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The Use of Cloned Sequences for the Identification of Coconut Foliar Decay Disease-associated DNA

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SUMMARY

Single-stranded circular DNA associated with foliar decay disease of coconut palm in Vanuatu (FDD-DNA) has been purified and three fragments have been cloned in plasmid pUC19. Clones labelled with ^{32}P by nick translation were used as specific probes for FDD-DNA in dot blot and Southern transfer hybridization assays. These assays were more sensitive than the polyacrylamide gel electrophoresis assay developed previously for diagnosis of FDD. Hybridization tests showed that FDD-DNA had no detectable sequence similarity to the geminivirus infecting *Digitaria sanguinalis* which grows alongside FDD-infected coconut palms; that *Hibiscus tiliaceus*, which is the host plant of the vector of FDD, *Myndus taffini*, is not infected with FDD-DNA; and that the symptomless coconut variety, Vanuatu Tall, is susceptible to infection with FDD-DNA. Native FDD-DNA migrates as two distinct bands in polyacrylamide gels, and both hybridize to the probe. Previous estimates and data presented here show that FDD-DNA is approximately half the size of the *D. sanguinalis* geminivirus DNA (approx. 2350 nucleotides) and support the view that FDD is caused by a virus not typical of any plant virus taxonomic group.

INTRODUCTION

The specific association of an unusual single-stranded circular DNA with foliar decay disease (FDD) of coconut palms in Vanuatu suggests that the disease has a viral aetiology (Randles *et al.*, 1986, 1987). The small size (approximately 1300 nucleotides) of the FDD-associated DNA (FDD-DNA), which is about half that of the single-stranded circular genomic DNA of the geminiviruses (Harrison, 1985) and the failure to observe geminivirus-like particles either in thin sections, extracts of infected tissue, or in FDD-DNA-enriched preparations, have suggested that the putative virus agent of FDD is unlike previously reported ssDNA plant viruses. The FDD-DNA most closely resembles the DNA of the porcine circovirus described by Tischer *et al.* (1982).

We now report the purification of FDD-DNA and the cloning of fragments for use in demonstrating the uniqueness of the FDD-DNA sequence. Moreover, while it is known that FDD is transmitted by the planthopper *Myndus taffini* (*Homoptera*, Cixiidae) (Julia, 1982), most aspects of disease epidemiology are unknown. We describe the development of a molecular hybridization assay which is more sensitive and versatile than the polyacrylamide gel electrophoretic assay developed previously for disease diagnosis (Randles *et al.*, 1986) and illustrate its application to elucidating the epidemiology of FDD.

METHODS

Plant tissue. Leaflets from uninoculated coconut palms, palms inoculated with *M. taffini* in insect-proof cages, or naturally infected coconut palms of a range of F_1 hybrids, as well as the other species exposed to FDD were collected at the IRHO Saraoutou Research Station, Espiritu Santo, Vanuatu. They were transported within 4 days to Adelaide, and stored at below −20 °C. Leaflets from healthy coconut seedlings grown at the Waite Institute, and leaves of other plant species tested were used without freezing.

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Extraction of plant and insect tissue. Leaf tissue was extracted for both polyacrylamide gel electrophoresis (PAGE) and dot blot hybridization assay as described previously (Randles *et al.*, 1987). Nucleic acids from *M. taffini* collected from infected palms were extracted by crushing 50 insects (approximately 100 mg) in 200 µl TNET (100 mM-Tris–HCl pH 7·2, 100 mM-sodium acetate, 10 mM-EDTA, 0·5% v/v thioglycerol), clarifying the extract by centrifugation, and adding three volumes of ethanol to precipitate the nucleic acids. Samples were dissolved in 20 µl of sterile water.

