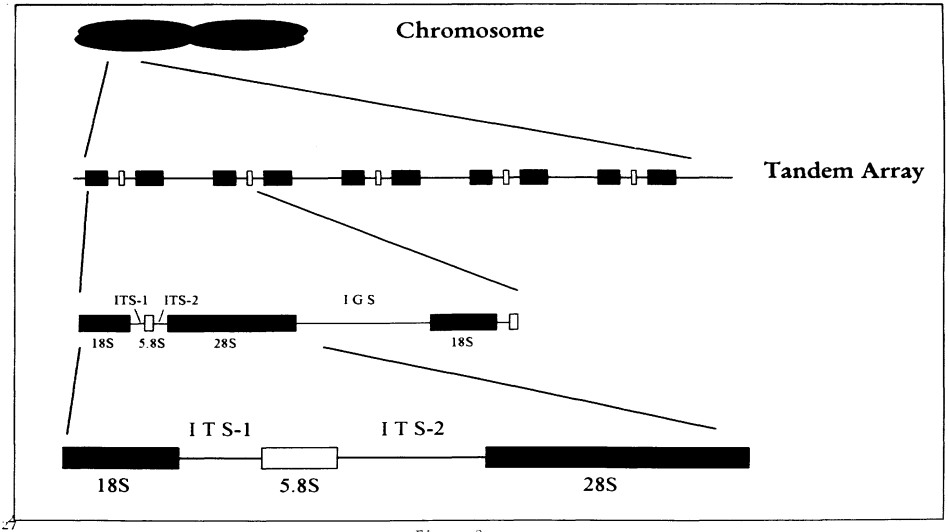


Figure 2

specific diagnostics (Carbone and Kohn, 1991; Xue *et al.*, 1992; Suh *et al.*, 1993; Zambino and Szabo, 1993). rDNA based diagnostic assays have several advantages. It is an extremely well studied gene family and its occurrence in many copies in an individual makes it a good target for PCR amplification from small amounts of DNA. It is also easily located, as the multiple copies are usually repeated end to end making it easily detectable by techniques such as fluorescent *in situ* hybridisation (Fenton *et al.*, 1994). The structure and sequence of the rRNA coding regions are highly conserved. The two ITS regions between the coding regions diverge quite rapidly between species, but are highly conserved within the species (Hills and Dixon, 1991). This has been confirmed in acarids (Navajas *et al.*, 1994; Fenton *et al.*, 1997).

Chapter 3

Studies on the Causal Agent of SMD

3.1. Pigeonpea Sterility Mosaic Material and Culture

Pigeonpea cultivars, ICP 8863, 7035 and 2376 (Table 5) were used for the maintenance of SMD-inoculum and mites in a growth chamber maintained at 27 ^{0}C with 55% humidity during day and 18 ^{0}C and 35% humidity during night. Leaf stapling technique (Nene and Reddy, 1976) was used to inoculate pigeonpea seedlings at two leaf stage (9-14 day old). The presence of mites was ensured before stapling to the healthy plants. Uninoculated pigeonpea plants maintained in another growth chamber were kept as controls.

Table 5

List of pigeonpea genotypes used in this study

Pigeonpea cultivar	Susceptible to
ICP 2376	SMD and mites
ICP 8863	SMD and mites
ICP 7035	Resistant to SMD and mites
ICP 8113	Resistant to mites only

3.2. Attempts for the Isolation of causal agent of SMD

For the isolation of causal agent of SMD two different approaches were made. (i) concentration of SMD-affected pigeonpea leaves and mechanical inoculation onto a range of herbaceous plants to transfer the pathogen and, (ii) purification of presumably a virus directly from SMD-affected samples. The experimental procedures in detail are given in the chapters 3.3 and 3.4. For this purpose SMD-affected samples maintained in a glass house and those obtained from experimental plots as well as from the farmers fields were used. Before extraction the leaf material was stored at least for 2 h at 4 ^{0}C. For long term storage leaf material was stored at -70 ^{0}C.

3.3. Virus Isolation Procedure - 1

Concentration of SMD-affected pigeonpea leaves and mechanical inoculation onto a range of experimentally herbaceous hosts to transfer a virus

3.3.1.1. Purification Procedure - 1

Twenty grams of SMD-affected leaf material obtained from ICRISAT field was macerated in 100 ml 0.05 M phosphate buffer, pH 7, containing 0.2% mono-thioglycerol and 1% egg albumin. Extract was filtered through cheese cloth and clarified by centrifuging at 10,000 rpm for 5 min at 4 ^{0}C in a Sorvall GSA rotor. The supernatant fluid was concentrated by centrifuging at 48,000 rpm for 60 min at 4 ^{0}C in a Beckman 50.2Ti rotor. The final pellets were resuspended overnight in 8 ml of phosphate buffer. This was clarified and centrifuged at 60,000 rpm for 60 min in a Beckman R75 rotor. The virus-containing pellets were dissolved in 500 µl of phosphate buffer and used to mechanically inoculate *Chenopodium quinoa, Phaseolus vulgaris, Cucurbita pepo, Nicotiana occidentalis* cv. P1, *N. tabacum* cvs Xanthi and Samsun, *N. benthamiana and N. clevelandii.*

Mechanical inoculation: Leaves of the selected plants were dusted with corundum. Using a muslin cloth piece sap was inoculated on to the leaves. The inoculated leaves were rinsed with tap water and the plants were covered with sheets of wet paper overnight.

Experimental Host Range: The virus host range was assessed using inoculum from infected *C. quinoa* leaves ground in 1 ml of 0.01 M phosphate buffer, pH 7. Following mechanical inoculation, plants were observed for 2-3 weeks. Infection of test plants was determined by return inoculations to *C. quinoa* from inoculated and uninoculated leaves.

Properties of Virus in Sap Extracts: Crude sap from infected *N. clevelandii* leaves was used for studying the different properties of the virus *in vitro*. The infected leaves were macerated and filtered through cheese cloth and used for studying the effects of different buffers, pH, additives and such properties like longevity *in vitro* (LIV) and

dilution end point (DIP), in addition to electron-microscopy (EM). *C. quinoa* or *C. amaranticolor* was used as test plant and plants were inoculated in a Latin Square design (Kleczkowski, 1968).

Electron Microscopy: Virus-infected leaves were extracted by grinding in 0.1 M citrate buffer, pH 6.5 (0.5 ml/1 sq.cm leaf), clarified with 1/5 volume of chloroform and centrifuged at 8,000 g for 3 min. A drop of the aqueous phase was mounted on carbon-coated EM grids and stained with eight drops of 2% aqueous uranyl acetate, pH 3.5 or 1.5% phosphotungstic acid, pH 6.5 or 2% ammonium molybdate, pH 7. Stained grids were observed under a JEOL JEM 100SX transmission electron microscope at 30,000X magnification. A catalase crystal grid was used to calibrate the microscope before photographing particles for measurements

3.3.1.2. Virus Purification from *N. clevelandii*

Virus was purified from infected *N. clevelandii* leaves harvested two weeks post-inoculation (pi) using a slightly modified protocol described by Lemmetty *et al.* (1997). Leaves were ground in 0.05 M phosphate buffer pH 7, containing 0.2% mono-thioglycerol (2 ml buffer/g leaf). The extract was squeezed through cheese cloth and clarified with an equal volume of chloroform before centrifuging at 10,000 rpm for 10 min in a Sorvall GSA rotor. The aqueous phase was concentrated by centrifugation at 48,000 rpm for 60 min in a Beckman 50.2Ti rotor. The pellets were resuspended in 8 ml of phosphate buffer overnight and, after low speed centrifugation to clarify the preparation, it was concentrated further by centrifugation at 65,000 rpm for 60 min in a Beckman R75 rotor. The pellets were resuspended in phosphate buffer and clarified by centrifuging at 10,000 rpm for 2 min. Partially purified particle preparations were layered on sucrose density gradient columns prepared by freezing and thawing of a 25% sucrose solution in 0.05 M phosphate buffer and then centrifuged at 36,000 rpm for 2.5 h in a Beckman SW41 rotor. Gradient tubes were observed under a narrow light beam. Light-scattering zones were fractionated by upward displacement in an ISCO ultraviolet gradient fractionator. Fractions corresponding to the main absorbance peak were pooled, diluted with 2.5 volumes of phosphate buffer and concentrated by centrifuging at 65,000 rpm for 90 min. Pellets were resuspended in a minimal volume of phosphate buffer and utilised for

determining UV absorption characters, buoyant density, protein and nucleic acid of the purified virus preparations (details given in results).

3.3.1.3. Production of Antibodies

An antiserum against virus was produced in a New Zealand White rabbit by subcutaneous injection of 100 µg of purified virus in 500 µl PBS emulsified in 500 µl of Freund's incomplete adjuvent on two occasions at two-week intervals. Antiserum was collected four weeks after the last injection and its titre determined by agarose gel double diffusion test (AGDD) using the protocol described by Ball (1990).

3.3.1.4. Serology

Purified virus preparations were tested by AGDD for reactivity with antisera to the following icosahedral viruses: cowpea mottle carmovirus, saguaro cactus carmovirus, cymbidium ringspot tombusvirus, galinsoga mosaic carmovirus, pelargonium ringspot virus, pelargonium flower break carmovirus, cowpea mosaic comovirus, tomato bushy stunt tombusvirus, carnation mottle tombusvirus, and hibiscus chlorotic ringspot carmovirus. For such tests, 0.8% agar gel was prepared in normal saline on 1.5x1.5" glass plates. A typical 8 well pattern was used, with an undiluted purified virus preparation in the central well and 1:20 antiserum dilution in the peripheral wells. Plates were incubated in a moist chamber at room temperature over night.

3.3.1.5. Virus Coat Protein

Purified virus particles were denatured by boiling for 3 min in an equal volume of Laemmli buffer (0.5M Tris-HCl, pH 6.8, 10% SDS, 5% 2-mono-thioglycerol, 10% glycerol, and 0.05% bromophenol blue) and the samples were electrophoresed in a 12% SDS-polyacrylamide gels (PAGE) in Laemmli's (1970) discontinuous buffer system. A BRL Model V16 was used (Bethesda Research Laboratories, Maryland, USA) for electrophoresis. Pre-stained protein markers (SeeBlue pre-stained standards, Noval Technologies, Cat.# SP-LC56251) were used as standards for estimating molecular weight. Mobility of the coat protein sub-units in different concentrations of polyacrylamide gels (7.5%, 10%, 12% and 14%; Appendix 8.1) and relative mobilities of the markers was determined. The molecular weight was estimated from the graph plotted on a semi-log graph sheet, against distance migration (x-axis) and corresponding molecular weights (y-axis).

3.3.1.6. Viral Nucleic Acid

The protocol described by Wood and Coutts (1975) was used for the initial analysis of viral nucleic acid. Purified virus particles were disrupted by incubating with an equal volume of disruption buffer (0.1M Tris-HCl pH 8.3, 1M urea, 5% sucrose, 1% SDS, 1% 2-mercaptoethanol and 0.1% bromophenol blue) at 52 ^{0}C for 10 min. The samples were electrophoresed in 1% agarose gel (Sambrook *et al.*, 1989) for 2 h at 80 V in TAE (0.04M Tris-acetate; 0.001M EDTA) buffer, pH 8. Gels were stained with ethidium bromide and viewed under an UV-transilluminator.

3.3.1.7. Characterisation of Viral Nucleic Acid

Viral nucleic acid was isolated from the purified virus preparations using the RNeasy^{TM} kit (Qiagen, Cat.# 74904). Extraction was done according to the manufacturers instructions. Purified particle preparations were mixed with 400 µl of RLT buffer containing 1% 2-mercaptoethanol. This was vortexed and transferred into a QIAshredder column and centrifuged for 1 min at 10,000 g. Eluent was collected without disturbing the pellet and, to this, 0.5 volumes of absolute ethanol was added and mixed well with the pipette and transferred to a RNeasy mini column and centrifuged at 10,000 g for 15 sec. Eluent was discarded and the column was washed once with 600 µl of RW1 buffer and twice with RPE buffer. RNA from the column was eluted into 40 µl of DEPC-treated sterile distilled water.

Molecular weight and sensitivity to nucleases: Isolated viral RNA was treated with DNase and RNase separately and incubated at 37 ^{0}C for 2 h. The samples were electrophoresed in a 1% TAE-agarose gel along with RNA molecular weight standards (IBI RNA size standards, Cat.# IB76200). Molecular weight was estimated from the graph plotted on a semi-log graph paper, against distance migrated (x-axis) and corresponding molecular weights (y-axis).

Infectivity of RNA: Viral RNA was diluted in bentonite buffer (10% bentonite in 0.01 M tris-HCl, pH 7.6; see Appendix 8.4) and inoculated onto *C. quinoa*. The infectivity of each RNA species was determined by eluting the separated viral RNA species on a 1% TAE-agarose gel. After electorphoresis, the gel corresponding to each band was excised and transferred into a 1.5 ml Eppendorf tube and kept at -70^{0}C for 10 min. To each gel slice 500 µl of bentonite buffer was added and crushed with an Eppendorf homogeniser and centrifuged at 10,000 g for 5 min. The supernatant fluid was inoculated to corundum-dusted *C. quinoa* leaves with a sterile muslin pad.

Test for 3' poly adenylated tract: To determine the presence of a poly A tract at the 3' end of viral RNA, the Oligotex™ mRNA kit (Qiagen, Cat.# 70022) was used. Purified viral RNA was processed as per the manufactures instructions. Briefly, viral RNA was mixed with oligo-dT_{30} linked latex particles and incubated for 10 min at room temperature. The mixture was centrifuged at 10,000 g for 3 min. The supernatant fluid was collected into a separate tube. The pellet was processed further by resuspending in a washing buffer^R and transferred to a spin column and centrifuged at 10,000 g for 1 min. The eluent was discarded and the RNA from the spin column eluted into 30 µl of DEPC-treated water. The eluent along with the supernatant from the first step was electrophoresed in a 1% TAE-agarose gel.

3.3.1.8. Complementary DNA (cDNA) Synthesis and Cloning

DNA complementary for the viral RNA was constructed using the Universal Riboclone Synthesis System™ (Promega, Cat.# C4360), purified RNA preparations and random hexameric primers as described by Gubler and Hoffmanm (1983).

First strand synthesis: Two µg of viral genomic RNA and 1 µg of random hexameric primers in a 15 µl sterile RNase-free water was heat treated at 65 ^{0}C for 15 min and chilled on ice for 5 min. To this was added, 2.5 µl of 40 mM sodium pyrophosphate, 40 U RNasin^R ribonuclease inhibitor, 30 U avian myeloblastosis virus (AMV) reverse transcriptase (RT), 5 µl 5x first strand buffer^R and sterile water to 25 µl and the mixture incubated at 37 ^{0}C for 60 min.

Second strand synthesis: To 20 µl of first strand reaction was added, 23 U of DNA polymerase I, 0.8 U RNase H, 40 µl of 2.5x second strand reaction buffer^R and sterile water to 100 µl and the mixture incubated at 14 ^{0}C for 4 h. Then reaction tube was heat treated at 70 ^{0}C for 10 min. To this 4 U of T4 DNA polymerase (2 U/µg of RNA) was added and incubated at 37 ^{0}C for exactly 10 min, to digest any 3' overhangs. This was extracted once with equal volumes of a TE-saturated phenol:chloroform:isoamylalcohol mixture and the DNA precipitated with ethanol and suspended in 30 µl of 0.1 M Tris-EDTA buffer, pH 9.2.

Cloning: Twenty µl of precipitated cDNA construct was size fractionated by electrophoresing in a 0.8% TAE agarose gel along with a DNA marker. The fraction corresponding to the 1.2 - 1.5 kbp was eluted using the Kristal Gelex Kit (Cambridge Molecular Technologies) as described in section 4.1.6.1. Eluted DNA was cloned into the 'end-filled' *Eco*R I site of a pCR blunt™ vector (Invitrogen, Cat.# K2700-20; see

Appendix 8.6 for vector map) by blunt-end ligation. The cloning reaction in a final volume of 10 µl of 1x ligation buffer (supplied with the enzyme) consists of 5 µl of DNA (80 -100 ng), 1 µl (25 ng/µl) vector, 4 U of T4 DNA ligase and distilled water to the final volume was added and incubated at exactly 16 ^{0}C for 1 h. This was transformed into *E. coli* TOP 10 competent cells (Invitrogen, Cat.# K2700-20) and plated on LB kanamycin plates as described in section 4.1.6. Similarly, in another experiment, 7 µl of the precipitated cDNA construct was directly used for cloning into the pCR blunt^{TM} vector. Plasmids from the transformed clones were isolated using the 'Wizard miniprep' kit (Promega, Cat.# A7100) and the presence of an insert was confirmed either by digesting with *Eco*R I enzyme or by PCR using F and R primers as described in the section 4.1.6.5.

Primer extension analysis: Full length cDNA for the viral RNA was constructed by RT-PCR using synthetic oligonucleotide primers (Genosys, UK) and purified viral RNA preparations (Ausubel *et al.*, 1994). Conditions for the RT-PCR were as described in section 3.3.1.14, except that instead of total plant RNA, 1:500 dilution of purified viral RNA preparation was used. RT-PCR products were cloned into the TOPO-TA vector (Invitrogen, Cat.# K4500-40; vector map given in Appendix 8.6) as described in section 4.1.6.

3.3.1.9. Northern Blotting and Hybridisation

Northern hybridisation to confirm the specificity of the cDNA construct was made using the cloned cDNA as a probe to hybridise with the viral RNA using protocols described in Molecular Cloning (Sambrook *et al.*, 1989) and Promega Protocols and Applications Guide (Promega, 1996).

Probe preparation: Universal forward (F) and reverse (R) primers were used to amplify and label the 1.6 kbp cDNA clone (named GB4.2) with digoxygenin (DIG; Boehringer Mannheim, Cat.# 1093 657) by PCR programme 13 (see Section 4.1.4). The PCR-reaction mix in a 50 µl of 1x PCR buffer (Promega) consisted of 5 µl of DIG-PCR mix (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.3 mM dTTP and 0.7 mM DIG-11-dUTP), 2.5 mM $MgCl_2$, 5 U *Taq* polymerase, 20 ng each of F and R primer and 3 µl of 1:100 diluted purified plasmid preparation. Incorporation of the DIG-label was confirmed by electrophoresing the PCR product along with the control reaction performed without label. The DIG-labelled PCR product was used as a probe in northern hybridisation.

Electrophoresis and blotting: Purified viral RNA, disrupted purified virus particles and total RNA isolated from uninfected *N. clevelandii* were diluted in denaturing buffer (see Appendix 8.2). Samples were heat treated at 65 ^{0}C for 5 min and quickly chilled on ice and electrophoresed for 2 h at 80 V in a formaldehyde agarose gel (Appendix 8.2) using 1x MOPS running buffer. The agarose gel was rinsed in DEPC-treated water (3x 15 min washes) and RNA was transferred on to Hybond N^{+} nylon membrane (Amersham, Cat.# RPN2222B) by vacuum blotting with 20x SSC buffer, pH 7.2 for 2 h (Appendix 8.2).

Hybridisation and detection: Hybridisation was done in a Techne Hybridiser IB-1D and the reporter molecule (DIG) was detected by the chromogenic detection system employing alkaline-phosphatase labelled-antibodies and BCIP-NBT substrate (Boehringer Mannheim, Cat.# 1093 657). After blotting, the membrane was prehybridised with DIG-1 buffer (Appendix 8.2) at 65 ^{0}C for 30 min. This was replaced with 10 ml of fresh DIG-1 solution. Five µl (50-60 ng) of the DIG-labelled PCR product (probe) was mixed with 50 µl of DIG-1 solution and denatured in a boiling water bath for 5 min and quickly chilled on ice. This was added to the hybridisation tube and hybridisation was continued at 65 ^{0}C overnight (8-14 h). The membrane was then washed three times as shown below at 65 ^{0}C for 20 min each time.

- Wash 1. 10 ml of 20x SSC, 5 ml 10% SDS and 85 ml sterile distilled water.
- Wash 2. 10 ml of 20x SSC, 1 ml 10% SDS and 89 ml sterile distilled water.
- Wash 3. 0.5 ml of 20x SSC, 1 ml 10% SDS and 98.5 ml sterile distilled water.

The membrane was equilibrated with DIG-2 buffer (Appendix 8.2) for 10 min at room temperature. This solution was discarded and 10 ml of freshly prepared blocking reagent (DIG-2 + 0.5% blocking agent; Boehringer Mannheim, Cat.# 1093 657) was added and incubated at room temperature for 30 min. This was replaced with 10 ml of blocking reagent containing 1:5000 anti-DIG antibodies and incubated at 37 ^{0}C for 30 min. The membrane was washed four times (each wash for 15 min) with DIG-2 solution containing 0.01% Tween-20. The membrane was equilibrated with DIG-3 buffer (Appendix 8.2) for 10 min. To the membrane 10 ml of DIG-3 buffer containing 50 µl of NBT (Boehringer Mannheim, Cat.# 1093 657) and 37.5 µl of x-gal (Boehringer Mannheim, Cat.# 1093 657) was added and incubated at 37 ^{0}C until the

signal was intense. The reaction was stopped by placing the membrane in 1x TBE buffer, pH 8.

3.3.1.10. Restriction Enzyme Analysis of cDNA Inserts

To identify overlapping clones and clones containing either similar or different sequence information, inserts were analysed with restriction enzymes as described in section 4.1.8. For this purpose, plasmids from the transformed clones were isolated and inserts were released by digesting with *Eco*R I enzyme. Alternatively, inserts were amplified by PCR using the programme 40 (see Section 4.1.4) with the F and R primers. Released/amplified inserts were analysed by digesting with *Eco*R I, *Hind* III or *Bam*H I either alone or in combination and the digests were analysed in a 12% non-denaturing PAGE gels.

3.3.1.11. Nucleotide Sequencing

Double-stranded sequencing of the reverse-transcribed RNA was performed on cDNA clones using virus specific, as well as F and R primers as described in section 4.1.9. Sequences were entered and analysed in SEQNET, HGMP and ICGEBnet computers. The various computer programmes used for sequence analysis are described in the results.

3.3.1.12. Oligonucleotide Primers

Sequence information from the viral RNA was used to design eight virus-specific synthetic oligonucleotide primers (Genosys, UK). These were used for primer extension analysis, sequencing and diagnostics.

3.3.1.13. DAC-Enzyme-Linked Immunosorbent Assay (ELISA)

Direct antigen coating (DAC)-ELISA was performed as described by Reddy *et al.* (1995). Briefly, leaf material was macerated in antigen coating buffer (Appendix 8.3) and 200 µl of the extract was added into the ELISA plates (Nunc, Denmark) and incubated at 37 ^{0}C for 2 h or at 4 ^{0}C overnight in a humid chamber. Plates were washed three times with PBS-T (Appendix 8.3) and 200 µl of an appropriate dilution of antibodies in antibody buffer (Appendix 8.3) was added to the wells and incubated at 37 ^{0}C for 1 h. Plates were washed with PBS-T and 200 µl of 1:1000 ALP-labelled (Sigma, Cat.# A4914) goat anti-rabbit antibodies in antibody buffer were added and

incubated at 37 ^{0}C for 1 h. Plates were washed with PBS-T and to this 200 µl/well of substrate mixture (Para-nitrophenyl phosphate (Sigma, Cat.# N2765) in diethanolamine buffer; see Appendix 8.3) was added and incubated at room temperature. The absorbance of the yellow colour of the reacting substrate was read at 405 nm in a Multiskan™ Plus (Labsystems) ELISA plate reader after 30 min to 2 h of substrate reaction time. Results were considered positive if the difference in absorbance value exceed 1 OD unit or double to that of OD readings of healthy sample.

3.3.1.14. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The procedure described by MacKenzie *et al.* (1997) for RT-PCR was followed with minor modifications. Total RNA from 100 mg leaf material from test plants was isolated using the RNeasy kit™ (Qiagen). Leaf material was ground in liquid nitrogen and to the extract 450 µl of RLC^{R} buffer containing 1% 2-mercaptoethanol was added and vortexed. This extract was transferred into the QIAshredder^{R} and processed further as described in section 3.3.7 and the RNA eluted into 25 µl DEPC treated water. First-strand cDNA synthesis was done using Moloney murine leukaemia virus (MMLV) reverse transcriptase (Promega, Cat.# M1701) and total RNA prepared from the leaf tissue.

In a 0.5 ml tube, 2 µl of RNA extract was mixed with 19 µl of RT master mix (2.5 mM $MgCl_2$, 1 mM each dNTPs, 10 ng primer-1, 50 U RT, 10 U *RNasin* in 1x *Taq* buffer) and incubated at 42 ^{0}C for 90 min. Following reverse transcription, 45 µl of PCR master mix (10 ng primer-2 and 2.5 U *Taq* polymerase (Promega, Cat.# M1865) in 1x *Taq* buffer (supplied with the enzyme) was added to each reaction mixture. The mixture was overlaid with 15 µl of paraffin oil and amplified in a Techne PHC3 thermal cycler using the PCR programme 40 (see Section 4.1.4). Ten µl of each of the amplified products was electrophoresed with DNA molecular weight standards (DNA Marker VIII, Cat.# 1336045, Boehringer Mannheim) in an 1% agarose gel (Sambrook *et al.*, 1989).

3.3.2. RESULTS

3.3.2.1. Pigeonpea Sterility Mosaic Material and Culture

Pigeonpea seeds took 8-10 days to germinate and for mite inoculation 15-20 days old plants at the two leaf stage were used . The infected plants developed SMD symptoms 10-15 days post inoculation (pi). The emerging leaves from mite inoculated plants showed severe mosaic, distorted leaves and drastic reduction in leaf size (Figures 3). These symptoms were absent on the leaves of control plants.

3.3.2.2. Virus Isolation

When a virus concentrated from SMD-affected pigeonpea leaf extract was inoculated to various hosts, in *C. quinoa*, local necrotic lesions of 2-5 mm in diameter were observed 7-12 days pi. *N. benthamiana* produced systemic wilting followed by death. In *C. quinoa* a maximum number of 5-8 local lesions per plant were produced, but not all of the inoculated plants showed symptoms, whereas in *N. benthamiana* most of the inoculated plants showed symptoms. Repetition of this experiment revealed that *C. quinoa* and *N. benthamiana* were the only two hosts to which virus could be passaged from concentrated pigeonpea sap. Out of ten attempts, virus was isolated by this means on six occasions (Table 6). However, inoculation of SMD-affected leaf sap without concentration did not result in virus isolation. For further studies a virus isolate from the material of ICRISAT field was used. This mechanically sap transmissible virus is designated as pigeonpea associated tombusvirus (PATV). *C. quinoa* and *N. benthamiana* leaves infected with this isolate were used as a virus source for the subsequent experiments.

Figure 3

Leaf symptoms of sterility mosaic disease in pigeonpea cv. ICP 8863. Healthy leaf (far left)

Table 6
Details of isolations attempted from SMD-affected samples from different locations of India

Location	Acronym	Virus recovery[1]
ICRISAT, Medak, Andhra Pradesh State	ICR	-
Badanapura, Maharasthra State	B	-
ICRISAT, Medak, Andhra Pradesh State	ICR	+
Kanukunta, Medak, Andhra Pradesh State	K	+
Gulbarga, Karnataka State	GUL	+
Rahuri, Maharasthra State	RM	+
Puddukkotai, Tamil Nadu State	PTN	+
Kanukunta, Medak, Andhra Pradesh State	KM	+
Samishtipur, Bihar State	PUI	-

[1] - Not recovered, + Recovered

3.3.2.3. Experimental Host Range

Of thirty different plant species and cultivars tested, 22 were infected with PATV (Table 7); local necrotic lesions on *Beta vulgaris, Brassica nepa, C. quinoa, C. amaranticolor, C. murale, N. silvestris, N. tabacum* cv. Samsun, *Petunia compacta* and *Spinacia oleraceae*; systemic necrotic lesions occurred in *Gomphrena globosa, N. clevelandii, N. tabacum* cvs. Xanthi n/n and Xanthi N/N, *N. hispens*, and *Tetragonia expansa*; and systemic wilting in *N. benthamiana*. Virus was recovered from only inoculated symptomless leaves of *Brassica perkinensis, Cucurbita pepo, Lycopersicon esculentum, N. occidentalis* 37B, *N. tabacum* cv.), and *N. debnyii, N. glutinosa, N. tabacum* cv. White Burley, *N. rustica, N. occidentalis* cv. P1, *Phaseolus vulgaris, Physalis floridana, Vicia faba* cvs The Sutton and Minden, were resistant to infection.

Manual inoculation of healthy pigeonpea plants (cv. ICP 8863) with infected sap of *C. quinoa* resulted in development of large necrotic areas on inoculated leaves 5 days pi, but symptoms typical of SMD were not noticed even after 4 weeks pi. However, the presence of virus in inoculated leaves was confirmed by back inoculation to *C. quinoa*.

Systemic infection of PATV was consistent in only three hosts; *N. clevelandii, N. hispens* and *N. benthamiana* and in five other hosts virus produced systemic infection only once in three trials (Table 7). Systemic infection in *N. benthamiana* resulted in wilting of the plant starting from the apical shoot spreading downwards and laterally and finally leading to plant death (Figure 4). In *N. hispens*, systemic infection resulted in necrosis which spread to the apical shoot and new growth and also led to plant death. In *N. clevelandii*, inoculated leaves showed dark brown necrotic lesions which later coalesced to form large necrotic patches. Systemic necrosis was confined to areas between lateral veins and often concentrated near the petiole (Figure 5). Systemic infection in *G. globosa* was confined to a few leaves only (Figure 6). At four weeks post inoculation, systemic hosts were dying, with the exception of *N. tabacum* cv. Xanthi N/N. Some *N. clevelandii* plants survived for longer periods, usually when the systemic infection was confined to a few leaves. Based on the host response and susceptibility to virus infection, *C. quinoa* was a sensitive local lesion host, *N. benthamiana* a sensitive systemic host and *N. clevelandii* a suitable propagative host. *G. globosa* was useful for long term maintenance of the virus because, in addition to being a good diagnostic host, the plants were alive for many months after infection.

