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Detection of coconut cadang-cadang viroid-like sequences in oil and coconut palm and other monocotyledons in the south-west Pacific

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Summary

Fractionation, electroblotting and molecular hybridisation of nucleic acids extracted from tissue of African oil palm and coconut palm and some other monocotyledonous species, collected in several areas of the south-west Pacific region, demonstrated the presence of small nucleic acids with nucleotide sequences and secondary structure similar to coconut cadang-cadang viroid (CCCVd). The oil palms which contained CCCVd-related molecules showed orange leaf spots resembling those described for oil palm naturally infected with CCCVd in the Philippines, and also characteristic of a condition known as "genetic orange spotting" (GOS). We provide preliminary evidence that GOS is an infectious disorder caused by a viroid. The coconut palms did not show symptoms typical of cadang-cadang disease, but sometimes were chlorotic, stunted, or had a reduced yield. The possibility that the isolates represent variants of CCCVd is discussed. The data suggest that viroids with nucleotide sequences similar to CCCVd occur widely in palms and other monocotyledons outside the Philippines.

Key words: Coconut cadang-cadang viroid, oil palm, coconut palm, genetic orange spotting, RNA hybridisation assay

Introduction

Cadang-cadang is a lethal disease of coconut palms (*Cocos nucifera* L.) in the central Philippines where it has been estimated to have caused losses exceeding 30 million palms (Zelazny, Randles, Boccardo & Imperial, 1982; Randles & Imperial, 1984; Randles, 1987). The causal coconut cadang-cadang viroid (CCCVd) occurs as a small (246 – 247 nucleotide) or large (296 – 297 nucleotide) form depending on the stage of infection (Imperial, Rodriguez & Randles, 1981; Haseloff, Mohamed & Symons, 1982). It has also been detected in naturally infected African oil palm (*Elaeis guineensis* Jacq.) and buri palm (*Corypha elata* Roxb.) in the Philippines and has been transmitted to these and other species of palm by mechanical inoculation (Randles, Boccardo & Imperial, 1980; Imperial, Bautista & Randles, 1985). A related viroid was found in coconuts with tinangaja disease in Guam (Boccardo, Beaver, Randles & Imperial, 1981). The tinangaja-associated viroid is a variant of CCCVd differing in symptom expression and size (254 nucleotides) and with about 64% nucleotide sequence homology to CCCVd (Randles & Imperial, 1984; Keese, Osorio-Keese & Symons, 1987). Despite the screening of a wide range of coconut varieties no genetic resistance has been detected (Randles, 1987). CCCVd spreads naturally by an unknown means and is regarded as a threat to coconut and oil palm production in south-east Asia. It is also a concern of quarantine authorities in this region (Randles, 1982).

Symptomatology is unreliable for disease diagnosis, and tests for the detection of CCCVd by polyacrylamide gel electrophoresis and molecular hybridisation have been developed (Schumacher, Randles & Riesner, 1983; Imperial *et al.*, 1985). These tests are sensitive and when used together are definitive for the viroid because they test for size, structure and nucleotide sequence. Using this method, a viroid closely resembling CCCVd has recently been reported for the first time outside the Philippines/Guam region (Hanold & Randles, 1989).

In this paper we report the results of an initial survey for nucleic acid sequences related to CCCVd in several species of palm and other monocotyledons in the region from Indonesia to Vanuatu. CCCVd-related sequences were found in all the countries surveyed indicating that viroid infection of monocotyledons may be quite common in areas outside the central Philippines.

Materials and Methods

Sample collection

A total of 259 samples were collected from 16 different sites in five western Pacific areas south of the equator. Sites were approximately 0.25 – 1 km^2 in area, and were either located on separate islands or separated by at least 10 km. Samples were tested from species of five families of monocotyledons belonging to four different superorders.

Leaflets of palm were harvested from fronds about 10 positions below the unopened spear leaf; for herbaceous plants young fully expanded leaves were chosen. Midribs were removed, and approximately 50 g of tissue was sealed in polythene bags for transport and stored at −20 °C on arrival.

Extraction

Ten to 20 g of leaf was chopped and blended in 120 ml of 100 mM Na_2SO_3. The brei was strained through cotton muslin, shaken for 30 min at 4 °C with 20 g/litre polyvinyl polypyrrolidone, mixed vigorously for 5 min with 50 ml of chloroform, then clarified by centrifugation at 10 000 ***g*** for 10 min. Polyethylene glycol 6000 was added to the aqueous supernatant at 80 g/litre and the resulting precipitate was collected after 2 h incubation at 4 °C by centrifugation as above (Randles, 1975). Nucleic acids were extracted from the precipitate by dissolving it in 2 ml of 10 g/litre sodium dodecyl sulphate (SDS) then adding 2 ml of aqueous phenol (900 g/litre) containing 1 g/litre 8-hydroxyquinoline, and shaking vigorously for 60 min. The aqueous supernatant phase was collected after centrifugation, and re-extracted with 1 ml of phenol and 1 ml of chloroform for 5 min. Nucleic acids were precipitated with 3.3 g/litre cetyl trimethyl ammonium bromide in 0.1 M NaCl (Imperial *et al.*, 1985), sedimented by centrifugation at 10 000 ***g*** for 30 min, washed three times with 0.1 M Na-acetate in 75% ethanol, then dissolved in TBE (90 mM Tris, 90 mM boric acid, 3 mM ethylene diamine tetraacetic acid (EDTA), pH 8.3) buffer with 50% glycerol and marker dyes ready for electrophoresis.

