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Characterization of the Different Electrophoretic Forms of the Cadang-Cadang Viroid

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SUMMARY

The relationship between symptom development and changes in the fast and slow electrophoretic forms of the two cadang-cadang disease-associated RNAs (ccRNA-1 and ccRNA-2) has been examined. The fast form of each is present in trees for up to 2 years before the appearance of symptoms, indicating an incubation period of about 2 years. Trees showing the first symptoms (on the nuts) contain only the fast form of the ccRNAs; the development of leaf symptoms coincides with the first detection of the slow form. After this time, both slow and fast forms are found in the newly developing fronds for the next 9 to 12 months but then, as symptoms develop, new fronds contain only the slow form. Therefore, at later stages of disease, only the slow form can be detected in all fronds. The fast form of ccRNA-1 and/or ccRNA-2 therefore appears to be the infectious form in nature. The number of nucleotides in the four ccRNAs as determined by polyacrylamide gel electrophoresis under denaturing conditions are: ccRNA-1 fast, 250; ccRNA-1 slow, 300; ccRNA-2 fast, 500; ccRNA-2 slow, 600. On the basis of two-dimensional fingerprints of ribonuclease A and T_1 digests of the four ccRNAs, it was concluded that the ccRNAs are composed of repeated sequences of ccRNA-1 fast, and each of the ccRNA-2 forms is a dimer of the respective ccRNA-1 form.

INTRODUCTION

Two circular viroid-like ribonucleic acids, ccRNA-1 and ccRNA-2, are associated with the cadang-cadang disease of coconut palms (Randles, 1975; Randles *et al.*, 1976); both RNAs have recently been shown to be infectious (N. A. Mohamed, unpublished results). Molecular hybridization experiments have shown that the two RNAs share nucleotide sequences (Randles & Palukaitis, 1979); their lengths, measured by electron microscopy, were estimated as 310 ± 3 and 438 ± 5 nucleotides for ccRNA-1 and ccRNA-2 respectively (Randles & Hatta, 1979). The two RNAs occur in infected palms as fast and slow electrophoretic variants (Imperial *et al.*, 1981): the fast forms of ccRNA-1 and ccRNA-2 predominate during early stages of the disease while the slow forms occur at later stages. Both forms occur as circular molecules differing in molecular weight but with nucleotide sequences in common, as shown by molecular hybridization. Preliminary studies showed that the fast form is more infectious than the slow (Imperial *et al.*, 1981).

The present study was therefore initiated to determine the relationship between disease development and changes in the electrophoretic variants of the cadang-cadang viroid. Fast and slow electrophoretic variants of ccRNA-1 and ccRNA-2 were also extracted from the same palms, their sequences compared by two-dimensional fingerprinting, and their sizes determined by gel electrophoresis.

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METHODS

Source of infected material. Detailed observations were made on naturally infected coconut palms in a plantation where detailed disease records have been kept since 1975. Samples were also obtained from naturally infected palms from a number of sites in the Philippines. Trees were sampled by taking leaflets from every frond starting with the spear leaf (frond 0).

Extraction and electrophoresis of nucleic acids. Nucleic acids were extracted from leaf samples as described by Imperial *et al.* (1981) using their Method 1 and analysed by electrophoresis on 5% polyacrylamide gels under non-denaturing conditions (Peacock & Dingman, 1968). For purification of individual ccRNAs, RNAs were extracted from bulk leaf samples and purified by 3 cycles of polyacrylamide gel electrophoresis (N. A. Mohamed, G. Buenaflor, Ma. J. Rodriguez, J. S. Imperial & J. W. Randles, unpublished results).

