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Assessment of variation in *Aceria cajani* using analysis of rDNA ITS regions and scanning electron microscopy: implications for the variability observed in host plant resistance to pigeonpea sterility mosaic disease

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Summary

Aceria cajani on pigeonpea (*Cajanus cajan*) is the vector of the agent of pigeonpea sterility mosaic disease (PSMD), a very damaging virus-like disease in the Indian subcontinent. PCR was used to amplify *A. cajani* nuclear ribosomal DNA (rDNA) internal transcribed spacers (ITS) and associated rDNA genes. They were assessed for variation in this genome region by nucleotide sequencing and RFLP. *A. cajani*-specific rDNA primers are described. Several *A. cajani* populations were collected from pigeonpea plants from various PSMD endemic locations in India, Nepal and Myanmar. No significant variation was identified in rDNA regions, or in morphological features. These results suggest strongly that *A. cajani* on pigeonpea across the Indian subcontinent constitutes one species and that no other *Aceria* species and probably no *A. cajani* biotypes that differ in vectoring ability are involved in the transmission of the agent of PSMD. The implications of these findings for the variability observed in PSMD-resistant pigeonpea genotypes across various locations in India are discussed.

Key words: pigeonpea, sterility mosaic, rDNA, PCR, morphology, biodiversity, resistance

Introduction

Eriophyid mites (Arthropoda: Acari) are amongst the smallest arthropods and are obligate plant pests in active stages of their life cycle. Several of them cause direct damage by affecting plant growth and some indirectly by acting as vectors of important plant viruses (Keifer *et al.*, 1982; Oldfield & Proeseler, 1996). *Aceria cajani* (Channabasavanna) (Acari: Eriophyidae) on pigeonpea (*Cajanus cajan*) causes no obvious damage to the host. However, it transmits the causal agent of pigeonpea sterility mosaic (PSMD), the most destructive disease of pigeonpea in the Indian subcontinent (Ghanekar *et al.*, 1992). When PSMD occurs early in the season yield losses can reach over 90%. Integrated management of PSMD includes the development of resistant cultivars, changing sowing dates and vector control using pesticides. However, the latter two options have limitations for field application. Work at ICRISAT, Patancheru, resulted in the identification of pigeonpea genotypes with field resistance to PSMD, but this resistance was found to be location specific (Reddy *et al.*, 1998). Resistant lines that performed well in field trials at ICRISAT, Patancheru, and surrounding regions, were much less resistant at other locations in the Indian subcontinent. The mechanism(s) of PSMD resistance is not known, but seems diverse (Saxena & Sharma, 1990). Presumably, the genotypes are resistant either to the disease agent, to the mite vector, or to both organisms (Reddy *et al.*, 1998). Interestingly, mites are not often found on PSMD-resistant genotypes and occur in only very low numbers on symptom-free pigeonpea plants (Reddy & Nene, 1980; Muniyappa & Nangia, 1982). It is possible that the breakdown in PSMD resistance at various locations is due to the occurrence of different *Aceria* species or biotypes of *A. cajani*, or of different strains of the PSMD pathogen. Understanding the factors that can contribute to diversity is vital to develop pigeonpea genotypes with durable resistance to PSMD. This present study sought to: (i) determine whether different species of *Aceria* mites are involved as vectors; (ii) assess the diversity amongst *A. cajani* populations and (iii) understand the variation in PSMD resistance shown by different pigeonpea genotypes with respect to the mite vector.

Accurate identification of eriophyid mites, particularly by morphological characters, is very difficult because of their very small size (~200 μm) and their morphological similarity. A diverse range

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of novel protein and DNA-based molecular markers has been used to analyse the occurrence of strains (biotypes) within species that differ physiologically, but not morphologically (Loxdale & Lushai, 1998). However, many of these techniques require relatively good DNA preparations, often from single individuals. This has restricted the use of such methods on eriophyid mites because of their microscopic size, soft body and because single individuals are difficult to manipulate. Recently, a polymerase chain reaction (PCR)-based rDNA analysis technique was developed for distinguishing morphologically closely related *Cecidophyopsis* mite species (Fenton *et al.*, 1995; Kumar *et al.*, 1999*a*). This was based on amplification of part of the primary transcription unit of the ribosomal (r) RNA encoding gene (3' end of the 18S gene, ITS1, 5.8S gene, ITS2 and 5' end of the 28S gene; collectively known as rDNA) and subsequent analysis by nucleotide sequencing and/or RFLP. rDNA is an extremely well studied gene family and its use in diagnostics has several advantages as the structure and sequence of the rDNA coding regions are highly conserved and the two ITS regions between the coding regions diverge rapidly between species, but are highly conserved within eukaryotic species and this has been confirmed in Acarids (Hills & Dixon, 1991; Navajas *et al.*, 1994, 2001; Fenton *et al.*, 1997). This technique was shown to be rapid and sensitive for the unambiguous identification of different *Cecidophyopsis* mite species.

To determine the potential involvement of different *Aceria* species and to understand the variation in *A. cajani* populations obtained from pigeonpeas from various locations of the Indian subcontinent, nuclear rDNA of mites was analysed. In addition, scanning electron microscopy was used to study morphological features of *A. cajani* from India, Nepal and Myanmar. The morphology was compared with the first description of *A. cajani* by Channabasavanna (1966).

Materials and Methods

Collection of mites and DNA extraction

Pigeonpea leaf samples from PSMD-affected and healthy (symptomless) plants were collected from India, Nepal and Myanmar (Table 1), placed in polythene bags or wrapped in aluminium foil and sent to the laboratory. With the aid of a binocular microscope, a fine needle was used to collect individual mites from leaves and to place them in a 1.5 ml Eppendorf tube containing 0.01% Decon (BDH, UK) in PBS. Mites were concentrated by centrifuging at 8,000 g for 1 min and the supernatant fluid was removed, and the tubes stored at -70°C or processed immediately for nucleic acid extraction.

Nucleic acid was extracted from 15-20 mites as described by Kumar *et al.* (1998). The final DNA pellet was dissolved in 15 µl of sterile TE (100 mM Tris-HCl and 1 mM EDTA) buffer, pH 9.2 and stored at -20°C. About 1-2 µl of this was used for rDNA amplification.