Hybridisation assay

A complementary RNA (cRNA) probe labelled with ^{32}P was prepared by Dr J. L. McInnes (Biochemistry Department, University of Adelaide) from a pSP64 plasmid containing a monomeric insert of the 246 nucleotide form of CCCVd at the *Bam*HI site (provided by Dr J. E. Visvader). Transcription was done with a kit from BRESATEC (Adelaide) and the RNA was isolated on a 5% polyacrylamide gel containing TBE and eluted for 16 h at 37 °C in 500 mM ammonium acetate, 1 mM EDTA, 1 g/litre SDS (Maxam & Gilbert, 1980). The probe was precipitated with ethanol, and resuspended in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 g/litre SDS.

For dot blot assays samples of 1 μl were applied to sheets of nitrocellulose filter (Schleicher and Schuell, 0.45 μm) which had been washed in distilled water, equilibrated in 20 × SSC (3 M NaCl, 0.3 M Na-citrate) and air dried (Thomas, 1980). After sample application, filters were baked for 2 h at 80 °C.

Nucleic acid samples were fractionated by electrophoresis in 5% or 20% polyacrylamide gels (PAGE) buffered in TBE. The CCCVd standard was prepared from infected palms in the Philippines and mixed to provide similar amounts of each of the 246, 247, 296 and 297 nucleotide forms with their respective dimers. Non-denaturing/denaturing two dimensional 5% PAGE was done according to Schumacher *et al.* (1983). Gels were equilibrated in TAE (9 mM Tris, 4 mM Na-acetate, 0.4 mM EDTA, pH 7.4) for 15 min and nucleic acids were transferred to nylon membranes (Zeta-probe, Biorad) in the TAE buffer at 60 V, 0.6 A for 5 h or 30 V, 0.3 A overnight followed by 60 V, 0.6 A for 1 h. Nucleic acids were fixed by baking for 2 h at 80 °C.

Pre-hybridisation was done for 15 – 20 h at 42 °C. For nitrocellulose, the pre-hybridisation buffer of Thomas (1980) was modified as follows: 0.75 M NaCl; 75 mM Na-citrate; 50 mM sodium phosphate at pH 6.5; 5 mM EDTA; 0.2 g/litre each of bovine serum albumin (BSA), Ficoll 400 (Pharmacia), polyvinyl pyrrolidone (PVP) of mol. wt 40 000; 2 g/litre SDS; 0.25 mg/ml denatured herring testis DNA; 50% deionised formamide. Nylon was washed for 1 h at 67 °C in 0.1 × SSC, 1 g/litre SDS and pre-hybridised in a modification of the above buffer according to the manufacturer's instructions (1 mg/ml denatured carrier DNA, 2 g/litre each of BSA, Ficoll and PVP).

The cRNA probe was heated at 80 °C for 1 min in 50% formamide and added to the hybridisation mixture (consisting of the nitrocellulose pre-hybridisation buffer with 110 g/litre dextran sulphate) at about 10^6 cpm/ml, and hybridisation was done at 42 °C or 37 °C for 20 – 40 h (Thomas, 1980). Filters were then washed for 5 min at 20 °C in 0.5 × SSC, 1 g/litre SDS, then by agitation for 1 h at 55 °C in 1 × SSC, 1 g/litre SDS for low stringency, or for 2 h at 67 °C in 0.1 × SSC, 1 g/litre SDS for high stringency, and then subjected to autoradiography at −70 °C using intensifying screens.

For the large scale survey non-denaturing PAGE in 20% gels was used to resolve the various forms of CCCVd. Each blot was first washed at low stringency, autoradiographed, re-washed at high stringency, and again autoradiographed adjusting the exposure times to obtain signal intensities similar to those of the low stringency wash.

Results

Samples collected

Table 1 shows the species collected and assayed in this survey, and their distribution.

The coconut palms sampled were sometimes shorter than their neighbours, and some showed either chlorosis, translucent leaf spots, narrowing of nuts or reduced bearing. However, none showed the disease syndrome typical of either cadang-cadang or tinangaja diseases.

Oil palms with bright orange spotting on leaflets were observed at an incidence of between 0.1 and 10% in plantations. These palms were generally smaller than their neighbours, bore smaller bunches, and appeared from a distance to be bronze-coloured to necrotic. The bright orange, non-necrotic spots were about 2 – 3 mm long and similar to those described for oil palm with cadang-cadang-like symptoms in the Philippines (Randles *et al.*, 1980; Imperial *et al.*, 1985), and for palms with "genetic orange spotting" (GOS; Turner & Bull, 1967). The youngest fronds were free of spots, whereas the size and number of spots increased with

Table 1. *Results of analyses for CCCVd-related sequences in samples of various species from five different geographical areas*

Family	Species	Area*	Symptoms**	Positive in dot blot†	Positive in electroblot after high-stringency wash†
Arecaceae	*Cocos nucifera*	I (1)	nil	7/8	5/8
	(Coconut palm)	II (1)	nil	4/22	4/4
		III (7)	nil	40/102	33/36
		IV (3)	nil	11/19	3/9
		V (1)	nil	9/34	8/9
	Elaeis guineensis	I (1)	GOS (1)	1/1	1/1
	(Oil palm)	II (2)	GOS (28)	–	–
		III (3)	nil	0/8	0/3
			nil††	5/10	5/10
			GOS (43)	12/12	12/12
		V (1)	nil	0/2	–
	Metroxylon sagu	IV (1)	CB (1)	1/1	0/1
	Metroxylon warburgii	V (1)	nil	0/3	–
	Areca catechu	III (1)	nil	0/2	0/1
		IV (1)	nil	1/1	0/1
			CB (many)	2/2	2/2
Pandanaceae	*Pandanus* sp.	III (1)	nil	–	1/1
Commelinaceae	Unidentified	II (1)	nil	–	1/1
		III (1)	nil	–	0/1
Zingiberaceae	*Alpinia* sp.	II (1)	nil	–	4/11
		III (3)	nil	–	2/7
	Zingiber sp.	III (3)	nil	–	3/9
Marantaceae	Unidentified	II (1)	nil	–	2/2

* Names of areas are confidential; number of sites surveyed per area given in parenthesis for each species.
** GOS = "genetic" orange spotting of oil palm; CB = chlorotic leaf blotches with concentric line pattern; total number of symptomatic palms observed given in parenthesis.
† Ratios in these columns are number positive/number tested.
†† Symptomless plants adjoining those with GOS in plantation shown in Fig. 8.

increasing age of the fronds, and the oldest fronds frequently showed distal necrosis of leaflets (Fig. 1).