Table 7
Experimental host range of PATV

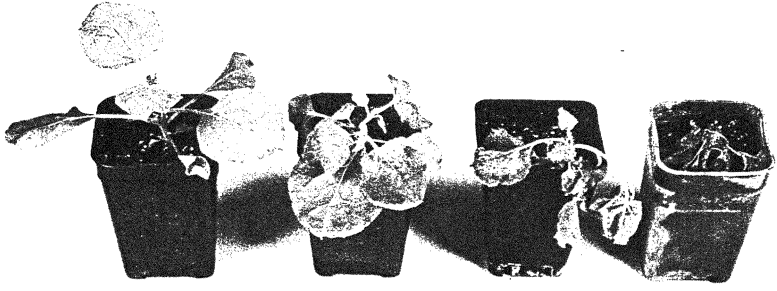
Host	Local[1]	Systemic[1]	Symptoms	Comments
Beta vulgaris	1/3	0/3	Necrotic local lesions	Local lesion host
Brassica nepa	2/3	0/3	Large irregular shaped necrotic local lesions on inoculated leaves which coaleced to form large necrotic areas	Local lesion host
Chenopodium amaranticolor	3/3	0/3	Round necrotic local lesions with purple margin	Local lesion host
Chenopodium murale	3/3	0/3	Pinpoint necrotic local lesions	Local lesion host
Chenopodium quinoa	3/3	0/3	Pinpoint to large necrotic local lesions 4 days p.i	Sensitive local lesion host
Brassica perkinensis	0/3	0/3	No symptoms. No virus recovery	Immune
Cucurbita pepo	0/3	0/3	No symptoms. No virus recovery	Immune
Gomphrena globosa	3/3	1/3	Large necrotic local lesions with purple margins followed by the death of inoculated leaves. Systemic infection confined to mid rib portion of a few leaves (veinal purpling)	Systemically infected host
Lycopersicon esculentum	2/3	0/3	No symptoms. Presence of virus on inoculated leaves confirmed	Infection restricted to inoculated leaves
Nicotiana benthamiana	3/3	3/3	Systemic wilting followed by death of the plant.	Systemically infected host
Nicotiana clevelandii	3/3	3/3	Large irregular shaped necrotic local lesions and pinpoint to large necrotic lesions on non-inoculated leaves. Death of the plant after 3-4 weeks post inoculation due to necrosis	Propagative host

Continued in next page

Nicotiana debnyii	2/3	0/3	No symptoms. Presence of virus on inoculated leaves confirmed	Infection restricted to inoculated leaves
Nicotiana glutinosa	0/3	0/3	No symptoms. Presence of virus on inoculated leaves confirmed	Infection restricted to inoculated leaves
Nicotiana hispens	3/3	3/3	Systemic necrotic infection resulting in death of apical shoot of the plant	Systamically infected host
Nicotiana occidentalis P1	0/3	0/3	No symptoms. No virus recovery	Immune
Nicotiana occidentalis 37B	0/3	0/3	No symptoms. Presence of virus in inoculated leaves confirmed	Infection restricted to inoculated leaves
Nicotiana rustica	0/3	0/3	No symptoms. No virus recovery	Immune
Nicotiana silvestris	3/3	0/3	Local necrotic spots	Local lesion host
Nicotiana tabacum cv. J	1/3	0/3	No symptoms. No virus recovery	Immune
Nicotiana tabacum cv. Samsun	2/3	0/3	Irregular shaped necrotic local lesions	Local lesion host
Nicotiana tabacum cv. White Burley	0/3	0/3	No symptoms. No virus recovery.	Immune
Nicotiana tabacum cv. Xanthi n/n	2/3	1/3	Irregular shaped white necrotic local lesions	Local lesion host
Nicotiana tabacum cv. Xanthi N/N	2/3	1/3	Irregular shaped white necrotic local lesions	Local lesion host
Petunia compacta	2/3	0/3	Few necrotic lesions	Local lesion host
Phaseolus vulgaris	0/3	0/3	No symptoms. No virus recovery	Immune
Physalis floridana	3/3	0/3	No symptoms. Presence of virus in inoculated leaves confirmed	Infection restricted to inoculated leaves
Spinacia oleraceae	3/3	1/3	Large round sunken necrotic lesions	Local lesion host
Tetragonia expansa	3/3	1/3	Local necrotic lesions and systemic necrotic spots on a few leaves	Systemic host
Vicia faba cv. Minden	0/3	0/3	No symptoms. No virus recovery	Immune
Vicia faba cv. The Suton	0/3	0/3	No symptoms. No virus recovery	Immune

[1]Number of occasions plants showed infection/number of tests made

Figure 4



Different stages of *Nicotiana benthamiana* plants infected with PATV showing progressive systemic wilting and death. Stages from left to right correspond to symptom expression after 2, 6, 12 and 16 days pi.

Figure 5

Systemic infection of PATV in *Nicotiana clevelandii* resulted in severe stunting, leaf wrinkling,downward leaf rolling of lamina, drastic reduction in size and curling of the newly formed leaves. Note that necrotic areas coalesce to form a large necrotic areas..

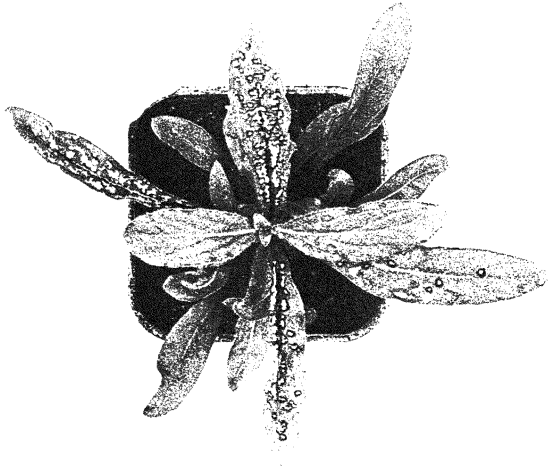


Figure 6

Gomphrena globosa infected with PATV showing necrotic local spots and purple veins. Necrotic spots spread laterally forming large necrotic patches.

3.3.2.4. Properties of PATV in Sap Extracts

Effect of different buffers: Infective sap from *N. clevelandii* was diluted to 1:100 in either 0.1 M, pH 7 phosphate, tris-HCl, citrate buffers or distilled water and inoculated to half leaves of *C. amaranticolor*. Large number (> 150 lesions/half leaf) of pin-pointed local necrotic lesions were noticed. No significant variation in the lesion numbers induced was noticed. Phosphate buffer was used for further studies.

Effect of different pH: Infective sap was diluted to 1:100 in 0.1 M phosphate buffer at either pH 5, 6, 7 and 8 and inoculated to half leaves of *C. amaranticolor*. Infectivity was maintained in all treatments (> 150 lesions/half leaf) without much variation, indicating PATV infectivity was not influenced by pH of phosphate buffer.

Effect of different additives to the inoculum: Infective sap was diluted in twice the volume of 0.05M phosphate buffer, pH 7 either alone or containing 0.05M EDTA or 0.05M DIECA or 0.2% mono-thioglycerol. Samples were incubated at 4 ^{0}C overnight before inoculation to half leaves of *C. quinoa*. PATV infectivity was unaffected (> 150 lesions/half leaf) by any of these treatments compared to the buffer control without any additives.

Effect of different organic solvents: Infective sap diluted in twice its volume of phosphate buffer was treated with equal volumes of buffer or organic solvent (chloroform, di-ethyl ether, n-butanol) and incubated on ice for 30 min with intermittent shaking. The mixtures were centrifuged at 10,000 rpm for 5 min. The aqueous phase collected and concentrated by centrifuging at 48,000 rpm for 60 min in a Beckman 50.2Ti rotor. The virus-containing pellets were resuspended in phosphate buffer and inoculated onto half leaves of *C. quinoa*. All treatments did not result in variation in lesion number, indicating that the virus was insensitive to the tested organic solvents. This results suggested that PATV had no carbohydrate or lipid associated components necessary for infectivity.

Longevity *in vitro*: Crude leaf sap of infected *N. clevelandii*, which had been stored at room temperature, 4 ^{0}C or -15 ^{0}C was tested for infectivity after 2, 4, 8, 16 and 32 days by inoculating samples onto *C. quinoa*. In addition, infected whole *N. clevelandii* leaves stored at 4 ^{0}C and -15 ^{0}C were tested for infectivity after 2, 4, 8, 16, 32, 46 and 90 days. Infectivity was retained without noticeable loss in all the treatments.

Dilution end point: Ten fold serial dilutions (up to 10^{-10}) of the infected *N. clevelandii* leaf sap were made in phosphate buffer and inoculated onto *C. quinoa* and *N.*

benthamiana. Symptoms developed in all the samples but lesion number decreased greatly after 10^{-8} dilution in *C. quinoa*, whereas in *N. benthamiana*, systemic wilting and death of the plant was delayed with increasing dilution. *Nicotiana benthamiana* was found to be the most sensitive host. This experiment was repeated three times, and on all the occasions, *N. benthamiana* plants showed symptoms incoulated with the highest dilution (10^{-10}) whereas in *C. quinoa* the end point varied between 10^{-7} and 10^{-9}.

3.3.2.5. Properties of Purified Virus Preparations

Infectivity: Purified virus particles were diluted to 1:1000 in phosphate buffer and inoculated onto *C. quinoa* and *N. clevelandii*. Typical symptoms of PATV developed 5 days pi, confirming that purified virus particles were infectious. Inoculation of pigeonpea (cv. ICP 8863) plants did not result in typical SMD symptoms. However, inoculated leaves developed necrosis, without systemic spread.

Sedimentation properties: The virus sedimented as a single zone in sucrose density gradients (Figure 7), corresponding to a single main peak of absorbance (Figure 8A). Sometimes, 1 or more fainter light scattering zones below the major band were observed and were probably due to particle aggregation.

Buoyant density determination: To determine the buoyant density of the virus, purified virus preparations in phosphate buffer were mixed with solid cesium chloride or cesium sulphate to an initial density of 1.36 and 1.34 g/cm^3, respectively, and filtered through glass wool before centrifuging in a Beckman SW 50.1 rotor at 40,000 rpm for 16 h. After centrifugation, the tubes were observed under narrow light beam for light scattering zones, then scanned and fractionated with an ISCO UV absorbance gradient fractionator. The refractive index for the alternative fraction was measured using an ATAGO digital refractometer and corresponding densities were calculated using the standard graph (ISCO tables, 1972). Virus preparations formed a single buoyant density band in cesium chloride and cesium sulphate gradients with densities of 1.34 and 1.27 g/cm^3, respectively (Figure 8B and 8C).

UV absorption spectrum: Purified virus preparations were scanned from 200-350 nm in a Pharmacia LKB Biochrom 4060 spectrophotometer. Particles had a maximum absorption at 258 nm, a minimum at 242 nm and the $A_{260/280}$ ratio was 1.78 (Figure 9). Virus concentration, determined by assuming an extension coefficient of 4.5 OD at 260 nm equal to 1 mg (Martelli *et al.*, 1971) was, 2 mg of virus recovered from 50 g of leaf material.

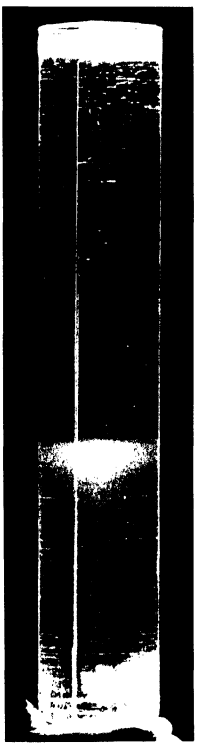
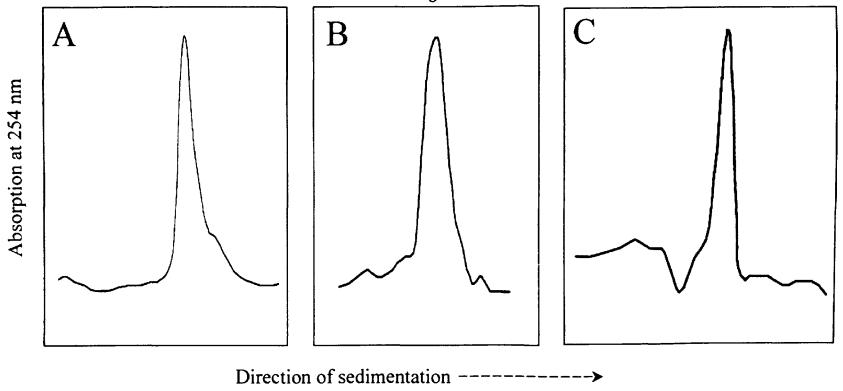


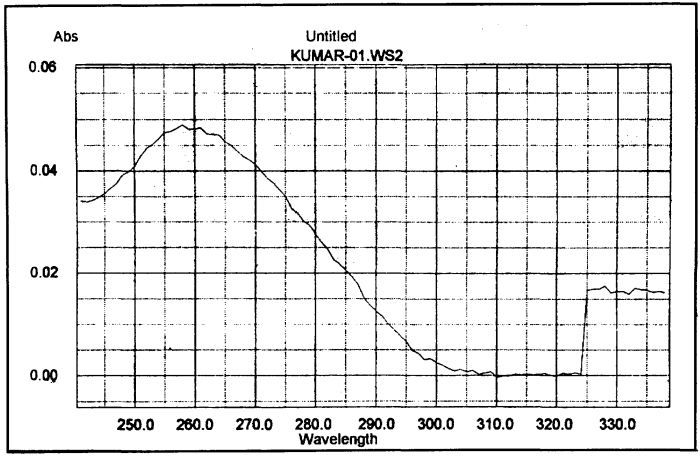
Figure 7
PATV separation in 10-40% sucrose density gradient after centrifugation at 36,000 rpm for 2.5 h in a Beckman SW41rotor. Light scattering zone indicated.

Figure 8



Ultraviolet trace of a purified PATV preparation in sucrose (A), cesium chloride (B), and cesium sulphate (C), gradients. The UV profile indicates that particles sediment as a single component.

Figure 9



Ultraviolet absorption spectrum of purified preparations of PATV particles showing a maximum absorption at 258 nm, a minimum at 242 nm and A260/280 ratio of 1.78

Particle size and morphology: Purified virus preparations were negatively stained with 2% uranyl acetate, pH 3.5 and 1.5% phosphotungstic acid, pH 6.5 and positively stained with 2% ammonium molybdate, pH 7. Preparations contained many isometric particles of 30-31 nm in diameter, with slightly angular edges and a knobbly surface (Figure 10). Very few empty particles were seen when stained with PTA, and these may be due to stain penetration rather than true empty particles as their number increased with increase in the amount of PTA stain employed.

3.3.2.6. Serology

Production of antibodies: Antiserum was collected four weeks after last injection and tested in agarose gel double diffusion test (AGDD) using the protocol described by Ball (1990). The antiserum reacted with virus in purified particle preparations and infected leaf sap of *N. clevelandii* to a titre of 1:512, but did not react with infected pigeonpea leaf sap or uninfected *N.clevelandii* sap. After another two booster injections antiserum titre was raised to 1:1024. Serum was mixed with equal volumes of sterile glycerol and stored at -20 ^{0}C.

Serological relationships: Purified virus preparations were tested by AGDD (Ouchterlony) for reactivity with antisera of several tombusviruses. PATV reacted with its homologous antiserum and antisera of pothos latent virus (PoLV), indicating that it was serologically related to PoLV, but not to other tested viruses belonging to the family *Tombusviridae* (see section 3.3.2.9).

3.3.2.7. Virus Coat Protein

A single protein species with an estimated molecular weight of 43-45 kDa was detected in the gels stained with Coomossie brilliant blue R-250 (Figure 11). Viral coat protein migrated as a single band of similar M_r in the different concentrations of SDS-PAGE.

3.3.2.8. Characterisation of PATV Nucleic Acid

Initial analysis by particle disruption method indicated the presence of 3 RNA species. The following viral nucleic acid properties were determined using purified viral nucleic acid preparations obtained from PATV.

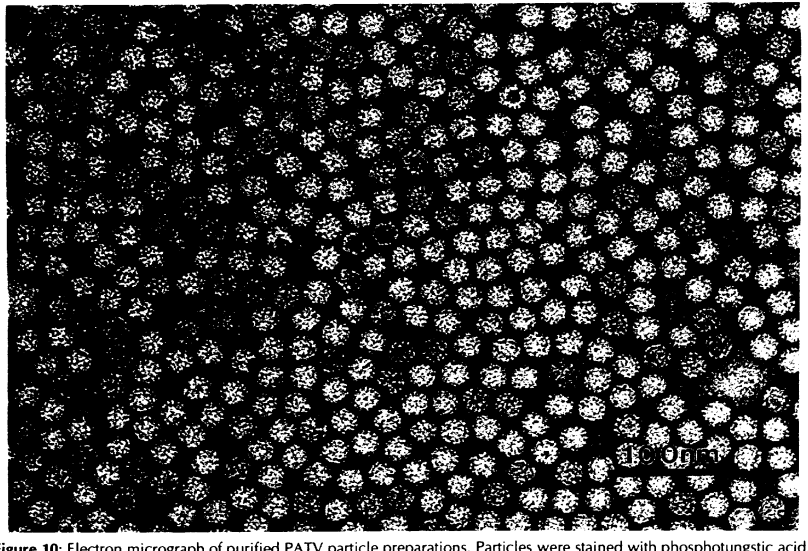
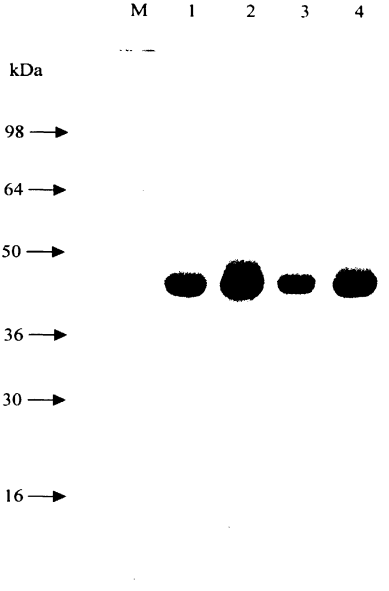


Figure 10: Electron micrograph of purified PATV particle preparations. Particles were stained with phosphotungstic acid.

Figure 11



Resolution of denatured coat protein sub-units of PATV in a 14% SDS-polyacrylamide gel. Denatured coat protein sub-units from different preparations in lanes 1-4. Lane M is the molecular weight marker (SeeBlue Pre-stained marker).

Molecular weight and sensitivity to nucleases: Three bands were resolved in DNase treated samples and a smear without any distinct bands in the RNase treated sample, indicating that the viral nucleic acid was RNA. The estimated size of the RNA species was about 4300, 2700 and 1500 nts. The concentration of the largest RNA species was greater than that of the other two species (Figure 12). Two or more number of small RNA species, presumably subgenomic RNAs, were detected in RNA preparations made from different batches of purified virus preparations. It was established that the genomic RNA of tombusviruses acts as mRNA for the expression of the 5' proximal genes (ORF1 and 2), whereas 3' half genomic RNA are expressed via the synthesis of two 3' coterminal subgenomic RNAs. Encapsidation of these subgenomic RNAs into virus particles is reported for several tombusviruses (Russo *et al.*, 1994). Further investigations to evaluate these small RNAs, and their possible packaging in virus particles was not performed.

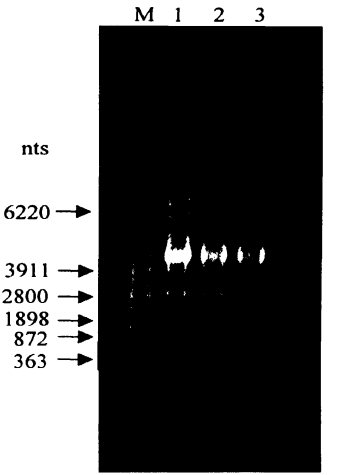
Infectivity of RNA: Purified nucleic acid preparations were infectious and produced typical PATV symptoms when inoculated onto *C. quinoa* and *N. clevelandii*. Plants inoculated with the large RNA species (4300 nts) showed local necrotic lesions 3 days pi, plants inoculated with small RNA species did not show any symptoms.

Test for 3' polyadenylated tract: Viral RNA did not bind with the oligo dT_{30} latex beads, indicating that the viral RNA lacks polyadenylation tract at the 3' end.

3.3.2.9. cDNA Synthesis and Sequencing

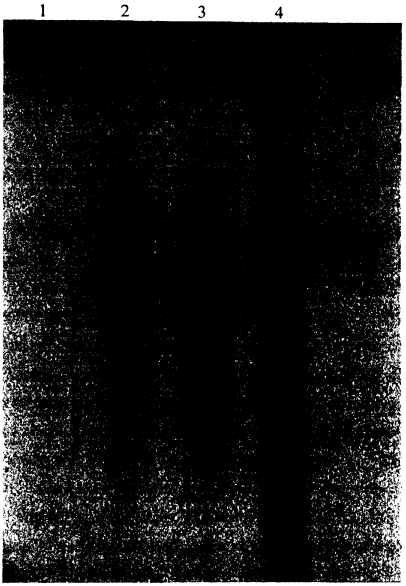
cDNA constructed to viral RNA was cloned into polylinker site in *cdd*B gene of pCR Blunt vector (vector map given in Appendix 8.6). This cloning vector allows direct selection of positive recombinants via disruption of the lethal *cdd*B gene (Bernard *et al.*, 1994). Clones were sub-cultured and analysed for inserts by PCR and restriction enzyme analysis. These clones contained inserts from 50 bp to1.6 kbp. Clones containing inserts of more than 200 bp were selected for further analysis. Specificity of the cDNA construct was confirmed by using DIG-labelled GB4.2 cDNA clone (1.6 kbp insert) as a probe in northern hybridisation to detect the viral RNA isolated from purified virus preparations and infected *N. clevelandii*. The probe reacted strongly with the largest RNA species of PATV as well as with disrupted virions but not with total RNA from uninfected *N. clevelandii*, suggesting its specificity to the viral RNA (Figure 13).

Figure 12



Resolution of PATV RNA in a 1% agarose gel. Lanes 1-3, RNA from different purified virus preparations. Lane M, RNA molecular weight marker.

Figure 13



Northern blot of RNA probed with a digoxygenin labelled cDNA probe constructed from a 1.6 kbp cDNA clone of PATV RNA. Total RNA from uninfected *N. clevelandii* (lane 1), disrupted purified particles (lanes 2 and 3) and RNA isolated from purified PATV particles (lane 4).

Sequencing: Sequencing of the reverse-transcribed viral RNA was performed using cDNA clones. A partial restriction map was constructed to identify overlapping clones. All clones were sequenced twice in both directions. DNA sequences were edited and aligned using the Sequence Navigator programme version 1.01. Aligned sequences were entered and analysed using several programmes of the University of Wisconsin Genetics Computer Group (GCG, 1994) software package on the Biological Biotechnological Sciences Research Council 'SEQNET' computer. Sequence similarity searches in the European Molecular Biological Laboratories (EMBL) data base was made with the programme 'FASTA' (Pearson and Lipman, 1988). Pair-wise comparisons were made with the programme 'PILEUP' and 'BESTFIT' (GCG, 1994).

Four overlapping clones (named as GB3.9, 4.2, 4.4, 5.5) were identified by restriction enzyme mapping which gave a sequence of 1847 bases. Two other overlapping clones (CD4.22 and 6.1) which did not overlap with GB clones contained 1341 bases, these giving a total of 3118 bases for the RNA, which is about 4.3 kb in size. The sequence corresponding to the 3118 nts of PATV RNA was assessed for homology with other sequences by comparing them with over 10 million sequences present in the EMBL sequence database. This showed that the PATV RNA sequence had about 90% homology with PoLV RNA and to a lesser extent to several members of the genera *Tombus-*, *Carmo-*, *Diantho*, *Necro-* and *Machlomo-viruses* of the family *Tombusviridae*. Besides this, some similarities were detected to barley yellow dwarf luteovirus, olive latent sobemovirus and carrot mottle umbravirus. Apart from PoLV, similarities detected with other members were not significant, other than those detected for the conserved domains of the replicase and capsid proteins, a characteristic feature of the family *Tombusviridae* (Russo *et al.*, 1994; Mayo and Pringle, 1998). The details of the sequence homologies are given in Table 8. When the determined sequence obtained from the four PATV clones was aligned with PoLV RNA (Rubino *et al.*, 1995), it revealed that the sequence obtained from the GB clones corresponded to the 5' end of the PoLV-RNA relating to ORF1 (unknown function) and the replicase gene; information from the CD clones corresponded to the capsid protein and movement protein genes. Nucleotide identities in most of the genomes between the two viruses was very high (85-90%). Thus, PATV resembles the recently described PoLV, a definitive member of the family *Tombusviridae* (Sabanadzovic *et al.*, 1995).

TABLE 8

i) Sequence similarity of 600 bases of PATV RNA corresponding to part of the replicase gene.

Sequences producing high-scoring segment pairs	Nucleotide overlap	% similarity
Pothos latent tombusvirus	579	91
Barley yellow dwarf luteovirus (serotype MAV)	128	64
Carnation Italian ringspot tombusvirus	217	62
Barley yellow dwarf luteovirus (serotype PAV)	145	62
Red clover necrotic mosaic dianthovirus	169	60
Panicum mosaic sobemovirus (Kansas strain)	204	59
Tobacco necrosis necrovirus	201	56
Olive latent sobemovirus 1	201	55
Cardamine chlorotic fleck carmovirus	332	54
Carrot mottle umbravirus	490	50

ii) Sequence similarity of 711 bases of PATV RNA corresponding to part of the coat protein gene.

Virus	Nucleotide overlap	% similarity
Pothos latent virus	630	83.65
Galinsoga mosaic carmovirus	454	58.81
Cymbidium ringspot tombusvirus	447	56.37
Carnation Italian ringspot tombusvirus	119	65.54
Artichoke mottled crinkle tombusvirus	116	62.93
Pelargonium ringspot carmovirus	268	56.34
Tomato bushy stunt tombusvirus	118	61.86
Carnation mottle carmovirus	115	62.6
Cucumber necrosis tombusvirus	118	59.32
Melon necrotic spot carmovirus	122	57.37

Primer extension analysis: As the longest overlapping clones gave the information for 3118 bases, cDNA for the remaining part of the genomic RNA was constructed by primer extension by RT-PCR using purified viral RNA preparation described by Ausubel *et al.* (1994). Six synthetic oligonucleotide primers (PS1, PS2, PS3, PS4, POL1 and POL2), were designed using the sequence information from GB and CD clones, and also from the PoLV RNA sequence information (Acc.# X87115). The sequence and properties of the primers are given in Table 9 and primer positions are shown in Figure 14. Primers PS2 and PS3 were derived from the complete PATV RNA sequence complementary to nucleotide positions 1898-1922 and 2434-2456, respectively, to amplify the gap between GB and CD clones. Following the high sequence similarity between PoLV and PATV, the regions towards the 5' and 3' ends of PoLV RNA were used to design POL1 and POL2 primers complementary to the positions 1-21 and 4336-4354, respectively in the complete PATV RNA sequence. These were used in combination with two other primers PS1 and PS4 designed from the PATV RNA sequence complementary to the position 254-275 and 3638-3658, respectively in the complete PATV RNA sequence. The cDNA strategy is depicted in Figure 14.

Primers PS2/PS3 amplified a product of 558 bp corresponding to the gap between GB and CD clones (Figure 15, lanes 3-4). Primers POL1/PS1 gave a product of 275 bp towards the 5' end (Figure 15, lanes 1-2); PS4/POL2 amplified a product of 678 bp towards the 3' end (Figure 15, 5-6). These were cloned into TOPO-TA™ vector (Invitrogen, Cat.# K4500-40) and transformed into *E. coli* TOP 10™ competent cells and plated on LB ampicillin agar plates. Three independent clones were sequenced on both strands. In this way a sequence spanning almost the full-length of virus genome was obtained, excluding few bases at the extreme 5' and 3' ends of the RNA. The determined sequence of the cloned PATV RNA is 4354 nucleotides and is predicted to contain 5 open reading frames (ORFs; see Figure 16). The ORF1 begins with AUG at 125 and terminates with amber codon (UAG) at nucleotide position 793, resulting in a product of 25K. If readthrough of the amber codon takes place a product of 84K would be synthesised terminating at opal codon (UGA) at 2344 (ORF2). ORF3, from positions 2356-3474 encodes a protein of about 41K. ORF4, from positions 3569-4297 encodes a protein of 27K. ORF5 completely overlapped by ORF4 encodes a protein in a different reading frame. It initiates with an AUG codon at nucleotide position 3700, encodes a putative protein of 14K before terminating at ochre codon (UAA) at position 4092. No attempts were made to determine the functions of the 5 ORFs or to study further the genome expression strategy of PATV.

TABLE 9
Nucleotide sequence and properties of the primers used for primer extension studies by RT-PCR

Primer	Length (Bases)	Sequence (5'–>3')	Annealing position in PATV sequence	Product size (bp)
POL1	21	GCCTAACCAGCTTGTTGAGAG	1-21	275
PS1	22	CCTTGAATCCAGCACACATGTC	254-275	
PS2	25	GAGATCGAGTTTTGCCAACAACACC	1898-1922	558
PS3	23	CCTGCTGGACTAAGCATAACTTG	2434-2456	
PS4	21	CTGTTGAGTCATAAAACTACC	3638-3658	678
POL2	19	CCAAGCGTCATTTATAGCC	4336-4354	
Rep-1	25	CAATCTAGCTCGTTCATACGTGGCG	1174-1198	222
Rep-2	21	GTCAACGTTTGTCGTGGTTTG	997-997	

Figure 14

Strategy for the synthesis of cDNA to the PATV genomic RNA

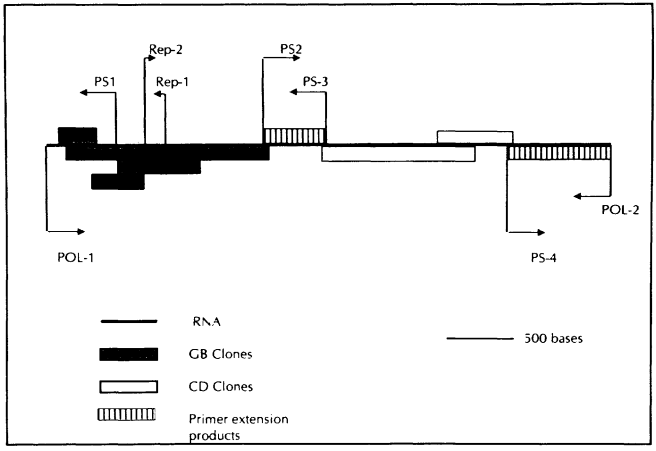
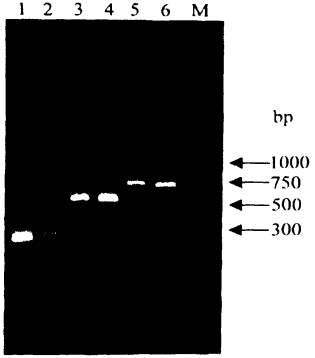
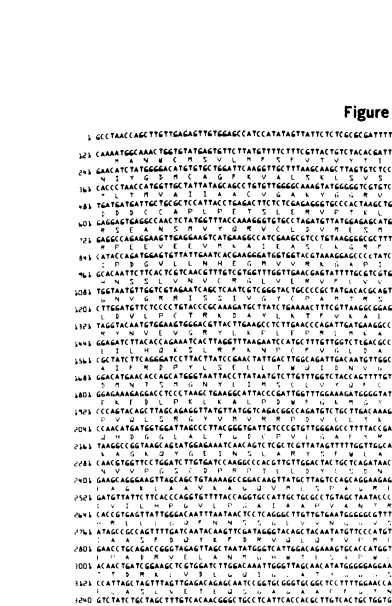


Figure 15



Resolution of RT-PCR primer extension products of PATV RNA in a 1% agarose gel. Lanes 1-2 with primers POL1 and PS1; lanes 3-4 with primers PS2 and PS3; lanes 5-6 with primers PS4 and POL2. Lane M, molecular weight marker (Promega, Cat.# G3161).