Ribosomal DNA amplification and cloning strategy

DNA corresponding to the 3' end of the 18S, the ITS-1, 5.8S, ITS-2 and the 5' end of the 28S rDNA genes was amplified by PCR using primers corresponding to the conserved regions of the 18S (primer C), 5.8S (primers B and Mb) and 28S rDNA (Primer E) regions (Fig. 1) (Fenton *et al.*, 1997). The primer annealing positions and direction of amplification are shown in Fig. 1. PCR was made in a final volume of 33 µl of 1 × *Taq* buffer (supplied with the enzyme) containing 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 2 U *Taq* (Promega, UK) and 10 ng of each primer. The conditions for amplification were those reported by Kumar *et al.* (1998). Amplified products were analysed in a 1% agarose gel (Sambrook *et al.*, 1989). The 'PCR Marker' (Promega, UK) was used as a DNA molecular weight marker. Amplified products were cloned into the TOPO TA vector (Invitrogen, Netherlands) and chemically transformed into *E. coli* TOP 10 competent cells (Invitrogen, Netherlands). For each PCR product, five to ten positive clones were selected and plasmids were purified using the Wizard Miniprep Kit (Promega, UK).

The universal M13 forward (F) and 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 (Fig. 1).

Nucleotide sequencing and computer programmes

Sequencing was done by the dideoxynucleotide chain termination method using the Dye-Prism™ cycle sequencing kit (Perkin-Elmer, UK), utilising M13 F and R and insert specific primers Mb, G and E (Kumar *et al.*, 1999*a*). The DNA sequences were analysed using the University of Wisconsin Genetics Computer Group (GCG) Package Version 8.1 (Anon., 1994) and CLUSTALW (Thompson *et al.*, 1994; Higgins & Sharp, 1988). For comparative analysis, plasmid clones containing the copies of ITS-1 sequence of the following six other eriophyid mite species available in our laboratory were also used. These were to, *Aceria tulipae, A. pongamia, Cecidophyopsis grossularia, Eriophyes insidiosus, Phyllocoptes fructiphilus and P. gracilis* (Table 2).

Table 1. *Locational details of PSMD-affected pigeonpea samples analysed for mites*

Location	Acronym	Location	Acronym
INDIA			
ICRISAT, Patancheru, Medak, AP state	ICR	ANGRAU, Tirupati, Andhra Pradesh state	RTPT
Badanpura, Maharasthra state	B	ANGRAU, Tirupati, Andhra Pradesh state	STPT1
ICRISAT, Patancheru, Medak, AP state	ICR2	ANGRAU, Tirupati, Andhra Pradesh state	STPT2
Kanukunta, Medak, AP state	K	ANGRAU, Tirupati, Andhra Pradesh state	ETPT
Buddipadiaga, Medak, AP state	BNM	ANGRAU, Tirupati, Andhra Pradesh state	YTPT
Arepalli, Medak, AP state	AKM	ANGRAU, Tirupati, Andhra Pradesh state	LTPT
Peddasamudrala, Karimnagar, AP state	PKK	Vempalli, Chittoor, Andhra Pradesh state	YEN
Antikapeta, Karimnagar, Andhra Pradesh state	AHK	Kurupalli-1, Chittoor, Andhra Pradesh state	KUR1
Immanaguda, Medak, Andhra Pradesh state	IGM	Kurupalli-2, Chittoor, Andhra Pradesh state	KUR2
Elkaturti, Karimnagar, Andhra Pradesh state	EEK	Hosamhpalli1, Karnataka state	HOS1
Gulbarga, Karnataka state	GUL	Hosamhpalli2, Karnataka state	HOS2
Rahuri, Maharashtra state	RM	Bangalore suburbs-1, Karnataka state	ARS1
Bilza fens, Patancheru, Andhra Pradesh state	BFI	Bangalore suburbs-2, Karnataka state	ARS2
Puddukkotai, Tamil Nadu state	PTN	GKVK, Bangalore, Karnataka state	BAL1
Kanukunta West, Medak, Andhra Pradesh state	KW	Balajiygpade, Karnataka state	BAL2
Kanukunta East, Medak, Andhra Pradesh state	KE	Bangalore suburbs-3, Karnataka state	BAL3
Balsapur, Medak, Andhra Pradesh state	BMM	Anchikacherla, Krishna, Andhra Pradesh state	KRI
Peddavura, Medak, Andhra Pradesh state	PSM	Nandigama, Krishna, Andhra Pradesh state	NAD
Kanukunta, Medak, Andhra Pradesh state	KM	Sidipeta, Karimnagar, Andhra Pradesh state	SID
Daupatpur, Dehat, Uttar Pradesh state	KAN	Patancheru, Medak, Andhra Pradesh state	ICR-N
Talakundu, Kolar, Karnataka state	TK	GKVK, Bangalore, Karnataka state	RSK
Malligonda, Vellore, Tamil Nadu state	MV		
Chittoor, Andhra Pradesh state	CT	*MYANMAR*	
Mydukuru-1, Cuddapaha, Andhra Pradesh state	MY	Plant Pathology Div., CARI, Yezn	BUR2
Mydukuru-2, Cuddapaha, Andhra Pradesh state	MD	Magliang Farm, Magliang	BUR8
Vijayapur, Chickballapur, Karnataka state	VC	Mygan Farm, Mygan	BUR10
Rayachoti, Cuddapaha, Andhra Pradesh state	RC	Nyaungoo Farm, Nyaungoo	BUR14
Ramateertham, Chittoor, Andhra Pradesh state	RAM	Kyaukpacaung	BUR20
Taticherla, Giddalur, Andhra Pradesh state	TAG		
Pernambatu, Tamil Nadu state	PER	*NEPAL*	
Dholi Agricultural College Farm, Bihar state	DF	Bhanupur	NEP-1
Mahamudpura, Iamashitipur, Bihar state	MB	Rajajna, Nepalgunj	NEP-2
Syadpura, Samishtapur, Bihar state	SAS	Agricultural Research Station, Nepalgunj	NEP-3
Pusa Station, IARI Gardens, Bihar state	PUI	Khairapur, Ward-2	NEP-4
Jaipur, Rajasthan state	JAI	Sanosari	NEP-5
ANGRAU, Tirupati, Andhra Pradesh state	MTPT	Jutepani, Ward-9	NEP-6

Restriction enzyme analysis of rDNA

RFLP was used to analyse a larger number of mite samples. About 15-20 ng of the PCR amplified product of genomic DNA and of rDNA-containing recombinant plasmids, were digested with *Dde* I, *Mse* I, *Taq* I, *Nde* II, and *Pst* I (Roche, Germany), either alone or in combination. Digested products were analysed by polyacrylamide gel electrophoresis (Kumar *et al.*, 1999*a*).