Purification of FDD-DNA. Nucleic acid extracts containing FDD-DNA were subjected to preparative electrophoresis in 3 mm thick 3·3% polyacrylamide gels containing 8 M-urea, for 4 h at 12 mA per cm^2. The position of the FDD-DNA was determined by silver staining of a lateral test strip, and a series of 2 × 120 mm horizontal slices of the gel including and adjacent to the DNA-containing zone were eluted in 1·5 ml of 0·5 M-ammonium acetate containing 1 mM-EDTA and 0·1% (w/v) SDS (Maxam & Gilbert, 1980) for at least 48 h at 40 °C. Two volumes of ethanol were added to the eluate, and precipitated material was collected by centrifugation. Aliquots of each eluate were assayed by analytical PAGE (3·3%, 8 M-urea) and those containing FDD-DNA were pooled and subjected to a second cycle of preparative PAGE as above. Following the second cycle, fractions which contained the single band of FDD-DNA were pooled.

Blot assays on membranes. For dot blot assays, nitrocellulose sheets (Schleicher & Schuell, 0·45 µm pore size) were washed in water, equilibrated in 20 × SSC (3 M-NaCl, 0·3 M-sodium citrate) and air-dried. One microlitre aliquots of nucleic acid samples (equivalent to about 0·5 g leaf extract or 5 mg insects) were applied as dots to the filters. To transfer electrophoretically separated nucleic acids from gels to membranes, samples were fractionated by electrophoresis in either 2% agarose gels, or in 3·3% or 5% polyacrylamide gels (with or without 7 M-urea) buffered in TBE (90 mM-Tris, 90 mM-boric acid, 3 mM-EDTA pH 8·3). Nucleic acids in gels were denatured by incubating in either 0·5 M-NaOH for 30 min (agarose) or 0·25 M-NaOH for 15 min (polyacrylamide). For capillary blotting (Southern, 1975) gels were neutralized in TBE, and equilibrated in 20 × SSC before transfer to nitrocellulose membrane filters (Schleicher & Schuell, 0·2 µm pore size). For electroblotting, gels were neutralized in 4 × TAE (TAE is 8 mM-Tris–HCl, 4 mM-sodium acetate, 0·4 mM-EDTA pH 7·4), equilibrated in 1 × TAE, and nucleic acids were transferred in the same buffer to nylon membranes (Zeta-Probe, Bio-Rad) for 5 h at 60 V and 0·6 A.

To prevent the 3·3% polyacrylamide gels sticking to the filters they were coated with 1% buffered agarose prior to transfer. Samples were fixed on membranes by baking *in vacuo* at 80 °C for 2 h.

Construction of FDD-specific DNA clones. Partially purified ss-FDD-DNA (10 ng) was mixed with 100 ng of random DNA primer (sheared calf thymus DNA), heat-denatured and then incubated in 20 µl at 37 °C for 90 min with two units of the Klenow fragment of *Escherichia coli* DNA polymerase I and 0·1 mM-dNTPs. The reaction was stopped and the DNA digested with the restriction endonuclease *Sau*3AI as recommended by the manufacturer (Pharmacia). This DNA was ligated directly into the *Bam*HI site of pUC19 (Vieira & Messing, 1982) and transformed into *E. coli* strain JM101. Transformants were selected on ampicillin plates and recombinants were identified as white colonies in the presence of X-gal and IPTG. The 300 colonies obtained were further screened by colony hybridization with primer-extended DNA labelled with [^{32}P]dCTP (Maniatis *et al.*, 1982). The labelled DNA was prepared by mixing 10 ng purified FDD-DNA with 100 ng random DNA primer, 20 µCi [α-^{32}P]dCTP, 50 µM each of dATP, dGTP and dTTP, 50 mM-Tris–HCl pH 7·6, 50 mM-NaCl, 10 mM-$MgCl_2$ and three units of the Klenow fragment of *E. coli* DNA polymerase I in a final volume of 30 µl. The reaction was incubated for 45 min at 37 °C and radiolabelled DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Only 12 colonies showed clear hybridization to the probe. Plasmids prepared from these colonies and digested with *Sau*3AI contained inserts of 200, 220 or 350 base pairs. These inserts did not cross-hybridize and are presumed to contain unique segments of FDD-DNA.