Sizing of ccRNAs. Sizes of the four ccRNAs were estimated by electrophoresis in 6% polyacrylamide gel slabs (40 × 20 × 0·05 cm) containing 98% formamide (Maniatis & Efstradiatis, 1980). The following were used as molecular weight markers. *Solanum nodiflorum* mottle virus (SNMV) RNA 2, 377 nucleotides (J. Haseloff & R. H. Symons, unpublished results); velvet tobacco mottle virus (VTMoV) RNA 2, 365 nucleotides (J. Haseloff & R. H. Symons, unpublished results); chrysanthemum stunt viroid (CSV), 356 nucleotides (Haseloff & Symons, 1981); Q-cucumber mosaic virus (CMV) RNA 4, 1047 nucleotides (A. R. Gould & R. H. Symons, unpublished results); CMV satellite RNA, 336 nucleotides (O. O. Iassiello & R. H. Symons, unpublished results); chicken 18S rRNA, 1800 nucleotides (Spohr *et al.*, 1976); alfalfa mosaic virus (AMV) RNA 4, 881 nucleotides (Brederode *et al.*, 1980); yeast 5·8S RNA, 158 nucleotides (Rubin, 1973); yeast 5S RNA, 121 nucleotides (Miyazaki, 1974), *Escherichia coli* phenylalanine tRNA, 76 nucleotides (Barrell & Sanger, 1969).

The circular RNAs (SNMV RNA 2, VTMoV RNA 2, CSV and the ccRNAs) were boiled for 15 min in distilled water before electrophoresis to produce the linear forms. After electrophoresis, gels were stained with 0·01% toluidine blue and destained with water.

Fingerprinting of ccRNAs. Purified ccRNAs (0·5 μg) were dried down, resuspended in 5 μl 5 mM-Tris–HCl pH 7·5, and digested with RNase A (0·1 μg RNase A at 37 °C for 1 h) or RNase T_1 (20 units RNase T_1 at 56 °C for 30 min). After digestion, the RNA samples were extracted with phenol, washed with ether and precipitated with ethanol. The resultant oligonucleotide fragments were labelled at the 5′-end with ^{32}P using [γ-^{32}P]ATP and polynucleotide kinase (Haseloff & Symons, 1981). For the first dimension, preparations were electrophoresed in 20 × 40 × 0·05 cm 10% polyacrylamide gels containing 25 mM-sodium citrate pH 3·5 (De Wachter & Fiers, 1972; Frisby, 1977). After electrophoresis for 2 to 3 h, gel strips were excised and embedded in a 20 × 40 × 0·05 cm 25% polyacrylamide gel containing 90 mM-Tris–borate, 1 mM-EDTA, pH 8·3 (Frisby, 1977). Polymerization of this gel was catalysed by the addition of 300 μl 10% (w/v) ammonium persulphate, 30 μl TEMED, 50 μl 10% (w/v) ascorbic acid and 70 μl 30% (w/v) H_2O_2 per 50 ml of gel solution to ensure complete polymerization in the region of the first-dimension gel strip. The ^{32}P-labelled oligonucleotides were detected after electrophoresis by autoradiography.

RESULTS

Symptom development

Three stages in the development of symptoms of cadang-cadang disease have been described (Zelazny & Pacumbaba, 1982): early (E), medium (M), and late (L). The early stage, which lasts 2 to 3 years (Zelazny & Niven, 1980), involves major changes in disease development both in terms of symptoms expressed and in changes in the ccRNA variants (Imperial *et al.*, 1981). This stage was therefore subdivided into four phases than can be distinguished on the basis of symptoms.

E_0: no visible symptoms but ccRNA can be detected in the youngest fronds. Symptoms usually develop in these trees 1 to 2 years after the first detection of ccRNA. Trees in this stage were located by random sampling of 'healthy' palms in an area containing 120 trees with a high infection rate (60%) with one or two new infections per year.

E_1: newly developed nuts are rounded with distinctive scarification around the equator. No leaf symptoms are apparent at this stage but develop within 6 months.

E_2: rounding and scarification of nuts is prominent. Chlorotic leaf spots with water soaking become apparent on leaves. Inflorescences are smaller than in healthy trees.