Low temperature scanning electron microscopy (LTSEM)

Specimens were prepared for LTSEM essentially as described by Lopez-Llorca & Duncan (1988) using an EMscope SP2000 Sputter Cryo System interfaced with a JEOL T200 scanning electron microscope. Mites were arranged on a piece of pigeonpea leaf or conductive, double-sided, sticky tape (5 mm × 5 mm) mounted on a copper stub with OCT Tissue Tek mounting medium (Miles Laboratories Inc., USA) and frozen rapidly by plunging the stub into nitrogen slush (-210 °C). Any surface ice was sublimed off by heating the frozen hydrated mites to -90°C for 5 min before sputter coating them with gold. These were then transferred under vacuum to the cold stage of the SEM and held at -168°C during viewing. Images were recorded at 10 KV using Kodak Tmax 100 film.

Results and Discussion

Collection of mites and DNA isolation

Mites were found on the under-side of pigeonpea leaves buried in dense trichomes. Young leaves contained most mites, which were concentrated around the veins towards the petiole end. Mites were not found on old and PSMD symptom-free leaves or on any samples from apparently healthy looking

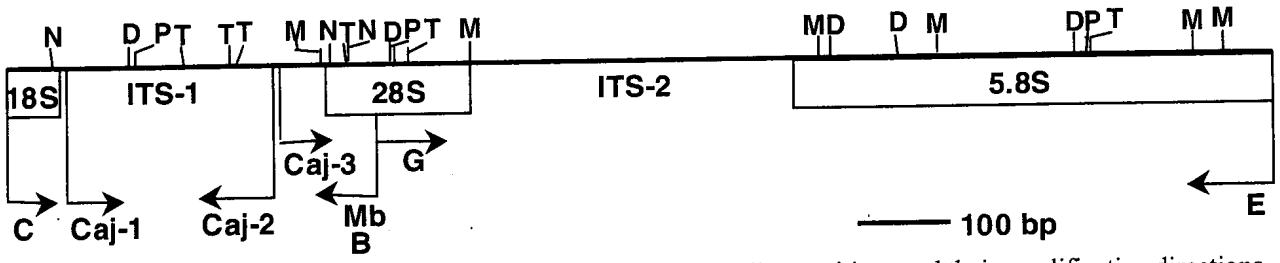

Fig 1. Map of rDNA analysed in this study showing the primer annealing positions and their amplification directions, and the restriction sites of the enzymes. (D) *Dde* I, (M) *Mse* I, (N) *Nde* II, (P) *Pst* I and (T) *Taq* I.

Table 2. *Details of eriophyid mite ITS-1 sequences used in this study for comparative analysis*

Species	Location	Host	Size of ITS-1 (bp)	Accession no.	Acronym
Aceria cajani	India	Pigeonpea	291	AJ251693	ACAJA
Aceria tulipae	Canada	Wheat	413	AJ251695	ATULI
Aceria pongamia	India	*Pongamia glabra*	521	AJ251696	APONG
Cecidophyopsis grossularia	UK	Gooseberry	356	X92640	CGROS
Phyllocoptes fructiphylus	USA	Rose	535	AJ251692	PFRUC
Phyllocoptes gracilis	UK	Wineberry	507	AJ251697	PGRAS
Eriophyes insidiosus	USA	Peach	554	AJ251694	EINSI

plants. Fresh leaf samples packed thinly in aluminium foil permitted survival of mites for up to a week at 4°C. In a few samples dead mites were found and molecular analysis of DNA from such mites was found to be difficult.

Amplification of A. cajani *rDNA*

Initially, primers were used in four combinations, C+E, C+B, C+Mb and G+E to amplify the *A. cajani* rDNA ITS regions. Amplification was obtained only with the C+Mb primer combination, resulting in a product of about 400 bp corresponding to the ITS-1 and flanking conserved regions (results not shown). The other primer combinations yielded no amplified product.

The C+Mb amplified products were cloned. Both strands of four independent clones were sequenced. From the sequence information, three regions in the ITS-1 were selected for primer design (see Fig. 1) resulting in a 21mer primer Caj-1 (5' GTA AAA AAC CAA ACG CGA GTC 3'; position 60 to 81 in Fig. 2), a 23mer primer Caj-2 (5' TTC CAC ACT GAT ATG GTA GTC GC 3'; position 264 to 287 in Fig. 2) and a 25mer primer Caj-3 (5' ACT ACC ATA TCA GTG TGG AAG CGCG 3'; position 267 to 292 in Fig. 2) overlapping with the Caj-2 primer, but with a downstream amplification direction. The three Caj primers were used together with universal primers (Caj-1+E; C+Caj-2 and Caj-3+E; see Fig. 1). Primer combinations Caj-1+E amplified a 1305 bp region corresponding to ITS-1/5.8S/ITS-2 and the 5' end of the 28S gene; Caj-3+E amplified a 1063 bp region corresponding to the 3' end of ITS-1/5.8S/ITS-2 and the 5' end of the 28S gene; C+Caj-2 amplified a 286 bp region corresponding to the 3' end of the 18S gene and part of ITS-1 (results not shown). No variation was observed in the size of the PCR products amplified by these primer pairs from mite samples from different locations in India, Nepal and Myanmar (results not shown).

The C+E primer combination amplified rDNA of 13 other eriophyid mite species, aphids, beetles, fungal, and other eukaryotic rDNA (Fenton *et al.*, 1994). Factors for the failure of this primer combination and that of G+E on *A. cajani* were not determined but variation in PCR reaction by altering concentrations of template DNA and $MgCl_2$ did not improve the results. It was found that *A. cajani* has a mutation in the B/G primer region, which resulted in the need to design a new primer, MiteB (Mb) which is similar to B, except for one base change (see Fenton *et al.*, 1997). Nevertheless, the new Caj primers together with the universal primers amplified rDNA ITS regions from almost all the *A. cajani* samples obtained from different locations of India and Nepal. For all DNA samples from Myanmar with the exception of the C+Caj-2 pair, all primer combinations failed to amplify rDNA. Myanmar mite samples were in the post for long periods and most mites were dead and decomposed on arrival. The DNA was therefore likely to be fragmented due to autolysis and this seriously affected amplification of large products. PCR amplification of products from degraded DNA is

