Variability of Kinetoplast DNA from Plant Trypanosomatids Responsible for Hartrot and Marchitez Diseases

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ABSTRACT

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The kinetoplast DNA of 27 plant trypanosomatid stocks (22 intraphloemic *Phytomonas* associated with coconut Hartrot, oil palm Marchitez, or decay of *Alpinia purpurata*, 3 *Phytomonas* isolates from latex, and 2 fruit trypanosomatids) was studied. Four classes of minicircle sizes were obtained: 1.6 and 1.8 kb for isolates originating from coconut palms, oil palms, *Alpinia purpurata*, and fruits; 850 bp and 2.8 kb for isolates from latex. Restriction endonuclease analysis showed that for almost all of the isolates minicircles were heterogeneous in base sequence. Cross-hybridization experiments were performed by Southern blot. A high sequence similarity only occurred between isolates of the same class of minicircle sizes, except for the 1.6-kb class, in which isolates from fruits and isolates originating from diseased coconut belonged to two hybridization groups. Moreover, the intraphloemic isolates that belonged to two classes, 1.6 and 1.8 kb, showed sequence similarity. These data confirm the existence of at least two groups of intraphloemic trypanosomatids associated with wilts, several groups of latex trypanosomatids, and one group of fruit trypanosomatids and could help in the classification of the "lower" trypanosomatids.

Additional keywords: epidemiology, South America.

Immediately following the discovery of trypanosomes in plants (17), the *Phytomonas* genus was created to distinguish them from trypanosomes isolated from animals (10). These *Phytomonas* can be found in the latex tubes of laticiferous plants and in the phloem of coffee plants affected by phloem necrosis and coconut palms or oil palms affected by the Hartrot or Marchitez diseases, respectively (6,9,25,36). They also can be isolated from fruits (5,15, 31). Only intraphloemic isolates, all from South America, are specifically associated with pathological syndromes in plants. Since their culture (8,22), these trypanosomes have been characterized, and the variability within the arbitrary *Phytomonas* genus has been studied by several research laboratories (1,2,14,27).

As a member of the family Trypanosomatidae and the order Kinetoplastidae, *Phytomonas* have in their sole mitochondrion a structure called a kinetoplast that contains highly concentrated DNA, kinetoplast DNA (kDNA), that has a specific molecular structure in the form of a network of thousands of catenated minicircles and a few maxicircles in which each individual circle is physically attached to another. In the Kinetoplastidae, mitochondrial genes are included in the maxicircles. The restriction cleavage patterns of minicircles and their sizes can be used to identify trypanosome species and even strains within a species (21,23,34).

In this study, we characterized the kDNA minicircles of intraphloemic *Phytomonas* to study the variability of these flagellates and to gain insight into the diseases caused by these organisms in coconut and oil palms in South America. An intraphloemic isolate from *Alpinia purpurata* (Vieill.) K. Schum. (Zingiberaceae) from Grenada in the Caribbean (12), linked to a type of decay and very similar in isoenzymatic analysis to certain coconut and oil palm isolates (24), was included in this study. Three latex and two fruit isolates were characterized to confirm the distinctions seen between them and the intraphloemic isolates.

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MATERIALS AND METHODS

Cells and cultures. The 27 primary isolates and clones studied are listed in Table 1. Cloning was carried out by limit dilution on five isolates (HART 1 and 3, MAR 2, TOMA, and EM 1). Briefly, cultures were diluted serially to obtain one or less parasite per culture tube; this process was repeated three times. Isolates obtained from laticiferous plants (*Euphorbia* and *Asclepias*) or fruits (*Annona cherimolia* Mill. and *Lycopersicon esculentum* Mill.) were cultured in the axenic medium of Grace (13) supplemented with 10% fetal calf serum (FCS). Intraphloemic *Phytomonas* were grown in insect cell cultures in Grace insect medium supplemented with 10% FCS (22). Culture media were centrifuged at 4,000 × *g* for 15 min, and the pellets were washed with 0.003 M KH_2PO_4, 0.012 M Na_2HPO_4, 0.15 M NaCl, pH 7.4.

Isolation of kDNA. Kinetoplast DNA was isolated and purified according to a technique already described (1), with some modifications. Briefly, cells were lysed by sarkosyl in a final concentration of 1% in SSC (1× is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) for 15 min at 37°C. After incubation with preheated pronase (1 mg/ml) for 2 h at 37°C, the lysate (1 volume) was layered onto 1 volume of sucrose (20% in SSC, wt/vol)

and centrifuged at 20,000 rpm for 1 h at 20°C (Ultracentrifuge Beckman, Rotor SW27, Beckman Instruments, Fullerton, CA). The kDNA collected in the pellet was fractionated in a CsCl-propidium iodide gradient (500 μg of propidium iodide per ml, 0.73 g of CsCl per ml to obtain n^{D}_{20} = 1.3840) at 40,000 rpm for 36 h at 20°C (Ultracentrifuge Beckman, Rotor 50Ti). The kDNA recovered from the lower band of the gradient (after removing propidium iodide with isopropanol) was dialyzed against 10 mM TrisHCl, pH 7.5, and 1 mM EDTA and precipitated with absolute ethanol.