Some betelnut palms (*Areca catechu* L.) and the sago palm (*Metroxylon sagu* Rottb.) showed chlorotic leaf blotches with concentric line patterns, crown size and frond number were reduced, and trees died prematurely (G. V. H. Jackson, personal communication).

None of the other species showed any abnormalities.

Hybridisation assays

Dot-blot hybridisation followed by high-stringency wash (Table 1) was used as a method for selecting some of the samples for further analysis. A number of leaf extracts from coconut and other palms, and monocotyledonous understorey plants also bound the cRNA probe, but the signals were weaker than those obtained for purified CCCVd which was used as a standard on all blots. Extracts from oil palms with orange spotting also bound the probe.

To test whether the probe was binding specifically to molecules of about the same size as viroids, leaf extracts were fractionated by polyacrylamide gel electrophoresis and electroblotted to nylon membrane before hybridisation (Table 1). Samples from some asymptomatic coconut palms run on 5% non-denaturing gels, showed two or three CCCVd specific bands

Fig. 1. Orange leaf spots ("genetic" orange spotting) associated with the detection of CCCVd related viroid in oil palm. Note their increasing frequency and size on fronds of increasing age (left to right) and the distal necrosis of the oldest leaflet shown.

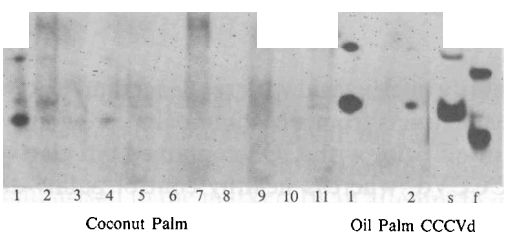

Fig. 2. Hybridisation of CCCVd-specific cRNA to an electroblot from a non-denaturing 5% polyacrylamide gel, followed by a high-stringency wash. Samples were: different coconut palms, and two different leaflets of a diseased oil palm. Monomers and dimers of the 296/297 nucleotide (s) and 246/247 nucleotide (f) forms of CCCVd were used as markers. Coconut samples 1, 2, 3, 4 and the oil palm were positive.

differing slightly in their electrophoretic mobility from the viroid marker bands (Fig. 2). Under the same conditions, samples from the diseased oil palms showed two CCCVd specific bands, corresponding approximately in electrophoretic mobility to the monomer and dimer of the large form of CCCVd. A high-stringency post-hybridisation wash of the blot failed to remove the CCCVd-cRNA probe from either the coconut or the oil palm samples, indicating a significant level of sequence homology to CCCVd. Extractions and assays were repeated several times to confirm these results.

To determine whether the CCCVd-related sequences were associated with viroid-like molecules, electroblots of two-dimensional non-denaturing/denaturing 5% PAGE were subjected to a hybridisation assay followed by a low-stringency wash. Two-dimensional PAGE identifies viroids and other small circular nucleic acids by their different relative electrophoretic mobilities under non-denaturing and denaturing conditions; circularity causes them to trail as a specific band behind the diagonal front (Schumacher *et al.*, 1983; Randles, Hanold & Julia, 1987; Sano, Hataya, Terai & Shikata, 1989). The cRNA probe was used,