E_3: number of nuts produced is markedly reduced; new inflorescences are stunted and sterile. Leaf spots are prominent and fronds appear yellow green when viewed from the ground.

Changes in electrophoretic forms of ccRNA

Changes in the electrophoretic mobilities of ccRNA-1 and ccRNA-2 occurring during disease development were followed by sampling every frond in selected trees at various stages of disease.

Table 1. *Fast (f) and slow (s) forms of ccRNA-1 and ccRNA-2 present in fronds of palms at different stages of cadang-cadang disease*

Column no.*:	1	2	3	4	5	6	7	8	9	10
Stage†:	E_0	E_0	E_1	E_2	E_2	E_3	E_3	M	M	L‡
Frond no.§										
1	(f)‖	f	f	f + s	s	s	s	s	s	s
2	f	f	f	f + s	s	s	s	s	s	s
3	f	f	f	f + s	s	s	s	s	s	s
4	f	f	f	f + s	f + s	s	s	s	s	s
5	f	f	f	f + s	f + s	s	s	s	s	s
6	f	f	f	f + s	f + s	s	s	s	s	s
7	f	f	f	f + s	f + s	s	s	s	s	s
8	f	f	f	f + s	f + s	s	s	s	s	s
9	f	f	f	f + s	f + s	s	s	s	s	s
10	–¶	f	f	f + s	f + s	s	s	s	f + s	s
11	–	f	f	f + s	f + s	s	s	s	f + s	s
12	–	f	f	f + s	f + s	s	s	s	f + s	s
13	–	f	f	f	f + s	f + s	s	s	f + s	s
14	–	(f)	f	f	f + s	f + s	s	s	f + s	s
15	–	f	f	f	f	f + s	s	s	f + s	s
16	–	f	f	f	f	f + s	s	s	f + s	s
17	–	f	f	f	f	f + s	s	s	f + s	s
18	–	f	(f)	f	f	f + s	s	s	f + s	s
19	–	f	f	f	f	f + s	f + s	s	f + s	s
20	–	f	f	f	f	f + s	f + s	s	f + s	
21	–	f	f	f	f	f + s	f + s	s	f + s	
22	–	f	f	f	f	f + s	f + s	s	f	
23	–	–	f	f	f	f	f + s	s	f	
24	–	–	f	f	f	f	f + s	s	f	
25	–	–	f	f	f	f	f + s	s	f	
26	–	–	f	f	f	f	f + s	s	f	
27	–	–	–	f	f	f	f + s	s	f	
28	–	–	–	f	f	f	f + s	s	f	

* Columns 1, 2 and 3 represent the same tree at three different samplings (March 1980, March 1981 and July 1981). Columns 4 to 10 each represent a different tree.

† Stage of disease: E_0 to E_3, early; M, medium; L, late.

‡ This late-stage palm had only 19 fronds.

§ Youngest opened frond is denoted '1' and progressively older fronds are numbered onwards.

‖ (f) indicates the position of one frond on this tree at the three samplings.

¶ – represents no ccRNA detected.

As coconut palms produce one new frond every 4 weeks, it was possible to relate changes in the ccRNA forms to the age of the fronds. Nucleic acids were extracted from samples and analysed on 5% polyacrylamide gels; results from trees representing each stage of the disease are presented in Table 1. Columns 1 to 3 represent sequential sampling of one tree over a period of 16 months; during that time the tree progressed from the symptomless stage (E_0) to the first appearance of leaf symptoms (E_2).