Figure 16



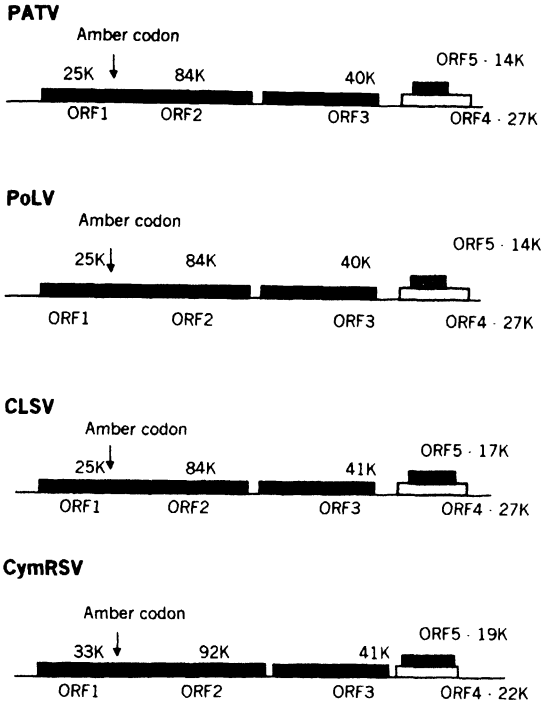
Nucleotide sequence of cDNA corresponding to PATV genomic RNA. The putative amino acid sequence deduced from the four ORFs (2,3,4 and 5) are coloured. Asterisks indicate a stop codon. Note that ORF2 is read through product of ORF1 which encodes a 25 K protein before terminating at nucleotide position 794. A few nucleotide bases corresponding to the 5' and 3' ends of the RNA are not determined in this study. Numbers correspond to the nucleotide sequence.

3.3.2.10. Comparison of PATV sequence with PoLV and CLSV

The putative genome organisation and deduced amino acid sequence of the 5 ORFs of PATV was compared with PoLV, a newly proposed tombusvirus species (Rubino *et al.*, 1995) and cucumber leaf spot virus (CLSV), recently reported as a virus with close similarities with PoLV (Miller *et al.*, 1997). PATV putative genome organisation is similar to that of PoLV and CLSV (Figure 17). PATV nucleotide sequence is 91% and 64% identical to PoLV and CLSV, respectively. All the five ORFs of PATV and PoLV encode proteins with high degrees of identity in their amino acid sequence (Table 10). The amino acid sequence of ORF2 of PATV and CLSV are 80% identical, whereas identity in the four other ORFs ranges from 39 to 77% (see Table 10). Because of the high similarity between PATV and PoLV, the 5 ORFs of PATV may be regarded as having an expression strategy and functions similar to the corresponding ORFs of PoLV. These are reported as the read through domain of the ORF2, which is part of the replicase encoding domain. ORF3 is the capsid protein encoding cistron, ORF4 encodes a virus movement protein and ORF5 encodes a protein responsible for symptom expression (Rubino and Russo, 1997). The functions of the proteins encoded by ORF5 in many tombusviruses are not clearly defined. However, plants inoculated with mutants of PoLV ORF5 were found to attenuate symptom expression in plants (Dalmay *et al.*, 1993; Rubino and Russo, 1997). In definitive tombusviruses, ORFs 1 and 2 encode a protein involved in viral replication, ORF3 encodes the coat protein, ORF4 encodes a virus movement protein and ORF5 encodes a protein whose precise functions are not clear (Russo *et al.*, 1994).

Comparative serological study of PATV and PoLV: For comparison with PATV, PoLV-infected *N. benthamiana* leaf and antisera were obtained as a gift from G. P. Martelli (Bari, Italy). The virus was propagated only once to minimise the risk of cross contamination in *N. clevelandii* and its properties determined. In agarose gel double diffusion tests using homologous and heterologous antisera, each virus reacted to the dilution end point of each antiserum (1:1024). In a separate test, the viruses reacted with each others' antiserum without the formation of spurs, indicating that the two viruses are serologically indistinguishable (Figure 18). However, PoLV and CLSV are serologically unrelated (G. P. Martelli, personal communication).

Figure 17

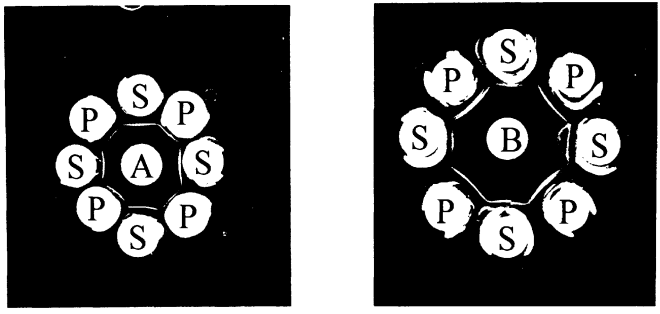


Structure and the genome organisation of PATV, PoLV, CLSV and CymRSV. The ORFs are shown with boxes. Non-coding regions represented with lines and the position of the amber codon is indicated with an arrow.

Table 10
Pair-wise comparison of PATV, PoLV and CLSV amino acid sequences

Virus Pair	% Amino acid sequence identity				
	ORF1	ORF2	ORF3	ORF4	ORF5
PATV/PoLV	96	96	92	97	98
PATV/CLSV	64	80	39	77	59
CLSV/PoLV	63	87	37	78	60

Figure 18



Agarose gel double diffusion test showing the serological relatedness of PATV (S) and PoLV (P). Central wells: PATV (A) and PoLV (B) antisera. Peripheral wells: PATV (S) and PoLV (P) infected *Nicotiana clevelandii* leaf sap.

3.3.2.11. PATV Variants

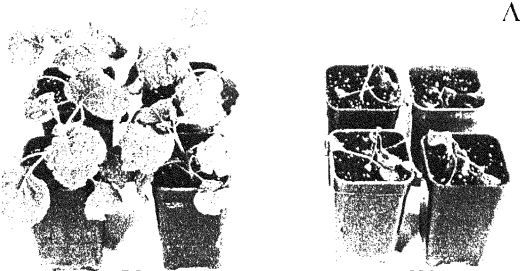
PATV was maintained by serial passages in *N. clevelandii* by inoculation with undiluted infected sap inoculum. After 8-10 serial passages variation in symptom expression was noted. Normally, the wild type virus induced local necrotic spots in *C. quinoa* (hypersensitive reaction), systemic necrosis and death of *N. clevelandii* and systemic wilting and death of *N. benthamiana*. Conversely, plants infected with inoculum after the 8th passage showed much less severe symptoms. *C. quinoa* developed necrotic local lesions which coalesced and spread systemically towards petioles and nodes, resulting in necrosis and death of the plant (Figure 19). Infected *N. benthamiana* showed apparently normal growth and developed only a mild systemic chlorosis (Figure 19). It was shown in tombus and carmoviruses that during undiluted passages of virus inoculum, lethal necrosis syndrome normally caused was prevented by defective interfering (DI) RNAs and persistent infections results (De Polo *et al.*, 1987; Knorr *et al.*, 1991; Havelda *et al.*, 1998). The apparent change in symptom expression in plants inoculated with PATV could be due to generation of DI RNAs. It is noteworthy that change in symptom expression can also occur due to the association of satellite (sat) RNAs. However, unlike DI RNAs, sat RNAs are not known to be spontaneously generated upon serial passages (Celix *et al.*, 1997). Further experiments to characterise DI RNAs associated with PATV was not performed.

3.3.2.12. Taxonomic Status of PATV

The high degree of nucleotide and amino acid sequence identity (>90%), similar genomic organisation and serological relatedness between PATV and PoLV, suggest that PATV is a variant or strain of PoLV. Although PATV and PoLV are genetically very similar, PATV is found to generate DI RNAs. It is noteworthy that PoLV is neither generated nor supported the replication of DI RNAs of other tombusviruses (Rubino and Russo, 1997). The high genomic similarities (about 64-70%) and high degree of inter-species molecular compatibility among definitive tombusviruses suggest that they should be consider as a related strains of the same virus (Gallitelli *et al.*, 1985; White and Morris, 1994; Russo *et al.*, 1994). Therefore, for specific identification and the separate species status of tombusviruses, it is essential to consider other characteristics (Koenig and Gibbs, 1986; Russo *et al.*, 1994). Detailed investigations of PATV DI RNAs and their ability to support other tombusvirus DI RNAs are essential to asses the precise status of PATV. The structural organisation of PATV, PoLV and CLSV

Figure 19

A



Nicotiana benthamiana inoculated with a variant of PATV (V) showing normal growth pattern with mild chlorosis. Plants inoculated with a wild type PATV (W) produced characteristic wilting and death.

B

Chenopodium quinoa inoculated with a wild type PATV (W) showing necrotic local lesions on inoculated leaves, and plants inoculated with a variant strain of PATV (V) showing systemic necrosis.

genomes is same as that of definitive tombusviruses (cymbidium ringspot virus (CymRSV), see Figure 17). However, sequence and size of the ORF1, ORF2, ORF4 and ORF5 are significantly different (Rubino and Russo, 1997; Miller *et al.*, 1997). It was, therefore, proposed that all the members of the genus *Tombusvirus* can be grouped into two major species, those related to PoLV (PATV and CLSV) and tomato bushy stunt virus (cucumber necrosis virus, artichoke mottle crinkle virus, cymbidium ringspot virus and other cross-hybridising members) (Miller *et al.*, 1997). Further, a proposal has been made to erect a genus *Aureovirus* with PoLV as a type species in the family *Tombusviridae* (Martelli *et al.*, 1998).

3.3.2.13. Detection of PATV

DAC-ELISA: PATV was detected in *N. clevelandii* and other infected experimental herbaceous hosts. For virus detection in herbaceous hosts antiserum diluted up to 1:8000 was used (see Table 11). Antiserum did not react with healthy leaf extract or buffer control. SMD-affected and uninfected pigeonpea samples collected from growth chamber culture and farmers fields were checked for virus in DAC-ELISA. Various dilutions of antiserum and antigen were used. There was no considerable difference between OD values obtained from pigeonpea healthy (asymptomatic), SMD-affected and buffer controls (Table 11). The results could be repeated in many independent tests. Therefore, DAC-ELISA may not be sensitive enough to detect the PATV or SMD samples did not contain PATV antigens. Leaf samples obtained from the pigeonpea plants (cv. ICP 8863) 2 weeks after mechanical inoculation with purified preparations of PATV were tested. Inoculated leaves gave positive results in ELISA. However, the infection restricted to inoculated leaves.

RT-PCR: The RNA sequence information was used to design two primers, Rep-1 and Rep-2, to amplify a 222 base product of PATV RNA corresponding to the 5' end of the RNA corresponding to replicase gene (see Table 9 and Figure 14). These primers were used to detect PATV in SMD-affected pigeonpea plants by RT-PCR. Initially, attempts made with PATV RNA and total RNA obtained from experimental herbaceous hosts yielded a PCR product of expected size (Figure 20). About 56 SMD-affected, and 37 healthy pigeonpea plants obtained from the material maintained in growth chamber and field-collected (ICRISAT; farmers fields, Sidipetta and Karimnagar, Andhra Pradesh; UAS, Bangalore) were tested for the virus. SMD-affected (6/32)and healthy

TABLE 11
Detection of PATV in *N. clevelandii* (A) and pigeonpea (B) leaf sap by DAC-ELISA

A. *Nicotiana clevelandii* leaf sap

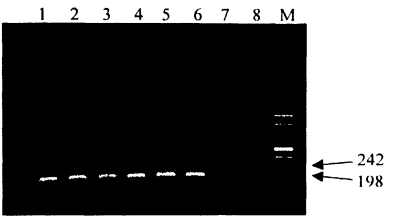
Antiserum dilutions	Antigen dilutions								
	1:100			1:500			1:1000		
	Buffer	Infected	Healthy	Buffer	Infected	Healthy	Buffer	Infected	Healthy
1:2000	0.433	2.716	0.506	0.464	2.697	0.483	0.425	2.703	0.542
1:4000	0.407	2.613	0.463	0.402	2.810	0.465	0.451	2.781	0.481
1:8000	0.201	2.683	0.200	0.197	2.738	0.178	0.155	2.72	0.259

B. Pigeonpea leaf sap

Antiserum dilutions	Antigen dilutions								
	1:10			1:25			1:50		
	Buffer	Infected	Healthy	Buffer	Infected	Healthy	Buffer	Infected	Healthy
1:100	0.431	0.599	0.560	0.434	0.719	0.612	0.397	0.653	0.592
1:500	0.333	0.340	0.296	0.279	0.409	0.311	0.233	0.402	0.309
1:1000	0.165	0.147	0.119	0.143	0.154	0.114	0.132	0.155	0.149

Note: Readings taken 1 h after adding substrate at 405 nm. OD values represents average from two replicates.

Figure 20



RT-PCR products of total RNA amplified by Rep-1 and Rep-2 primers in a 1% agarose gel. Total RNA from PATV infected *C. quinoa* (lanes 1 and 2), *N. benthamiana* (lanes 3 and 4), *N. clevelandii* (lanes 5 and 6), and uninfected N. clevelandii (lane 7). Lane 8, control reaction without target DNA and lane M, molecular weight marker (DNA Marker VIII, Boehringer Mannheim Cat.# 1336045).

(3/37) samples gave positive results. From plants which did not give positive results, RNA was extracted from 25 g material (both from healthy and SMD-affected pigeonpeas) and processed by PCR. None of them yielded the PCR product of expected size.

3.3.2.14. Association of PATV with Sterility Mosaic Disease

Purified preparations of PATV failed to produce typical SMD symptoms when inoculated onto healthy pigeonpea plants (cv ICP 8863). Furthermore, this virus was found to be localised to inoculated leaves. Attempts to detect this virus by DAC-ELISA and RT-PCR in a large proportion of SMD-affected samples were consistently negative. Additionally the virus was detected in apparently healthy samples. This indicates that PATV may not be the causal agent of SMD *per se* and is regarded as a benign virus restricted to few locations.

3.4. Virus Isolation Procedure – 2

Purification of a virus directly from SMD-affected samples

3.4.1.1. Purification Procedure

A protocol developed based on the procedures described by Peterschmitt *et al.* (1991) and Gispert *et al.* (1998) was used for the purification of a virus from SMD-affected pigeonpea leaf material. Hundred g of infected and healthy leaf material was macerated in 400 ml of 0.01 M tris buffer, pH 9, containing 0.25 M sodium sulphite, 0.02 M EDTA, 0.02 M DIECA, 1% polyvinyl-pyrrolidone (PVP) 40,000 and 2% monothioglycerol (this buffer will be referred to as extraction buffer). Extract was squeezed through double layered muslin cloth and clarified by centrifuging at 4,000 rpm for 5 min in a Sorvall GS3 rotor. To the supernatant, NaCl, polyethylene glycol-8,000 and Triton-X 100 were added to the final concentration of 0.1 M, 6% and 25%, respectively, and stirred at 4 ^{0}C for 1 h. This was centrifuged at 8,000 rpm for 30 min in a Sorvall GSA rotor. Supernatant was discarded. Pellets were suspended in 100 ml of 1:10 diluted extraction buffer (this will be referred as to resuspension buffer). This was clarified and layered over 7 ml of a 12% sucrose cushion and centrifuged at 24,000 rpm for 2 h in Beckman SW28 rotor. Supernatant was discarded and pellets were resuspended in resuspension buffer. This was overlaid on pre-formed 10-40% sucrose gradient prepared in resuspension buffer and centrifuged for 2 h at 24,000 rpm in a Beckman SW28 rotor. Gradients were observed under a narrow light beam and light scattering zones were collected using a bent needle. This was diluted with 2.5 volumes of resuspension buffer and virus was concentrated by centrifuging at 24,000 rpm for 2.5 h in a Beckman SW28 rotor. Pellets were dissolved in a 300 µl of 0.01 M tris buffer, pH 7.5 and analysed for protein and nucleic acid.

Purification using cesium chloride gradients: A 40% CsCl stock solution (density = 1.420 g/cc; ISCOtables, 1972) was prepared in 0.01 M tris buffer, pH 7.5. From this 35, 30 and 25% (w/v) solutions were prepared and used to prepare a CsCl gradient ranging in concentration from of 25-40%. The final purified preparation was layered over CsCl gradient column and centrifuged for 4.5 h at 10 ^{0}C in a Beckman SW50 rotor. Using a bent needle light scattering zone was collected and CsCl was removed by dialysing overnight in a 0.01 M tris buffer, pH 7.5.

3.4.1.2. Protein Analysis

The final purified preparations were analysed in a 12% SDS-PAGE gel as described in section 3.3.1.5. The electrophoresis was performed in a HSI SE 600 unit (Hoefer Scientific Instruments, San Francisco, USA). Purified virus preparations were denatured by boiling for 3 min in an equal volume of Laemmli buffer and the samples were electrophoresed in a 12% SDS-PAGE. Dalton Marker VII-L (Sigma, Cat.# SDS7) or SDS-PAGE Standards (Low Range, BioRad, Cat.# 161-0304) were used as protein molecular weight standards for estimating molecular weight. PAGE gels were stained with silver using a modified procedure of Morrissey (1981) as described in Reddy *et al.* (1995).

3.4.1.2.1. Silver staining procedure: After electrophoresis gel was placed in 200 ml of a fixing solution (1.5% glacial acetic acid, 25% methanol in distilled water) for 30 min with gentle shaking. Gel was rinsed in distilled water for three times, allowing 10 min for each wash. This was replaced with freshly prepared 200 ml of 1:100 DTT solution from a stock (5 mg in 10 ml dH_20) for 30 min. Followed by additions of 200 ml of 0.2% silver nitrate solution and gently agitated for 30 min. Gel was quickly rinsed with distilled water and placed in a developer solution (3% sodium carbonate and 0.05% formaldehyde in distilled water) until bands appeared clearly (10-15 min). Reaction was stopped by adding 1% glacial acetic acid.

3.4.1.3. Nucleic Acid Analysis

Nucleic acid was isolated from the purified virus preparations using TRIzol reagent (Gibco, Cat.# 15596-018). To 200 µl of purified virus preparation, 1 ml of TRIzol was

added and mixed well. To this 200 µl of chloroform was added and vortexed. This was centrifuged for 15 min at 14,000 rpm in an Eppendorf microcentrifuge (model 5410). Upper aqueous phase was collected and 500 µl of isopropanol was added and incubated at room temperature for 10 min. This was centrifuged at 14,000 rpm for 10 min. Pellets were washed with 500 µl of 70% ethanol, dried and dissolved in minimal volume of DEPC-treated water. This was analysed in methylmercuric hydroxide denaturing agarose gels (Sambrook *et al.*, 1989).

3.4.1.3.1. 1.5% Methylmercuric hydroxide (MMH) denaturing agarose gel electrohporesis: Agarose (750 mg/ 50 ml buffer) was dissolved in MMH gel running buffer, pH 8.1 (50 mM boric acid, 5 mM sodium borate and 10 mM sodium sulphite) and allowed to cool to 50 ^{0}C before adding MMH (Alfa Reserach Chemicals, Frankfurt) to a final concentration of 5 mM. Equal volumes of 2x gel loading solution (25 µl of 1 M MMH, 500 µl 4x gel running buffer, 200 µl glycerol, 0.2% w/v bromophenol blue and 275 µl of water) was mixed with RNA and loaded into gel. Electrophoresis was carried by applying 6 V/cm length of the gel. After electrophoresis, RNA was stained by placing the gel in 0.1 M ammonium acetate containing 0.5 mg/ml ethidium bromide and viewed on UV-transilluminator. (Note: (Note: MMH is highly extremely toxic and ethidium bromide is suspected carcinogen. Care must be take during handling and disposing solutions containing these substances)

3.4.1.4. Electron Microscopy

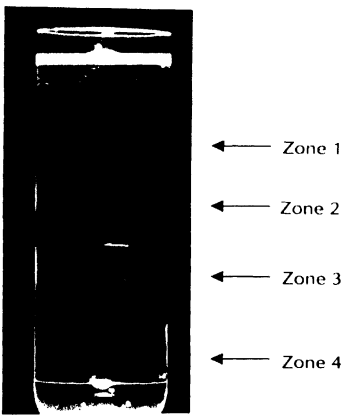
A drop of purified preparation was mounted on carbon-coated EM-grids and stained with 2% UA or PTA, pH 6.5 and observed under a Phillips CM110 transmission electron microscope (x34,000 magnification). Particles were also stained positively with UA in ethanol (25 µl 2% UA, 50 µl distilled water, 100 µl absolute ethanol).

3.4.2.1. Isolation of a Highly Flexuous Filamentous Virus

The extraction of highly flexuous filamentous virus (named as pigeonpea sterility mosaic virus-PPSMV) from SMD-affected leaf material was accomplished by using a buffer with high pH (tris buffer, pH 9) which contained chealting (EDTA and DIECA), reducing (sodium sulphite and 2-monothioglycerol), anti-aggregating (PVP) agents to reduce the activity of host polyphenols and endonucleases. Clarification with high concentration (25%) of Triton X-100 aided in solubilisation of membrane associated proteins. Organic solvents were not used for clarification, thus the pellets were relatively greenish due to the presence of chlorophyll pigment. Four diffused light scattering zones were observed in sucrose gradients layered with both infected and healthy preparations (Figure 21). These zones were collected separately and concentrated by high speed centrifugation. Analysis of the pellets in SDS-PAGE gels revealed the presence of two major polypeptides of molecular weight 52 and 32 kDa in SMD-affected samples (Figure 22). The 52 kDa protein was present in high concentrations in all the four fractions of healthy and infected extracts, whereas, the 32 kDa protein was confined to only extracts from infected plants (Figure 22). Occasionally a minor band of 30 kDa was noticed in infected, which could be a break-down product of 32 kDa protein. There was no variation in size of the disease-specific protein observed in samples drawn from all the 4 light scattering zones.

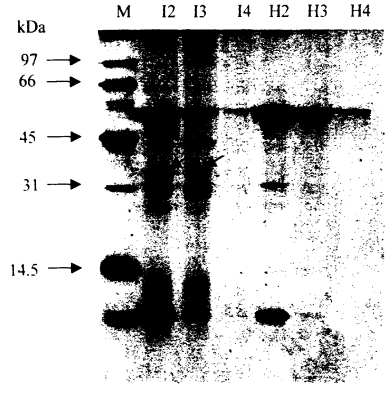
Separation of final purified preparations in CsCl density gradient resulted in single light scattering zone. The zone was collected and dialysed to remove CsCl and analysed in SDS-PAGE gel. Three bands of 54, 32 and 30 kDa were noticed in silver stained gel (Figure 23). The 54 kDa protein might be of host origin that was always detected in high concentrations. The protein (32 kDa) was virus-specific and 30

Figure 21

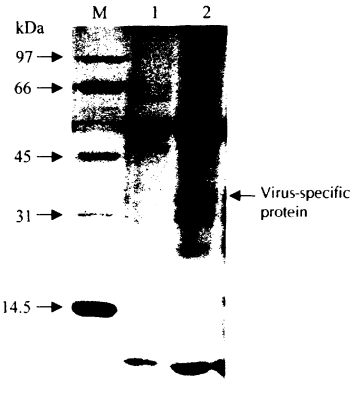


Four light scattering bands obtained in a 10-40% sucrose density gradient of partially purified virus preparations from infected plants after centrifugation for 2 h at 24,000 rpm in a SW28 rotor. Note that partially purified preparations from healthy also separates into four zones at similar positions.

Figure 22

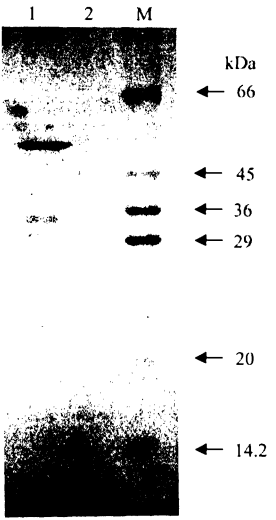


Separation of proteins from the three light scattering zones (zones 2, 3, 4 of sucrose density gradient) in a 12% SDS-PAGE gel. Fractions from infected (I) and healthy (H) purified preparations. M is the molecular weight marker (BioRad). SMD-specific proteins indicated with an arrow.



Migration following SDS-PAGE in 12% gel from preparations purified from healthy (lane 1), infected (lane 2) plants. Lane M is a molecular weight marker (BioRad). Virus specific protein is shown an with arrow.

Figure 23



Silver stained 12% SDS-PAGE showing virus-specific proteins purified through CsCl gradient. Lanes 1 SMD-affected pigeonpea extract and lane 2 healthy extract. Lane M is the molecular weight marker (Sigma).

kDa may be a degradation product of the 32 kDa protein. However, concentration of 32 kDa protein obtained from CsCl preparations was very low. This may be due to protein degradation in CsCl or during dialysis. No bands were observed in the gradients layered with healthy preparations. Occasionally some light scattering zones were observed in healthy preparations, but they were at different positions. Analysis of such fractions in EM and PAGE revealed either virus-like particles or proteins, respectively.

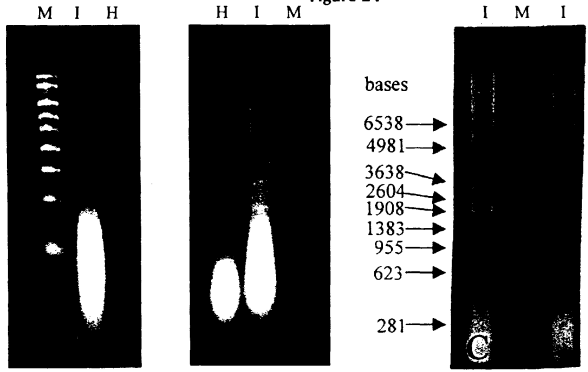
3.4.2.2. Nucleic Acid Analysis

Nucleic acids extracted from the light scattering density gradient fraction of both healthy and infected leaf extracts were separated by electrophoresis (Figure 24). A consistent pattern of 4-5 bands of estimated 3.5, 2.6, 1.9 and 0.8 kb in size were observed in SMD-affected material, but not in any of the healthy preparations. These were found to be sensitive to RNase treatment. Few minor bands greater in size than 3.5 kb were occasionally observed in infected preparations. The thickness of the nucleic acid bands varied suggesting it may contain a mixture of single and double stranded molecules. In all these preparations some material (possibly polysaccharieds and/or polyphenols) which interfered with clear visibility of the nucleic acid species, when stained with ethidium bromide, was observed.

3.4.2.3. Electron Microscopic Studies

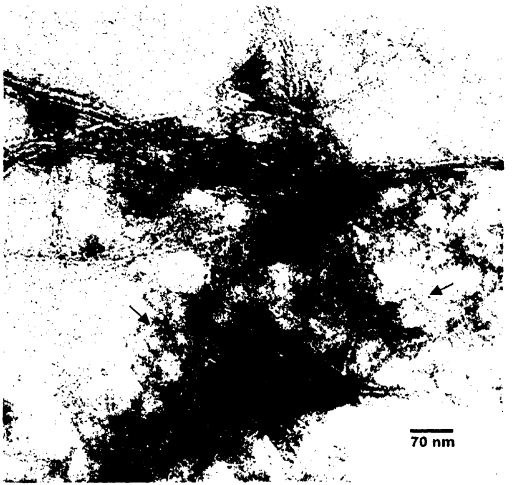
Initial electron microscopic studies showed the presence of bundles of needle shaped rigid particles and positively stained highly flexuous thin filaments when stained with PTA, pH 6.5 (Figures 25). Positive staining of these preparations with UA in ethanol, revealed the presence of thread like flexuous filaments of about 3 nm diameter, varying in lengths (Figure 26). The positive staining may be due to the uptake of stain by viral nucleic acids, as reported for tenuiviruses (Toriyma, 1982b; Toriyama, 1995). Observation of CsCl purified preparations revealed thin filamentous particles of unusual morphology with diameter of 8-11 nm (Figures 27 and 28). These particles are often short and branched irregularly (boxed regions in Figure 27). Sometimes closed circular forms were observed (marked regions in Figure 28). The filaments seem to be unstable in UA stain and the degenerated to small knob like structures.

Figure 24



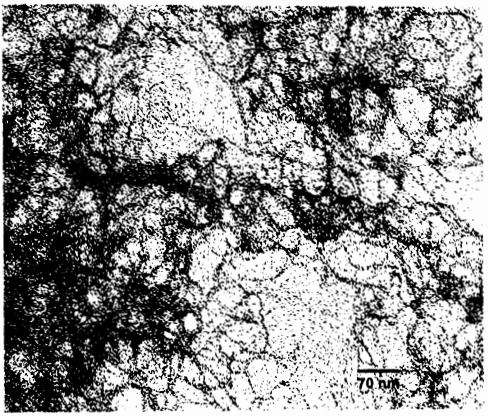
Resolution of various RNA preparations isolated from from SMD-affected (I) and healthy (H) purified preparations in methylmercuric hydroxide denaturing agarose gels. Lane M is the RNA molecular weight marker (Promega).