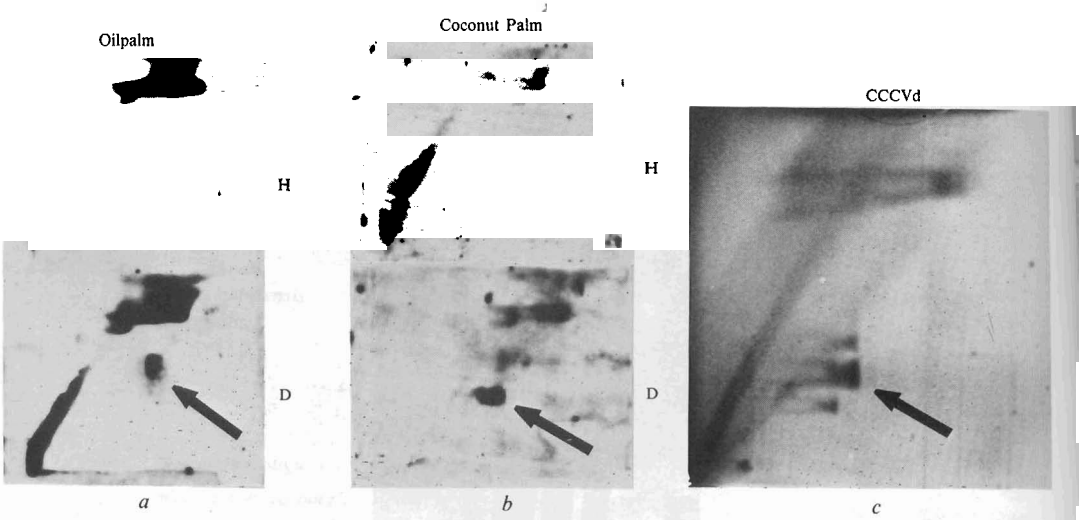

Fig. 3. Two dimensional 5% PAGE with non-denaturing conditions in the first dimension and denaturing conditions in the second. Circular molecules migrate in this system behind the diagonal front of linear nucleic acids and arrows indicate the positions of the CCCVd-related viroids.
(*a*) An example of electroblot hybridisation with low-stringency wash for oil palm samples from healthy asymptomatic (top) and orange-spotted (bottom) trees.
(*b*) An example of electroblot hybridisation with low-stringency wash for coconut samples from healthy control (top) and from asymptomatic palm containing CCCVd-related viroid (bottom).
(*c*) Silver stained gel of CCCVd marker run under conditions similar to (*a*) and (*b*). The arrow indicates the 246 nucleotide form of CCCVd.

instead of staining, to identify nucleic acid with sequence similarity to CCCVd in the position expected for a viroid. Leaf extracts from a coconut palm, previously shown to give a positive signal by probing, and an oil palm with GOS contained circular viroid-like molecules with sequence similarity to CCCVd, whereas healthy control palms did not (Fig. 3). The non-specific binding of probe to material, other than the viroid-like band, in both infected and healthy plants can be explained by the use of a low-stringency wash to obtain a strong signal from the viroid-like molecules in this experiment.

Incidence of viroid-like sequences

Of 185 coconut samples collected and assayed by the dot blot procedure, 71 (38%) were positive (Table 1). A group of 66, including dot blot positives and a small number of dot blot negatives, were subjected to electroblot assay under high-stringency conditions. All samples that were positive by electroblot (53 samples) were positive by dot blot. Those negative by dot blot were also negative by electroblot. In the few cases where samples were recorded as positive by dot blot but negative by electroblot, the dot blot signal was weak.

A total of 72 palms with GOS were recorded. Of these, 13 were assayed both by dot blot and electroblot, and an absolute correlation was observed between the presence of GOS and the detection of CCCVd-related bands (13/13, Table 1 and Fig. 5*a,b*). We failed to detect CCCVd-related sequences in symptomless oil palms either by dot blot (0/10, Table 1) or by electroblot (0/3, Table 1 and Fig 5*c*).

Of the nine samples of *Metroxylon* and *Areca*, four were positive in dot blot, but in electroblot only the two *Areca* samples with symptoms were positive.

Other species collected were assayed by electroblot only and positives included one of one *Pandanus* sp., one of two Commelinaceae, two of two Marantaceae, and nine (33%) of 27 Zingiberaceae.

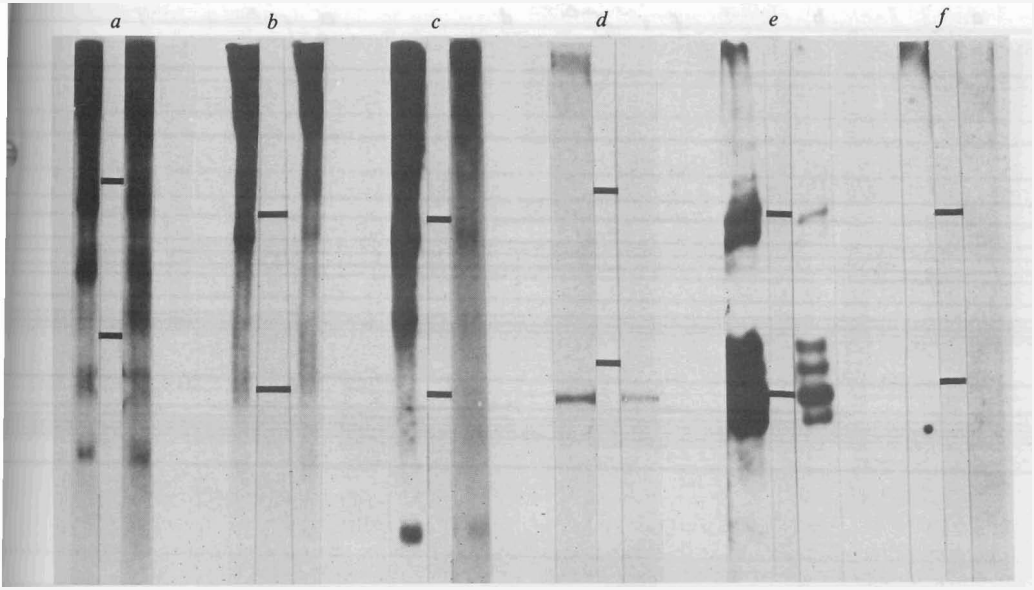

Fig. 4. Hybridisation assay of electroblots from 20% non-denaturing PAGE. Each pair is a comparison of selected *C. nucifera* samples following a low-stringency (left) and high-stringency (right) wash. Monomeric and dimeric CCCVd marker bands of 246 and 492 nucleotides, respectively, are indicated for each by a bar between each gel pair. *a, b*: palms from area III. *c*: palm from area V. *d*: palm infected with CTiVd. *e*: palm infected with CCCVd. *f*: healthy control.

Variation in band number, mobility, and binding of probe

Examples of assays of a selection of samples from all the families included in the survey are shown in Figs. 4 – 6 and Table 2. The loss of probe from the samples after high-stringency wash was estimated in comparison to the marker. In an attempt to quantify the loss of probe, densitometer traces of autoradiographs were made in selected cases to assess relative heights of peaks compared to the marker (Fig. 7).