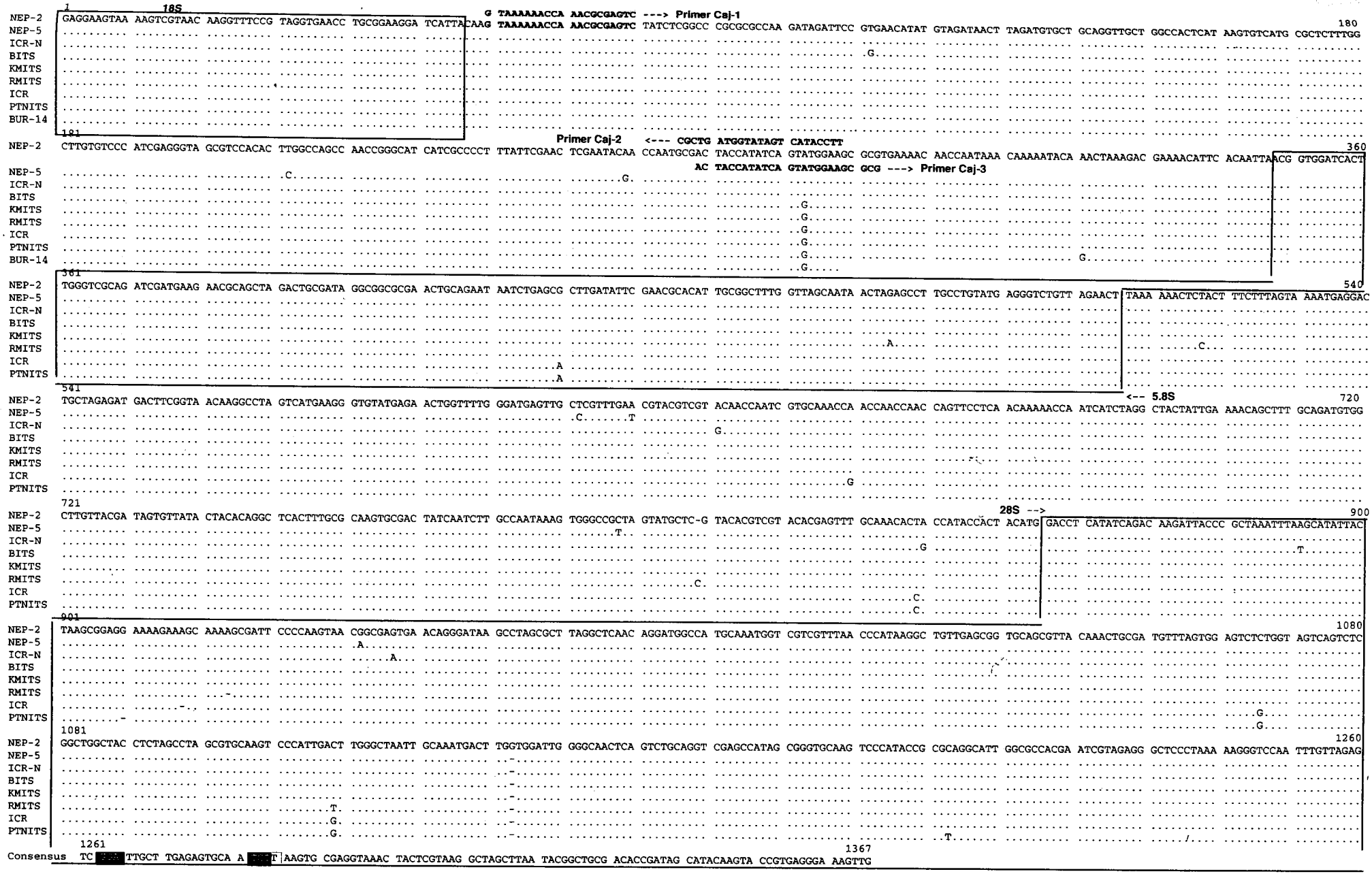

Fig 2. Alignment of rDNA sequences of *A. cajani* from various locations. Dots signify the sequence similarity, letters correspond to the single base differences relative to other sequences; dashes signify single base deletions. The rRNA encoding regions are boxed. The sequence of BUR-14 could be aligned to only a short section of ITS-1.

difficult but smaller products of < 300 bp may be amplified because of the shorter template requirement (Golenberg *et al.*, 1996; Kumar *et al.*, 1999*a*). As the C+Caj-2 amplicon size is small, this primer set resulted in amplification. Amplified products were cloned for further analyses. Analysis of insert-containing clones by PCR and electrophoresis alone indicated a lack of variation in size within the population (results not shown) in agreement with the results from genomic DNA.

rDNA analysis by sequencing and RFLP

Nucleotide sequence information was obtained for seven samples from India (B, ICR, K, KM, PTN, RM and ICR-N), and two each from Nepal (Nep-2 and Nep-5) and Myanmar (Bur-14 and Bur-20) (Table 1). The sequences of mite samples from different locations were aligned using the program CLUSTALW and are presented in Fig. 2. The full length of the rDNA ITS region was 1366 bp. This started 56 bp from the 3' end of the conserved 18S gene to 511 bp at the 5' end of the 28S gene. Sizes of *A. cajani* mite ITS-1, 5.8S and ITS-2 regions were 291, 159 and 349 bp respectively. The boundaries of various rDNA regions were defined by comparing them with other eriophyid mite rDNA sequences reported previously (Figs. 2 and 3). The per cent composition of the four nucleotides A, C, G and T were 29.4, 22.7, 24.6 and 23.3, respectively. Comparisons of *A. cajani* rDNA sequences from the eleven different locations showed little or no sequence divergence amongst them (Fig. 2). There were no major deletions/insertions in the sequences studied, but there were 22 single base substitutions, some of which were in the conserved rRNA genes. This is equivalent to 0.06% variation. These single base mutations could have arisen due to errors in *Taq* polymerase replication, reading errors during sequencing or low levels of intra-specific variation. However, there are five mutations, which are found in more than one sequence. The chance of two independent clones containing the same randomly generated mutation is very small (1/1367). Therefore, these changes probably represent genuine point mutation variants within the population. Thus the level of within-species variation in the nucleotide sequences of *A. cajani* ITS-1 examined in this study was comparable to the variation observed within different species of *Cecidophyopsis* mites (Fenton *et al.*, 1997). Although, only partial sequences corresponding to the 18S and ITS-1 were available for mites from Myanmar, the determined sequences had 100% identity with mites studied from other locations of India and Nepal and it is therefore unlikely that other regions would be variable. This assumption can be substantiated from the fact that the ITS regions of all the *A. cajani* samples and within all other eriophyid mite species studied so far, are almost identical (discussed below).