In trees sampled during the early part of the symptomless stage, the fast forms of ccRNA-1 and ccRNA-2 were detected in the younger fronds (column 1, Table 1) while the older fronds did not contain detectable levels of ccRNA. Subsequent sampling of trees at 4-monthly intervals indicated that there was no detectable movement of ccRNA into the older fronds: those that were free of ccRNA at the beginning of the sampling period were also free 12 months later (columns 1 and 2, Table 1), but their position in the crown had altered as new fronds developed. The new fronds that developed contained the fast form of ccRNA-1 and ccRNA-2 so that after 12 months, 22 fronds were infected (column 2, Table 1). The first symptoms of cadang-cadang disease (scarification and rounding of nuts) were usually apparent in trees where at least 16 to 24 fronds contained ccRNA, indicating that this stage (E_0) lasts between 15 and 24 months.

All trees sampled during this stage, E_1, when nut symptoms are apparent but no leaf symptoms have developed, contained only the fast form of ccRNA-1 and ccRNA-2. In addition to those trees being studied in detail, over 50 palms from all over the region were sampled and in all

cases only the fast form was present. These results indicate that the fast form is always the first one to be found in natural infections.

Stage E_1 usually lasts about 6 months before the first leaf symptoms become apparent. The development of leaf symptoms coincided with the first detection of the slow forms of ccRNA-1 and ccRNA-2 in the youngest fronds (column 4, Table 1). On a qualitative basis (i.e. intensity of bands on gels), the slow form of ccRNA was usually present as a minor component in the first fronds to contain the slow forms. However, as new fronds developed, there was a progressive decline in the amount of the fast form and a corresponding increase in the slow form so that, within a year of the appearance of the slow form, the newly developing fronds contained only the slow form (column 5, Table 1). Typically, between 10 and 13 fronds contained both the fast and slow forms (Table 1, columns 4, 5 and 6).

In order to obtain a quantitative estimate of the amounts of slow and fast forms of ccRNA present in the fronds of a tree in this transition stage, the circular forms of ccRNA-1 were extracted and purified from two sets of fronds: numbers 4 to 7 and 11 to 14 (column 5, Table 1). In the older fronds (11 to 14), the fast form was the major component (76% of the circular ccRNA-1) while in the younger fronds (4 to 7), the slow form predominated (81% of circular ccRNA-1). These results support the qualitative estimates made on analytical gels.

Palms in the initial phase of the next stage (E_3) contained the slow form of ccRNA in the first 10 to 12 fronds (column 6, Table 1) while the next 11 fronds containing both fast and slow forms had matured. This stage lasts about 1 year so that palms in the last phase of this stage contained the slow variant in the first 18 to 20 fronds (column 7, Table 1).

Trees in the medium stage of the disease usually contained only the slow form in all fronds (column 8, Table 1) as the fronds containing the fast form have senesced and been shed. This happens when the early stage (E_1 to E_3) lasts the average of 2 to 3 years. However, in some trees, symptoms develop faster than usual and the early stage lasts less than 2 years. Therefore, in these medium-stage trees, it is possible to detect the fast form in the older fronds (column 9, Table 1). This observation explains the low incidence of palms containing fast ccRNA even in the medium stage as reported by Imperial *et al.* (1981).

In trees in the late stage of the disease, only the slow form of ccRNA can be detected in the surviving fronds (column 10, Table 1); trees in this stage usually have only a few fronds (about 12 to 20).

Sizing of ccRNAs

The sizes of the linear fast and slow forms of ccRNA-1 and ccRNA-2 were estimated by electrophoresis in polyacrylamide gels containing 98% formamide, using linear RNAs of known molecular weight as markers (Fig. 1). No differences in mobility were observed between different isolates from the same area. The sizes of the RNAs were estimated to be: ccRNA-1 fast, 250 nucleotides; ccRNA-1 slow, 300 nucleotides; ccRNA-2 fast, 500 nucleotides; ccRNA-2 slow, 600 nucleotides (Fig. 1). Because ccRNA-2 fast and ccRNA-2 slow are approximately twice the size of the corresponding ccRNA-1 forms, the ccRNA-2 forms may be dimers. The slow forms of ccRNA-1 and ccRNA-2 were about 20% larger than the fast forms.