Figure 25



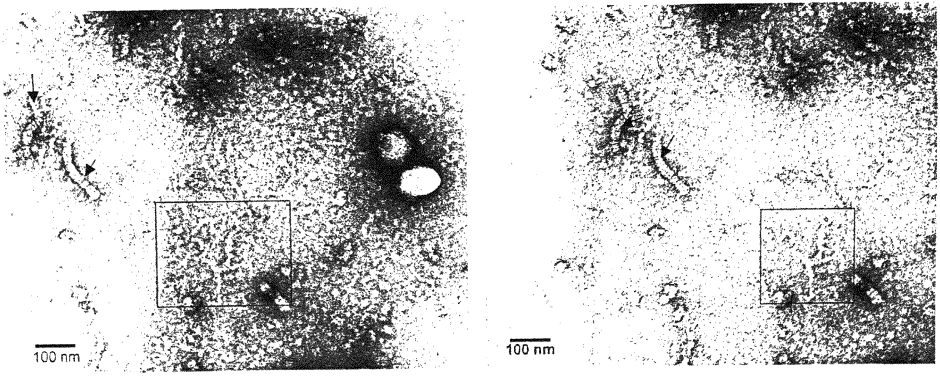
Electron micrograph of a purified virus preparations from SMD-affected plants showing aggregates of slender flexuous filamentous particles (indicated with arrows).

Figure 26



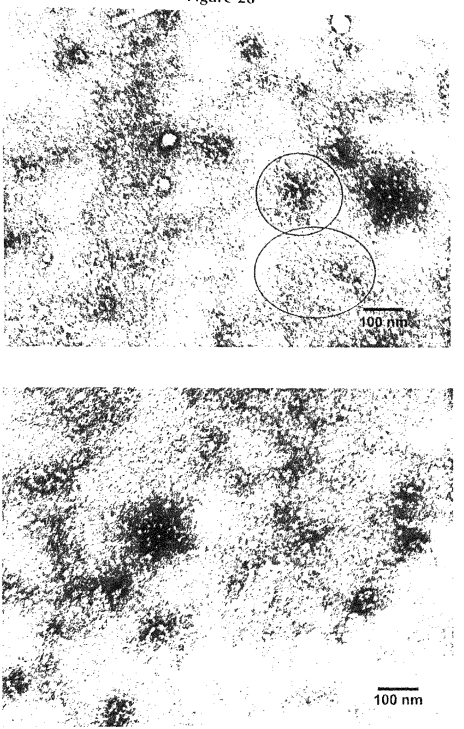
Electron micrograph of positively stained purified virus preparations from SMD-affected pigeonpea leaf material.

Figure 27



Electron micrographs of CsCl gradient purified preparations from SMD-affected leaf material.Branched particles are boxed and RDP components were showd with arrows.

Figure 28



Electron micrograhs of CsCl purified preparations from SMD-affected plants. Closed circular forms are marked.

The features of the particles obtained from SMD-affected plants resembled to those reported for tenuiviruses (Falk and Tsai, 1998). Such particles were not found in the purified preparations of the healthy pigeonpea extracts. However, both SMD-affected and healthy pigeonpea preparations contained rod shaped structures (indicated with arrows in Figure 27), suggesting they may be host related components, presumably ribulose diphosphate carboxylase. In addition to this, phytoferritins were also noticed in the purified virus preparations.

3.2.2.4. Relation of PPSMV with SMD

PPSMV was consistently isolated from all the 36 SMD-affected samples collected from four different locations in South India (ICRISAT, Patancheru, AP State; GKVK, Bangalore, Karnataka State, ANGRU Pulse Research Station, Tirupati, AP State and Sidipetta, Kharimnagr, AP State), but not in any of the 30 comparable healthy samples. Importantly, the same virus was isolated from SMD-affected samples inoculated previously with *A. cajani* at the two leaf stage and maintained subsequently in the growth chambers. This suggests that PPSMV is a mite-transmitted virus. The consistent isolation of PPSMV from SMD-affected plants, especially from laboratory maintained cultures, and the similarity of its properties with HPV, another mite-transmitted virus reported recently form maize (discussed in section 5.1.2), suggests that the virus from pigeonpea is probably the causal agent of SMD, hence named PPSMV. The information obtained in this preliminary studies indicate that PPSMV has several similarities with the members of the genus *Tenuiviruses*. Further characterisation of this virus by bio-chemical methods is essential.

Chapter 4

Analysis of Aceria cajani population Diversity

4.1. MATERIALS AND METHODS

4.1.1. Collection and Storage of Mites

SMD-affected pigeonpea samples were collected from various locations of India, Nepal and Myanmar (see Table 12). Leaf samples were collected randomly from the infected plants, stored in polythene bangs or wrapped with aluminium foil (8x10" and 0.5" thickness) and placed in manila covers. For long term storage host material containing mites was stored in 95% ethanol.

In addition to mites from pigeonpea, other eriophyid mite species or its recombinant plasmid clones containing rDNA copies were included for comparison and phylogentic studies (Table 13).

Using a fine needle mites were collected from leaves and buds, and placed in a 1.5 ml Eppendorf tube containing 0.01% Decon (non-frothing detergent, BDH Chemicals, UK) in distilled water. Mites were concentrated by centrifuging at 8,000 g for 1 min, the supernatant fluid was removed, and the tubes containing mites were stored at -70 ^{0}C or processed immediately for nucleic acid extraction. All observations and manipulations of mites were done with the aid of a stereoscopic microscope (Kyowa Opticals, Model SDZ-PL, Japan).

Ethanol preserved samples: Host tissue was vigorously shaken to release mites into ethanol. Mites from ethanol solution were pelleted by centrifuging at 8,000 g for 2 min. Using a micropipette alcohol was removed and tubes were dried in a vacuum drier (Savant Speedvac, Farmingdale, USA) for 5-10 h and stored at -70 ^{0}C.

4.1.2. Mite DNA Extraction

Nucleic acid was extracted from approximately 15-20 mites as described in Kumar *et al.* (1998). Mites were frozen by immersing the tubes in liquid nitrogen. Frozen mites were crushed with an Eppendorf homogeniser, 400 µl of extraction buffer (100 mM

Table 12
Locational details of SMD-affected pigeonpea samples analysed

	Date	Location	State	Country	Acronym
1.	30-10-96	ICIRSAT, Patancheru, Medak	Andhra Pradesh	India	ICR
2.	8-11-96	Badanpura	Maharasthra	India	B
3.	11-11-96	ICRISAT, Patancheru, Medak	Andhra Pradesh	India	ICR2
4.	21-11-96	Kanukunta, Medak	Andhra Pradesh	India	K
5.	21-1-97	Buddipadiaga, Medak	Andhra Pradesh	India	BNM
6.	21-1-97	Arepalli, Medak	Andhra Pradesh	India	AKM
7.	21-1-97	Peddasamudrala, Karimnagar	Andhra Pradesh	India	PKK
8.	21-1-97	Antikapeta, Karimnagar	Andhra Pradesh	India	AHK
9.	21-1-97	Immanaguda, Medak	Andhra Pradesh	India	IGM
10.	21-1-97	Elkaturti, Karimnagar	Andhra Pradesh	India	EEK
11.	23-1-97	Gulbarga	Karnataka	India	GUL
12.	23-1-97	Rahuri	Maharasthra	India	RM
13.	23-1-97	Bilza fens, ICRISAT, Patancheru	Andhra Pradesh	India	BFI
14.	27-1-97	Puddukkotai	Tamil Nadu	India	PTN
15.	3-2-97	Kanukunta West, Medak	Andhra Pradesh	India	KW
16.	3-2-97	Kanukunta East, Medak	Andhra Pradesh	India	KE
17.	3-2-97	Balsapur, Medak	Andhra Pradesh	India	BMM
18.	3-2-97	Peddavura, Medak	Andhra Pradesh	India	PSM
19.	22-2-97	Kanukunta, Medak	Andhra Pradesh	India	KM
20.	17-3-97	Daupatpur, Dehat	Uttar Pradesh	India	KAN
21.	29-4-97	Talakundu, Kolar	Karnataka	India	TK
22.	29-4-97	Malligonda, Vellore	Tamil Nadu	India	MV
23.	29-4-97	Chittoor	Andhra Pradesh	India	CT
24.	29-4-97	Mydukuru-1, Cuddapaha	Andhra Pradesh	India	MY
25.	29-4-97	Mydukuru-2, Cuddapaha	Andhra Pradesh	India	MD
26.	29-4-97	Vijayapur, Chickballapur	Karnataka	India	VC
27.	29-4-97	Rayachoti, Cuddapaha	Andhra Pradesh	India	RC
28.	29-4-97	Ramateertham, Chittoor	Andhra Pradesh	India	RAM
29.	29-4-97	Taticherla, Giddalur	Andhra Pradesh	India	TAG
30.	29-4-97	Pernambatu	Tamil Nadu	India	PER
31.	6-5-97	Dholi Agricultural College Farm	Bihar	India	DF
32.	6-5-97	Mahamudpura, Iamashitipur	Bihar	India	MB
33.	6-5-97	Syadpura, Samishtapur	Bihar	India	SAS
34.	6-5-97	Pusa Station, IARI Gardens	Bihar	India	PUI
35.	3-6-97	ICRISAT, Patancheru	Andhra Pradesh	India	ICR-3
36.	15-8-97	Jaipur	Rajasthan	India	JAI
37.	15-9-97	ANGRAU, Tirupati	Andhra Pradesh	India	MTPT
38.	15-9-97	ANGRAU, Tirupati	Andhra Pradesh	India	RTPT
39.	15-9-97	ANGRAU, Tirupati	Andhra Pradesh	India	STPT1

40.	15-9-97	ANGRAU, Tirupati	Andhra Pradesh	India	STPT2
41.	15-9-97	ANGRAU, Tirupati	Andhra Pradesh	India	ETPT
42.	15-9-97	ANGRAU, Tirupati	Andhra Pradesh	India	YTPT
43.	15-9-97	ANGRAU, Tirupati	Andhra Pradesh	India	LTPT
44.	15-9-97	Vempalli, Chittoor	Andhra Pradesh	India	YEN
45.	15-9-97	Kurupalli-1, Chittoor	Andhra Pradesh	India	KUR1
46.	15-9-97	Kurupalli-2, Chittoor	Andhra Pradesh	India	KUR2
47.	15-9-97	Hosamhpalli1	Karnataka	India	HOS1
48.	15-9-97	Hosamhpalli2	Karnataka	India	HOS2
49.	15-9-97	Bangalore suburbs-1	Karnataka	India	ARS1
50.	15-9-97	Bangalore suburbs-2	Karnataka	India	ARS2
51.	15-9-97	GKVK, Bangalore	Karnataka	India	BAL1
52.	15-9-97	Balajiygpade	Karnataka	India	BAL2
53.	15-9-97	Bangalore suburbs-3	Karnataka	India	BAL3
54.	24-9-97	Plant Pathology Div., CARI	Yezin	Myanmar	BUR2
55.	24-9-97	Magliang Farm	Maghang	Myanmar	BUR8
56.	24-9-97	Mygan Farm	Mygan	Myanmar	BUR10
57.	24-9-97	Nyaungoo Farm	Nyaungoo	Myanmar	BUR14
58.	24-9-97	Kyaukpacaung		Myanmar	BUR20
59.	17-12-97	Kanchikacherla, Krishna	Andhra Pradesh	India	KRI
60.	17-12-97	Nandigama, Krishna	Andhra Pradesh	India	NAD
61.	22-12-97	Sidipeta, Karimnagar	Andhra Pradesh	India	SID
62.	29-1-98	ICRISAT, Patancheru, Medak	Andhra Pradesh	India	ICR-N
63.	2-2-98	Bhanupur		Nepal	NEP-1
64.	2-2-98	Rajajna	Nepalgunj	Nepal	NEP-2
65.	2-2-98	Agricultural Research Station	Nepalgunj	Nepal	NEP-3
66.	2-2-98	Khairapur, Ward-2		Nepal	NEP-4
67.	2-2-98	Sanosari		Nepal	NEP-5
68.	2-2-98	Jutepani, Ward-9		Nepal	NEP-6
69.	24-5-98	GKVK, Bangalore	Karnataka	India	RSK

Table 13

Details of the various eriophyid mites analysed in this study

Mites	Geographic distribution	Host	Localisation	Acronym
1. *Aceria cajani*	Indian-subcontinent	Pigeonpea	Under surface of the leaf	ACAJA
2. *A. tulipae*[1]	Canada	Wheat	Unfolded young leaves	ATULI
3. *Cecidophyopsis aureum**	Finland	Goldencurrants	Buds	AUREA
4. *C. alpina**	Finland	Alpinecurrants	-do-	ALPIN
5. *C. grossulariae**	UK	Gooseberries	-do-	GROSS
6. *C. psilaspis**	Canada and UK	Yew tree	-do-	SILAP
7. *C. ribis**	UK	Blackcurrants	-do-	RIBIS
8. *C. selachodon**	Finland	Redcurrants	-do-	SELAC
9. WC mites*[*3]	UK	Whitecurrants	-do-	REDIT
10. *Eriophyes insidiosus*[2]	USA	Peach	-do-	ERIOP
11. *Phyllocoptes fructiphilus*[4]	USA	Rose	-do-	PHYLO
12. *Phyllocoptes gracilis**	UK	Wineberries	Under surface of the leaf	PGRAC
13. *Nalepella halourga*[*3]	Norway	Norway spruce	Buds	SPRUS

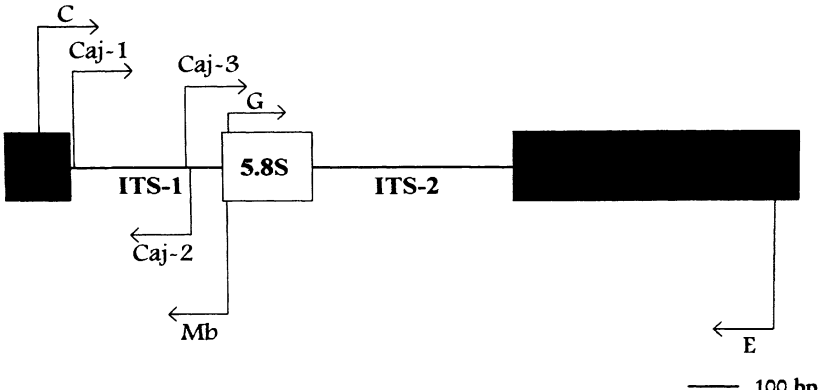
*Recombinant plasmid clones containing rDNA copies and nucleotide sequence information supplied by Dr Brian Fenton, SCRI.
*New species of *Ribes* infesting *Cecidophyopsis* mites (Formally not yet described).

1. Mite specimens supplied by Dr Hiruki, University of Alberta, Canada.
2. Mite specimens supplied by Dr Oldfield, University of California, USA.
3. Mite specimens supplied by Stuart Gordon, Unit of SFPC, SCRI, UK.
4. Mite specimens supplied by Dr Jim Amrine Jr. University of West Virginia, USA.

Tris-HCl, pH 7.5, 10 mM EDTA, 350 mM NaCl, 2% SDS and 7 M urea) was added to the homogenate and mixed well. This suspension was extracted twice with an equal volumes of tris-saturated phenol (pH 7.8):chloroform: isoamylalcohol (25:24:1 v/v) mixture and once with an equal volume of chloroform:isoamylalcohol (24:1). To the aqueous phase 2.5 volumes of cold absolute ethanol were added and the mixture was stored at -20 ^{0}C for 3 h. Nucleic acid was pelleted by centrifuging at 10,000 g for 15 min. The pellet was washed twice with 500 µl of 70% ethanol. DNA pellet was dried in a vacuum dryer (Savant Speedvac, medium heat for 5 min) and resuspended in 15 µl of sterile TE (100 mM tris-HCl and 1 mM EDTA) buffer, pH 9.2 and stored at -20 ^{0}C until analysed.

4.1.3. PCR Primers

Oligonucleotide primers designated C, B, Mb, E and G for the amplification of mite rDNA regions were previously reported by Fenton *et al.* (1994; 1997). Primer C (23mer) has annealing sequence towards the 3' end of the 18S gene and amplifies downstream; Mb (19mer) has annealing region in 5.8S gene and amplifies upstream; primer G (23mer) also has annealing sequence in 5.8S gene amplifies downstream; and E (22mer) has annealing sequences in 28S gene and amplifies upstream. Universal M13 24mer forward (F) and 22mer reverse (R) primers were used for the amplification of fragments cloned into plasmid vectors. Primers Caj-1, Caj-2 and Caj-3 designed in this study (discussed in results) were used in combination with other conserved primers for specific amplification of *A. cajani* rDNA. Primer properties, such as melting temperature, G + C content and $MgCl_2$ requirement were determined using the GCG (1994) computer programmes PRIME and MELT. Random sequence similarities in the primer annealing sequences to non-target sties were tested using the programme FINDPATTERNS to search the EMBL DNA database. When matches were found with significant sequence similarity, especially towards the 3' end of the primer, primer sequences were altered. The primer positions and direction of amplification are shown in Figure 29 and primer sequences and properties are given in Table 14. All the primers were synthesised in a 3 OD scale by a Genosys Custom oligonucleotide synthesiser (Genosys Biotechnologies, Pampisford, UK). Lyophilised primers were resuspended in sterile TE (100 mM tris-HCl and 1 mM EDTA, pH 8) and dispensed into 1:10 aliquots and stored at -20 ^{0}C.



The map of rDNA regions used for studies on the genetic variation of *Aceria cajani* populations. The positions and orientations of various primers used for the amplification are shown by arrows.

Table 14
Properties of the PCR primers used for rDNA amplification and sequencing

Primer	Length (bases)	Sequence (5'-->3')	%G+C Content	Amplification Direction	Annealing Site
C	23	GACCAACTAAAAGTCGTAACAAG		Downstream	18S rRNA
Mb	19	GCTGCGTTCTTCATCGATC		Upstream	5.8S rRNA
G	20	GGATCGATGAAGACCGCAGC		Downstream	5.8S rRNA
E	22	CAACTTTCCCTCACGGTACTTG		Upstream	28S rRNA
Caj-1	21	GTAAAAAACCAAACGCGAGTC		Downstream	ITS-1
Caj-2	23	TTCCACACTGATATGGTAGTCGC		Upstream	ITS-1
Caj-3	25	ACTACCATATCAGTGTGGAAGCGCG		Downstream	ITS-1
Old-1	17	GTCATGTCACTATTCGC		Downstream	ITS-2
Old-2	16	GTTGAGTGAAAAAGTG		Downstream	ITS-2
M13 F	24	CGCCAGG(GTTTTCCCAGTCACGAC)		Downstream	Plasmid DNA
M13 R	22	TCACA(CAGGAAACAGCTATGAC)		Upstream	Plasmid DNA

4.1.4. PCR Programmes

Three different PCR programmes were used in this study for the amplification of different fragments:

- **PCR programme cycle 13:** This programme was used for the amplification of rDNA from mite DNA extracts and also for the amplification of the long DNA fragments. Cycle parameters are:- 94 ^{0}C for 1 min denaturation, 58 ^{0}C for 2 min primer annealing and 72 ^{0}C for 2 min extension for 25 cycles, followed by 10 cycles of amplification by denaturation at 94 ^{0}C for 1 min, primer annealing at 58 ^{0}C for 3 min, extension at 72 ^{0}C for 3 min and finally 72 ^{0}C for 7 min for extension. Ramp rate was set to 20 ^{0}C/min while cooling down to annealing temperature.
- **PCR programme cycle 40:** This programme was used for the amplification of products cloned into plasmid vectors. Cycle parameters are:- Initial denaturation at 94 ^{0}C for 5 min, followed by 35 cycles of amplification by denaturation at 94 ^{0}C for 30 sec, primer annealing at 58 ^{0}C for 30 sec and polymerisation at 72 ^{0}C for 1 min, and finally 5 min at 72 ^{0}C for extension.
- **PCR programme cycle 18:** This was used for the nucleotide sequencing by dideoxy chain termination cycle sequencing. Cycle parameters of this programme for 25 cycles are:- 96 ^{0}C for 10 sec denaturation, 50 ^{0}C for 5 sec primer annealing and 60 ^{0}C for 4 min polymerisation.

4.1.5. Ribosomal DNA Amplification by PCR

DNA sequence corresponding to the 3' end of the 18S/ITS-1/5.8S/ITS-2/5' end of the 28S rDNA genes were amplified by PCR (see Figure 29). Oligonucleotide primers corresponding to the conserved regions of the 18S (primer C), 5.8S (primers B and Mb) and 28S rDNA (Primer E) regions were used as described by Fenton *et al.* (1994) (Figure 29). PCR reaction was performed using Promega PCR reagents (Cat.# M1865, Promega, Southampton, UK).

dNTP mixture: 25 µl of each of dATP, dCTP, dTTP and dGTP from a 100 mM stock (Promega, Cat.# U1330) were mixed together. The final concentration of each dNTP in this mixture is 25 mM.

4.1.5.1. PCR reaction mixture:

The following components was added into a sterile 0.2 ml tube:

10x PCR buffer (supplied with the enzyme)	3 µl
25 mM MgCl2	3 µl
dNTP mixture	0.3 µl
Taq polymerase	2 U
Primer 1 (upstream)	1 µl (10 ng)
Primer 2 (downstream)	1 µl (10 ng)
1-2 µl of mite DNA	
sterile dH_20 to 33 µl	

The reaction contents were overlaid with 15 µl of mineral oil and amplification was performed in a Techne PHC3 thermal cycler using PCR programme 13. Mineral oil was not used when the amplification was performed in thermal cyclers, GeneAMP PCR System Model 9700 and 2400 (PE Applied Biosystems, USA) as these are fitted with heated covers to prevent evaporation.

4.1.5.2. Analysis of PCR products

Aliquots (8 µl) of amplified products were mixed with 3 µl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in distilled water) and electophoresed in an 1% agarose gel using TBE (0.045 M tris-borate and 1 mM EDTA) buffer system, pH 8 (Sambrook *et al.*, 1989). Gels were stained with ethidium bromide and viewed on a UV trans-illuminator (Spectroline TR-312A, Spectronic corporation, Westbury, USA). DNA 'Marker VIII' (114, 900, 692, 501, 489, 404, 320, 242, 190, 147, 124, 110, 67, 37, and 34 bp; Cat.# 1336045, Boehringer Mannheim, Germany) was used as molecular weight marker.

4.1.6. Cloning and Transformation

Three methods were used to clone amplified PCR product into the plasmid vectors.

- When the amplified product concentration is optimum (~5-10 ng/µl), 2 µl of the PCR product was used for cloning.
- Low concentration of PCR products were enriched by precipitating with 2.5 volumes of cold absolute ethanol. Pellet was washed with 70% ethanol and dissolved in minimal volume (5-10 µl) of sterile distilled water. Two µl (5-10 ng) of this was used for cloning.

- In the presence of non-specific products along with targeted PCR product, desired fragment was eluted after separating in low gelling agarose gels (Sigma, Cat.# A-4018) with a Kristal Gelex DNA purification kit (Cambridge Molecular Technologies LTD., Cat.# KX-50). Amplified products were electrophoresed in 1% agarose as described in section 4.1.5.2. and DNA was eluted as described below.

4.1.6.1. DNA elution from agarose gels (All reagents were supplied with the kit): Agarose gel corresponding to the target product was sliced under UV-light and transferred into an Eppendorf tube. To this 4.5 volumes of sodium percholate and 0.5 volumes of Gelex^{R} modifier were added and kept at 55 ^{0}C until the gel slice was completely melted. To this 10 µl of Gelex^{R} resin was added, incubated at room temperature for 5 min with intermittent shaking and centrifuged at 10,000 g for 30 sec. Supernatant was discarded and pellet was washed twice with wash buffer and dried by keeping the tube at 40 ^{0}C for 1 min. Ten µl of sterile distilled water was added directly to the pellet, mixed well and kept at room temperature for 1 min. This was centrifuged at 10,000 g for 30 sec and DNA containing supernatant was collected and used for cloning.

4.1.6.2. Cloning: PCR products were cloned into an *Eco*R I linearised TOPO-TA vector (Invitrogen, Cat.# K4500-40; vector map given in Appendix 8.6). This vector utilises ligation property of vaccinia DNA topoisomerase attached to the linearised ends (Shuman, 1994). PCR products were cloned into the end-filled *Eco*R I digested polylinker site. Ligated products were chemically transformed into *E. coli* TOP 10 competent cells (Invitrogen, Cat.# K4500-40).

4.1.6.3 Ligation reaction

The following components were added into a 0.5 ml tube:

2 µl of DNA (10 ng/µl)

1 µl of vector (10 ng/µl)

Sterile distilled water to 5 µl

Reaction mixture was incubated at 25 ^{0}C for exactly 5 min. Reaction was stopped by placing the tubes in ice.

4.1.6.4. Transformation

Fifty µl of *E. coli* Top 10 competent cells were thawed on ice. To this 2 µl of 0.5 M 2-mercaptoethanol was added and gently mixed with a pipette tip. To these cells, 3 µl of ligation reaction mixture was added and incubated on ice for 30 min and heat treated at 42 ^{0}C for exactly 30 sec. Immediately tubes were chilled on ice for 2 min.

To this 300 µl of SOC medium (Appendix 8.4) was added and vials were kept at 37 ^{0}C in a shaking incubator set at 225 rpm for 30 min. Contents of the tube were spread on LB ampicillin (50 µg/ml) agar plates containing 40 µl of 40 mg/ml X-gal (Sigma, Cat.# B4252) and incubated overnight at 37 ^{0}C.

4.1.6.5. Analysis of Positive clones

E. coli Top-10 cells containing the recombinant plasmid appear white due to the inactivation of α-complementation process by the insertion of an inset into the polycloning site, which otherwise gives blue coloured colony due to the uptake and catabolism of x-gal by *lacZ* gene (Ullmann *et al.*, 1967). The white clones were sub-cultured for plasmid isolation. About 65% of the clones were found to contain recombinant clones.

Selected clones were sub-cultured overnight in LB medium containing 50 µg/ml ampicillin (Appendix 8.4). Presence of insert was confirmed either by releasing the insert by restriction enzyme digestion or by PCR using insert specific primers or M13 F and R primers. Compared to the insert confirmation by RE digestion, confirmation by PCR method was rapid. Furthermore, 2 h bacterial culture could directly be used as a template for PCR. In the absence of specific primers, plasmid specific F and R primers facilitated the amplification of any cloned insert.

- Plasmid isolated from the bacterial cultures (described in section 4.1.7) were used for digesting with *Eco*R I to release the insert (see section 4.1.8). Digested products were analysed in an 1% agarose gels along with DNA molecular weight marker as described in section 4.1.5.2.
- Conditions for PCR were the same as given in section 4.1.5, except that instead of high molecular weight DNA, 2 µl of bacterial culture was used as target. PCR was performed in Techne PHC3 thermal cycler using the programme 40. Amplified products were analysed in agarose gels.

4.1.7. Isolation of Plasmid DNA

Plasmids from the positive clones were isolated with one of the methods described below:

- **Boil-lysis method** (Sambrook *et al.*, 1989): Cells from 2 ml over night bacteria culture were pelleted by centrifuging at 10,000 g for 2 min, resuspended in 300 µl of STET (Appendix 8.4) buffer, pH 8, and vortexed. To this 9 µl of lysozyme (from the stock of 10 mg/ml in 10 mM tris-HCl, pH 8; Sigma, Cat.# L6876) was added

and incubated on ice for 10 min. These were heat treated by placing in a boiling water bath for 40 sec, centrifuged at 10,000 g for 15 min and bacterial debris was removed with a pipette tip. To the supernatant 450 µl of cold isopropanol was added and the mixture left at room temperature for 10 min. This was centrifuged at 10,000 g for 15 min and nucleic acid pellet was resuspended in 300 µl of TE buffer, pH 8. This was treated with 3 µl of *DNase* free pancreatic *RNase* (10 mg/ml in 10 mM tris-HCl, pH 7.5; Boehringer Mannheim, Cat.# 1119915), incubated at 37 °C for 30 min and re-extracted with phenol:chloroform as described in section 3.1.2. The plasmid DNA was precipitated by ethanol and pellets were dissolved in 50 µl of TE buffer, pH 9.2.

- **Plasmid isolation using affinity columns:** For sequencing purpose plasmid from the bacterial culture was isolated using the Wizrd™ *Plus* miniprep plasmid purification system (all reagents supplied with the kit; Promega, Cat.# A7100). Bacterial cells from 2 ml overnight culture was pelleted by centrifuging at 10,000 g for 1 min. Supernatant was discarded and pellet resuspended in 200 µl of cell Resuspension™ buffer. To this 200 µl of Cell Lysis™ solution was added and mixed by inverting the tubes. Then 200 µl of Neutralisation™ buffer was added, mixed by inverting the tubes and centrifuged at 10,000 g for 10 min. Supernatant was collected and to this 1 ml of DNA binding Resin™ solution was added and kept at room temperature for 1 min. This was transferred into a mini column using 5 ml syringe (Cat.# 309603, Becton-Dickinson, USA). The column was washed with 3 ml of wash buffer and plasmid from the column was eluted with 100 µl of sterile distilled water.

Selected plasmids were stored at -70 °C after adding glycerol to 20% final concentration.

4.1.8. Restriction Enzyme Analysis of rDNA

Amplified DNA obtained by PCR of genomic DNA as well as from rDNA containing recombinant plasmids were digested with *Dde* I, *Mse* I, *Taq* I *Nde* II + *Pst* I (Boehringer Mannheim), either alone or in combination.

Reaction mixture: Enzyme digestions in a final volume of 20 µl of 1x Onephorall™ reaction buffer (Pharmacia) consists of 10-15 µl (15-20 ng) of PCR product and 1 U enzyme. Reaction tubes were incubated at 37 °C for 3 h to overnight for all enzyme

digests, except for *Taq* I, which was incubated at 66 ^{0}C after overlaying with mineral oil.

Analysis of digested products: Digested products were analysed in a Model V16 electrophoresis apparatus (Bethesda Research Laboratories, Maryland, USA) on a 14 cm long 12% non-denaturing polyacrylamide gel (30:1 Acrylamide/Bis acrylamide; BioRad) for 7 h at 150 V in a Laemmli's (1970) discontinuous buffer system, with TG (0.05 M tris-HCl and 0.384 M glycine, pH 8.3) running buffer. Gels were stained with ethidium bromide and viewed on a UV trans-illuminator. Marker VIII (Boehringer Mannheim) was used as molecular weight marker.