Hybridization assay. Nitrocellulose filters were incubated for 15 to 20 h at 42 °C in prehybridization buffer containing 0·75 M-NaCl, 75 mM-sodium citrate, 0·02% (w/v) each of bovine serum albumin (BSA), Ficoll and polyvinylpyrrolidone (PVP), 0·25 mg/ml denatured sheared herring testis DNA, 50 mM-sodium phosphate buffer pH 6·5, 50% (v/v) formamide, 5 mM-EDTA and 0·2% (w/v) SDS (Maniatis *et al.*, 1982). Nylon filters were washed for 1 h at 67 °C in 0·1 × SSC, 0·5% (w/v) SDS and then were prehybridized as above except that the buffer was modified to include 1 mg/ml DNA and 0·2% (w/v) each of BSA, Ficoll and PVP.

Plasmids containing FDD-DNA inserts were nick-translated (Maniatis *et al.*, 1982) and the resulting ^{32}P-labelled probe was extracted with equal volumes of phenol and chloroform and precipitated with ethanol. Probe was resuspended in 50% (v/v) formamide, heated at 100 °C for 3 min, and added to hybridization buffer which then contained 0·8 × prehybridization buffer, 10% (w/v) dextran sulphate, and approximately 0·5 × 10^6 c.p.m./ml. Hybridization was for 20 to 40 h at 42 °C (Maniatis *et al.*, 1982). Filters were washed for 5 min at 20 °C in 0·5 × SSC, 0·1% (w/v) SDS, and for at least 2 h at 67 °C in 0·1 × SSC, 0·1% (w/v) SDS with constant agitation.

A probe specific for the *Digitaria sanguinalis* geminivirus (Dollet *et al.*, 1986; Donson *et al.*, 1987) was prepared by nick translation of an essentially full-length clone inserted in the vector M13 mp9 (a generous gift from Dr J. Donson, John Innes Institute, Norwich, U.K.).

RESULTS

Specificity of FDD-DNA probes

Each of the three probes reacted similarly with extracts from diseased but not healthy palms in the dot blot assays. Fig. 1 shows a test with one of the probes. The post-hybridization wash at 67 °C for over 2 h was found to be necessary to remove non-specifically bound probe. The intensity of the signal varied between samples (Fig. 1) and it is not known whether failure to observe a signal with some palms selected as being diseased was due to low concentrations of FDD-DNA in extracts or to misdiagnosis of infection by symptoms.

Further extracts were assayed with both PAGE (Randles *et al.*, 1986) and dot blot hybridization. Good agreement was obtained between the assays (Table 1) but with more FDD-DNA-positive samples being detected by hybridization than by PAGE. Southern blot analysis in an agarose gel showed that the probe was specific for purified FDD-DNA (Fig. 2, 3). The strongest signal was obtained for inoculated Malayan Red Dwarf (MRD) palms which were sampled within 8 months of inoculation and within 2 months of symptom appearance. These samples also showed minor bands of higher molecular weight. Nucleic acids from field-infected MRD reacted more weakly. Specimens of the Vanuatu Tall variety (VTT), which is regarded as tolerant to FDD because it is symptomless in the field (Calvez *et al.*, 1980), were shown to contain FDD-DNA.

Table 2 shows comparative results from several palms which indicate that both PAGE and hybridization assays are more sensitive than monitoring of symptoms for diagnosis of FDD, and that the hybridization assay is more sensitive than PAGE.

FDD-DNA migrates as a single band in denaturing PAGE, but migrates as two bands with similar intensity of staining under non-denaturing conditions. Both of these bands hybridized to the 220 nucleotide probe (Fig. 4).

Lack of homology between FDD-DNA and DNA of D. sanguinalis *geminivirus*

The understorey of coconut plantations at Saraoutou frequently contains *D. sanguinalis* showing striate mosaic symptoms. Dollet *et al.* (1986) have identified a geminivirus, possibly a strain of maize streak virus, in these plants. The vector is a leafhopper, *Nesoclutha declivata* (J. F. Julia & M. Dollet, personal communication). The possibility therefore arose that the *D. sanguinalis* geminivirus, with single-stranded genomic DNA of about 2350 nucleotides, could have been associated with the disease in the adjacent coconut palms. Although the vectors of the two diseases differ, the physical similarity of the two DNAs led us to test for nucleotide sequence homology between them.