The rDNA ITS1 sequences of *A. cajani* mites were aligned and compared with the sequences of other eriophyid mites (Table 2; Fig. 3). Of the regions between primer positions C and Mb analysed, sequences corresponding to the 18S and 5.8S gene were 100% identical (boxed region in Fig. 3). The ITS-1 sequence length of *A. cajani* mites is the smallest of any reported eriophyid, with a large number of sequence deletions and variations compared to other mite species (Table 2). A major feature of the rDNA regions is that, between species, there is a high level of conservation in the regions encoding rRNA genes, but variation both in nucleotide sequence and length of ITS regions (Fig. 3). Sequences at two positions in the *A. cajani* 28S gene, GGA and TCT (blocked regions in Fig. 2) were different from those reported for other mite species. In many animal species, including higher vertebrates, the nucleotide sequence is GAG and CTT, respectively (Christen *et al.*, 1991). Although this conflicting information could be due to sequencing errors, this seems unlikely because the variation was consistent in all the *A. cajani* samples sequenced.

The sequences were used to design a PCR/restriction enzyme analysis of rDNA so that more samples could be examined. When *A. cajani* DNA obtained from different locations was analysed by this method, no restriction site polymorphism was detected in digests using *Taq* I, *Nde* II + *Pst* I (results not shown) or *Dde* I, and *Mse* I (Fig. 4). The restriction patterns of ITS regions amplified from the mite genomic DNA, and the recombinant plasmids containing single copies of the same sequences were also similar, indicating that there was little or no variation within the populations. However, occasional variation in the restriction pattern was observed (see Fig. 4A, lanes RM, BMM; Fig. 4B, lane ICR-N; and Fig. 5), and this was found to be due to point mutations in the restriction site or to the digestion of terminated/non-specific products during PCR. Only two examples of changes in restriction site were observed:- Firstly in the *Mse* I digest profile of ICR-2, clone 4, one point mutation of A to T (Fig. 5A) in the cleaving site eliminated a *Mse* I site, resulting in a larger product. Secondly, the variation in *Taq* I and *Nde* II+*Pst* I restriction profiles in B, clone 6 (Fig. 5B,C) was due to a transition from C to T which affected the overlapping restriction sites of *Nde* II and *Taq* I.

The existence of low levels of intra-specific variation within populations can never be completely ruled out. Some subtle variation in RFLP patterns were found in the RFLP profiles (for example, see Fig. 4, lanes RM and BMM). Such variation was also detected in digests of the PCR products of plasmids containing single copies of rDNA (results not shown). This suggests that digestion of non-

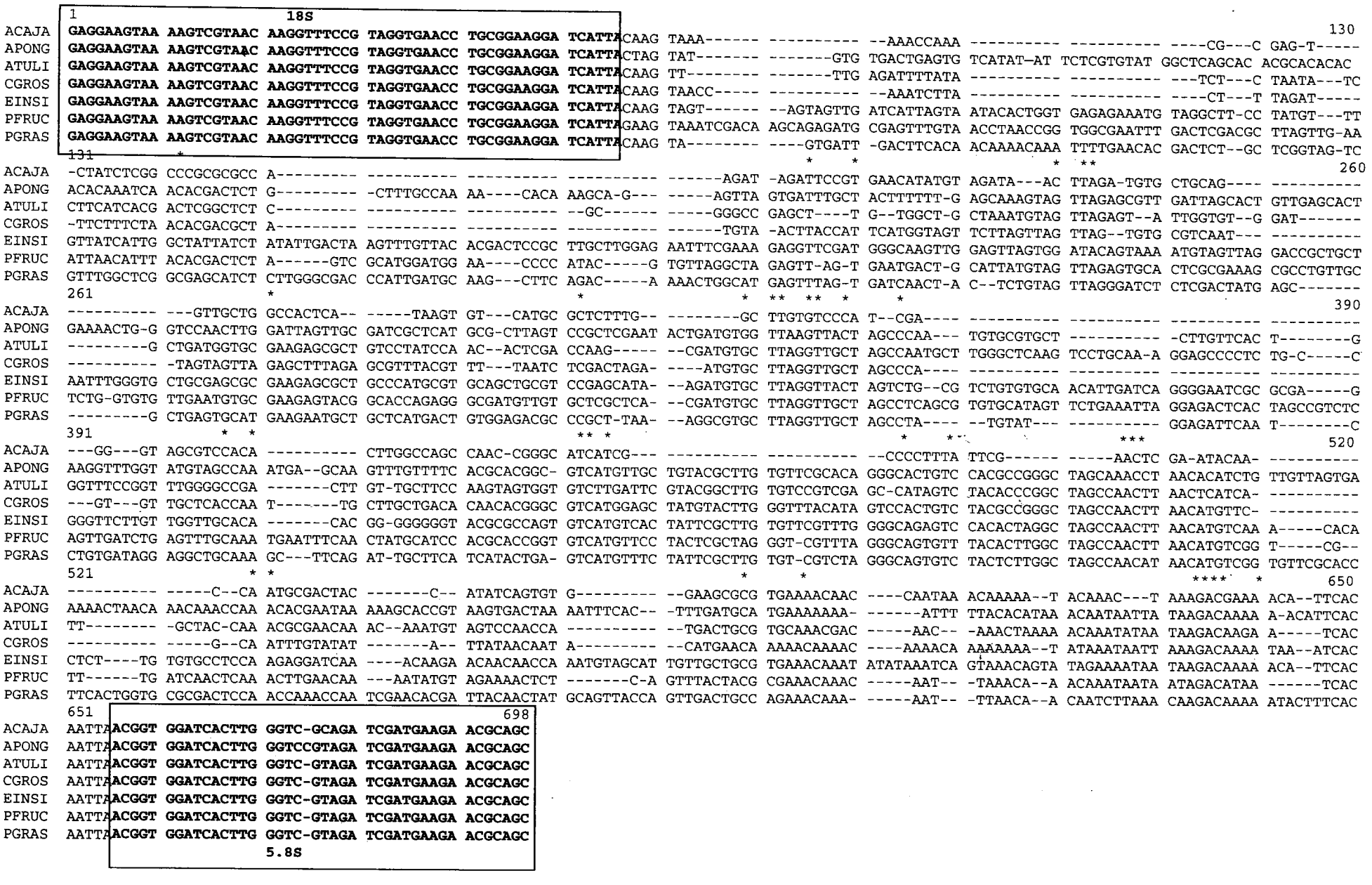

Fig 3. CLUSTALW alignment of ITS-1 sequences of various eriophyid mite species. Parts of the rRNA encoding genes (18S and 5.8S) are boxed and the conserved domains are indicated with *.