The number and position of bands and their strength of binding to probe varied markedly (Table 2), but all samples showed multiple bands. No consistent patterns were observed with respect to either species or area of symptoms. All positive *Cocos* and orange-spotted *Elaeis* samples analysed had bands resistant to high-stringency washing conditions in the region of the CCCVd dimers, and some had, in addition, bands in or below the monomeric region. Some samples of the other species had bands only in and below the monomeric CCCVd region.

Evidence for natural spread of CCCVd-like sequences

Although the above results provide circumstantial evidence that a viroid similar to CCCVd causes orange leaf spotting in oil palms, final proof will depend on showing that the putative viroid is infectious in its purified form, and is pathogenic. However, we have obtained evidence that the viroid-like molecule is transmissible in the field from a survey of oil palms surrounding a cluster of four palms with GOS in a 12 year old commercial plantation. Five out of 10 asymptomatic trees as shown in Fig. 8 contained CCCVd-related bands, and the distribution of the positive and negative palms would be consistent with spread of viroid from the diseased palms. Because CCCVd can be detected by hybridisation in coconut palms in the Philippines about one year before the appearance of symptoms (M J. B. Rodriguez, personal communication) the site was revisited after one year. Two of the five positive palms had

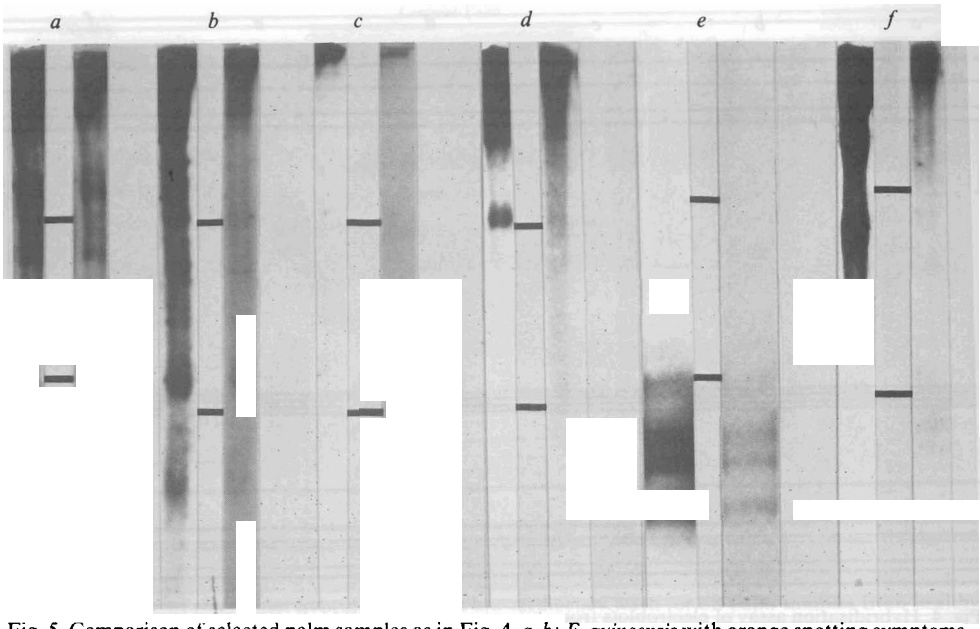

Fig. 5. Comparison of selected palm samples as in Fig. 4. *a, b*: *E. guineensis* with orange spotting symptoms (area III), *c*: symptomless oil palm (area III), *d*: symptomless *A. catechu* (area III). *e*: betelnut palm exhibiting chlorotic leaf blotches with concentric line pattern (area IV). *f*: *M. sagu* with chlorotic leaf blotches (area IV), an example of loss of bands following the high stringency wash.

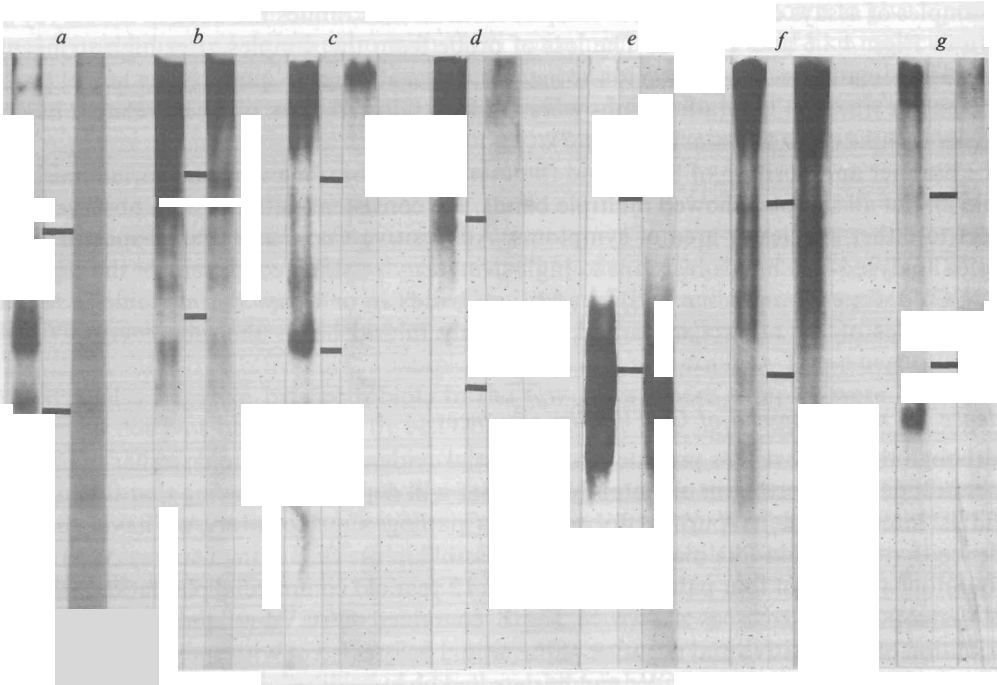

Fig. 6. Comparison of samples from assorted asymptomatic monocot species as in Fig. 4. *a*: *Pandanus* (area III). *b, c*: *Alpinia* (area II). *d*: *Zingiber* (area III). *e, f*: samples of Marantaceae (area II). *g*: sample of Commelinaceae (area II).