Fingerprinting of the ccRNAs

The sequence relationships between the ccRNAs were further investigated by the technique of RNase fingerprinting using RNase A or RNase T_1 to digest the ccRNAs. The RNase A fingerprints of the four forms of ccRNA extracted from the same tree show that an essentially identical pattern of labelled fragments was obtained (Fig. 2). This indicates that the three larger RNAs contain the same sequences as the smaller ccRNA-1. Hence, ccRNA-2 fast and slow are most likely dimers of the ccRNA-1 fast and slow respectively, on the basis of their relative molecular weights (Fig. 1). Furthermore, it is likely that each slow ccRNA species contains only repeated sequences of the respective fast ccRNA species.

These results and conclusions were supported by the RNase T_1 fingerprints of the same four RNAs since an essentially identical pattern of labelled fragments was also obtained (data not shown). Three extra oligonucleotides were found in the RNase T_1 fingerprints of the fast ccRNA forms which were not in the fingerprints of the slow ccRNA forms: the significance of

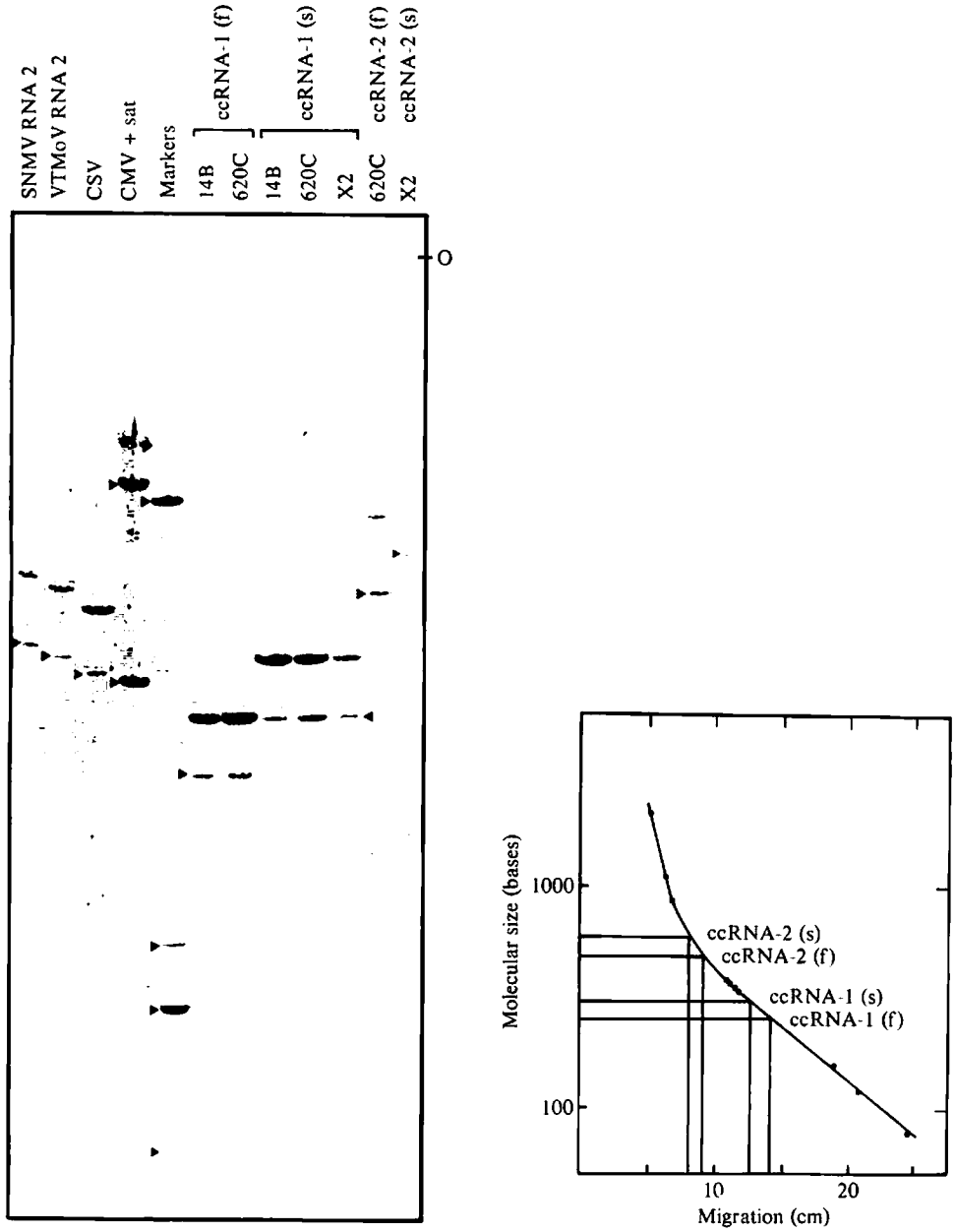

Fig. 1. Molecular weight estimation of fast and slow forms of ccRNA-1 and ccRNA-2 by electrophoresis in a 6% polyacrylamide gel containing 98% formamide. 14B, 620C and X2 were different isolates of ccRNA. The sizes and descriptions of the molecular weight markers are given in Methods. A standard curve of the mobility (cm from origin) of these markers as measured from the stained gel was plotted against the size of the marker RNAs (number of bases) on a logarithmic scale and the sizes of the ccRNAs determined. Only the linear RNAs, and not the circular RNAs, were used for this estimation. For SNMV RNA2, VTMoV RNA 2 and CSV, the arrow indicates linear forms. For CMV plus satellite (sat) RNA, the bands are (in order from the top) RNAs 1, 2 and 3 running as a broad band, RNA 4 and satellite RNA. Marker RNAs, in order from the top, are chicken 18S rRNA, AMV RNA 4, yeast 5·8S RNA, yeast 5S RNA and *E. coli* phenylalanine tRNA. For ccRNA-1 and ccRNA-2, fast (f) and slow (s) forms, the linear form of each is marked by an arrow. O, Origin.