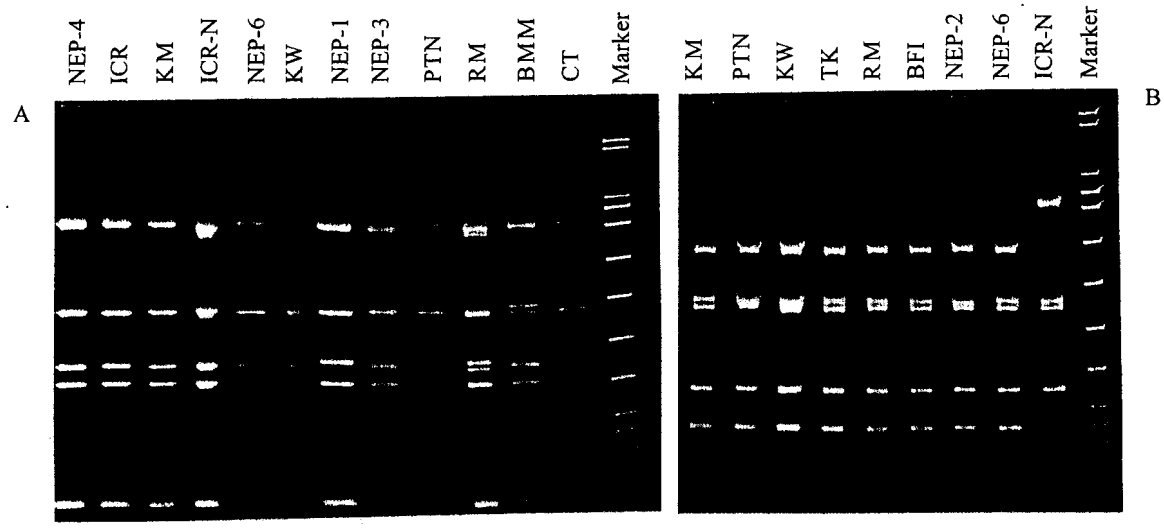

Fig 4. RFLP patterns obtained after digestion with *Dde* I (A) and *Mse* I (B), of *A. cajani* rDNA from the various locations indicated above the lanes.

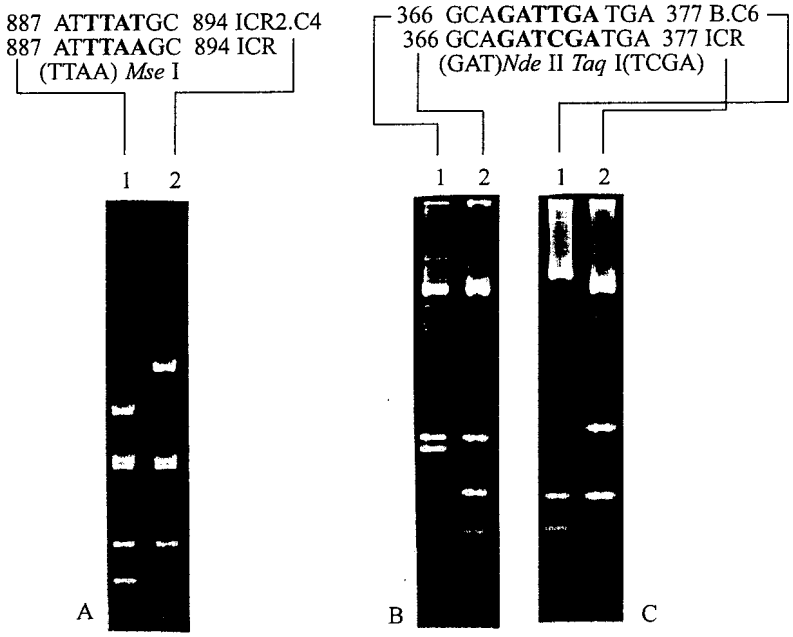

Fig 5. Variation in RFLP profile due to a mutation in *Mse* I (lane 2, A), *Taq* I (lane 1, B) and *Nde* II (lane 1, C) restriction sites.

specific and/or terminated products generated during the PCR process are most likely to be responsible for these minor variations in RFLP patterns.

Very low levels of intraspecific variation in rDNA ITS regions is not restricted to *A. cajani*, but is reported in *Cecidophyopsis* mites (Fenton *et al.*, 1997; B Fenton & P L Kumar, unpublished data), and in cassava green mites (Navajas *et al.*, 1994). Although, *A. tulipae, A. pongamia, E. insidiosus, P. gracilis* and *P. fructiphilus* were not studied extensively, their ITS sequences are very distinct from each other, but again within population variation was not observed (results not shown). The rDNA sequences of *C. ribis* studied from UK and New Zealand, *C. psilaspis* from UK and Canada, and *C. grossularia* from USA and Europe, were also identical within their respective groups (Fenton, *et al.*, 1996; Kumar *et al.*, 1999a; B Fenton & P L Kumar, unpublished). In the *Cecidophyopsis* mites it has been possible to study, in detail, inter-specific differences as there are seven closely related species. The differences amongst these mites were found mainly in variable simple sequence repeats (vSSRs) in the ITS-1 region. Morphological differences between these mites are minor, but they are distinct biologically, indicating that rDNA markers can distinguish such closely related species (Fenton *et al.*, 1996; Kumar *et al.*, 1999*a*). Although sequences similar in composition to *Cecidophyopsis* vSSRs were found in ITS-1 of *A. cajani*, these

sequences, like the rest of the rDNA, were homogenous in the samples analysed from India and Nepal, suggesting strongly that *A. cajani* from throughout these countries is a single species. For the Myanmar samples, only an incomplete sequence was available (Fig. 2). Nevertheless, the sequenced ITS-1 region contained 80% of the variable ITS1 region (Fig. 3; Fenton *et al.*, 1997) and had 100% identity with *A. cajani.* Furthermore, these mites have a morphology and ecology indistinguishable from *A. cajani* in India and Nepal (discussed below) suggesting these mites too are *A. cajani.* This suggestion is supported by the finding that there is no significant within species variation in ITS-1 or vSSRs in all the *A. cajani* samples examined or within any of the other eriophyid mite species studied (Fenton *et al.*, 1997; Kumar *et al.*, 1999). This indicates that the rDNA sequence of each eriophyid mite species is unique, is extremely homogeneous between individuals and populations of that species, and reflects its biological separation.