4.1.9. Nucleotide Sequencing

Nucleotide sequencing was done with dideoxynucleotide chain termination method using the Dye-Prism^{TM} sequencing kit (Perkin Elmer, Cat.# 402079) on the plasmid clones containing the ITS copies by PCR cycle sequencing (Craxton, 1991; DNA Sequencing, 1995). Sequencing was done with M13 F and R primers, as well as with the insert specific primers. PCR was performed in a GeneAMP 2400 thermal cycle (PE-Applied Biosystems) using programme 18. Sequencing reaction in a 10 µl final volume consisted of:- 4 µl of Dye-Terminator mix, 10 ng of primer, 100 ng of template DNA and sterile distilled water to the final volume. To the extension products, 1 µl of 3 M ammonium acetate, pH 5.2 and 30 µl of cold absolute ethanol were added and incubated on ice for 10 min. This was centrifuged at 10,000 g for 20 min. Supernatant was discarded and pellet was washed once with 200 µl of 70% ethanol. The pellet was dried and resuspended in 10 µl of 5:1 formamide: 50 mM EDTA and resolved on a 48 cm long 4% polyacrylamide gel (19:1 acrylamide:bis acrylamide) containing 8.3 M urea in an automated sequencer (PE-Applied Biosystems) for 15 h at 40 W. For DNA sequence analysis and refinement of basecalls, the ABI Sequence Analysis Software Version 2.1.1, and ABI Sequence Navigator Programme Version 1.0.1, respectively were used.

4.1.10. Computer Programmes for DNA Sequence Analysis

The DNA sequences were analysed using the University of Wisconsin Genetics Computer Group (GCG) Package Version 8.1 (GCG, 1994), and phylogenetic analyses were carried out using programmes in PHYLIP version 3.57c (Felsenstein, 1995; Felsenstein and Churchill, 1996; Wright, 1997a,b,c), on the Biological and

Biotechnological Sciences Research Council, SEQNET computer, Daresbury, UK; Medical Research Council HGMP computer, Hinxton, Cambridge, UK; and International Centre for Genetic Engineering and Biotechnology, ICGEBnet computer, Trieste, Italy. Various sequence analyses programmes used in this study are discussed in the relevant sections and schematically presented in Appendix 8.7. Nucleotide sequences determined in this study were deposited in European Molecular Biology Laboratory (EMBL) data base.

Sequence alignments: Raw data from the automatic sequencer was refined using sequence Navigator programme. Uncertainties in base callings were corrected using electrophorogram as the template. These data were saved in GCG format and transferred to SEQNET computer using the programme 'SEQED'. Sequence information corresponding to the vector arms was identified and omitted. Information obtained from the two strands of a single clone was aligned and per cent similarity was determined using 'BESTFIT' programme. Multiple sequences were aligned for sequence similarity using 'PILEUP' programme. Any conflicts in the sequence information was corrected based on the consensus information from the other strands using multiple sequence editor programme 'LINEUP'. Refined sequence was used for further applications.

Pylogenetic analysis: For phylogenetic analysis about 330 bases of the 5' end of 28S gene from the primer E direction was used. DNA sequences were first aligned with CLUSTALW (Higgins and Sharp, 1988). Default parameters were used to obtain plausible alignments. The nucleotide substitution via transition and transversion ratios (Ts/Tv) were estimated using the programme PUZZLEv3. Genetic distances between pairs of species were calculated using the programme DNADIST, using the Kimura 2-parameter method with a Ts/Tv rate 1 and one category of substitution rate 1. Phylogenetic trees were constructed using the original data set and 100 bootstrap data sets generated by the programme SEQBOOT from the original data set using a DNA-parsimony method (DNAPARS) and distance methods (DNADIST, FITCH and NEIGHBOR). In all cases consensus tree was generated by the programme CONSENSE and trees were displayed using the programme RETREE.

4.1.11. Scanning Electron Microscopy (SEM) for Mite Morphology

For SEM studies, mite specimens were prepared in an EMscope Sputter-Cryo low temperature system, as described by Lopez-Llorca and Duncan (1988) with minor modifications. Using a fine needle mites were picked off the leaf material and carefully arranged on the conductive sticky tape pasted on a copper stub. Specimens were rapidly frozen by immersing the copper stub in a slushing chamber containing sub-cooled liquid nitrogen (less than -196 ^{0}C). Surface ice was sublimed off the hydrated specimen by etching at -90 ^{0}C for 5 min. Specimens were sputter coated with gold in a coating chamber. Gold coated specimens were transferred onto the cold stage of a JEOL T200 SEM for observations. Images were recorded on a Kodak Tmax 100 film.

4.2. RESULTS

4.2.1. Collection of Mites and DNA Isolation

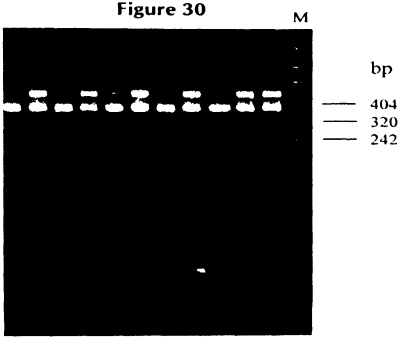
Mites were found on the under-side of the pigeonpea leaf buried in the dense trichomes. Immature and young leaves contained the maximum number of mites and they were concentrated around the veins towards the petiole end. Old and asymptomatic leaves were devoid of mites. Leaf samples collected following rains (in monsoon season) contained very few or no mites. Leaf material packed in aluminium foil remained fresh for longer time than in polythene bags. Fresh leaf samples thinly packed in aluminium foil permitted survival of mites up to a week at room temperature and 2-3 weeks at 4 ^{0}C. On few samples dead mites were found. Further manipulations of such mites were difficult due to degradation by saprophytic fungi.

The protocol used for mite genomic DNA isolation was simple and effective. Freezing mites in liquid nitrogen assisted in easy grinding. High salt concentration and presence of denaturing agents like urea and SDS in extraction buffer were effective in lysing cells and releasing the DNA. Subsequent treatments with phenol and chloroform and precipitation with ethanol yielded DNA useful for rDNA amplification by PCR. Isolated DNA resolved as a single high molecular weight band and 3 fast moving smaller bands in 1% agarose gel (results not shown). It was found that 1 µl (from 15 µl final volume) of DNA isolated from 15-25 mites was sufficient for amplification of rDNA in PCR.

4.2.2. Amplification of *A. cajani* rDNA

Initially to amplify the *A. cajani* rDNA ITS regions universal primers were used in three combinations, C + E, C + Mb and G + E. Amplification was obtained only with the primer combination C + Mb, resulting in a product of about 400 bp corresponding to the ITS-1 and flanking conserved regions (Figures 30). This was cloned and the insert containing clones were identified either by releasing the insert from isolated plasmids after digesting with *EcoR* I or by PCR using the F and R primers. Four independent

Figure 30



PCR amplified product of *A. cajani* rDNA ITS-1 region with the primers C + Mb (400 bp). Lane M is the molecular weight marker.

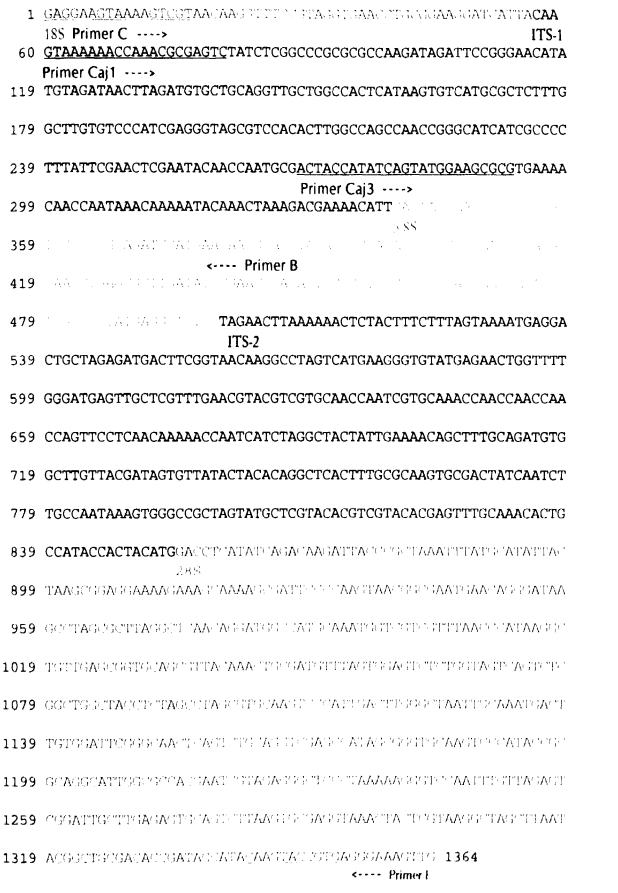
clones were sequenced on both strands. The information obtained was used for designing three oligonucleotide primers, Caj-1, Caj-2 and Caj-3 (discussed in section 4.2.3). Another two primer combinations (C+E and G+E) yielded no amplification. Slight alteration in $MgCl_2$ concentration did not improve the results. DNA isolated from 15-25 mites was found to be optimum for the amplification of rDNA in PCR. DNA obtained from decomposed mites resulted often in non-specific amplification and sometimes no amplification in PCR. rDNA from ethanol preserved samples often did not amplify. This could be due to inefficient recovery of DNA from ethanol preserved samples.

Other eriophyid mite species, *A. tulipae, E. insidiosus, P. fructiphilus* and WC mites were successfully amplified with the primers C+E. The size of amplified product of these mites was 1431, 1810, 1662 and 1377 bases, respectively (results not shown). These were cloned and sequenced and the information was used for comparison with *A. cajani.*

4.2.3. Design and Testing of *A. cajani* Specific Primers

The plasmid clones containing *A. cajani* C+Mb primer pair amplified products were sequenced and aligned. The consensus sequence was used for finding similar sequences in the EMBL database using BLAST search programme. This comparison aided in confirming the identity of the regions corresponding to the ITS-1 and flanking 18S and 5.8S genes. Three regions were selected in the ITS-1 region for primer designing (see Figures 29, 31 and Table 14). A 21mer primer Caj-1 (position 60 to 81 in Figure 31), a 23mer primer Caj-2 (position 264 to 287 in Figure 31) and a 25mer primer Caj-3 (position 267 to 292 in Figure 31) overlapping with the Caj-2 primer but with downstream amplification direction (Figure 29) were designed for specific amplification of *A. cajani.* These were checked against the EMBL database using FINDPATTERNS and they do not recognise other sequences.

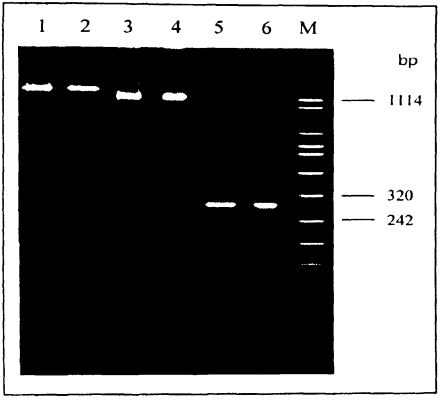
The three Caj primers were used together with universal primers (Caj-1+E; C+Caj-2 and Caj-3+E; Figure 32) for *A. cajani* rDNA amplification. Primer combinations Caj-1+E amplified a 1305 bp region corresponding to ITS-1/5.8S/ITS-2 and 5' end of 28S gene; C+Caj-2 amplified 286 bp region corresponding to 3' end of 18S and part of ITS-1; Caj-3+E amplified a1063 bp region corresponding to 3'end of ITS-1/5.8S/ITS-2 and 5' end of 28S genes. No variation was observed in sizes of the PCR products corresponding to ITS-1, ITS-2 or conserved regions from mite samples



rDNA sequence of *Aceria cajani* (location ICR) from the 3' 18S (56 bp) to the 5' 28S (510 bp) genes, covering ITS-1 (280 bp), 5.8S (160 bp) and ITS-2 (355 bp) regions. rRNA coding sequences are coloured. Primer annealing sites are underlined and the amplification direction shown with arrows.

Figure 31

Figure 32



PCR amplification of *A. cajani* rDNA with specific primers. 1-2 with primers Caj-1 and E (1305 bp); 3-4 with primers Caj-3 and E (1063 bp); 5-6 with primers Caj-2 and C (286 bp); Lane M, DNA molecular weight marker.

from different locations in India, Nepal and Myanmar. It was interesting to note that primer E when used with Caj-1 and Caj-3 primers efficiently amplified the targeted region, but failed to amplify when used with primer C. The annealing sequence of the Caj-1 primer was 56 bp downstream to that of the primer C. Nucleotide sequencing had revealed that there was no variation in annealing sequences of universal rDNA primers, C, Mb, G and E. The universal primer combination C+E amplified rDNA of 13 different eriophyid mites species, aphids, beetles, fungal and other eukaryotic rDNA (Fenton *et al.*, 1994; 1995; 1997). Factors for the failure of C+E combination on *A. cajani* to work could be due to interference of secondary structures during primer annealing process.

The Caj primers together with universal primers amplified all the *A. cajani* samples obtained from different locations of India and Nepal, but no amplification was obtained from the Myanmar samples except with the primes C+Caj-2. The latter samples were obtained in poor condition. DNA derived from decomposed mites may have affected amplification of large products. Amplified products were cloned for further analyses. Analysis of insert containing clones by PCR alone indicated a lack of variation in size within the population.

4.2.4. Sequence Analysis of *A. cajani* rDNA

Sequence information was obtained by double stranded sequencing of the different recombinant clones containing rDNA regions. At least two and a maximum of 5 independent clones were sequenced for each mite sample. Sequencing was done on plasmids constructed from PCR products of seven samples from India (B, ICR, K, KM, PTN, RM and ICR-N), two each from Nepal (Nep-2 and Nep-5) and Myanmar (Bur-14 and Bur-20). The full rDNA sequence of the samples from ICR is given in Figure 31. The sequences of mite samples from different locations were aligned using the programme 'CLUSTALW' and are presented in the Figure 33. The full length of rDNA analysed for the species identification studies was 1364 bp. This started 56 bp from the 3' end of conserved 18S gene to 510 bp at the 5' end of the 28S gene. Sizes of *A. cajani* mite ITS-1, 5.8S and ITS-2 regions were 283, 160 and 355 bp, respectively. The boundaries of various rDNA regions were defined by comparing with other rDNA sequences reported previously for other eriophyid mites (Figure 34). The per cent composition of the four nucleotides A, C, G and T were 29.4, 22.7, 24.6 and 23.3, respectively. The rDNA of *A. cajani* was predominantly A+T rich (52.7%) due to the short poly (A)n stretches towards 3' end of ITS-1 region.

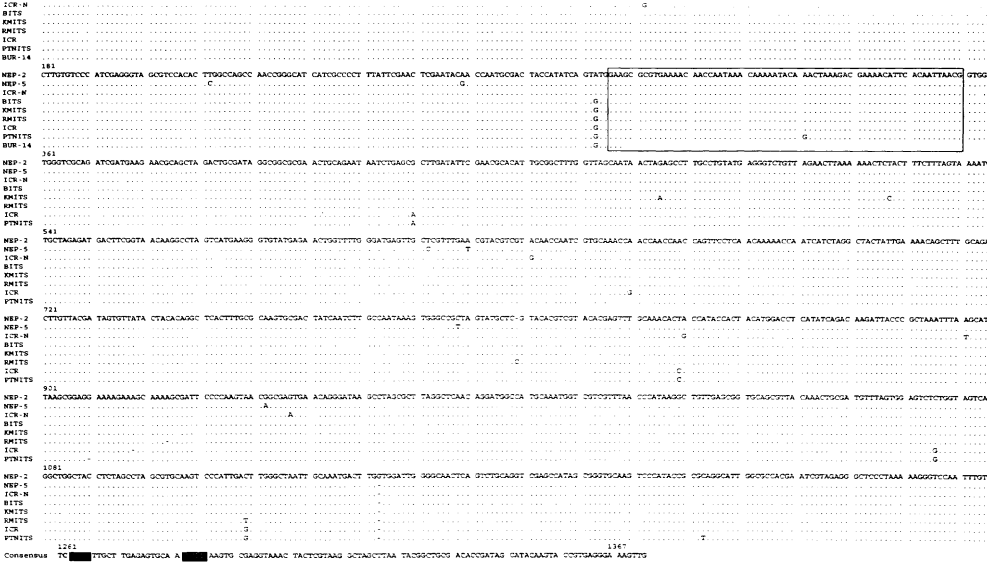
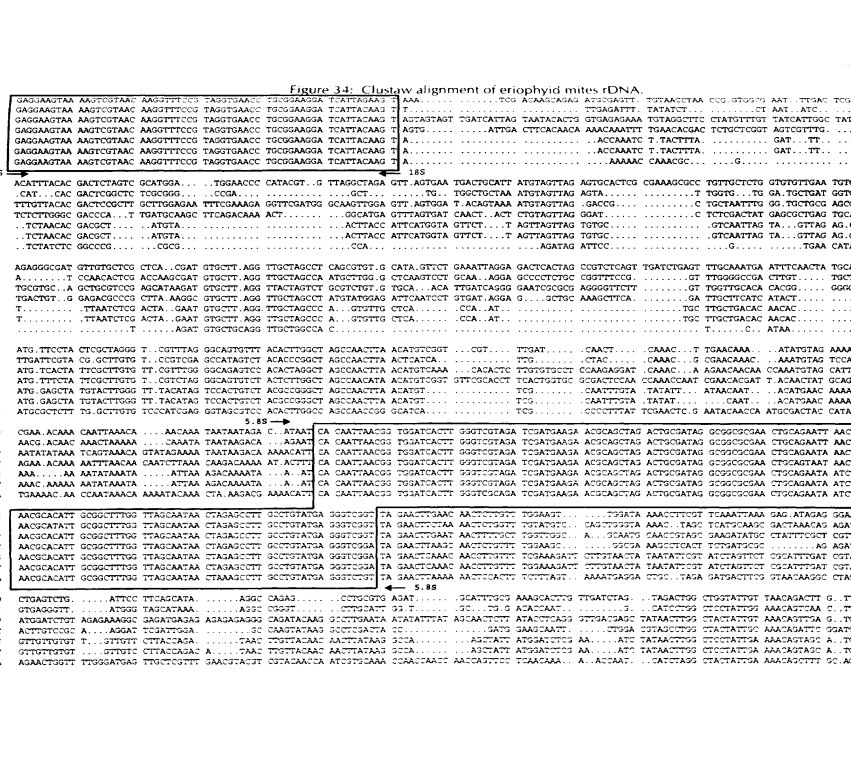


Figure 33: Alignment of rDNA sequences from *Aceria cajani* obtained from different locations (ICR, ICR-N, B, KM, PTN, RM, NEP-2, NEP-5 and BUR-14). Dots signify the sequence similarity; letters correspond to single base differences relative to other sequences; dashes signify single base deletions.

Figure 34: Clustaw alignment of eriophyid mites rDNA.



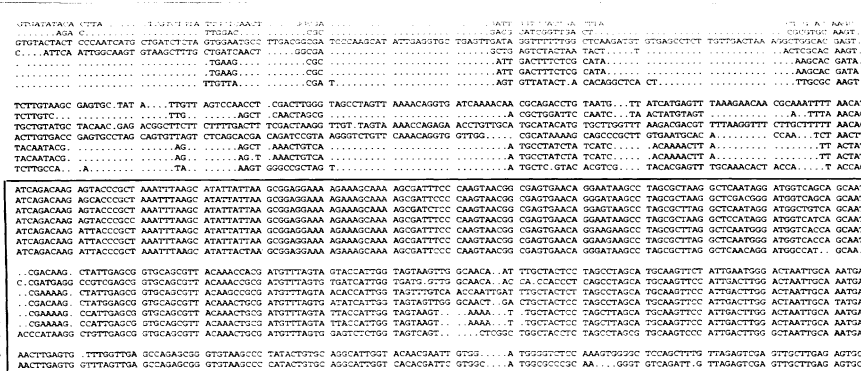


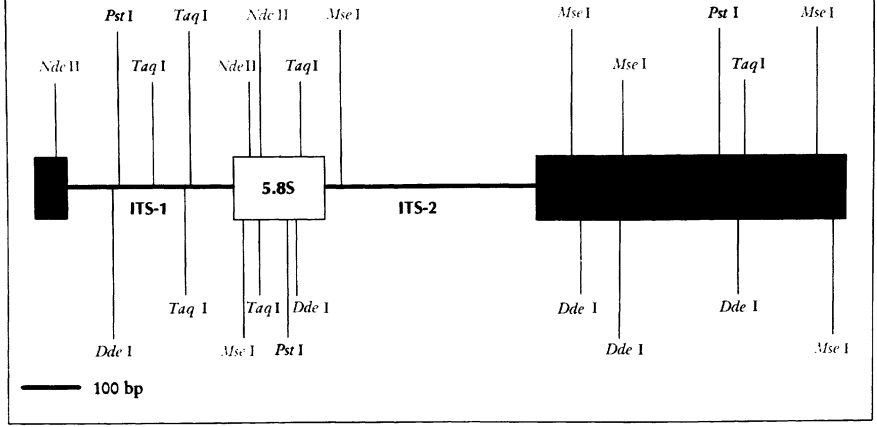
Figure 34: CLUSTALW alignment of various eriophyid mites rDNA sequences. Regions corresponding to the 18S, 5.8S and 28S genes are boxed. PHYLO: *P. fructiphilus*; ATULI: *A. tulipae*; ERIOP: *E. insidiosus*; PGRAC: *P. gracilis*: GROSS: *C. grossulariae*; REDIT: WC Mites; ACAJA: *A. cajani*.

Comparisons of the *A. cajani* rDNA sequences from the eleven different locations suggested that there was little or no sequence divergence amongst them (Figure 33). There were no major deletions/insertions in the sequences studied, except for a few single base substitutions, some of which were in the conserved rRNA genes. The assignment of these single base mutations were difficult, as a few could arise due to the errors in *Taq* polymerase replication and reading errors during sequencing. To determine whether the ITS sequence could uniquely identify the mite species (*A. cajani*) from which it was obtained, the rDNA sequences of *A. cajani* mites were aligned and compared with the sequences of other eriophyid mites (Figure 34). Of the regions between primer positions C and E analysed, sequences corresponding to the 18S gene were 100% identical, about 98% conservation was found in the 5.8S gene and 94% conservation in 28S gene. The ITS-1 sequence length of *A. cajani* mites was the smallest of all, with a large number of sequence deletions and variations compared to other mite species. The level of within species variation in the nucleotide sequences of ITS-1 examined in this study was comparable to the variation observed by Fenton *et al.* (1997) within different species of *Cecidophyopsis* mites, indicating that *A. cajani* mites from different regions of Indian subcontinent were all one species, distinguishable from other species in the family *Eriophyidae*. The unique feature of the rDNA regions of eriophyid mites was that there was high level of conservation in the regions encoding rRNA genes within the speices, but their ITS regions varied in both nucleotide sequence and length between species (see Figure 34). No two species showed significant level of conservation in these regions. In the *Ribes* infesting *Cecidophyopsis* mites, sequence variation is mainly confined to variable simple sequence repeat (vSSRs) regions in ITS-1 region (Fenton *et al.*, 1997). Though, sequences similar to vSSRs were found in ITS-1 of *A. cajani* (boxed region in figure 33), no variation was detected in samples analysed from three countries again suggesting that *A cajani* from throughout Asia is a single species. Sequences at two positions in the *A. cajani* 28S gene, GAG and TTC (blocked regions in Figure 33) were different from other mite species. Indeed in many species, including higher vertebrates, the nucleotide sequence is GGA and CTT, respectively (Christen *et al.*, 1991). This conflicting information could well be due to sequencing errors, but the variation found at that positions in *A. cajani* were consistent in all the samples sequenced.

4.2.5. Restriction Enzyme Analysis

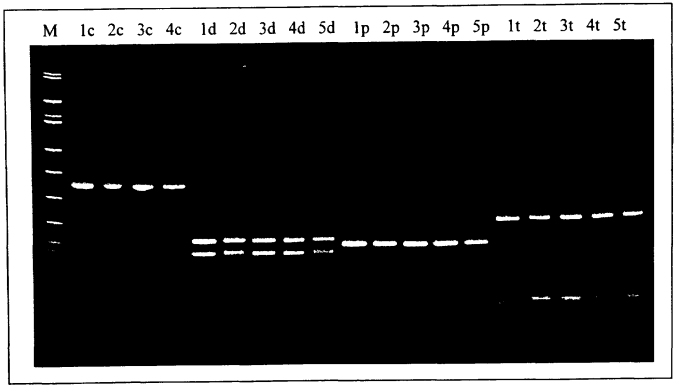
For routine analysis, *A. cajani* rDNA obtained from mites collected from different locations of India was analysed by restriction enzymes. The complete rDNA sequence of ICR mites was analysed using GCG (1994) computer programme 'MapPlot' which graphically displays restriction sites of all the restriction enzymes which cut the target sequence. From the data 5 enzymes:- *Mse* I, *Dde* I, *Taq* I, *Pst* I and *Nde* II with recognition sequences T^TAA, CT^NAG, T^CGA, CTGCA^G and ^CATC, respectively, were selected (Figure 35). Restriction digestion patterns of amplified *A. cajani* rDNA from different locations of Indian subcontinent revealed no variation in lengths of the fragments (Figures 36-39). There was no restriction site polymorphism in the ITS-1 regions digested with *Pst* I, *Taq* I and *Dde* I (Figure 36). The digestion patterns of ITS-2 with *Taq* I, and *Nde* II and *Pst* I double digests were also similar for all the *A. cajani* rDNA analysed (Figure 37). The restriction patterns of genomic and the recombinant plasmids containing the copies of ITS sequences were similar, indicating there was no variation within the populations (Figures 38 and 39). However, occasional variation in the restriction patterns was observed (see Figure 37, lanes 1a, 1b and Figure 39, lane 21), which was found to be due to the errors made by *Taq* polymerase during PCR (discussed below).

4.2.5.1. Anomalies in Restriction Patterns: rDNA of *A. cajani* showed no variation in its digestion profile. On a few occasions some extra bands mostly shadowing the major bands appeared. This could be due to digestion of non-specific and/or terminated targeted products generated during PCR process. Sometimes partial enzymatic digestion resulted in varied restriction patterns. In recent studies it was found that primer concatamers generated during PCR process can contribute to the variation in the length of the fragments and thus the profile (B. Fenton, unpublished). When RFLP analysis was performed on PCR products obtained from recombinant clones, on two occasions point mutations at cleaving site was found to be responsible for major variation in restriction patterns. In *Mse* I digest profile of ICR-2, clone 4 (See Figure 39, lane 21) one point mutation A to T (see Figure 40A and 40B) in the cleaving site has eliminated a *Mse* I site resulting in a larger product. Variation in *Taq* I and *Nde* II + *Pst* I restriction profile in B, clone 6 (see Figure 37, lanes 1a and 1b) was due to a transition (C to T) which affected overlapping restriction sites of *Nde* II and *Taq* I (Figure 40A and 40C). The apparent variation due to PCR artefacts and other conditions warrants for characterisation of the selected sequences before using for population analysis by PCR/RFLP, in order not to misinterpret as the real variation in populations.



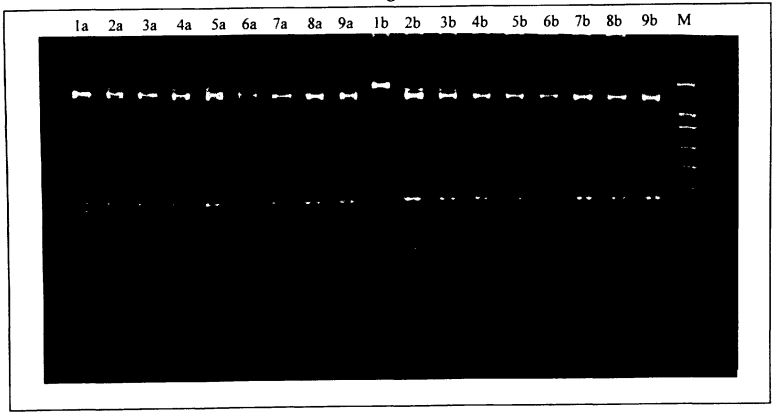
A restriction map of *Aceria cajani* rDNA deduced from the digests using *Dde I, Mse I, Nde II, Pst I and Taq I.*

Figure 36



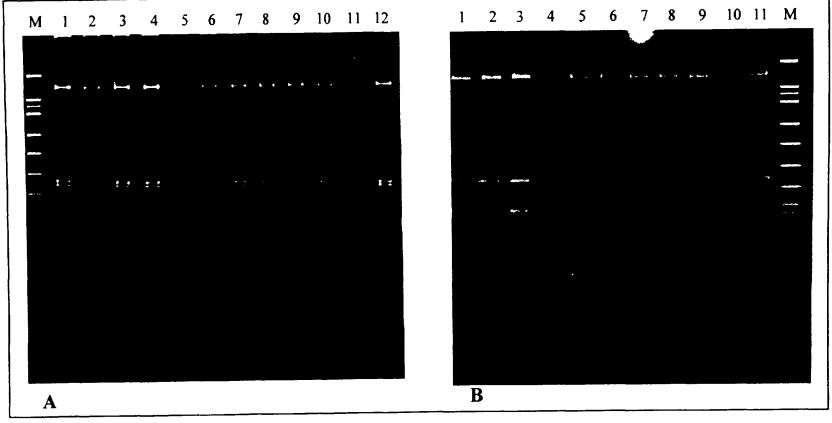
The 3' 18S and ITS-1 regions of *A. cajani* rDNA digested with *Dde* 1 (d), *Pst* I (p), and *Taq* I (t). M is the molecular weight marker and C, are undigested PCR products. Lanes 1-5 represents samples from K, B,ICR, PTN and BFI locations, respectively.