Table 1. *Association between expression of symptoms of FDD and detection of FDD-DNA by both PAGE and dot blot hybridization assays*

Sample	Appearance of plants	Total	Number positive by PAGE	Dot blot
Experimental seedlings*				
Inoculated by *M. taffini*	Symptoms†	27	24	25
Uninoculated	Symptomless	6	0	2
Palms exposed to infection‡				
	Symptoms†	37	25	31
	Symptoms appeared after assay†	5	2	4
	Symptomless†	47	2	12

* Palms of MRD indicator variety caged for inoculation, then placed in field for 6 to 8 months before assay; uninoculated seedlings therefore possibly subject to natural inoculation.

† As described in Randles *et al.* (1986).

‡ Eight year-old palms from a range of F_1 hybrids of varying susceptibility (Calvez *et al.*, 1980) in an area of high incidence.

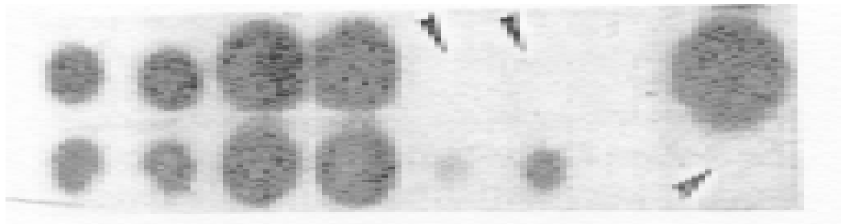


Fig. 1. Dot blot hybridization of nucleic acid extracts from symptomatic and healthy (arrowed) palms, with an FDD probe containing an insert of 350 base pairs.