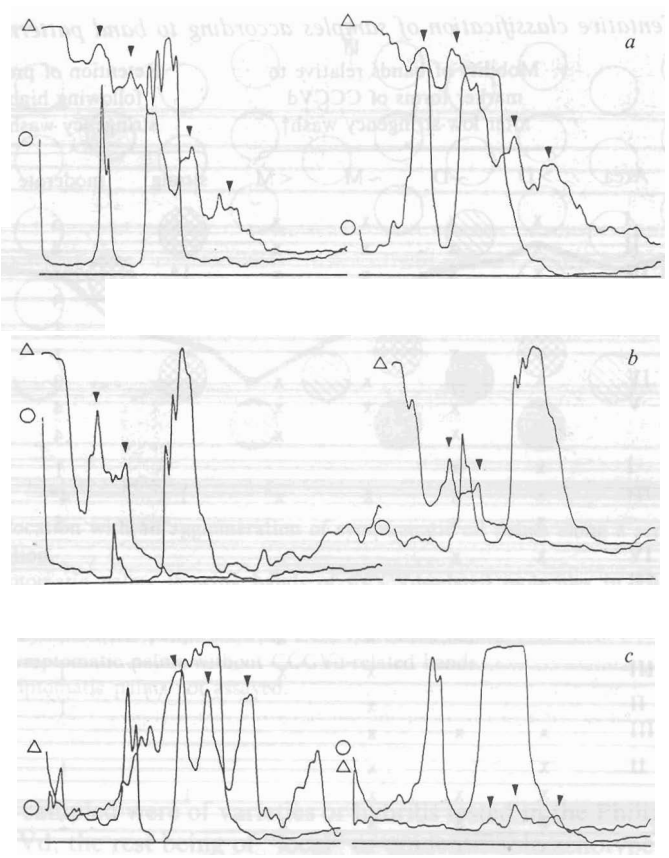

Fig. 7. Densitometer traces of selected pairs from area III: for each sample track (lines starting at triangles), the corresponding CCCVd control (lines starting at circles) is superimposed after an identical exposure time. Arrows indicate bands in sample tracks with sequence homology to the CCCVd probe. Signal intensity relative to the marker can be observed in the traces.
a: Coconut palm; no relative decrease of signal intensity at high stringency (right).
b: Oil palm (Fig. 5*b*); small relative probe loss.
c: *Pandanus* (Fig. 6*a*); much of the probe is lost at high stringency.

started to develop orange spotting at this second observation indicating that detection of viroid-like RNA may precede development of this symptom.

Thirty plantlets raised from seeds (in collaboration with J. Saelea and R. Liloquila) of two symptomatic oil palms showed an incidence of GOS of 20% after 18 months. This demonstrates seed transmissibility of the condition in the region of our survey, in agreement with earlier reports from other areas (Forde & Leyritz, 1968; Gascon & Meunier, 1979).

Discussion

Our results show that viroid-like nucleic acid similar to CCCVd occurs in coconut palms in the areas surveyed. Sequence similarity has been demonstrated by hybridisation in electroblots, but the bands are weaker and bind probe less strongly than those obtained with marker CCCVd. As differences in sequence will cause differences both in band mobility and strength of binding of probe, we conclude that there is some degree of sequence heterogeneity between the nucleic acids detected and the CCCVd probe. Since, in our assay, CTiVd with 64% sequence homology to CCCVd (Koltunow & Rezaian, 1989) retains nearly as much probe at high stringency (Fig. 4*d*) as the marker, and chrysanthemum stunt viroid with 44%

Table 2. *Tentative classification of samples according to band pattern in 20% PAGE*

Species	Area	Mobility of bands relative to marker forms of CCCVd after low-stringency wash†				Retention of probe following high-stringency wash††			Example in Fig.
		>D	~D	~M	<M	strong	moderate	low	
C. nucifera	I	x	x	x	x		5	1	
	II	x	x	x	x		4		
	III	x	x	x	x	1*	22**	2	7*a**,4*a***
		x	x		x		3		4*c*
		x	x	x			1		
		x	x				1		4*b*
	IV	x	x	x	x		3		
	V	x	x	x	x		4		
		x	x		x		4		
E. guineensis	I	x	x				1		5*a*
	III	x	x	x	x	1	4*	2	5*b**
		x	x				5		
M. sagu	IV	x	x					1	5*f*
A. catechu	III	x	x					1	5*d*
	IV	x	x	x	x		1	1	
				x	x	1			5*e*
Pandanus sp.	III			x	x		1		6*a*
Commelinaceae	II			x			1		6*g*
	III	x	x	x				1	
Alpinia sp.	II	x		x			1		6*c*
		x	x	x		1		2	
		x	x	x	x		2		6*b*
		x	x					3	
	III	x	x	x	x		1	2	
				x	x		1		
Zingiber sp.	III	x	x	x	x		3	1	
		x	x	x				1	
		x	x					1	6*d*
Marantaceae	II	x	x	x		1			6*f*
				x	x	1			6*e*

*,** Denotes appropriate figure.
† D,M: Dimers and monomers, respectively, of marker CCCVd. Bands in same regions are not necessarily identical.
†† Number of samples.

homology (Koltunow & Rezaian, 1989) does not show a signal even at low stringency (data not shown), we conclude that the extent of homology of the samples assayed can be estimated to be in the range of these values. Purification and sequencing will be necessary to compare isolates and determine their degree of relatedness to the oil palm isolates and CCCVd.