Figure 37



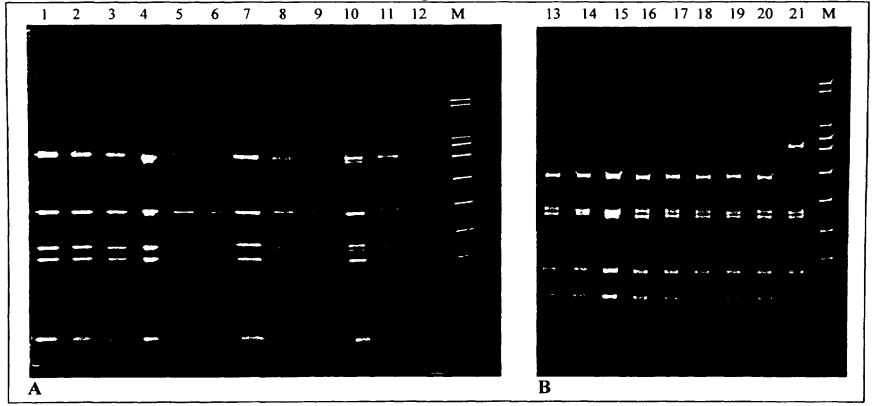
The 3' ITS-1 to 5' 28S regions of *A. cajani* rDNA digested with *Taq* I (a) and *Pst* I + *Nde* II double digests (b). Lanes 1-9 represent samples from the locations B, PSM, BMM, BFI, RM, PTN, KE, KW and K, respectively. Lane M is the molecular weight marker. The variation in restriction profiles of 1a and 1b samples were found to be due to the occurrence of point mutation in the restriction sites of *Taq* I (1a) and *Nde* II (1b).

Figure 38



The restriction profile of complete *A. cajani* rDNA digested with *Pst* I + *Nde* II (A) and *Taq* I (B). Lanes 1-12 represent samples from BNM, KW, EEK, AKM, PSM, BFI, PER, RM, PTN, TK, KW and SID, respectively. Lane M is the molecular weight marker.

Figure 39



The restriction enzyme profile of *A. cajani* rDNA digested with of *Dde* I (A) and *Mse* I (B) digests. Lanes 1-21 represents samples from NEP4, ICR, KM,ICR-N, NEP6, KW, NEP1, NEP-3, PTN, RM, BMM, CT, KM, PTN, KW, TK, RM, BFI, NEP-2, NEP6 and ICR-N, respectively. M is the molecular weight marker. The variation in restriction profile in lane 21 was found to be due to the point mutation in the *Mse* I site (lane 21).

Figure 40

A. Restriction sites of the five enzymes *Mse* I, *Taq* I, *Dde* I, *Pst* I and *Nde* II used for digesting *A. cajani* rDNA.

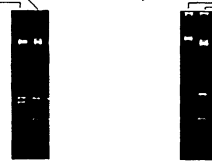
```
                                                                                                                                Dde I
   1 GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAAGTAAAAAACCAAACGCGAGTCTATCTCGGCCCGCGCGCCAAGATAGATTCCGGGAACATATGTAGATAACCTTAGATGTG
     Pst I                                                 Taq I                                                             Taq I Taq I
 139 CTGCAGGTTGCTGGCCACTCATAAGTGTCATGCGCTCTTTGGCTTGTGTCCCATCGAGGGTAGCGTCCACACTTGGCCAGCCAACCGGGCATCATCGCCCCTTTATTCGAACTCGAATACAACCAATGCGACTACCAT
                                                                       Mse I     Nde II        •Nde II•Taq I                                 Pst I
 277 ATCAGTATGGAAGCGCGTGAAAACAACCAATAAACAAAAATACAAACTAAAGACGAAAACATTCACAATTAACGGTGGATCACTTGGGTCGCAGATCGATGAAGAACGCAGCTAGACTGCGATAGGCGGCGCGAACTG
                 Dde I  Taq I                                                                      Mse I
 415 CAGAATAATCTGAGCGCTTGATATTCGAACGCACATTGCGGCTTTGGTTAGCAATAACTAGAGCCTTGCCTGTATGAGGGTCTGTTAGAACTTAAAAAACTCTACTTTCTTTAGTAAAATGAGGACTGCTAGAGATGA

 553 CTTCGGTAACAAGGCCTAGTCATGAAGGGTGTATGAGAACTGGTTTTGGGATGAGTTGCTCGTTTGAACGTACGTCGTGCAACCAATCGTGCAAACCAACCAACCAACCAGTTCCTCAACAAAAACCAATCATCTAGG

 691 CTACTATTGAAAACAGCTTTGCAGATGTGGCTTGTTACGATAGTGTTATACTACACAGGCTCACTTTGCGCAAGTGCGACTATCAATCTTGCCAATAAAGTGGGCCGCTAGTATGCTCGTACACGTCGTACACGAGTT
                                                                  *Mse I      Dde I
 829 TGCAAACACTGCCATACCACTACATGGACCTCATATCAGACAAGATTACCCGCTAAATTTAAGCATATTACTAAGCGGAGGAAAAGAAAGCAAAAGCGATTCCCCAAGTAACGGCGAATGAACAGGGATAAGCCTAGC
       Dde I                       Mse I
 967 GCTTAGGCTCAACAGGATGGCCATGCAAATGGTCGTCGTTTAACCCATAAGGCTGTTGAGCGGTGCAGCGTTACAAACTGCGATGTTTAGTGGAGTCTCTGGTAGTCAGTCTCGGCTGGCTACCTCTAGCCTAGCGTG
                                                    Dde I Pst I Taq I
1105 CAAGTCCCATTGACTTGGGCTAATTGCAAATGACTTGTGGATTGGGGCAACTCAGTCTGCAGGTCGAGCCATAGCGGGTGCAAGTCCCATACCGCGCAGGCATTGGCGCCACGAATCGTAGAGGGCTCCCTAAAAAGG
                                   Mse I                     Mse I
1243 GTCCAATTTGTTAGAGTCGGATTGCTTGAGAGTGCAGTCTTAAGTGCGAGGTAAACTACTCGTAAGGCTAGCTTAATACGGCTGCGACACCGATAGCATACAAGTACCGTGAGGGAAAGTTG 1364
```

***B.** Variation in RE profile due to mutation in *Mse* I site

```
            (TTAA)Mse I
879 CCGCTAAATTTATGCATATTAC 901 ICR2.seq (Mutated)
879 CCGCTAAATTTAAGCATATTAC 901 ICR.seq (Normal
```

•C. Variation in RE profile due to mutation in *Taq* I and *Nde* II sites

```
359 TTGGGTCGCAGATTGATGAAGAACGC 385 B.seq   (Mutated)
359 TTGGGTCGCAGATCGATGAAGAACGC 385 ICP.seq (Normal)
          (GATC)Nde II Taq I(TCGA)
```



4.2.6. *Aceria cajani* Morphology

The results of SEM analysis indicated that there are no consistent differences in morphological features of *A. cajani* from 3 different regions (ICRISAT, Patancheru, India; Nepalgunj, Nepal; and Mygan farm, Myanmar) of the Indian sub-continent. No obvious morphological differences were apparent between male and female mites. Only one form of females were found in the populations suggesting absence of deutogyny (alternate generations of females that are morphologically distinct) in *A. cajani*. However, in a population there were mites with different sizes. These could be due to the occurrence of mites of different stages in life cycle which include, nymphs, adults and gravid females. Structural features of nymphs and adults were similar, except that lesser waxation occurred in immature stages. The morphological features are discussed below.

Structure of A. cajani (see Figures 41-44)

The *A. cajani* body is cylindrical and can be divided into a prodorsum and opisthosoma (Figures 41A and 42A). The opisthosoma is vermiform shaped with broader anterior compared to the caudal region. The posterior region is slightly curved inside giving an oblique shape (Figure 4.12A). The dorsoventral regions of opisthosoma are covered with elongated ridge shaped annuli (also known as microtubercles), which cover the body, forming ring like structures. Some of them are arched covering either dorsal or ventral regions. Because of this, their numbers on the dorsal side and ventral side are not even. The opisthosoma bears four pairs of ventral setae, which include one pair each of genital, lateral, caudal and accessory setae, but no subdorsal setae (Figures 42A and 42B). None of the setae are segmented.

The prodorsum is connate with ornamentation (Figures 41B and 42C). The anteromedian frontal lobe is straight, ending just over the rostrum, and does not contain spinules. No spines are present at the anterior margin of the frontal lobe. The gnathosoma is shorter than the legs. The median line on the dorsal shield is incomplete towards the anterior end (Figure 43 A). The amedian line, originating from the frontal lobe, is slightly wavy and complete, touching the margin of the opisthosoma (Figure 43A). The sub-median line is incomplete with no clear branches.

The area between sub-median line and the margin of prodorsum is irregularly ornamented with dot-like spicules (Figures 43A and 43B). The dorsal tubercle positioned at the posterior end of the prodorsum is ovate with a diagonal axis directing scapular setae towards the posterior end (Figures 43A and 43B).

The genital region of the adults is located towards the anterior end of the opisthosoma (Figures 41C, 41B). In the adult females the genital chamber is broader than their length and covered by a single broad sub-triangular epigynium (also known as genital flap) hinged anteriorly to the body surface (Figures 42B and 43D). The epigynium is devoid of body spicules, but ornamented with longitudinally arranged single row of striae (Figure 43D). Some of the striae are incomplete. The area at the hinge region of the epigynium is ornamented with microspicules. In adult male the progenital chamber is exposed, slightly elevated, ovate and covered with microspicules, without a clear epigynium like structure (Figure 43C). A pair of knob-like protrusions on the male progenital chamber are the external openings of the ejaculatory ducts. Genital opening is absent in nymphs or larvae. Two coxisternal plates are present at the region anterior to the epigium (epimeral region), and bears three pairs of setae (two pairs on fore-coxa and one pair on hind-coxa; Figures 41C, 44A). The coxisternal plates are fused without any clear demarcation.

All stages of *A. cajani* have two pairs of 5 segmented legs, consisting of trochanter, femur, genu, tibia and tarsus. All segments are devoid of spicules and bear setae with the exception of those of the trochanter (in both pairs of legs) and tibia (in second pair of legs only). The setae on femur are placed ventrally. The setae on the genu are the longest and positioned anteriorly. Only the tibia on the first pair of legs bear a short setae. The tarsus bears a pair of setae positioned dorso-laterally and solenidia are positioned between them (Figures 41D, 43B and 44B). The solenidion possess a stout base tapering towards the end with a slightly enlarged apex. Tarsus bears an empodium (also known as featherclaw) at its tip (Figures 41E, 44B and 44C). The empodium is branched symmetrically into 5 rays (Figures 44B and 44C). The empodial rays, with the exception of those placed terminally are branched secondarily into 3 apical rays which possess enlarged pad like tips (Figure 44C).

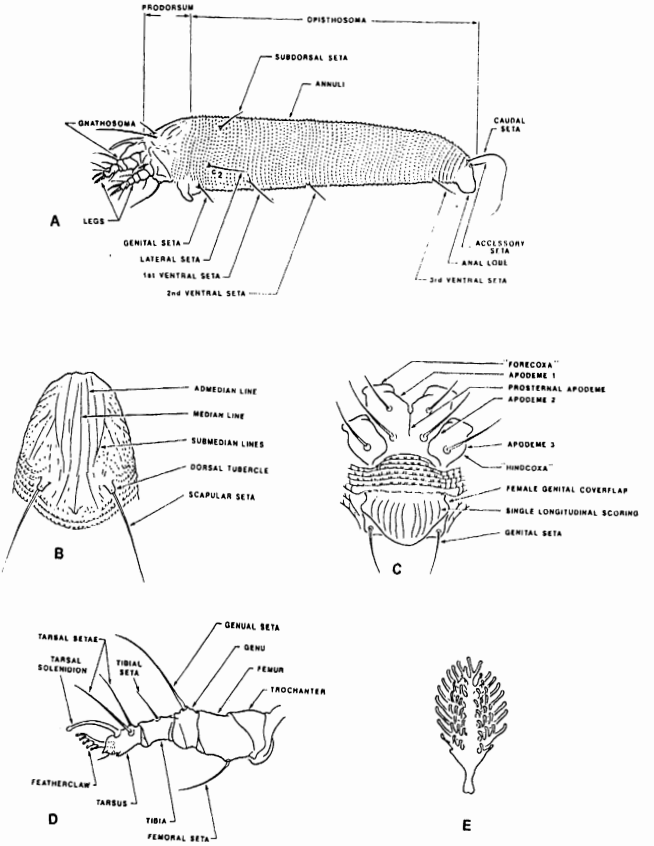
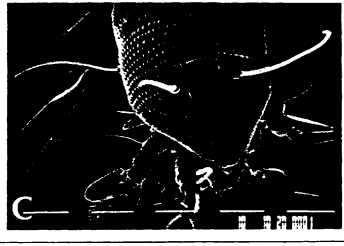
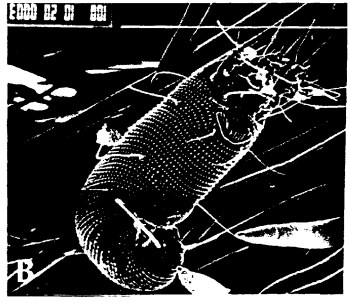


Figure 41 External morphology and notation of structures of *Eriophyoidea* mites adopted from Lindquist and Amrine (1996). A. Body form; B. Prodorsal shield; C. Coxisternal and genital region; D. Leg; E. empodium

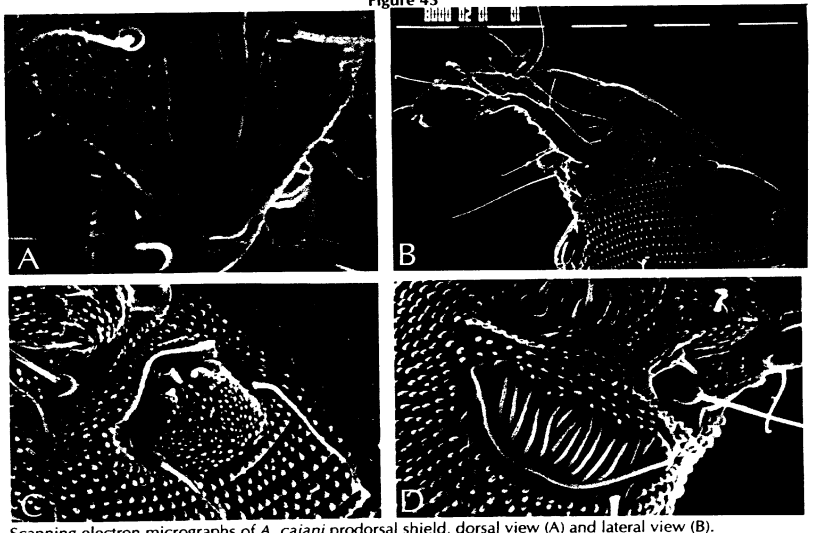
Figure 42





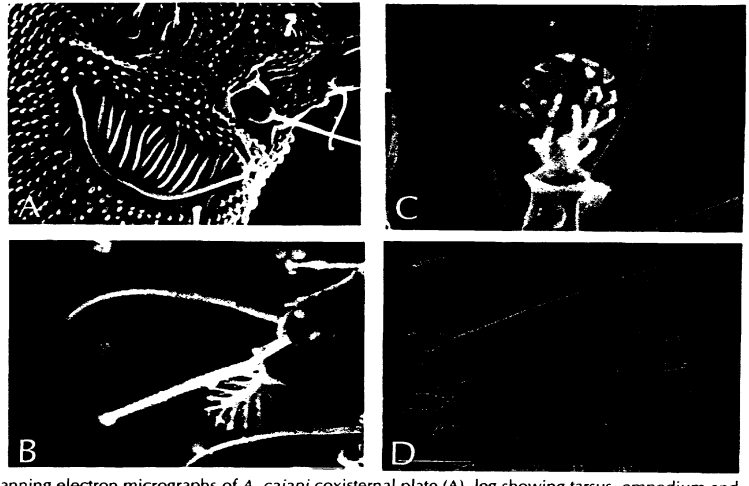
SEM micrographs of *A. cajani*, dorso-lateral view (A), Ventral View (B), and anterodorsal shield showing prodorsal shield, gnathosoma and legs (C).

Figure 43



Scanning electron micrographs of *A. cajani* prodorsal shield, dorsal view (A) and lateral view (B). Anteroventral view of *A. cajani* male (C) and female (D) showing progenital chamber. Note that female progenital chamber covered with epigynium (D).

Figure 44



Scanning electron micrographs of *A. cajani* coxisternal plate (A), leg showing tarsus, empodium and solenidia (B), empodium ventral view showing the apical rays (C). D is SEM of *A. tulipae* leg showing solenidia and empodial rays. Note 5 rays on *A. cajani* (C) and 6 rays on *A. tulipae*.

4.2.6.1. Classification of *A. cajani*

Based on the characters described, *A. cajani* taxonomic position is re-evaluated using a key described for *Eriophyoidae* classification (Amrine and Stasny, 1994; Lindquist and Amrine, 1996).

- Body divided into prodorsum and opisthosoma. Prodorsum covered with a shield and sclerotised plates absent on opisthosoma. Transverse genital opening present. Caudal structure terminates into an adhesive structure. Equal number of setae on nymphs as well as adults.

  Super Family: Eriophyoidea

- Prodorsal shield with 2 setae, gnathosoma curved downwards and ornamentation present on epigynium. Complete coxal and leg setation present.

  Family: Eriophyidae

- Prodorsal shield possess setae; a narrow lobe present at the anterior end of the prodorsal shield. Opisthosoma vermiform. Annuli undifferentiated dorsoventrally. First coxal plate contains two pairs of setae delineated from each other by midsternal line. Epigynium has single row of striae. Tibia distinct from tarsus.

  Sub-family: Eriophyini

- Prodorsal shield setae tubercles located close to the rear margin of the shield, diverging setae posteriorly.

  Tribe: Aceriini

- Gnathosoma shorter than legs. No anterior spines on prodorsal shield.

  Genera: Aceria

- No branches on submedian line of prodorsal shield. Frontal lobe wedge shaped. Spicules present on the lateral side of the prodorsal shield. Empodium with five rays.

  Species: cajani

Aceria cajani was compared with *A. tulipae*, the type member of the genus *Aceria* (Figures 44D; SEMs of *A. tulipae* photographs courtesy Dr. Don Griffiths, Former Director, Central Science Laboratory, UK). These two mites have the same generic characters. Compared to *A. cajani, A. tulipae* possess branched sub-median lines, additional accessory lines on the prodorsum, prodorsal tubercles with horizontal axis, anterofrontal lobe slightly beaked, epigynium with complete striae, and 6 rayed empodium with thread like apical rays (see Table 15; Figure 44D). However, five rayed empodium was noticed in *Phyllocoptes gracilis,* (genera

Table 15
A comparison of morphological features of *Aceria cajani, Aceria tulipae* and *Phyllocoptes gracilis*

Morphological feature	*A. cajani*	*A. tulipae*	*P. gracilis*
Median line	Incomplete	Incomplete	Nearly complete
Sub-median line	Not branched	Branched	Branched
Accessory lines	Absent	Present	Present
Prodorsal tubercles	Ovate with diagonal axis	Horizontal axis	Longitudinal axis
Projection of prodorsal setae	Posterior	Posterior	Upright
Frontal lobe	Wedge shaped	Slightly beaked	Beaked
Striae on epigynium	Single row and few are incomplete	Single row and complete	Single row and complete
Empodial rays	5	6	5
Shape of apical rays	Pad-like	Curved and thread-like	Pad-like

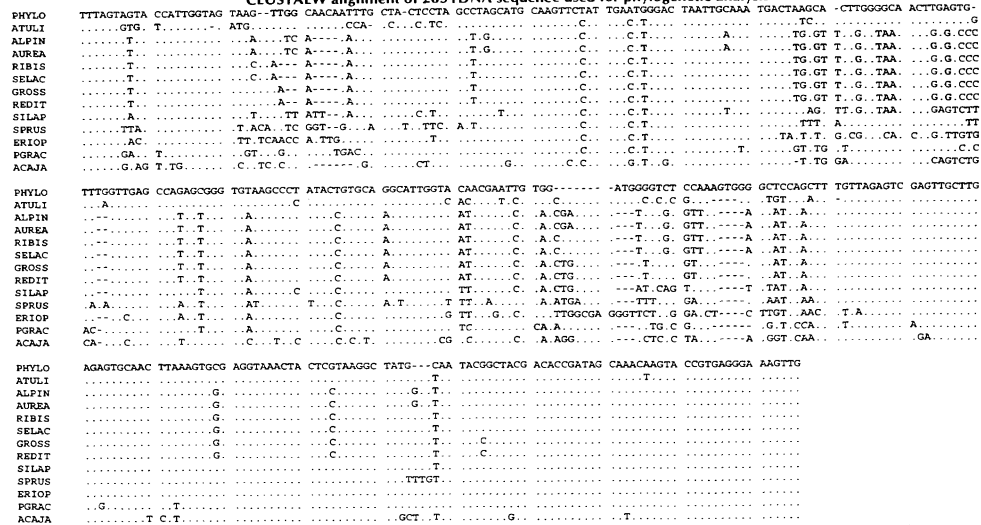
Phyllocoptes; tribe *Phyllocoptini* and sub-family *Phyllocoptinae*). Interestingly, *P. gracilis* shares several features of *A. cajani* and *A. tulipae*. The *P. gracilis* differs in the position and axis of the prodorsal tubercle and the projection of the prodorsal setae (Table 15). It is worthy to note that the division of the sub-families Eriophyinae and Phyllocoptinae are not based on any derived characteristics and they cannot therefore form natural groupings and thus there is scope for reclassification (Lindquist and Amrine, 1996).

4.2.7. Phylogenetic Relations

The rDNA region of *A. cajani* was compared with sequences of other eriophyid mites obtained from different countries and crops (see Table 13). These were from 4 genera of the Eriophyidae family. These include 7 members from the genus *Cecidophyopsis*, 2 each from *Aceria* and *Phylocoptes* and one from *Eriophyes* genus. The only other member outside the family Eriophyidae included was *Nalepella halourga*, (belongs to the family Phytoptidae), which was used as an out-group member to root the tree. The rDNA sequences of these mites were aligned with CLUSTALW. The variation in ITS-1 and ITS-2 sequences among these mites was very high affecting the sequence alignments (see Figure 34). Therefore, regions of sequence ambiguity and positions that were not available for all the sequences for comparison were omitted (i.e. 18S /ITS-1/5.8S/ITS-2 and 3' end of the 28S gene). For phylogenetic analysis about 330 bases of the 5' end of 28S gene from the primer E direction was used. A CLUSTALW alignment of these sequences obtained with default parameters was used for further analysis (Figure 45). The pair-wise distance values estimated using DNADIST method between these mite species are shown in Table 16. The phylogenetic trees obtained using the 3 tree construction programmes (DNAPARS, FITCH and NEIGHBOR) are shown in Figure 46. The results show good support from bootstrapping for the *Cecidophyosis* group and Aceria and Phylocoptes groups. The analysis indicates that *Ribes* infesting mites (*C. ribis, C. grossulariae, C. selachodon, C. aureum, C. alpina* and WC mites) form a close cluster with 100% bootstrap value in all the trees, with *C. psilaspis*, a gymnosperm infesting *Cecidophyopsis* mite rooting out (Figure 46). The *Phyllocoptes* and *Aceria* members group together with the bootstrap value of 74% in the FITCH analysis (Figure 46B), 79% in NEIGHBOR analysis (Figure 46B), but with DNAPARS analysis these members clustered together with only 61% bootstrap value

Figure 45

CLUSTALW alignment of 28S rDNA sequence used for phylogenetic analysis



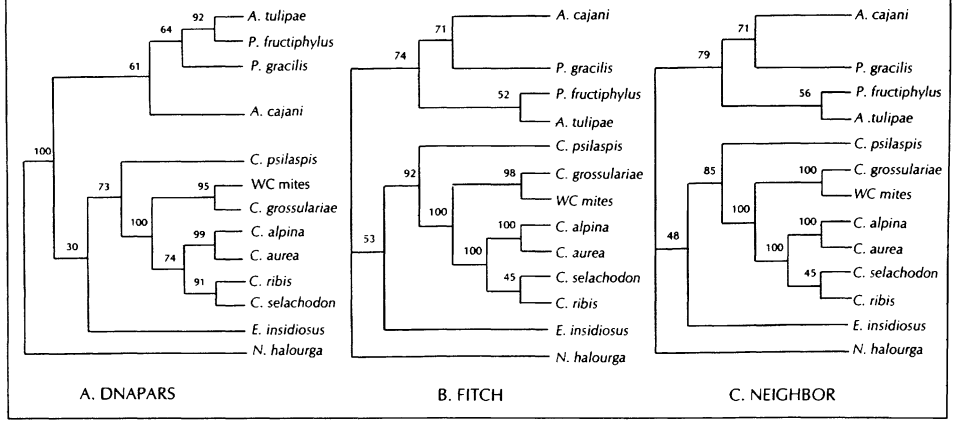
PHYLO: *P. fructiphilus*; ATULI: *A. tulipae*; ALPIN: *C. alpina*; AUREA: *C. aurea*; RIBIS: *C. ribis*; SELAC: *C. selachodon*; GROSS: *C. grossulariae*; REDIT: WC Mites; SILAP: *C. psilaspis*; SPRUS: *N. halourga*; ERIOP: *E. insidiosus*; PGRAC: *P. gracilis*; ACAJA: *A. cajani*

Table 16
Pairwise distance values between various eriophyid mites studied in their 28S gene.

	PHYLO	ATULI	ALPIN	AUREA	RIBIS	SELAC	GROSS	REDIT	SILAP	SPRUS	ERIOP	PGRAC	ACAJA
PHYLO	0.000												
ATULI	0.131	0.000											
ALPIN	0.246	0.267	0.000										
AUREA	0.246	0.267	0.000	0.000									
RIBIS	0.246	0.268	0.016	0.016	0.000								
SELAC	0.246	0.268	0.016	0.016	0.000	0.000							
GROSS	0.226	0.237	0.049	0.049	0.040	0.040	0.000						
REDIT	0.226	0.237	0.049	0.049	0.040	0.040	0.000	0.000					
SILAP	0.224	0.214	0.156	0.156	0.152	0.152	0.142	0.142	0.000				
SPRUS	0.232	0.240	0.245	0.245	0.257	0.257	0.225	0.225	0.196	0.000			
ERIOP	0.301	0.274	0.299	0.299	0.268	0.268	0.258	0.258	0.196	0.284	0.000		
PGRAC	0.190	0.231	0.248	0.248	0.237	0.237	0.240	0.240	0.200	0.217	0.289	0.000	
ACAJA	0.261	0.270	0.332	0.332	0.316	0.316	0.309	0.309	0.266	0.313	0.320	0.258	0.000

PHYLO: *P. fructiphilus*; ATULI: *A. tulipae*; ALPIN: *C. alpinum*; AUREA: *C. aurea*; RIBIS: *C. ribis*; SELAC: *C. selachodon*; GROSS: *C. grossulariae*; REDIT: WC Mites; SILAP: *C. psilaspis*; SPRUS: *N. halourga*; ERIOP: *E. insidiosus*; PGRAC: *P. gracilis*; ACAJA: *A. cajani*

Figure 46



The phylogenetic tree of eriophyid mite 28S gene obtained using DNAPARS (A), FITCH (B) and NEIGHBOR (C) analyses. The values at the forks indicate the number of times out of 100 trees that this grouping occurred after bootstrapping the data.

(Figure 46A). In all these analysis *A. tulipae* clustered with *P. fructiphylus* and *A. cajani* with *P. gracilis,* these groupings are unequivocal, but with low bootstrap values. In all these analyses *E. insidiosus* associated with *Cecidophyopsis* group, supported by a low bootstrap value and *N. halourga* was separated from all the other members.

The trees obtained using DNAPARS, NEIGHBOR and FITCH all gave essentially identical results with the 13 eriophyid mite species from the four genera (*Aceria, Cecidophyopsis, Eriophyies* and *Phyllocoptes),* forming two clusters, when the tree is rooted with *N. halourga*. Groups of closely related species (like *Cecidophyopsis* mites) are well-defined with good support from bootstrapping. The internal structure of the branching pattern (relationships between the member species) in all the trees were almost similar, but with varied bootstrap values. In all the cases *A. tulipae* grouped with *P. fructiphylus* and *A. cajani* with *P. gracilis*. *E. insidiosus* grouped with *Cecidophyopsis* mites, but with low bootstrap value. It is interesting to note that these two groupings correlate with the morphological features. *Ribes* infesting *Cecidophyopsis* are morphologically very similar (Amrine *et al.*, 1994) and in the tree these mites are separated with short branch lengths forming one cluster. *C. psilaspis* which was from a gymnosperm, *Taxus brevifolia,* has all the key morphological features of *Cecidophyopsis* genus (unpublished observation). Though *A. cajani, A. tulipae* and *P. gracilis* belong to different genera as per the present classification, they are forming one cluster. In this and other studies at SCRI on eriophyid mite morphology, similarities were found between these mites (unpublished). *P. fructiphylus* is always closely associated with *A. tulipae* with distinct morphological features on the prodorsal shield (results not shown). *E. insidiosus* has many characteristics similar to the *Aceria* genus, except for the direction of prodorsal setae projection and 4 rays on the empodium. Further studies which include more representative members from these genera are essential to determine the classification of these mites.

Chapter 5

Discussion

5. DISCUSSION

Sterility mosaic disease is one of the major diseases affecting pigeonpea production worth over US$ 100 million per annum in India alone. Options used for integrated management of SMD include; (i) development of resistant cultivars, (ii) cultural practise, which include adjustment of date of sowing and (iii) chemical sprays for controlling eriophyid mites. However, the later two options have limitations and are not likely to be adopted especially by small scale farmers. Therefore, at ICRISAT in collaboration with ICAR centres in India, extensive work has been done for more than three decades to identify sources of resistance to SMD. As a result of these efforts many pigeonpea genotypes with field resistance to SMD have been identified, but the underlying resistant mechanism(s) are not known. However, the majority of them did not support mite multiplication (Reddy and Nene, 1980). In various studies it was found that the resistance offered by various genotypes are location specific (for review see Ghanekar et *al.*, 1992; Reddy et *al.*, 1998).