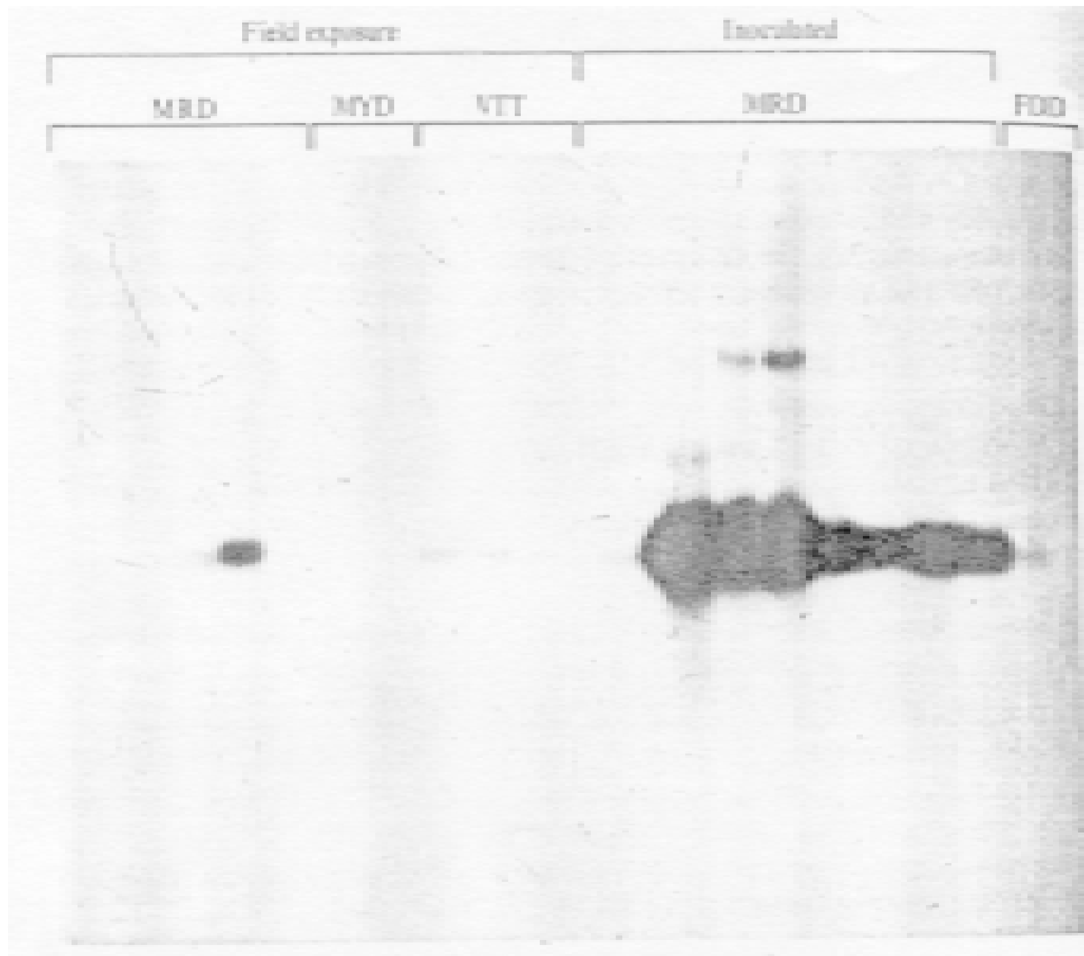
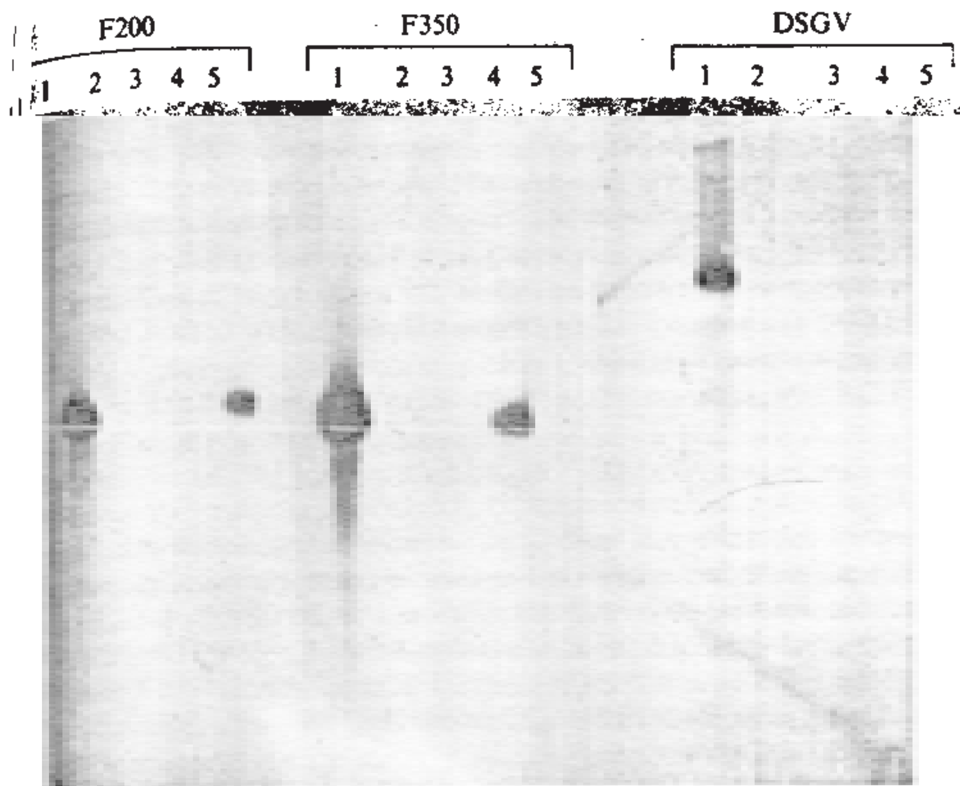


Fig. 2. Southern blot analysis of nucleic acid extracts of MRD, Malayan Yellow Dwarf (MYD), and VTT palms and purified FDD-DNA. Nucleic acids were fractionated on 2% agarose, blotted and hybridized with probe containing an insert of 350 base pairs.