In contrast to eriophyid mites, ITS regions of some other organisms such as ticks (Rich *et al.*, 1997; McLean *et al.*, 1995), tiger beetles (Vogler & DeSalle 1994), and mosquitoes (McLean *et al.*, 1995; Beebe *et al.*, 1999) show greater levels of intraspecific and intra-individual variation. This indicates that ribosomal arrays in different organisms are not always homogenous and that careful studies are needed to understand variation before utilising the data for species identification.

Aceria cajani *morphology*

LTSEM observations detected no significant differences in the morphological features of *A. cajani* from three different locations (Patancheru, Andhra Pradesh state, India; Nepalgunj, Nepal; and Mygan farm, Myanmar) of the Indian sub-continent. Except at the progenital chamber, no obvious morphological differences were apparent between male and female mites. Only one form of female was found in populations, suggesting the absence of deutogyny in *A. cajani.* However, populations contained mites of different sizes that probably represented different developmental stages (larvae, nymphs, adults and gravid females). Structural features of immatures and adults were similar, except that nymphs lacked genital openings and had less waxation. The present study was restricted to structural observations rather than a strict morphometrical analysis and these were used to compare the three mite samples with the type description of *A. cajani* and also to evaluate the taxonomic position using the features listed by Amrine *et al.* (1994) and Lindquist & Amrine (1996). The morphological details studied, following the notations of Lindquist (1996), are discussed below.

Structure of A. cajani *and its classification*

The body of *A. cajani* is cylindrical with a prodorsum and opisthosoma (Fig. 6A, and B). The opisthosoma is vermiform with a broader anterior and covered with elongated annuli. Some of them are incompletely arched, covering either dorsal or ventral regions. Because of this, their numbers on the dorsal and ventral sides 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 (Fig. 6A, and B).

The prodorsum is connate with ornamentation (Fig. 6C, and D). The anteromedian frontal lobe is straight, ending over the rostrum. Spines are absent at the anterior margin of the frontal lobe. The gnathosoma is shorter than the legs (Fig. 6C). The median line on the dorsal shield is incomplete towards the anterior end (Fig. 6D). The admedian line, originating from the frontal lobe, is wavy and complete (Fig. 6D). The sub-median line is incomplete with no clear branches. The area between the sub-median line and the margin of the prodorsum is irregularly ornamented with small tubercles (Fig 6C, and D). The paired dorsal tubercles positioned at the posterior end of the prodorsum are ovate with their longitudinal axes directing the scapular setae posteriorly (Fig. 6C).

In adult females the genital chamber is broader and covered by a broad sub-triangular epigynium (Fig. 6F). The epigynium is ornamented with a longitudinally arranged single row of striae; some of them are incomplete (Fig. 6F). The area at the hinge region is ornamented with microtubercles. In adult males the epigynium is absent. The progenital chamber is exposed, slightly elevated, ovate and covered with microtubercles (Fig. 6E). The ejaculatory ducts opened as a pair of knob-like protrusions on the progenital chamber (Fig. 6E). The genital opening is absent in larvae and nymph. Two pairs of coxisternal plates are present in the region anterior to the epigynum (epimeral region), and they bear three pairs of setae (two pairs on the 'fore-coxa' and one pair on the 'hind-coxa') (Fig. 6F). The coxisternal plates are fused without any clear external demarcation.

All active instars of *A. cajani* have two pairs of five-segmented legs consisting of trochanter, femur, genu, tibia and tarsus. All segments are devoid of tubercles and bear setae with the exception of the trochanter (in both pairs of legs) and tibia (in the second pair of legs only). The seta on the femur is inserted ventrally. The seta on the genu is the longest and positioned dorsally. The tarsus bears a pair of setae positioned dorso-laterally, a solenidion positioned between them and a short seta ventro-distally (Fig. 6G). The solenidion possesses a stout base tapering towards the end with a slightly enlarged apex. The tarsus bears an empodium at its tip (Fig. 6G and H). The empodium is branched symmetrically

into five rays (Fig. 6G and H). The empodial rays, with the exception of those placed terminally are branched secondarily into two or three apical rays, which possess enlarged pad like tips (Fig. 6H).

The taxonomic position of *A. cajani,* based on morphological characters, was assessed using a key for Eriophyoidae classification described by Lindquist & Amrine (1996) and compared with the previous description of *A. cajani* (Channabasavanna, 1966) (summary given in Table 3). The description of these