Earlier, SMD is known to be a serious problem in the northeastern (especially Bihar and Uttar Pradesh) and southern states (particularly Tamil Nadu) of India (Kannaiyan et *al.*, 1984). Recently, severe out breaks of SMD have been reported from Marathwada region of Maharashtra state (Zote et *al.*, 1997) and Gulbarga region in Karnataka state (Dr Jayalakshmi, personal communication). Occasional incidence of SMD has been noted in this regions, but large scale epidemics covering the entire region have never until recently occur. Furthermore, reports of SMD resistant genotypes (eg ICP 2376, ICPL 85073, ICP 7035, Bahar, Maruti) succumbing to infection were attributed to the appearance of new strains of the SMD pathogen (Reddy et *al.*, 1991). Several resistant genotypes previously identified as resistant to SMD were found to be susceptible to SMD (Reddy, 1987; MV Reddy, personal communication). In the recent multilocational trails at different locations in India, out of 102 selections tested, 29 lines were found to be resistant or moderately resistant to

SMD (AICRPP Report, 1999). At least four factors could be contributing to the variation in resistance to SMD at different locations in India. These include; (i) variation in causal agent, (ii) involvement of different mite vectors, other than *A. cajani* in pathogen transmission, (iii) biodiversity among the *A. cajani* populations and (iv) environmental factors. Knowledge on SMD pathogen is essential to understand its biodiversity. This study was undertaken to isolate the causal agent of SMD, and to address the role played by the mite vector to understand the differential host response to SMD.

5.1. Studies on the SMD-Pathogen

Isolation and characterisation of the pathogen(s) associated with a particular disease is the fundamental step to devise a method for identification, to understand its distribution, its host range, variants and to develop suitable resistant sources. Causal agents of the majority of economically important diseases have been identified. There are few important diseases known to be caused by agents of uncertain etiology (Randles, 1993). The SMD of pigeonpea was listed one among them. Though the disease was described nearly 7 decades ago, not much is known about the causal agent, except that it may be due to involvement of a virus. Extensive studies were made at ICRISAT to isolate the SMD pathogen (ICRISAT Ann Rep 1983, 1984, 1985, 1988, 1991, 1992; for reviews Ghanekar *et al.*, 1992; Reddy *et al.*, 1994; Reddy *et al.*, 1998). The summary of findings from these studies are:- (i) SMD is not caused by a fungus, bacteria, phytoplasma, spiroplasma or rickettsia-like organisms or it is caused by a sub-viral agent like viroid or by mite toxaemia, (ii) SMD agent is not mechanically transmissible, (iii) the causal agent could be a virus, (iv) various purification protocols were failed to yield any virus or virus-like agent, (v) SMD resistant pigeonpea genotypes were identified, but the resistance was found to be location specific, (vi) resistance mechanism was not characterised, and (vii) based on differential host reaction, occurrence of 5 strains of the SMD-pathogen were reported.

During the last four years considerable progress has been achieved in understanding the causal agents of mite transmitted diseases (see Table 4). All known mite transmitted viruses are flexuous rod shaped, belong to the families *Potyviridae* and *Closteroviridae*. Recently, association of a spherical virus with reversion disease of blackcurrants, transmitted by *C. ribis* was reported (Lemmetty *et al.*, 1997; Latvala *et al.*, 1997). All these mite-transmitted viruses are mechanically transmissible.

However, unlike mite-transmitted viruses infecting monocotyledonous hosts, mechanical transmission of virus infecting dicotyledonous hosts, such as PMV, CMLV and BRAV infecting peach, cherry and blackcurrants, respectively, were difficult due to the presence of polyphenols and other interfering compounds in the leaves. However, HPV, a tenui-like virus transmitted by *A. tosichella* was not mechanically transmissible (Jensen and Hall, 1995; Jensen *et al.*, 1996). This is more likely to be due to the nature of the virus involved. Unlike luteoviruses and badnaviruses, viruses transmitted by mites are known to be present in all tissues of the plant. Eriophyid mites possess short stylets. In order to acquire viruses, the particles should present in the epidermal and mesophyll cells. It is known that except those viruses that have negative sense genome (eg: tenuiviruses, rhabdoviruses) and restricted to conductive tissues (eg: luteoviruses, badnaviruses), others can be mechanically transmissible.

With this background, it can be assumed that the interference of host polyphenolic compounds or involvement of virus with negative sense genome could be the major factors responsible for preventing mechanical transmission of the agent. In this study two methods were used to elucidate the causal agent of SMD;- (i) Application of a protocol to minimise the host interfering compounds and to transfer pathogen to herbaceous hosts and (ii) Isolation of agent directly from SMD-affected plants, assuming that the causal agent may be a non-mechanically transmissible virus.

5.1.1. Virus isolation method 1

The assumption is that, SMD causal virus exists in low titre and its distribution is erratic in the host plant and polyphenolic compounds are interfering with virus infectivity. Preparation of leaf material in a high volume of a buffer (10 ml buffer/ 1 g leaf material) dilutes the polysaccharides and other host components and concentration by differential centrifugation enriches the virus. The final pellets were used for mechanical inoculation to various experimental hosts. This resulted in transmission of PATV from pigeonpea to *C. quinoa* and *N. benthamiana* only. Subsequent characterisation of PATV was achieved by using infected *C. quiona* as virus source. Mechanical inoculation of PATV onto various herbaceous hosts resulted in chlorotic or necrotic spots on inoculated leaves (see Table 7). Systemic infection was confined to few hosts like *N. clevelandii, N. hispens* and *N. benthamiana* (Figures 4 and 5). Initially systemic infection resulted in mosaic and distortion of the leaf, followed by necrotic spots in the interveinal tissue that expand to entire leaf and

then spreading to entire plant leading to the death of the host. PATV exhibited characteristic features of the genus *Tombusvirus* and *Carmovirus*, which include; icosahedral shaped particles (Figure 10), monopartite ssRNA without a poly(A) tail, presence of two subgenomic RNAs in the purified preparations (Figure 12), production of high number of virus particles in few experimental hosts, tendency to remain localised in infected hosts and invasiveness of host tissues when it spreads systemically (Martelli *et al.*, 1988). Except with PoLV antiserum, PATV did not react with antisera of the several members of the family *Tombusviridae* (Figure 18). PATV genomic RNA was cloned and sequenced. It has very high sequence similarity (94-98%) with the PoLV (see Table 10), a member of the newly proposed genus *Auresovirus* of the family *Tombusviridae* (Rubino and Russo, 1997; Martelli *et al.*, 1998). PATV genome contains 5 ORFs (Figure 17). *In vitro* translation studies were not performed. However, putative amino acid sequence deduced from the 5 ORFs showed high sequence similarity with PoLV (92-98%; see Table 10). The placement of PATV ORFs is similar to that of PoLV and other tombusviruses, indicating that the functional properties of the proteins expressed from the 5 ORFs are similar (Russo *et al.*, 1994; Rubino *et al.*, 1995; Rubino and Russo, 1997).

The most intriguing feature of PATV was generation of DI RNAs. The attenuated symptoms observed in PATV inoculated *N. benthamiana* was attributed to the involvement of DI RNAs (Figure 19). In previous studies differences in biological properties of two viruses having very high sequence homology (95-99%) was reported (d'Aquino *et al.*, 1995; Revers *et al.*, 1997a and 1997b; Hamamoto *et al.*, 1997). Viruses are subjected to processes which generate variation between individuals. Variants, which can be recognised by some characteristic of the phenotype (such as changes in the symptoms) may be classed as distinct strains (van Regenmortel *et al.*, 1997). The existence of virus strains creates problem for classification and identification, particularly, if the strains show considerable variation in biological properties. Sometimes new strains break resistance (eg: Revers *et al.*, 1997a; 1997b) or adopt to new hosts (Hamamoto *et al.*, 1997) or cause altogether new symptoms (eg: d'Aquino *et al.*, 1995). Generation of DI RNAs by PATV significantly distinguishes it from PoLV. It was established that some tombusviruses were able to generate and/or support the replication of either homologous or heterologous DI RNAs, and those infected plants usually develop typical attenuated symptoms. Ability to support or generate DI RNA multiplication is one of the features considered to

differentiate strains (Russo *et al.*, 1994). DI RNAs are thought to be generated by aberrant RNA synthesis by the viral RNA-dependent RNA polymerase (RDRP) resulting in the introduction of deletions into nascent RNA strands (Lazzarini *et al.*, 1981; Perrault, 1981). Despite high similarity between PoLV and PATV at nucleotide and amino acid sequence level, unlike PATV, PoLV was neither generated or supported DI RNAs of CymRSV (Rubino and Russo, 1997). Virus can be demarcated as species by considering number of properties that are not shared by the other members of the genus. PATV and PoLV are similar, except that PATV generates DI RNAs. Therefore, PATV should be considered as a strain of PoLV. Information on aspects leading to the generations of DI RNAs in PATV was not studied.

PATV was isolated from 6 different locations of India (Table 6). Though PATV was isolated from SMD affected plants, experiments using ELISA and RT-PCR failed to detect the virus consistently in the infected plants. Furthermore, this virus was found in few apparently healthy looking plants. This indicates that SMD is not caused by PATV. The results suggests that PATV was found to be associated with pigeonpea at particular locations without causing any overt symptoms. Tombusviruses are very stable viruses and are often found in natural environments (i.e. surface waters and soils) from which host can acquire without the assistance of vectors. These viruses often remained confined to the tissues. However, when systemic infection occurs in the host plant, they become invasive colonising all types of tissues (Martelli *et al.*, 1988). Inoculation of purified PATV preparations to pigeonpea did not result in systemic spread of the disease. PATV isolations were made from the material obtained from the fields. The conditions under which PATV is associated with pigeonpea is not known. Nevertheless this is the first report of a tombusvirus occurring in pigeonpea. This study indicates the need for careful evaluation of viruses isolated from diseases of unknown etiology.

5.1.2. Virus isolation method 2

Attempts to purify a virus directly from SMD-affected leaves using a newly developed protocol resulted in isolation of a PPSMV. This was achieved using a high pH buffer, containing high concentration of sodium sulphite, EDTA, monothioglycerol, DIECA and PVP, followed by clarification with 25% Triton X-100 (alkylphenoxypolyethoxy ethoanol), a non-ionic detergent. The difficulty of purification of virus from pigenpea is thought to be due to the presence of large amounts of mucilage (a herteropolysaccharide), and such phenolic substances as tannins. In addition to this,

cell constituents consisting of proteins, polysaccharides and enzymes, especially ribonucleases, may inactivate the virus. Presence of reducing agents like $NaNO_3$, EDTA, DIECA and monothioglycerol inhibits oxidases and reacts with quines reducing them to phenols (Pierpoint, 1996). PVP reduces the particle precipitation by aggregation and also reduces the activity of tannins. High pH (9) aids in inactivating several enzymes. Clarification of the extract with non-ionic detergent Triton X-100, aids in solubilisation of membrane associated proteins (Han *et al.*, 1999). Furthermore, this also aids in inactivation of endonucleases and prevents particle aggregation. Further purification of the partially purified preparations was achieved by centrifuging over linear sucrose gradients. Centrifugation of preparations derived from sucrose gradients in CsCl gradient improved the quality of the purified preparations.

The characterisation of PPSMV appeared to be more complex than its isolation due to its presence in low concentration in purified preparations. Additionally, the particles were highly unstable and interference of host material occurred when nucleic acids were extracted (see Figure 24). The nucleoproteins obtained from purified preparations contained highly flexuous filamentous particles of unusual morphology in various forms (branched, filamentous, spiral and circular) of 8-11 nm in diameter (Figures 27 and 28), a 32 kDa protein (Figure 23) and 5-8 RNA species (Figure 24C). Several aspects of the molecular characterisation of PPSMV suggested similarities to tenuiviruses (Kumar *et al.*, 1999b and 1999c). A coat protein of 32-35 kDa, termed as ribonucleoprotein particles (RNPs) is consistent with tenuiviruses (for review see Falk and Tsai, 1998). PPSMV particles, similar to that of tenuiviruses, sediment often as 3 and sometimes 4 diffused bands in sucrose density gradients (Figure 21). Sedimentation of maize strip virus (MSpV) as 4 components, and rice grassy stunt (RGSV), rice stripe (RSV) and rice hoja balnca (RHBV) viruses as 3-5 components in sucrose density gradients were reported (Falk and Tsai, 1984; Hibino *et al.*, 1985; Ishikawa *et al.*, 1989; de Miranda *et al.*, 1996). In contrast to separation of PPSMV particles as multiple bands in sucrose gradients, they band together as single zone in CsCl gradients, another feature of tenuiviruses (Gingery *et al.*, 1981). The isolation of 5-8 nucleic acid species would also be indicative of tenuiviruses. Isolation of different-sized RNAs from different components and presence of single and double stranded RNAs in purified tenuivirus preparations were reported (Toriyama, 1982a; Falk and Tsai, 1984 and 1998; Ramirez and Haenni, 1994). However, strandedness of the RNA isolated from PPSMV purified preparations was

determined. PPSMV particle morphology in various forms, but similar to those reported for tenuiviruses is an important feature that relate PPSMV to tenuiviruses (Gingery, 1985;Toriyama, 1995). However, all currently known tenuviruses infect monocotyledons plants, transmitted by delphacid plant-hoppers and induce cellular inclusion bodies. To our knowledge PPSMV is the first 'tenui-like' virus isolated from a dicotyledonous plant, transmitted by an eriophyid mite. Nucleotide sequence information of genomic RNA is essential to understand the taxonomic status and relation with the existing tenuiviruses.

PPSMV have similarities with recently described HPV (Jensen *et al.*, 1996). Both these viruses have a 32 kDa protein, 5-8 RNA species, transmitted by eriophyid mite vectors and are not mechanically transmissible. However, in AGDD test PPSMV did not react with HPV antiserum (results not shown). DMBs were reported to be associated with HPV. Association of DMBs with SMD-affected pigeonpea leaves have been reported (ICRISAT Ann. Rep., 1989). In addition to PPSMV and HPV, DMBs were found with other mite-transmitted diseases such as, fig mosaic (Bradfute *et al.*, 1970), thistle mosaic (Ahn *et al.*, 1993), rose rosette (Kim and Gergerich, 1994) and redbud yellow ringspot (Ahn *et al.*, 1996), for which no causal agent has been identified. It is likely that 'tenui-like' viruses are involved with the etiology of these diseases. The method used for PPSMV isolation can be applied for the isolation of these agents.

Unequivocal evidence that PPSMV is the causal agent of SMD depends on the ability to fulfil Koch's postulates by reinfecting pigeonpea with the purified virus and producing the disease. Several technical difficulties prevent this. Firstly, PPSMV is highly unstable, secondly, mechanical inoculation onto pigeonpea is difficult process due to involvement of high levels of tannins and polyphenols in leaves, as well as the nature of the virus. Despite the absence of a diagnostic test, the consistent detection of virus in the infected plants collected from different places, especially from the cultures established in growth chambers inoculated with mites, indicates that PPSMV is transmitted by *A. cajani*. Further characterisation of PPSMV and development of diagnostic tools are underway.

Funding has been obtained from Natural Resource International, United Kingdom [Project No. ZA0321 (R7452)], to further characterise the PPSMV, develop diagnostic tests, to study vector relationships and cytological effects, and to understand its variants and interaction with various pigeonpea genotypes.

5.2. Studies on *Aceria cajani* Population Diversity

Eriophyid mites are obligate plant pests in all stages of their life cycle. Due to this habit, they are potentially destructive to agriculture and hamper production to a great extent. Several of them cause direct damage by affecting plant growth in various ways and some, indirectly by acting as vectors to several important plant viruses (Keifer *et al.*, 1982). *Aceria cajani* inhabits pigeonpea, but causes no obvious damage to the host. However, it is important as it transmits the causal virus of SMD.

The occurrence of biological strains within pest species (often referred to as "biotypes" or "pathotypes") that differ physiologically, but not morphologically, has been known in several organisms (Milgrooom and Fry, 1997), but little is known about such forms in eriophyid mites. A diverse range of novel protein and DNA-based molecular markers have been applied to investigate this problem, especially to understand the evolution, ecology and population dynamics of agricultural pests (Loxdale and Hollander, 1989; Smith and Wayne, 1996; Symondson and Liddell, 1996). However, with Eriophyid mites formidable technical difficulties due to their microscopic size, soft body and presence in low numbers on host plants prevented application of many of these methods to *A. cajani*. Studies on another group of Eriophyids, the *Cecidophyopsis* mites, have examined the genetic variability and species status using particularly suited molecular methods suggest that these provide a useful alternative tool for mite identification.

For example, amplifying nuclear rDNA ITS regions and subsequently digesting with restriction enzymes revealed the existence of species-specific differences amongst what had previously been considered biotypes (Fenton *et al.*, 1995). Ribosomal DNA based diagnostic assays have several advantages, especially for eriophyid mite analysis. It is an extremely well studied gene family and its occurrence in many copies (> 100) in an individual makes it a good target for PCR amplification from small amounts of DNA. The rDNA is transcribed to generate rRNA which is then processed further to form part of the functional ribosome. Between the rRNA genes are spacer regions (ITS and IGS; see Figure 2) which are either transcribed or are processed out of the mature rRNA (Eichler and Craig, 1994). The structure and sequence of the rRNA coding genes are highly conserved. The two ITS regions between the coding regions diverge quite rapidly between species, but are highly conserved within several eukaryotic species (Hills and Dixon, 1991). Different copies of rDNA evolve together and are found to be homogenous. This is due to processes

such as unequal crossover and gene conversion, collectively known as molecular drive (Dover, 1982). Due to this many organisms show little intra-specific ITS-sequence variation (Navajas *et al.*, 1994; Fenton *et al.*, 1997). However, other organisms display very high levels of variation suggesting molecular drive had not caused sufficient homogenisation (Wesson *et al.*, 1992; Vogler and DeSalle, 1994; McLean 1995). PCR has revolutionised isolation and analysis of sequenced genomes (Saiki *et al.*, 1988). The rDNA multigene family is one of the simplest targets as it offers multiple templates and conserved sequences can be used to design primers that can be used for the amplification of rDNA of several species. Because of this, rDNA ITS regions are extensively used in the examination of taxonomic status of species, as well as for diagnostic purpose (Collins and Paskewitz, 1996). Further, its utility has been confirmed in Acarids (Navajas *et al.*, 1994) and Eriophyids in particular (Fenton *et al.*, 1997; Kumar *et al.*, 1999a).

In this study, the *A. cajani* population diversity in SMD endemic regions was assessed using rDNA as a marker. Universal as well as *A. cajani*-specific primers designed in this study, were used to amplify the regions between the 18S and 28S genes, covering two ITS regions and part of the 5' end of the 28S gene (Table14). Isolation and purification of DNA by effectively lysing the cells was the first step, which should be accomplished under conditions to prevent action of contaminating nucleases and by applying gentle method to minimise DNA shearing. A simple protocol used in this study for isolating DNA from mites was found to be effective. Though DNA isolated from 20 mites was found to be sufficient for successful amplification of target region, from some samples that number could not be obtained. Further, transportation of samples from distant places took more than two weeks. Because of the soft body mites tend to be decomposed rapidly by saprophytic fungi (McCoy, 1996). DNA derived from such degraded tissue is often highly fragmented due to autolysis, and to the degradation by micro-organisms. These factors can seriously affect the PCR efficiency (Golenberg *et al.*, 1996). Failure of amplification of long PCR products, but successful amplification of short products suggests that DNA degradation is occurring in such samples. This was a constraint preventing analysis of some of the current samples.

Aceria cajani samples from various locations of India, Nepal and Myanmar were analysed (Table 12). These were mainly from southern, north-western and south-central regions of India and selected sites of Nepal and Myanmar, represent:- samples

from a similar location collected over different seasons and within the same location at different places. This samples include from farmers fields and experimental plots. Many of the samples analysed were from pigeonpea crops sown in early (June-Aug) or late (Sep-Dec) monsoon season during the year 1996, 1997 and 1998. Analysis of these *A. cajani* by rDNA PCR/restriction enzymes digestion revealed no variation in the rDNA finger-print patterns among populations (see Figures 36-39). Lack of intra-population variation was also confirmed by analysing different numbers of plasmid clones generated from the rDNA of mites from each location. However, on a few instances intra-population variation in RFLP patters was observed (Figure 40). This was later confirmed to be due to point mutations, presumably occurring during the PCR process, and not due to natural variation (discussed below). Nucleotide sequence comparisons of rDNA of *A. cajani* mites from several locations of India also revealed very little variation (Figure 33). However, when the sequence of *A. cajani* mites were compared with other eriophyid mites, the extent of sequence variation observed between them is significant enough to discriminate *A. cajani* as a distinct species (Figure 34). The absence of intraspecific variation was noted not only in *A. cajani*, but also in other *Cecidophyposis* mites studied so far (Fenton *et al.*, 1997), and in cassava green mites (Navajas *et al.*, 1994). Restriction enzyme profiles of the rDNA of the Nepal mite sample was similar to that of the *A. cajani* populations analysed from India (Figure 39). Due to the difficulty in getting full rDNA regions from the Myanmar mite samples, no RFLP analysis was done. Comparison of the nucleotide sequence of the rDNA of mites from Nepal with those obtained from India showed a high sequence homology between them. With the exception of a few point mutations, no major variation was detected between the sequences. Sequence information corresponding to the 5' end of 18S and ITS-1 regions of rDNA from the Myanmar samples was also identical to that of other *A. cajani* sequences (Figure 33). Despite the incompleteness of the rDNA sequence from the Myanmar samples, these results confirm the lack of variability in rDNA regions of *A. cajani* population from different locations in India, Nepal and Myanmar.

Lack of intraspecific variation in rDNA regions is not restricted to *A. cajani*, but in other eriophyid mites studied so far (B. Fenton and L. Kumar, unpublished). Studies on the rDNA regions of *Ribes* infesting *Cecidophyopsis* mites revealed that the sequences of the different mite species are 92-99% identical (Fenton *et al.*, 1997; Kumar *et al.*, 1999). Inter-specific, but not intra-specific differences were found in the

variable simple sequence repeat regions in the ITS-1 region of these mites. It is to note that the morphological differences between these mites are minor, and previously they were considered as host specific biotypes of the same species (Easterbook, 1980; Amrine *et al.*, 1994). While the morphological differences between these mites are minor these mites are biologically quite distinct. For example, *C. spicatum* and *C. selachodon* were found on the same host (wild redcurrants) without interbreeding (Fenton *et al.*, 1996). Further analysis found that *C. selachodon* was host specific to redcurrant but *C. spicata* could colonise blackcurrant in addition to redcurrant (Fenton *et al.*,1996). The rDNA sequences can also indicate how closely mites are related, for example *C. selachodon* and *C. ribis and C. grossulariae* and WC mites are 99% similar within the pairs, but only about 93% between the pairs, suggesting that they are two distinct groups (Kumar *et al.*, 1999a). The rDNA sequences of *C. ribis* studied from UK and New Zealand; *C. psilaspis* from UK and Canada; and *C. grossulariae* from USA and Europe are 100% identical within their respective groups (Fenton *et al.*, 1996; Kumar *et al.*, 1999a; B. Fenton and L. Kumar, unpublished). All these examples, and the studies on *A. cajani* rDNA suggest that rDNA sequence of an individual eriophyid mite species is unique and homogeneous. Differences in biological properties, such as variation in stylet length, are therefore likely to be due to genetic variation within a species and not due to the presence of different species.

Several factors associated with life cycle of eriophyid mites should be contributing to lack of intraspecific variation at the rDNA locus. These mites reproduce using arrhentoky with haploid eggs developing as males (Evans, 1992). They are wingless and therefore have limited natural mobility, they are also highly host specific and it can be predicted that the rDNA arrays in these organisms are confined to a single chromosome (Note: only report of Eriophyid chromosomes is that there are only N = 2; Helle and Wysoki, 1984). These factors might significantly reduce the levels of intraspecific variation. Studies on ITS regions of *Drosophila melanogaster* showed that ITS copies are more likely to be homogenous when they were on the same chromosome, suggesting molecular drive is more efficient within homologous chromosomes than between them (Schlotterer and Tautz, 1994). In contrast to eriophyid mites, ITS regions of ticks (Rich *et al.*, 1997), tiger beetles (Vogler and DeSalle, 1994), and mosquitoes (McLain *et al.*, 1995) showed greater levels of intraspecific and intra-individual variation. This indicates that ribosomal arrays in different organisms are not always homogenous. Careful studies to

understand variation in different regions of rDNA is essential before utilising the data for the identification of species.

In this study it was found that variation in RFLP patterns observed in few samples could be due to the errors made by *Taq* polymerase during the PCR process (Figures 37, 39 and 40). A single base mutation in RE site eliminates or creates a RE site contributing to the variation in RE profile (Figure 40A). Some thermostable enzymes such as *Taq* polymerase lack proof reading (3'->5' exonuclease) activity. Different thermostable enzymes used in PCR have different error incorporation rates. The most popular enzyme, *Taq* polymearase is estimated to have 1.1x10-4 to 2.4x10-4 errors/base (Keohavang and Thilly, 1989; Barnes, 1992); 1.1x10-4 base substitutions/base (Tindall and Kunkel, 1988) and 2.4x10-5 frameshift mutations/bp (Tindall and Kunkel, 1988). This suggests that characterisation of the selected rDNA or other targeted sequence is essential before utilising the region for PCR/RFLP based analysis to understand the population structure and to eliminate real variation from the variation due to artefacts during the experimental process.

In addition to rDNA analysis, morphological characteristics of the mites obtained from SMD-affected pigeonpea samples from India, Nepal and Myanmar were also studied. The structures considered for *Eriophyoidea* taxonomy are derived from body parts and its appendages (Figure 41). However, due to considerable reduction and simplification of body features and lack of ontogenic diversity and convergent evolution, structures available for taxonomy are relatively few. Furthermore, different authors have considered different structures to classify *Eriophyoid*ae mites into families, sub-families, tribes and genera. (Keifer, 1975; Manson, 1984; Schevchenko *et al.*, 1991; Lindquist and Amrine, 1996). In the present study SEM was used for studying the morphology of *A. cajani* and the study is restricted to structural observations than for morphometrics (Figures 42-44). The morphological features observed in this study were used to classify mites according to the classification of Amrine and Stasny (1994). Note that, Channabasavanna (1966) has studied samples from three locations in India, Hebbal, Bangalore, Karnataka state; Poona, Maharashtra state and IARI, New Delhi. In this study, samples observed were from ICRISAT, Patancheru, Andhra Pradesh state, India; Nepalgunj, Nepal; and Mygan farm, Myanmar. The description of mites obtained from different places of India, Nepal and Myanmar, is similar to that described for *A. cajani* by Channabasavanna (1966), indicating that the mites inhabiting pigeonpea is only one species. However,

some minor differences were noted. For instance, in the previous description *A. cajani* was described as having a nearly complete median line, bare coxae and 6 rayed empodium (feather claw). In this study we observed that the median line is incomplete (see Figure 43A), the coxa is ornamented (Figure 43D) and possesses 5 rayed empodium (Figure 44C). SEM provided high resolution pictures of various structures which facilitated observation up to minute details. Some other members of the genus *Aceria* were described as having 6 rayed empodium (Channabasavanna, 1966). However, Amrine and Stansey (1994) did not consider empodial rays to delineate mites into families or genera. Channabasavanna (1966), in his description has mentioned *A. cajani* as morphologically close to *A. neocynarae* Keifer. However, *A. cajani* could not be compared with *A. neocynarae* due to the lack of a full description of the latter.

Interestingly the mite populations derived from the regions where pigeonpea genotypes showed variation in reaction to SMD (Puddukkotai, Tamil Nadu state, Badanpura, Maharastra state and ICRISAT, AP state) were similar to those elsewhere. However, it should be noted that mite samples were obtained from SMD affected cultivars, the host genotype was not known for the field collected samples, as most of them are local varieties. Nevertheless, the present data suggest that *A. cajani* mites are genetically uniform, and the observations recorded are not likely to be influenced by the host genotype. The variability in pigeonpea resistance may be due to the occurrence of different strains of the SMD pathogen and not due to different strains of the mite vector. The same genotype, identified as resistant to SMD at one location, was susceptible at a different location. Since mite populations from these locations showed similarity, the pathogen variability is likely to be contributed to the differential reaction by these genotypes. Environmental factors may not be having a profound influence, because the host reaction at a particular site is independent of different growing seasons.

From the published information available on mite vectors, it is possible to generalise the relationship between pathogens and their Eriophyid vectors as being highly intimate and very specific (Oldfield, 1994). Although *A. tulipae* and *Abacarus hystrix* are reported as vectors of two plant pathogens, no other mite vector has been shown to transmit more than one pathogen and no virus has been shown to be transmitted by two different mite species (Oldfield and Proeseler, 1996). Virus-vector specificity in mite transmission was first demonstrated by Slykhuis (1969). Thereafter,

Paliwal (1980) showed *A. tulipae* can only transmit the WSMV, although *Abacarus hystrix* (a vector of RGMV) can multiply on WSMV infected wheat, but cannot transmit WSMV. Blackcurrant reversion agent is vectored only by *Cecidophyopsis ribis*, though in the recent studies it was found that *C. grossulariae* can colonise blackcurrants, although there is no evidence that it can transmit the reversion agent (AT Jones, personal communication). These findings suggest involvement of some receptors in vectors which determine vector specificity. This suggests that other mite species are unlikely to be involved in the transmission of SMD virus .

Studies on population characteristics of *Nephotettix virescens,* (Hemiptera: Cicadellidae) colonies revealed that their behaviour varied on different cultivars with the same resistant mechanism, indicating variation in resistance observed could be due to host factors (Dahal *et al.*, 1997). Involvement of minor genes in determining resistance in various cultivars were suspected to be the possible reason (Heinrichs and Rapusas, 1985). The resistance mechanisms to leaf hoppers and mites may be different. Though studies were not performed to understand the mechanisms adopted by plants to resist eriophyid mites, it was assumed to be similar to those used against other arthropods (Westphal *et al.*, 1996). Both constitutive (morphological and chemical factors) and induced (operative after the invasion by specific organism) resistance are involved in host plant resistance. One of the reason for the non-preference of *A. cajani* to some pigeonpea genotypes is a degree of cuticle thickness (Reddy *et al.*, 1995). In pigeonpea, resistant mechanism involving host gene(s) is not known, but it is likely that multiple genes might be involved in determining this resistance. Further clarification of the genetics of resistance to mites is desirable, in order to develop promising SMD resistant cultivars.