The Southern analysis in Fig. 3 clearly shows that the FDD-DNA and *D. sanguinalis* geminivirus DNA lack detectable homology. Both FDD-DNA probes hybridized only with a single band in the preparation of purified FDD-DNA, and a band of the same size in nucleic acids extracted from diseased coconut palms. No nucleic acid homologous to FDD-DNA was detected in healthy coconut or *D. sanguinalis* infected with the geminivirus. Conversely, the *D. sanguinalis* geminivirus probe hybridized only to the extract from infected *D. sanguinalis*. The molecular weight of the geminivirus DNA was about twice that of the FDD-DNA, in agreement with our previous estimate of the size of FDD-DNA (Randles *et al.*, 1987). The minor higher molecular weight bands homologous to the main band of the geminivirus DNA are possibly the circular and linear dimers (Dollet *et al.*, 1986).

Assay of a range of plant species and the insect vector for sequences homologous to FDD-DNA

The host plant of the insect vector *M. taffini* is *Hibiscus tiliaceus* (Julia, 1982) which is generally found adjacent to coconut palms with high incidence of FDD. To test whether it is a host of the FDD agent, we assayed nucleic acid extracts from 11 large *H. tiliaceus* trees growing



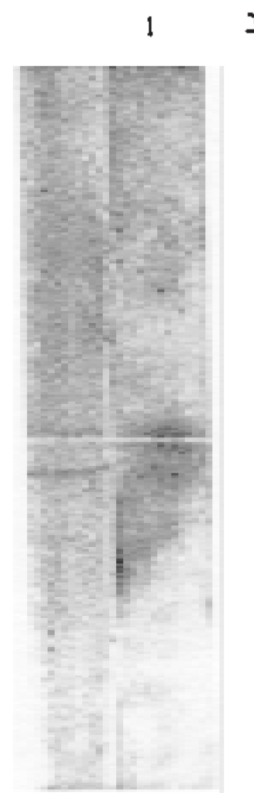


Fig. 3 Fig. 4

Fig. 3. Southern blot analysis of nucleic acid extracts from diseased (lanes 5) and healthy (lanes 4) coconut palm, *H. tiliaceus* (lanes 3) and *D. sanguinalis* with striate mosaic symptoms (lanes 2) separated on a 2% agarose gel. The marker was purified FDD-DNA (lanes 1). The FDD probes contained 200 (F200) or 350 (F350) base pairs; the *D. sanguinalis* geminivirus probe (DSGV) was essentially full-length.

Fig. 4. Separation of two bands from a preparation of purified FDD-DNA subjected to electrophoresis in a non-denaturing 3·3% polyacrylamide gel. The silver-stained bands (lane 1) both hybridize with the FDD-DNA probe (lane 2) after electrophoretic transfer to nylon membranes.

Table 2. *A comparison of PAGE, dot blot hybridization and Southern blot assays (SH) for detecting FDD-DNA in selected samples*

Palm	Symptom	PAGE	Dot blot	SH
VTT*				
1	−	+	−	+
2	−	+	+	+
3	−	−	−	−
MYD × RLT†				
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	−	+	−	NA‡
5	−	+	−	NA
6	−	+	+	NA
7	−	−	+	NA
8	−	−	+	NA
9	−	−	−	NA

* Tolerant, does not develop symptoms.
† Malayan Yellow Dwarf × Rennel Tall hybrid; sensitive and developing typical leaf symptoms of FDD.
‡ NA, Not assayed.

adjacent to coconut palms which had a high incidence of FDD. Neither dot blot assays nor Southern analysis (Fig. 3) detected FDD-DNA in this species. Other plant species were collected either at a site adjacent to coconut palms with high disease incidence (15 plants of *D. sanguinalis*, seven of *Axonopus compressus* and two of *Paspalum paniculatum*), or they were grown in isolation from FDD (two plants each of *Nicotiana tabacum*, *Lycopersicon esculentum*, *Phaseolus vulgaris*, *Vicia faba*, *Pisum sativum*, *Chenopodium amaranticolor*). The plant species were tested by dot blot assay with the FDD-DNA probe and all were negative. Moreover, extracts of the grass *Chloris gayana* infected with chloris striate mosaic geminivirus (Francki & Hatta, 1980) showed no homology with FDD-DNA; this provides further evidence for a lack of relatedness between FDD-DNA and another geminivirus.

Attempts to detect FDD-DNA sequences in the insect vector were unsuccessful.