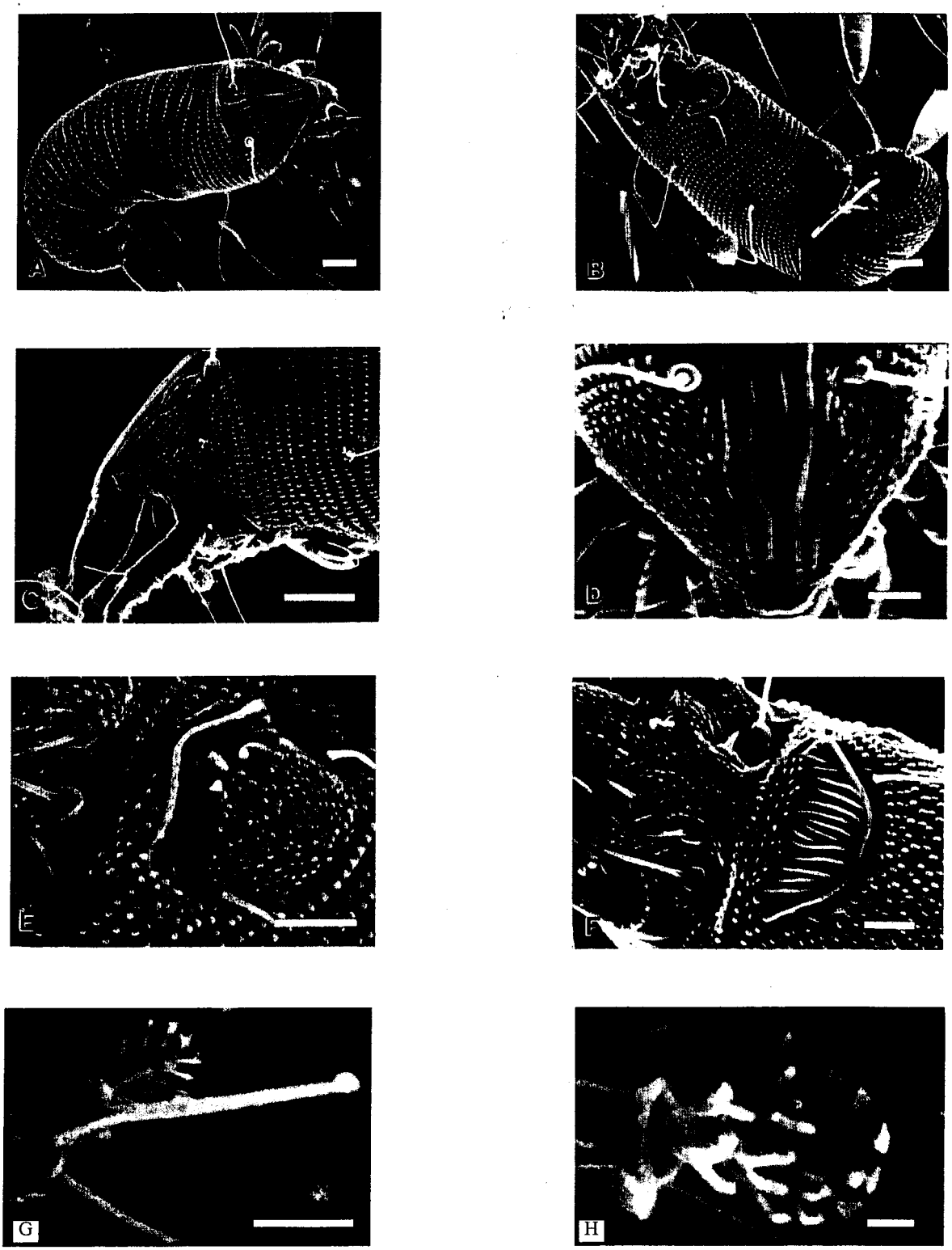

Fig 6. Scanning electron micrographs of morphological features of *A. cajani*. A. Lateral view; B. Ventral view; C. Lateral view; D. Prodorsal shield; E. Male progenital chamber; F. Female progenital chamber and coxisternal plate; G. Fore leg and solenidium; H. Empodium. Bar = 10 μm on A, B & C; 5 μm on D, E, F & G; and 1 μm on H

Table 3. *Assessment of* Aceria cajani *taxonomic position*

Morphological features	Taxonomic position
Body divided into prodorsum and opisthosoma. Prodorsum covered with a shield. 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. Ornamentation on epigynium. Complete coxal and leg setation present.	Family: *Eriophyidae*
Prodorsal shield possess setae and a narrow lobe present at the anterior end. 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 marginof the shield, diverging setae posteriorly.	Tribe: *Aceriini*
Gnathosoma shorter than legs. No anterior spines on prodorsal shield.	Genera: *Aceria*
No branches on the submedian line of the prodorsal shield. Frontal lobe wedge shaped. Tubercles present on the lateral side of the prodorsal shield. Empodium with five rays.	Species: *cajani*

mites is very similar to that described for *A. cajani* from Bangalore, Karnataka state; Poona, Maharashtra state and New Delhi, India, by Channabasavanna (1966), indicating that the mite inhabiting pigeonpea is one species. However, some minor differences from the earlier report were noted. *A. cajani* was described as having a nearly complete median line on the prodorsal shield, bare coxae and six rayed empodium (Channabasavanna, 1966), but we observed that the median line is incomplete, the coxa is ornamented and possesses a five-rayed empodium. This was confirmed by our high-resolution LTSEM images that allowed observation of minute detail (Fig. 6). Although some other members of the genus *Aceria* were described as having six rayed empodium, this feature is not considered for delineating mites into families or genera (Lindquist & Amrine, 1996).

A. cajani *and host plant resistance*

In this study, the mite samples analysed were from diverse geographical locations spanning three countries, different agro-ecological conditions, and were sampled over different seasons, and from pigeonpea crops sown in the early (June-August) or late (September-December) monsoon season. Furthermore, mite populations were obtained from the regions where pigeonpea genotypes showed variation in reaction to PSMD (Puddukkotai, Tamil Nadu State; Bangalore, Karnataka State; Badanpura, Maharashtra state; Patancheru, Andhra Pradesh state, India; and Nepalgunj, Nepal) (Reddy *et al.*, 1998). The results of this present study using sensitive rDNA analysis indicate clearly that all mites on PSMD-affected pigeonpea in the Indian subcontinent are indistinguishable and are morphologically the same as *A. cajani.* There was no evidence from these different locations of the involvement of other eriophyid species in the transmission of the PSMD agent or that mites differed significantly in their ability to transmit the agent. Thus, genotypes, such as ICP8863, that are susceptible to both mites and virus, become affected with PSMD at all locations in the Indian subcontinent indicating that mites at all these locations can transmit the PSMD agent. Some pigeonpea genotypes differ in their resistance to field infection with PSMD and this is due to either resistance to: (i) mites and virus (e.g. ICP7035); (ii) virus only (e.g. ICP8136, mites multiply but not virus); (iii) mites only (e.g. ICP15650); (iv) mild resistance to virus (e.g. ICP2376, shows localised/mild mosaic symptoms). Genotype ICP2376 showed moderate resistance (mild mosaic symptoms) to PSMD at Patancheru but severe mosaic at Bangalore and this reaction pattern remained the same when this genotype was infected by grafting with these different PSMD sources. This suggests that these different reactions are determined by the pathogen strain. Studies on the inheritance of resistance to PSMD using two strains of the pathogen also suggest that variation in host reaction is pathogen dependent (Srinivas *et al.*, 1997*a*, *b*).

It is noteworthy that under natural conditions none of the asymptomatic plants examined contained mites, whether or not they were resistant or

susceptible to the PSMD agent. Furthermore, on PSMD-affected plants, mites were found mostly on symptomatic leaves. Even under experimental conditions, it is difficult to maintain mites on PSMD-free pigeonpea, even on genotypes that are susceptible to mites alone. All these observations suggest that infection with the PSMD agent increases mite multiplication, confirming earlier studies (Reddy & Nene, 1980; Muniyappa & Nangia, 1982; Reddy *et al.*, 1989). A similar situation is reported in blackcurrant (*Ribes nigrum*) where the numbers of eriophyid gall mites (*Cecidophyopsis ribis*) on plants increases greatly if plants are infected with the agent of reversion disease that the mites transmit (Thresh, 1964).

This work shows therefore that the variability observed in PSMD resistance in pigeonpea in different regions of the Indian subcontinent is not due to the occurrence of different mite species and probably not to *A. cajani* that may differ in vectoring ability. It seems most likely therefore that this variation in resistance is due to the occurrence of strains of the PSMD agent and the host interactions with these strains. Recently, a breakthrough was made in the identification of the probable PSMD agent (Kumar *et al.*, 1999*b*, 2000) and work is progressing to characterise this agent and to distinguish its variants.

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