5.2.1. Phylogenetic studies

Phylogenetic relationships among 13 mite species of 4 genera of *Eriophyoidea* family were inferred from nucleotide variation in part of the 28S rDNA gene (Table 13; Figure 45). Although the complete rDNA sequences (region between primers C and E) for these 13 mite species are available the variation in length of the ITS regions makes unambiguous alignment of the sequence impossible (results not shown). It has been suggested that regions of DNA that are greater than 70% but less than 100% similar are best suited for phylogenetic studies (Hills and Dixon, 1991). Moreover, different regions of rDNA evolve at different rates and regions of rDNA arrays that are

particularly likely to yield informative data for systematic question can be selected for phylogenetic analysis. In the initial studies to assess relationships among these mites, complete rDNA amplified by primer pair C+E was aligned and analysed (results not shown). This posed a problem for determining species-level relationships. Various segments of rDNA was tested and about 350 bases from primer E direction of 28S gene was selected for assessing the relations among these mites (Figure 45). The advantages of using this region are:- it has many divergent domains and it varies considerably among phyla (Hassouna *et al.*, 1984). Further, these regions have been used to examine late and recent evolutionary events (eg: Hills *et al.*, 1991). Two methods were used to estimate phylogenetic trees (Felsenstein, 1993). The DNA parsimony programme, DNAPARS, carries out unrooted parsimony on DNA sequence data sets. DNA-distance based method DNADIST computes a distance matrix. The distance for each pair of species estimates the total branch length between the two species, which were used in the distance matrix programmes FITCH and NEIGHBOR. The trees generated (see Figure 46) using the three tree construction programmes were supported with good bootstrap values and indicated that:- (1) the 13 mites formed 3 groups, and to an extent supports the classification based on morphological features, (2) *Aceria* and *Phylocoptes* species share a common clade showing close affinities between these two genera, suggesting that the morphological features used to distinguishe these genera may not be based on truly derived characters, (3) *Ribes* infesting *Cecidophyopsis* mites grouped closely, and separated clearly from a gymnosperm infesting *C. psilaspis*, suggesting a common origin, (4) *N. halourga* of the family *Phytoptidae* rooted out from the other members belong to the family *Eriophyidae*, (5) phylogenetic estimation using sequences of the 28S region is suitable for classification of Eriophyid mites up to the levels of genus and species.

Further studies by including more representative members from these genera is essential to assess the classification of these mites. Nevertheless, in this study with the economically important eriophyid mites, clearly showed the importance of rDNA sequences for unambiguous identification of eriophyid mites. It also highlighted the importance of studying morphological features especially by SEM and rDNA, for delineating the species differences and to determine which are the important taxonomical morphological features.

Chapter 6

Summary and Conclusions

6. SUMMARY AND CONCLUSIONS

6.1. Summary

Pigeonpea is an important pulse crop in the semi-arid tropics of Asia, Africa and the Caribbean. Production of pigeonpea in India and several other Asian countries is seriously affected by sterility mosaic disease (SMD). This disease was described in the early 1930s. Despite intense efforts, especially during the last fifteen years, the causal agent has remained elusive and enigmatic. It is transmitted by the eriophyid mite, *Aceria cajani* and experimentally by grafting, but not my mechanical inoculation of sap. Diagnostic tools are not available for accurate confirmation of the disease. Losses due to SMD, if infection is early in the season, may be >90%. Several new pigeonpea varieties with field-resistance to SMD have been identified. The mechanism(s) underlying this resistance have not been characterised. However, field-resistance was assumed to be due to resistance to the mite vector and to the pathogen or to both. In different localities in India several resistant genotypes released in the 1980s have become infected. This breakdown in resistance is attributed to the occurrence either of different *A. cajani* biotypes, or different species of *Aceria* mites acting as vectors, or to the occurrence of different strains of the causal agent. This study was undertaken to isolate the agent causing SMD, and to understand the variation in *A. cajani* populations in SMD endemic regions.

I. Studies on the isolation of the causal agent of SMD

Two different approaches were used for the isolation of the causal agent of SMD assuming it is a virus. (i) Mechanical transmission from SMD-affected plants to herbaceous hosts and subsequent characterisation of the virus, and (ii) purification and characterisation of virus directly from infected pigeonpea plants (see Figure 47). For this purpose SMD-affected samples maintained in a glasshouse and those obtained from experimental plots, and from farmers' fields were used.

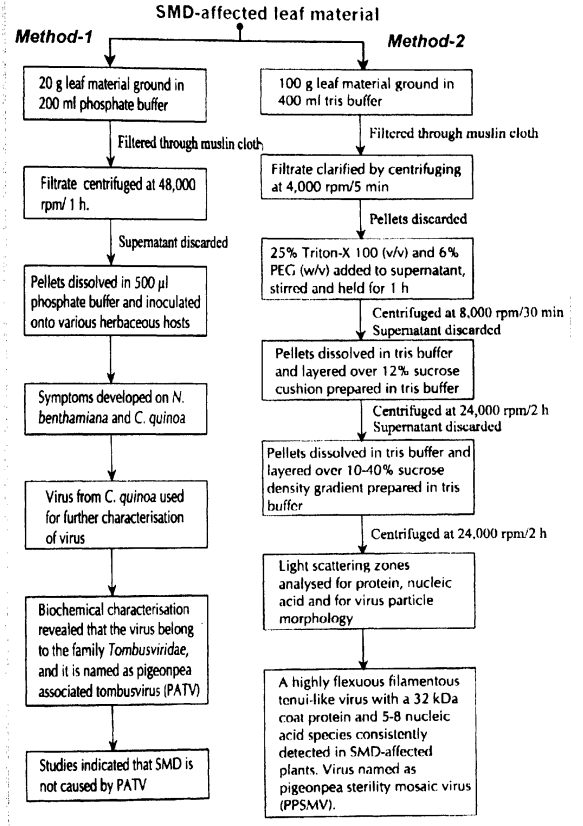
Approach 1:

- Attempts were made to transmit the causal agent from concentrated SMD-affected pigeonpea leaves by mechanical inoculation of sap on to a range of herbaceous test plants.
- This resulted in the isolation of a tombusvirus (named as PATV). This virus was isolated from the samples obtained from six different locations in India.
- The virus was stable, occurred in only very low concentration in pigeonpea extracts, but reached very high concentration in some experimental herbaceous hosts such as *N. clevelandii* and *N. benthamiana*. For subsequent characterisation PATV was propagated in *N. clevelandii*.
- Purified virus particles were isometric with a diameter of ~30 nm. Virus particles contained a single coat protein of about 43 kDa and a positive sense, single stranded RNA of about 4.4 kb; in some virus preparations smaller RNA species of about 2.7 and 1.5 kb were also present, presumably due to the encapsidation of sub-genomic RNAs. The virus genomic RNA was sequenced (Acc.# AJ243370) and was shown to contain 4354 nts, coding for 5 ORFs.
- PATV is closely related serologically to *Pothos latent virus* (PoLV) of the family *Tombusviridae*. PATV and PoLV RNA sequences are 90% identical. However, unlike PoLV, serial passages of PATV in herbaceous hosts resulted in alteration in symptom expression, presumably due to the generation of defective interfering (DI) RNA's.
- On the basis of the biological and physico-chemical properties, the virus was identified as a member of the family *Tombusviridae*.
- Attempts to detect PATV in a range of SMD-affected plants by ELISA and RT-PCR have failed to yield consistent results.
- Mechanical inoculation with purified preparations of this virus to pigeonpea resulted in infection and necrosis of the inoculated leaves but without systemic spread. Symptoms therefore did not resemble SMD symptoms. Furthermore, PATV was detected in few apparently healthy looking pigeonpea plants
- These studies suggest that PATV is not specifically associated with SMD and its role in pathogenesis is to be determined.

Approach 2:

- Various protocols reported for the successful isolation of mite-transmitted viruses were initially tested.
- Samples made during various steps of the purification procedure were monitored by electron microscopy and polypeptide analysis by SDS-polyacrylamide gel electrophoresis.
- A modified protocol derived from the procedures used for the isolation of peach mosaic virus and maize stripe virus was developed.
- It involved the use of tris buffer, pH 9.0, containing DIECA, sodium sulphite, 1-monothioglycerol and Triton X-100 for extraction and precipitation of virus particles by polyethylene glycol.
- Further purification was achieved by two cycles of quasi-equilibrium zonal density gradient centrifugation in a 10-40% sucrose solution in tris buffer.
- Four light scattering zones were observed. All light scattering fractions from SMD-affected and healthy pigeonpea leaves were collected and analysed for protein and nucleic acid.
- In electron microscopic studies, purified preparations from SMD-affected pigeonpea plants revealed aggregates of highly flexuous filamentous virus particles of 8-11 nm diameter.
- Three major polypeptides of molecular weight 52, 32 and 30 kDa were observed in all pigeonpea samples, but the 32 kDa protein was only present in extracts from SMD-affected plants.
- Purified virus particle preparations contained five to eight RNA species of size 3.5 to 0.5 kb.
- Comparable healthy pigeonpea leaves, processed by using the same protocol as that used for virus purification, were found to be free from virus particles and these RNA species.
- This virus was isolated consistently from 36 different SMD-affected plant samples collected from four different locations in India, but not from any of 30 apparently healthy plants sampled from the same locations.
- A similar virus was isolated from SMD-affected pigeonpea samples inoculated at the 2-leaf stage with infective mites (*A. cajani*) and maintained subsequently in a growth chamber.
- This evidence indicates that this virus is most likely the causal agent of SMD, and is provisionally named pigeonpea sterility mosaic virus (PPSMV).
- PPSMV has many similarities with the virus recently described from cereals in North America (HPV), and transmitted by the eriophyid mite, *Aceria tosichella*. However, in agar gel double diffusion and immunoblot assays, HPV antiserum failed to react with PPSMV.

Figure 47
Summary of the approaches used to isolate virus causing SMD



II. Analysis of *Aceria cajani* Populations

- For population analysis, mites were obtained from SMD-affected leaves from 58 different locations in India, 5 locations in Myanmar and 6 locations in Nepal.
- The scheme applied to study *A. cajani* population diversity is shown in Figure 48.
- Variation within and between mite populations was addressed by studying variation in the nuclear ribosomal encoding genes and associated transcribed spacers, known collectively as ribosomal DNA (rDNA).
- A PCR-based method was standardised to amplify rDNA with universal primers corresponding to the conserved regions (18S and 28S sub-units) and *A. cajani*-specific primers corresponding to unique sequences in the ITS-1 region, developed in this study.
- Total nucleic acid extracted from the mites was used for rDNA amplification by PCR.
- Amplified products were cloned into plasmid vectors and transformed into bacteria. Transformed clones containing copies of rDNA were selected and used for restriction enzyme analysis and sequencing.
- Variation in the amplified products was analysed by digestion with various restriction enzymes and also by nucleotide sequencing.
- rDNA analysis by restriction enzymes of different mite samples from India, Nepal and Myanmar revealed virtually no significant variation within or between populations. On few occasions variation observed in RFLP patterns were found to be due to errors produced during PCR.
- Nucleotide sequence comparison of rDNA of various isolates of *A. cajani* confirmed the high homogeneity within and between populations.
- Variation in rDNA regions was significant when compared with other eriophyid mite rDNA sequences.
- Mite morphological studies by scanning electron microscopy revealed no major differences in structural features among populations of *A. cajani*.
- In particular, the *A. cajani* populations derived from the regions where pigeonpea genotypes have shown variation in reaction to SMD, were found to be very similar to those elsewhere.
- This study also showed no significant variation in mite populations collected from selected sites in Nepal and Myanmar, suggesting that there were no other eriophyid mite species which could vector the causal agent of SMD and contribute to the breakdown in resistance. It is also presumed that biotypes of *A. cajani* do not exist.
- Phylogenetic trees constructed using part of the 28S gene for mite species from 5 genera indicated a correlation between conventional classification of eriophyid mites and the DNA-based methods using rDNA as a marker.

The approaches used to study *Aceria cajani* population diversity

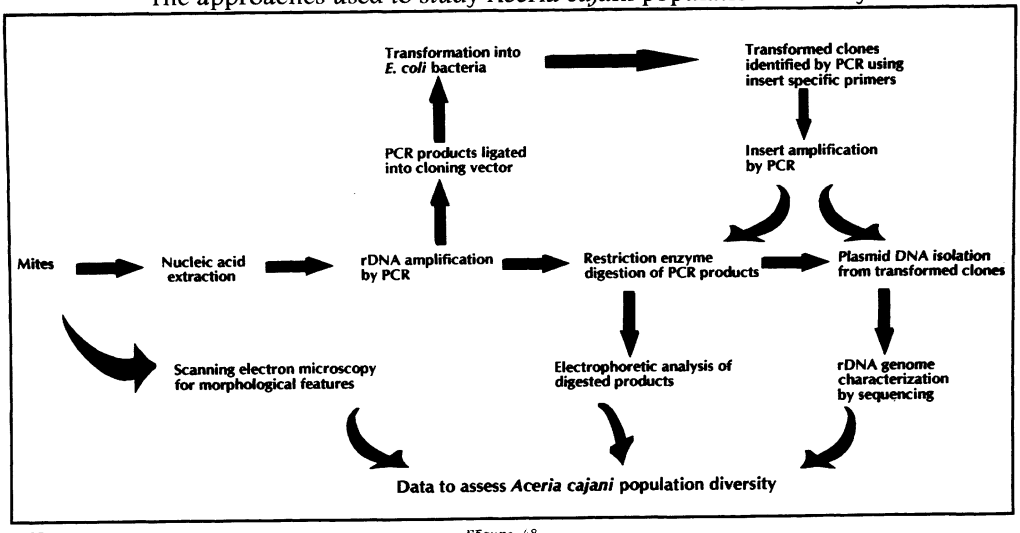


Figure 48

6.2. Conclusions

The two objectives of this project, 1. Isolation of the causal agent of SMD and 2. Assessment of genetic variation between *Aceria cajani* populations, have been accomplished. The following conclusions are drawn from this study.

- As a result of application of a protocol for isolating a mechanically transmissible virus, PATV, a highly stable tombusvirus was isolated from pigeonpea plants from certain locations of India. PATV was fully characterised and shown to be a member of the genus *Tombusvirus*. Because of its slightly different encoding sequence it is best placed in the newly proposed genus *Auresovirus*, along with PoLV and CLSV. Though PATV is serologically related to and has very high (>90%) sequence similarity with PoLV, unlike PoLV, PATV generates DI RNA's. Although PATV is associated with pigeonpea at certain locations, it is not specifically associated with SMD. This is the first report of the association of a tombusvirus with pigeonpea.

- Application of a newly developed protocol resulted in the isolation of, a 'tenui-like' virus (PPSMV) from SMD-affected material. This virus is highly unstable, and is present in only very low concentration in plants. This has hindered rapid progress in its characterisation and in the production of antibodies to it. However, PPSMV was found to be consistently associated with SMD-affected material and its isolation from the SMD cultures established in growth chambers following mite inoculation suggests that it is most likely to be the causal agent of SMD. PPSMV, like HPV, has many similarities with members of the genus *Tenuivirus* is the first report of a tenui-like virus in dicotyledonous plants.

- Studies on mite nuclear rDNA and morphology showed virtually no significant variation in *A. cajani* populations analysed from India, Nepal and Myanmar, suggesting that there are no other eriophyid mite species and probably no biotypes of *A. cajani* involved in the transmission of the SMD agent. This suggests therefore that the variation in pigeonpea genotype response to SMD is more likely to be due to the occurrence of different strains of the SMD pathogen or to variation in host genotype or to environmental factors. Nevertheless, this is the first study that has used both rDNA and morphological data from SEM to assess

biodiversity within a species. This study on eriophyid mite rDNA regions has shown that the ITS sequences of each eriophyid mite species are unique, bur that within the species they were highly conserved. The phylogeny inferred using part of the sequence of the 28S gene and using the maximum likelihood method, was similar, apart from some minor taxonomic revisions, with traditional classification based on morphological characters suggesting that rDNA could be used as a rapid tool for eriophyid mite identification and classification.

6.3. Scope for further work

- PPSMV requires further characterisation, and the development of reliable diagnostic tools for its identification and the differentiation of its biotypes. This is necessary to understand the variation in host reaction and to develop pigeonpea cultivars with broad-based resistance to SMD

- Recently, funding has been obtained [Project ZA0321 (R7452)] from the Crop Protection Division of Natural Resource International (NRI), United Kingdom, under the Renewable Natural Resource Knowledge Strategy (RNRKS) programme, to characterise further the PPSMV; to develop versatile diagnostic tools for its detection; to understand its biodiversity; and to select pigeonpea genotypes with broad based resistance to SMD.

Chapter 7

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Chapter 8

Appendix

8.1. Polyacrylamide gel electrophoresis reagents and buffers

Stack gel buffer (1 M Tris-HCl, pH 6.8)
Tris base 12.1 g
Dissolve in 70 ml distilled water, adjust pH to 6.8 with 1 N HCl and make up to 100 ml with distilled water.

Resolving gel buffer (1 M Tris-HCl, pH 8.8)
Tris base 12.1 g
Dissolve in 70 ml distilled water, adjust pH to 8.8 with 1 N HC land make up to 100 ml with distilled water.

Acrylamide/Bis (30:0.8 w/w) mixture
Acrylamide 30 g
Bis acrylamide 0.8 g
Distilled water to 100 ml
Store this solution at 4 °C in amber coloured bottle or wrap the bottle with aluminium foil to avoid exposure to light.
Precaution: Acrylamide is a neurotoxin. Direct contact with skin or inhalation of acrylamide should be avoided. Prepare this solution in fume hood and always wear gloves.

10% ammonium persulphate (APS)
APS 100 mg
Distilled water 1 ml
Note: Always prepare fresh solution before use.

TEMED (Sigma, Cat.# T9281)
Store at 4 °C.

Electrode (running or tank or TG) buffer, pH 8.3
Tris base (25mM) 3 g
Glycine (250 mM) 14.4 g
SDS* 1 g
Distilled water to 1 litre. No need to adjust pH. Store at room temperature.

Plug gel composition (Optional)		**Stacking gel composition (4%)**	
Acrylamide: Bis mixture	1.75 ml	Acrylamide: Bis mixture	1.5 ml
Resolving gel buffer	1 ml	Stacking gel buffer	1.25 ml
Distilled water	1 ml	Distilled water	7 ml
TEMED	20 µl	TEMED	15 µl
10% APS	40 µl	10% APS	200 µl
		10% SDS*	100 µl

Resolving gel composition

	10%	12%	14%	16%
Acrylamide: Bis mixture	10 ml	12 ml	14 ml	16 ml
Resolving gel buffer	11.25 ml	11.25 ml	11.25 ml	11.25 ml
Distilled water	9 ml	7 ml	5 ml	3 ml
TEMED	20 µl	20 µl	20 µl	20 µl
10% APS	100 µl	100 µl	100 µl	100 µl
10% SDS*	300 µl	300 µl	300 µl	300 µl

Note: Mix acrylamide:bis solution, gel buffer, distilled water and TEMED mix well, then add APS, swirl the flask and immediately pour into the gel mould.
Precaution: Unpolymerised acrylamide is a neurotoxin. Gloves should be worn when handling this solution.
*** For denaturing gel only**

8.2. Buffers and reagents for northern blotting, hybridisation and DIG-detection system

5x MOPS buffer

200 mM MOPS	83.72 g
50 mM sodium acetate	8.23 g
DEPC-treated water	1.6 l
0.5 M EDTA	20 ml

Adjust pH to 7 with 10 N NaOH make up to 2 l with dH_20 and sterilise by autoclaving.

Formaldehyde gel

5x MOPS	28 ml
DEPC-dH_20	87 ml
Agarose	1.39 g

Melt agarose by heating in a microwave, cool to 55 °C and then add 25 ml formaldehyde (37%v/v) mix and cast the gel.

Gel loading (sample) buffer

Formaldehyde (37%)	0.7 ml
Formamide	2 ml
5x MOPS	0.4 ml

20X SSC buffer, pH 7.0

3 M Sodium chloride	175.32 g
300 mM tri-Sodium citrate	88.23 g

Dissolve in 500 ml DEPC-dH_20, adjust pH to 7, then make up to 1 l and sterilise by autoclaving.

10% SDS

Dissolve 10 g SDS in 100 ml water.

Pre-hybridisation (DIG-1) buffer

20x SSC	30 ml
10% SDS	5 ml
Denhardt's solution	5ml
Milk powder	1 g

Sterile-dH_2O to 100 ml

Denhardt's solution

2% Polyvinyl pyrrolidone (PVP)-40,000
2% BSA
2% Ficoll

Hybridisation buffer

DIG-1 buffer containing labelled probe.

Maleic acid (DIG-2) buffer, pH 7.5

0.1 M maleic acid
0.15 M NaCl

Adjust pH with concentrated or solid NaOH. Autoclave.

Blocking buffer

DIG-2 buffer containing 0.5% blocking agent

Antibody buffer

Blocking buffer containing Anti-DIG antibodies

Washing buffer

DIG-2 buffer containing 0.3% Tween-20.

Detection (DIG-3) buffer, pH 9.5

100 mM tris-HCl
100 mM NaCl

Colour substrate

45 µl NBT solution and 35 µl BCIP solution added to detection buffer.

8.3. Buffers and reagents for ELISA

Carbonate (coating) buffer, pH 9.6

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
dH_2O	1 l

Phosphate buffer saline (PBS), pH 7.4

Na_2HPO_4	2.38 g
KH_2PO_4	0.4 g
KCl	0.4 g
NaCl	16.g
dH_2O	2 l

PBS-T (washing) buffer

PBS	1 l
Tween-20	0.5 ml

Antibody buffer

PVP-40,000	2 g
Ovalbumin	0.2 g
PBS-tween	100 ml

Diethanolamine buffer, pH 9.8 (10% v/v)

Diethanolamine	100 ml
dH_2O	1 l

Adjust pH to concentrated HCl

Substrate buffer

Diethanolamine buffer	30 ml
PNP substrate (0.5%)	15 mg

8.4. Miscellaneous reagents

Bentonite buffer

1. Dissolve 20 g bentonite in 400 ml 10 mM Tris-HCl, pH 7.6.
2. Mix and centrifuge at 2,500 rpm/15 min
3. Collect supernatant and centrifuge at 8,500 rpm/20 min
4. Collect pellet and resuspend in 100 ml Tris buffer.
5. Autoclave before use.

TAE buffer, pH 8

Tris base	4.8 g
Glacial acetic acid	1.1 ml
EDTA	0.327 g
Sterile-dH_2O	1 l

TBE buffer, pH 8.3

Tris base	5.4 g
Boric acid	2.75 g
0.1 M EDTA	10 ml
Sterile-dH_2O	1 l

DEPC treated water

DEPC 10 ml (1% v/V)
dH_2O 1 l
Leave the water at 37 0C overnight, then autoclave for 15 min at 15 lb/sq.
Note: DEPC is a suspected carcinogen.

SOC medium

Tryptone	2 g
Yeast extract	500 mg
NaCl	58 mg
KCl	18 mg
MgCl2.6H2O	203 mg
MgSO4.7H2O	246 mg
Glucose	36 mg

Distilled water to 100 ml.
Sterilise by autolclaving

LB medium

Tryptone	1 g
Yeast extract	500 mg
NaCl	1 g

Distilled water to 100 ml
Adjust pH to 7. Sterilise by autoclaving

STET buffer, pH 8

NaCl	584 mg
100 mM tris-HCl, pH 8	10 ml
100 mM EDTA	1 ml
Titon-X 100	5 ml

Distilled water to 100 ml.

8.5 Geographical map of India subcontinent

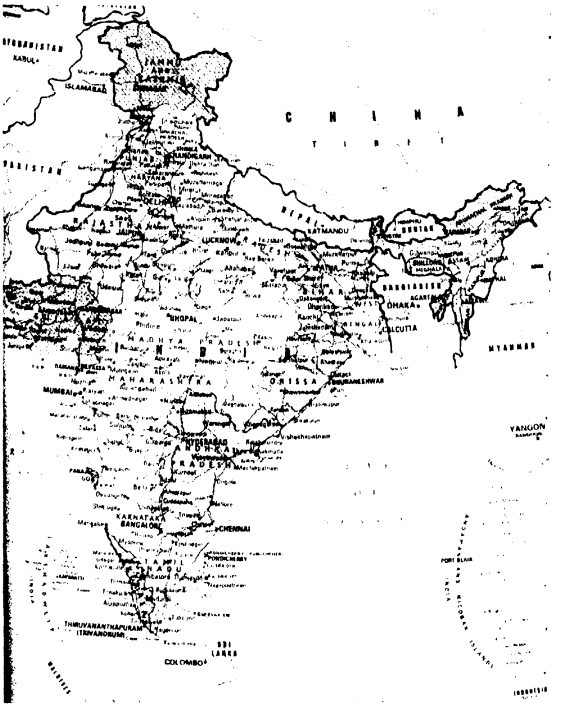
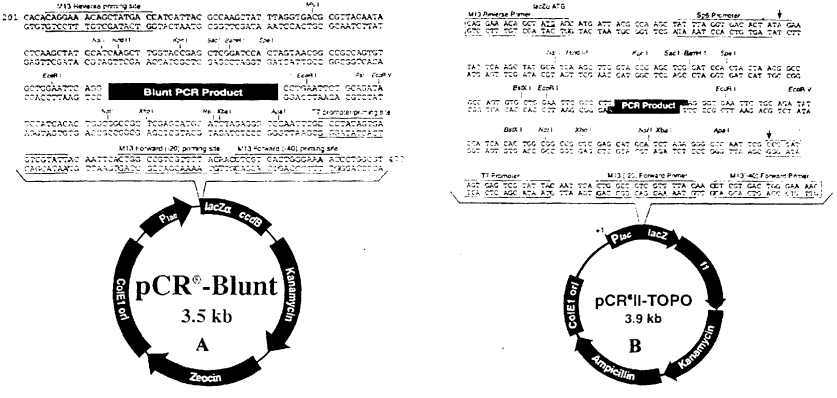


Figure 50

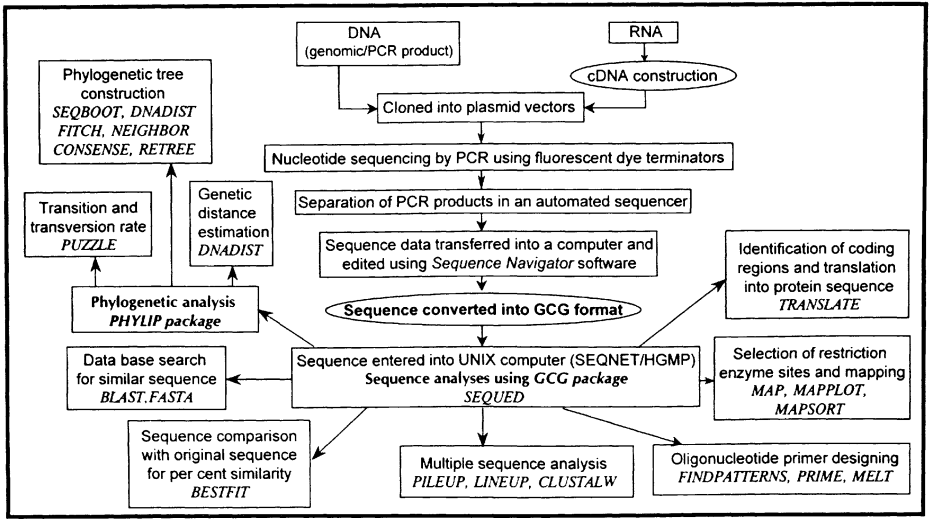
8.6. Plasmid Vector Maps



Plasmid vectors used for cloning blunt ended DNA (A), and polyA tailed PCR products (B). (Invitrogen, The Netherlands)

Figure 51

8.7. Pictorial Representation of Strategy for Nucleotide Sequence Analysis



8.8. Addresses of suppliers

Amersham Pharmacia Biotech, White Lion Road, Amersham, Bucks Hp7 9LL, UK; FF3 Palani Centre, 32 Venkatnarayana Road, T Nagar, Chennai 600 017, India.

Beckman, Wipro Biomed Division, 903 Prakash Deep, 7 Tolstoy Marg, New Delhi-1, India.

BioRad, BioRad House, Maylands Avenue, Hemel Hempstead, Hertfordshire HP2 7TD; Biogen Technologies, Shalibanda, Hyderabad 500 265, India.

Boehringer-Mannheim, Postfach 310120, D-6800, Mannhem 31, Germany; 54-A Boehringer Mannheim Diagnostics, Mathuradas Vasanji Road, Chakala, Andheri East, Bombay 400 093, India.

Cambridge Molecular Technologies Limited, Babraham Hall, Babraham, Cambridge CB2 4UL, UK.

Eppendorf, PO Box 650670, D-2000 Hamburg, Germany

Gibco BRL Life Technologies Inc., PO Box 35, Trident House, Renfrew Road, Paisley, PA3 4EF, UK; Gibco BRL India, 4F, Gopala Tower, 25 Rajendra Place, New Delhi 110 008, India.

Hybaid, 111-113 Waldegrave Road, Teddington, Middlesesx, TW11 8LL, UK.

Hoefer Scientific Instruments, 654 Minnesota Street PO Box 77387, San Francisco, CA 94107.

Invitrogen, De Schelp 12, 9351 NV Leek, The Netherlands.

Perkin Elmer Cetus, Maxwell Road, Beaconsfield, Bucks, HP9 1QA, UK; Labindia Instruments Pvt Ltd. B-1 Alsa Regencey, 16 Eldams Road Alwarpet Chennai 600 018, India.

PE-Applied Biosystems Inc., Biotech instruments Ltd., Unit A, Caxton Hill Extension Road, Caxton Hill, Hertford, SG13 7LS, UK; Labindia Instruments Pvt Ltd. B-1 Alsa Regencey, 16 Eldams Road Alwarpet Chennai 600 018, India.

Promega, Spiscon House, Enterprise Road, Chilworth Research Centre, Southampton SO1 7NS, UK; Hysel India Pvt. Ltd. 41 DDA Shopping Centre, Sukhdev Vihar, New Delhi 110 025, India.

QUIAGEN, Max-Volmer-Streabe4, 40724, Hilden, Germany; Genetix, C-88 Kirti Nagar, New Delhi 110 015, India.

Sigma Chemical Company, Fancy road, Poole, Dorset BH17 7NH, UK; SIGMA-ALDRICH CORPORATION, Bangalore, India.

Sorvall-DuPont Company, Biotechnology Systems Division, BRML, G-50986, Wilmington, DE19898m USA; Kendro Laboratory Products, B-5/75 Safdarjung Enclave, New Delhi 110 029, India.

Whatman Labs, Unit 1, Coldred Road, Parkwook, Maidstone, Kent, ME15 9XN, UK.