The coconut palms showed bands in the range of both the large and small forms of CCCVd. As the large form of CCCVd appears at the medium/late stage of cadang-cadang disease (Imperial *et al.*, 1981; Mohamed, Haseloff, Imperial & Symons, 1982) it would be expected that symptoms of cadang-cadang should occur on these palms. The absence of typical symptoms of cadang-cadang in coconut implies that either the isolates are variants of CCCVd with low pathogenicity, or that some unknown environmental factor influences disease development. Genetic resistance can almost certainly be ruled out since many of the

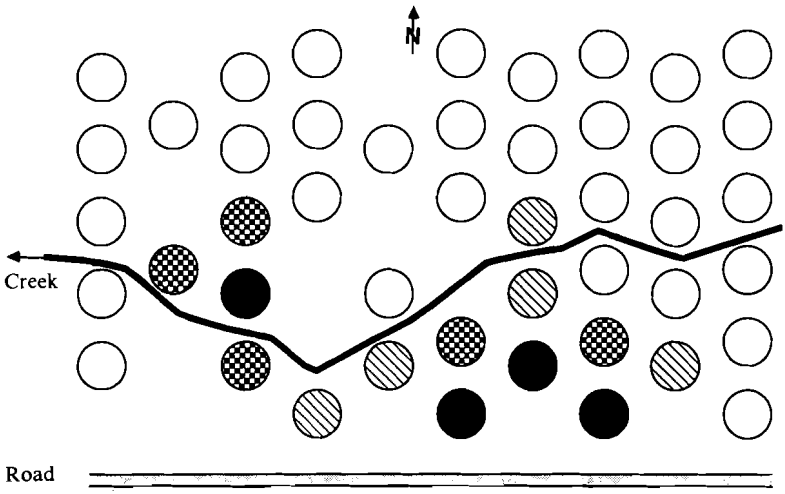

Fig. 8. Map of a location with an agglomeration of symptomatic oil palms along a service track in a commercial plantation.
Solid circles: symptomatic palms showing bands of CCCVd-related molecules in PAGE-electroblot hybridisation.
Checkered circles: asymptomatic palms showing CCCVd-related bands.
Hatched circles: asymptomatic palms without CCCVd-related bands.
Empty circles: asymptomatic palms not assayed.

symptomless palms sampled were of varieties or hybrids tested in the Philippines and found susceptible to CCCVd, the rest being of "local" or unidentifiable genotype. Further work is needed to test the two hypotheses. Low-pathogenicity variants of viroid could be used in tests of cross-protection for the control of CCCVd, whereas identification of a possible environmental factor may provide control measures for CCCVd-affected areas.

The orange spotting of oil palm occurring as a gradient of increasing severity downwards through the crown and with even distribution on all sides of the tree, is a well known disorder tentatively attributed to genetic causes and named GOS. It has been reported from both West Africa and the Malay peninsula, and is associated with reduced productivity (Forde & Leyritz, 1968). It is, however, transferred through the seed from parent to progeny in a pattern unlike any genetically inherited trait (Gascon & Meunier, 1979). The recommendation has been made that palms with progeny showing GOS (occasionally also referred to as "confluent orange spotting") should not be used for seed production (Robertson, Prendergast & Sly, 1968; Gascon & Meunier, 1979). The orange-spotted palms observed in this survey closely resembled palms at the Albay Research Center, Philippines which were either naturally infected (Randles *et al.*, 1980) or experimentally inoculated with CCCVd (Imperial *et al.*, 1985). Our analyses of a number of palms with GOS symptoms (Fig. 1) have demonstrated an association between the orange spotting and the detection of viroid-like nucleic acid. The only non-spotted palms tested which had detectable viroid-like sequences were five trees adjoining GOS palms, and two of these later developed spots resembling early symptoms of the disorder (Fig. 8).

The GOS-associated viroid-like molecules in oil palm had electrophoretic mobilities in 5% PAGE approximating those of the monomers and dimers of the large form (296 or 297 nucleotides; Haseloff *et al.*, 1982) of CCCVd (Fig. 2). The bands had significant but not complete nucleotide sequence homology to a full-length cRNA probe for CCCVd, and the behaviour of selected samples in two-dimensional gel electrophoresis was typical of a viroid-

like nucleic acid molecule. The detection of monomeric and dimeric viroids in similar amounts has only been reported for CCCVd (Randles, 1975) and we therefore conclude that oil palm "genetic" orange spotting is probably an infectious disorder caused by a viroid allied to CCCVd. Continuing surveys are under way to further test the correlation between GOS and CCCVd-related molecules observed here, in other geographical areas. Preliminary results obtained in collaboration with the Institute de Recherches pour les Huiles et Oléagineux (M. Dollet, Montpellier) indicate that similar sequences are also present in oil palm in South America and West Africa. Viroid purification, inoculation, and induction of disease are required to prove the viroid hypothesis, while sequencing should allow the examination of differences between different isolates and their degree of relatedness to CCCVd. The data in Fig. 8 show that palm-to-palm spread of the putative viroid may be occurring, and this is the first evidence of such an event for a CCCVd allied viroid. Seed transmission of the orange spotting indicates that the putative viroid is probably seedborne and such seedborne infections may be primary foci for secondary spread of viroid to adjacent palms. Secondarily infected palms may be presymptomatic and may act as a source of infected seed for carryover of viroid into the next generation. In this way, viroid infection could be perpetuated in propagating material even if symptomatic palms are excluded as a seed source. Alternatively, the putative viroid may spread into nurseries or plantations from an outside source. Further mapping of outbreak areas may show the patterns of spread and identify origins of infection.