DISCUSSION

The aetiology of FDD is unknown, and in our attempts to determine whether a virus causes the disease we have adopted the strategy of seeking and characterizing disease-associated molecules. We have previously identified a DNA molecule with unusual physical properties which is specifically associated with diseased palms and which can be identified by PAGE and used as a diagnostic marker of infection of coconut palms (Randles *et al.*, 1986). The DNA is about 1300 nucleotides in length, is circular and single-stranded (Randles *et al.*, 1987). Although we concluded tentatively that it is probably of viral origin it is unlike that of previously described plant viruses. It is about half the size of the circular ssDNA of geminiviruses (Harrison, 1985), similar in size to the DNA of porcine circovirus (Tischer *et al.*, 1982), and larger than the circular ssDNA of subterranean clover stunt virus which has a size in the range 850 to 880 nucleotides (Chu & Helms, 1987).

Cloning of FDD-DNA was therefore attempted to determine whether the nucleotide sequence of FDD-DNA was represented in either the DNA of the host plant or the DNA of a geminivirus infecting *D. sanguinalis* in the plantation understorey. The sequences cloned were uniquely associated with FDD-infected but not healthy palms and showed no homology with the *D. sanguinalis* geminivirus DNA. FDD-DNA appears to be unique and we therefore concluded, on the basis of the unique size and sequence of FDD-DNA, that the putative virus agent of FDD is a member of a previously undescribed taxonomic group.

The availability of cloned FDD-DNA sequences has allowed the development of specific and sensitive molecular hybridization tests for identifying and characterizing FDD-DNA. The hybridization tests appear to be more sensitive than PAGE assays for coconut palms, and probably have broader applicability to other species for which the PAGE nucleic acid preparation procedure developed for coconut palms may not be appropriate. Variation in the strength of the signal is presumably due to differences in FDD-DNA concentration in tissue, sample preparation, and assay procedures. Palms assayed within about 8 months of inoculation just after the first appearance of symptoms generally had higher concentrations of FDD-DNA than palms infected by natural exposure in the field (Fig. 2). Unreliability of diagnosis for FDD by symptoms is expected because of the variation in responses by different palm cultivars (Calvez *et al.*, 1980). Only MRD palms show characteristic symptoms of the disease.

Detection of FDD-DNA independently of symptomatology allows a more reliable evaluation of FDD epidemiology to be undertaken. Hybridization assays indicate that the symptomless VTT coconut palm variety can be infected, and raise the possibility that this variety which is the main component of plantations in Vanuatu could act as a latent reservoir of virus infection for sensitive varieties. Neither *H. tiliaceus*, the host plant of *M. taffini*, nor geminivirus-infected *D. sanguinalis* contain detectable sequences of FDD-DNA, and neither appears to be implicated as a virus source. Further development of more reliable and faster dot blot assays will allow other species to be assayed on a large scale as potential natural hosts of FDD-DNA. The lack of homology between the *D. sanguinalis* geminivirus DNA and FDD-DNA is in agreement with the observation that the two disease agents have different vectors (*N. declivata* and *M. taffini* respectively). Further development of the assay will be necessary to test for FDD-DNA in *M. taffini*.

The probes have allowed several structural features of FDD-DNA to be evaluated. FDD-DNA migrates as a single band in denaturing polyacrylamide gels, but generally as two bands in non-denaturing gels (Randles *et al.*, 1987). Hybridization of both bands to the same probe indicates that they share sequences and may represent either different conformations of the same molecule, or two species of DNA with a common region and very similar molecular weight.

In agarose gel electrophoresis, heavy loadings of nucleic acid extracts from infected palms sometimes showed additional minor higher molecular weight bands with homology to FDD-DNA (Fig. 2). The identity of these is unknown, but they could represent different forms of FDD-DNA, such as linear or circular dimers, similar to those apparent in the *D. sanguinalis* geminivirus DNA preparation (Fig. 3; Dollet *et al.*, 1986). These higher molecular weight bands have been observed only in agarose gels but not in polyacrylamide gels where denaturing conditions existed, and therefore are not an FDD-DNA species with the size of geminivirus DNA.

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