Decatenation of kDNA networks and preparation of free minicircles. Free minicircles were obtained by decatenation of the kDNA network with topoisomerase II (Topo II) from calf thymus (Topogen, Columbus, OH). The reaction was performed at 37°C for 30 min under the conditions recommended by the manufacturer. Samples were loaded onto 1.5% agarose slab gel for electrophoresis in 0.36 M Tris, 0.29 M Na_2PO_4, and 10 mM EDTA. The gels were stained with ethidium bromide (10 μg/ml) and photographed under UV light. Minicircle sizes were estimated by comparing their mobilities with those of isolates HART 1 and 3 already studied (1) and λ-phage DNA digested by *Eco*RI and *Hin*dIII.

The purified minicircles were prepared according to a technique described by Ahomadegbe et al. (2). After decatenation of the kDNA network, electrophoresis onto 1.5% agarose slab gel was performed in 89 mM Tris-borate, pH 8, and 2 mM EDTA and run for 2 h at room temperature at 6 V/cm. The agarose gel containing the free minicircle bands was excised, inserted in a dialysis bag in the presence of 8.9 mM Tris-borate and 0.2 mM EDTA and subjected to an electric field for 2 h under 120 V. Free minicircles were released into the buffer, dialyzed against 10 mM Tris HCl, pH 7.5, and 1 mM EDTA, precipitated by absolute ethanol, dried under vacuum, and resuspended in 10 mM Tris HCl, pH 7.5, and 1 mM EDTA. The free minicircles from the different *Phytomonas* were used as probes for hybridization experiments.

Restriction enzyme digestion and agarose gel electrophoresis. Four restriction enzymes were selected for use in this study (1): *Hin*fI, *Hae*III, *Hpa*II, and *Taq*I. For isolates HART 9, 12, and 13, restriction enzyme *Msp*I, which is not sensitive to base methylation and which recognizes the same site as *Hpa*II, was used to compare restriction patterns with those obtained with *Hpa*II to study the hypothesis of base methylation. Restriction endonucleases purchased from Boehringer (GmbH, Mannheim, Germany) were used according to the conditions recommended by the manufacturer. Reaction mixtures were analyzed by electrophoresis in 1.5% agarose slab gel for electrophoresis in 0.36 M Tris, 0.29 M Na_2PO_4, and 10 mM EDTA. Fragment sizes were estimated by comparing their mobilities with those of λ-phage DNA digested by *Eco*RI and *Hin*dIII. The gels were stained with ethidium bromide and photographed under UV light.

Southern blot hybridization. Gels containing restriction fragments of minicircles or decatenated minicircles were used for Southern blot hybridization. DNA fragments were partially depurinated, denatured, and transferred by vacuum onto a Biodyne B nylon membrane (Pall Industries, Portsmouth, UK) in 20× SSC using standard procedures (19). Blots were prehybridized at 65°C for 6 h in hybridization buffer (hybridization buffer tablets, Amersham, Little Chalfont, UK) and then hybridized at 65°C for 18 h in the same solution plus about 10^6 cpm/ml denatured probes ^{32}P-dCTP labeled by the nick translation method (19).The probes used were the purified minicircles obtained from different *Phytomonas* (HART 1, 10, and 13, MAR 1 and 2, ALP I, and TOMA). The membranes were washed twice in 2× SSC at 20°C for 15 min, twice in 0.5× SSC at 65°C for 30 min, twice in 1× SSC and 1% sodium dodecyl sulfate at 20°C for 30 min, and exposed at –70°C for autoradiography on Hyperfilm MP (Amersham) for different periods of time.

Electron microscopy of kDNA. Topo II-reacted kDNA was precipitated by absolute ethanol and solubilized in 1 mM Tris HCl and 0.5 mM EDTA, pH 7.5. Intact kDNA and free minicircles were observed in separate experiments after absorption to carbon-coated grids prepared according to the technique of Dubochet et al. (11) and observed in annular dark field mode in a Zeiss 902 electron microscope (Carl Zeiss, Thornwood, NY). The minicircle contour length was measured from electron micrographs (21 to 28 molecules for each preparation), and standard deviation was calculated for each measure.