A limited number of samples in the Commeliniflorae and Zingiberiflorae were included in the survey because members of these superorders growing in the vicinity of cadang-cadang-infected coconuts have been shown to contain CCCVd-related sequences in the Philippines (Hanold, Rodriguez & Randles, 1989; M. J. B. Rodriguez & D. Hanold, unpublished data). The CCCVd sequences found in the understorey monocotyledons may represent either different forms of the respective palm viroids, or additional related members of the same viroid family. Whether there is any viroid movement between understorey plants and palms remains to be determined.

Our results indicate that a certification program for propagating material of coconut and oil palm needs to be established using molecular methods, and not symptoms as a basis for viroid indexing, and that the risk of CCCVd to palm production in the Pacific region should be further evaluated.

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References

Boccardo, G., Beaver, R. G, Randles, J. W. & Imperial, J. S. (1981). Tinangaja and bristle top, coconut diseases of uncertain etiology in Guam, and their relationships to cadang-cadang disease of coconut in the Philippines. *Phytopathology* **71**, 1104–1107.

Forde, S. C. M. & Leyritz, M. J. P. (1968). A study of confluent orange spotting of the oil palm in Nigeria. *Journal of the Nigerian Institute for Oil Palm Research* **4**, 371–380.

Gascon, J. P. & Meunier, J. (1979). Anomalies of genetic origin in the oil palm, *Elaeis*. Description and results. *Oléagineux* **34**, 437–447.

Hanold, D., Rodriguez, M. J. B. & Randles, J. W. (1989). Detection of sequences related to coconut cadang-cadang viroid in new species and in new locations. In *Proceedings, 7th Australasian Plant Pathology Society Conference, Brisbane*, p. 73.

Haseloff, J., Mohamed, N. A. & Symons, R. H. (1982). Viroid RNAs of cadang-cadang disease of coconuts. *Nature* **99**, 316–321.

Imperial, J. S., Bautista, R. M. & Randles, J. W. (1985). Transmission of the coconut cadang-cadang viroid to six species of palm by inoculation with nucleic acid extracts. *Plant Pathology* **34**, 391–401.

Imperial, J. S., Rodriguez, M. J. B. & Randles, J. W. (1981). Variation in the viroid-like RNA associated with cadang-cadang disease: evidence for an increase in molecular weight with disease progress. *Journal of General Virology* **56**, 77–85.

Keese, P., Osorio-Keese, M. W. & Symons, R. H. (1987). Coconut tinangaja viroid; sequence homology with coconut cadang-cadang viroid and other potato spindle tuber related RNAs. *Virology* **162**, 508–510.

Koltunow, A. M. & Rezaian, M. A. (1989). A scheme for viroid classification. *Intervirology* **30**, 194–201.

Maxam, A. M. & Gilbert, W. (1980). In *Methods in Enzymology* Vol. 65, Part 1, pp. 499–560. Eds L. Grossman and K. Moldave. New York: Academic Press.

Mohamed, N. A., Haseloff, J., Imperial, J. S. & Symons, R. H. (1982). Characterization of the different electrophoretic forms of the cadang-cadang viroid. *Journal of General Virology* **63**, 181–188.

Randles, J. W. (1975). Association of two ribonucleic acid species with cadang-cadang disease of coconut palm. *Phytopathology* **65**, 163–167.

Randles, J. W. (1982). Cadang-cadang disease – a potential threat to palms in South-east Asia. In *Proceedings, International Conference in Plant Protection in the Tropics, Kuala Lumpur*, pp. 135–139.

Randles, J. W. (1987). Coconut cadang-cadang. In *The Viroids*, pp. 265–277. Ed. T. O. Diener. New York and London: Plenum Press.

Randles, J. W. & Imperial, J. S. (1984). Coconut cadang-cadang viroid. *CMI/AAB Descriptions of Plant Viruses* No. 287.

Randles, J. W., Boccardo, G. & Imperial, J. S. (1980). Detection of the cadang-cadang RNA in African oil palm and buri palm. *Phytopathology* **70**, 185–189.

Randles, J. W., Hanold, D. & Julia, J. F. (1987). Small circular single-stranded DNA associated with foliar decay disease of coconut palm in Vanuatu. *Journal of General Virology* **68**, 273–280.

Robertson, J. S., Prendergast, A. G. & Sly, J. M. (1968). Diseases and disorders of the oil palm (*Elaeis guineensis*) in West Africa. *Journal of the Nigerian Institute for Oil Palm Research* **4**, 381–409.

Sano, T., Hataya, T., Terai, Y. & Shikata, E. (1989). Hop stunt viroid strains from dapple fruit disease of plum and peach in Japan. *Journal of General Virology* **70**, 1311–1319.

Schumacher, J., Randles, J. W. & Riesner, D. (1983). A two-dimensional electrophoretic technique for the detection of circular viroids and virusoids. *Analytical Biochemistry* **135**, 288–295.

Thomas, P. (1980). Hybridisation of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proceedings of the National Academy of Science USA* **77**, 5201–5205.

Turner, P. D. & Bull, R. A. (1967). *Diseases and disorders of the oil palm in Malaysia*. Incorporated Society of Planters, Kuala Lumpur.

Zelazny, B., Randles, J. W., Boccardo, G. & Imperial, J. S. (1982). The viroid nature of the cadang-cadang disease of coconut palm. *Scientia Filipinas* **2**, 45–63.

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