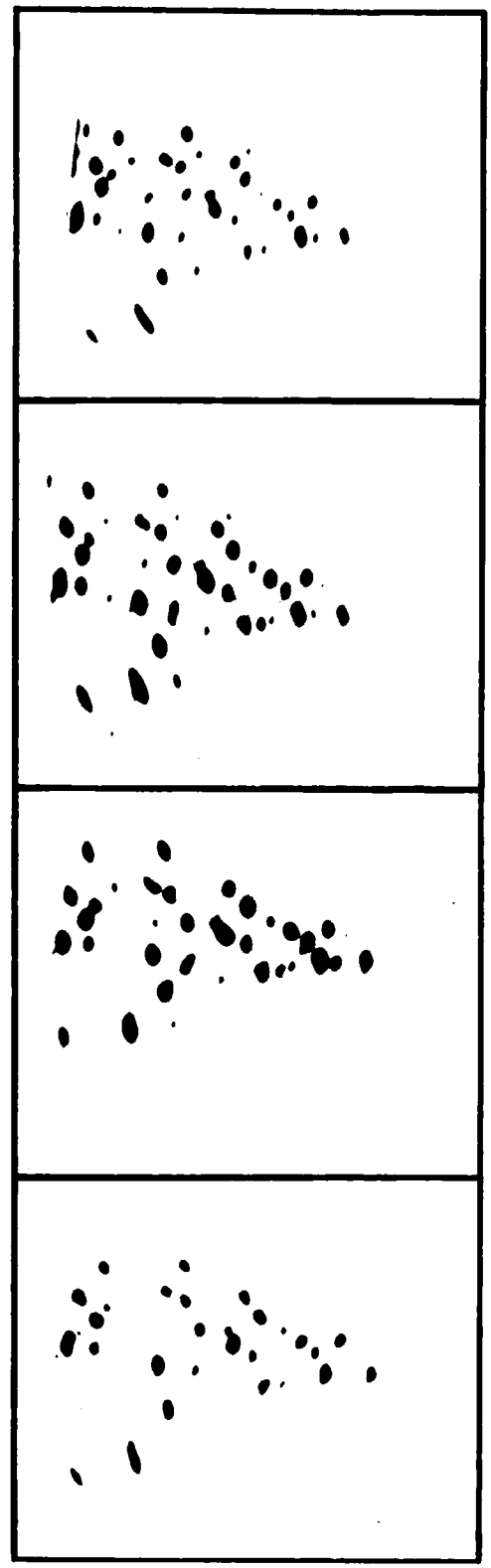

Fig. 2. Two-dimensional fingerprint analysis of RNase A digests of the fast and slow forms of ccRNA-1 and ccRNA-2. Details are given in Methods. Migration in the first dimension is from left to right and in the second dimension from bottom to top.

this is not known, but may be related to the difficulty we have experienced in making sure that RNase T_1 digestions, in contrast to RNase A digestions, always go to completion.

DISCUSSION

The results of this study show that the fast forms of the cadang-cadang RNAs always occur first in naturally infected palms and that the slow form is found only after the appearance of leaf symptoms. This indicates that the fast form is involved in the natural spread of cadang-cadang disease. Furthermore, since fast forms of ccRNA-1 and ccRNA-2 are only found in the early stages of the disease, it can be concluded that trees in this stage would be sources of inoculum for natural spread. This conclusion is supported by field observations in which cutting down and removal of medium- and late-stage palms had no effect on spread of the disease while the cutting down of palms in the early stage reduced spread significantly (B. Zelazny, personal communication).

The detection of fast ccRNAs in trees before the appearance of symptoms indicates that the incubation period (time from first infection to the appearance of symptoms) is between 1·5 and 2 years. This is based on the observation that 15 to 24 fronds of a palm contain detectable levels of ccRNA before the first symptoms (rounding and scarification of nuts) are apparent. As there is no detectable movement of ccRNA into mature fronds during disease development, and newly developing fronds contain high levels of ccRNA, it appears that ccRNA replicates in the meristematic tissues of the trees. Hence, when a palm is first infected, the leaves developing after infection would be expected to contain detectable levels of ccRNA while mature fronds would remain free of ccRNA. Thus, the number of infected fronds on a tree in the early stage, combined with the fact that one new frond is produced every 4 weeks, gives an indication of when a tree first became infected. This estimate of 18 months to 2 years for the incubation period is in good agreement with the estimate of between 1 and 2 years for the latent period obtained from field studies (B. Zelazny, personal communication).

The ccRNA-2 fast and slow forms are estimated to be twice the molecular weights of their respective ccRNA-1 forms (Fig. 1), and, since the RNase fingerprints show that ccRNA-1 and ccRNA-2 possess similar sequence complexity (Fig. 2), it can be concluded that the ccRNA-2 fast and slow forms are dimers of ccRNA-1 fast and slow forms respectively. This conclusion is consistent with extensive sequence data available for the four ccRNAs (J. Haseloff, N. A. Mohamed & R. H. Symons, unpublished results). These estimates of size differ from those obtained by length measurements in the electron microscope (Randles & Hatta, 1979). However, Randles & Hatta (1979) did not differentiate between the fast and slow forms and therefore they may have measured the slow form of ccRNA-1 and the fast form of ccRNA-2. Their values of 310 $\pm$ 3 nucleotides for ccRNA-1 and 438 $\pm$ 5 for ccRNA-2 are comparable with our values of 300 nucleotides for ccRNA-1 and 500 for ccRNA-2; the differences are probably due to the different techniques used.

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