RESULTS

kDNA minicircles. Intact kDNA networks did not penetrate agarose gels. Free minicircles were obtained by decatenating the

TABLE 1. *Phytomonas* isolate origins

Isolate[a]	Host	Isolation date	Country Location
HART 1	*Cocos nucifera*	1986	French Guiana Saut Sabbat
HART 1, cl. 1	*C. nucifera*	1986	French Guiana Saut Sabbat
HART 1, cl. 2	*C. nucifera*	1986	French Guiana Saut Sabbat
HART 3	*C. nucifera*	1987	Brazil Moju (Para)
HART 3, cl. 1	*C. nucifera*	1987	Brazil Moju (Para)
HART 3, cl. 2	*C. nucifera*	1987	Brazil Moju (Para)
HART 4	*C. nucifera*	1987	French Guiana Saut Sabbat
HART 6	*C. nucifera*	1990	French Guiana Combi
HART 9	*C. nucifera*	1990	French Guiana Organabo
HART 10	*C. nucifera*	1990	French Guiana Combi
HART 11	*C. nucifera*	1990	Venezuela Irapa (Sucre)
HART 12	*C. nucifera*	1991	Venezuela San Augustin (Sucre)
HART 13	*C. nucifera*	1992	Brazil Moju (Para)
MAR 1	*Elaeis guineensis*	1989	Colombia La Cabaña (Meta)
MAR 2	*E. guineensis*	1989	Ecuador Shushufindi (Oriente)
MAR 2, cl. 1	*E. guineensis*	1989	Ecuador Shushufindi (Oriente)
MAR 2, cl. 2	*E. guineensis*	1989	Ecuador Shushufindi (Oriente)
MAR 4	*E. guineensis*	1991	Colombia Acacias (Meta)
MAR 5	*E. guineensis*	1992	Colombia Manuelita (Meta)
MAR 6	*E. guineensis*	1992	Venezuela Casigua el Cubo (Zulia)
MAR 7	*E. guineensis*	1993	Venezuela Palmonagas (Maturin)
ALP I	*Alpinia purpurata*	1991	Grenada Grand Etang
TOMA, cl.*[b]	*Lycopersicon esculentum*	1992	Spain Andalousia
CHERI*	*Annona cherimolia*	1993	Spain Almuñecar (Andalousia)
ASCL 1	*Asclepias curassavica*	1991	Venezuela Irapa (Sucre)
ASCL 3	*Asclepias curassavica*	1993	Venezuela Irapa (Sucre)
EM 1, cl.	*Euphorbia pinea*	1980	France Montpellier

[a] cl. = clone.
[b] * = isolated by Sanchez-Moreno et al. (31).

kDNA networks with Topo II. Several bands corresponding to relaxed or nicked and supercoiled minicircles were observed after electrophoresis in agarose gels (Fig. 1). Four minicircle size classes were obtained by electrophoresis: about 0.85, 1.6, 1.8, and 2.8 kb (Table 2). No difference was seen in minicircle size between the clones and the corresponding primary isolate (for isolates that have been cloned). For a given *Phytomonas* isolate, the minicircles were of uniform size. The sizes obtained by electrophoresis and electron microscopy for isolates HART 13, ASCL 3, and TOMA were in good agreement. Isolates ASCL 1 and 3 had very small minicircles that had yet to be detected for plant trypanosomes but which corresponded to the size of the minicircles in certain *Leishmania* species (35).

Analysis of *Phytomonas* kDNA minicircles by restriction endonucleases. For the three isolates tested, identical restriction patterns were obtained for *Hpa*II and *Msp*I, suggesting that variability of restriction patterns was not due to differential methylation between isolates. Total cleavage with restriction endonucleases was obtained when the kDNA disappeared from the top of the gel (Fig. 2). Minicircle sizes estimated by decatenating the kDNA networks were confirmed by the size of kDNA minicircles when they were cleaved by restriction endonucleases in only one site.

For all the isolates, except HART 1 (2), when the kDNA minicircles were incubated with the restriction enzymes, they released numerous DNA fragments whose sum of lengths was much higher than the minicircle length. This was seen for both primary isolates and associated clones (data not shown). These data indicate that the minicircles of these isolates are heterogeneous with regard to their sequence, as reported for most Trypanosomatidae (29,35).

For isolate HART 1, the minicircles seemed to be a homogeneous group with regard to the sequence based on the restriction enzymes tested, in agreement with previous work by Ahomadegbe et al. (2). However, when these restriction cleavage patterns were hybridized with HART 3 minicircles (with high sequence similarity as demonstrated previously [2]), faint bands were visible and revealed the presence of small quantities of other classes of minicircles with a sequence that differed from the major class seen in the restriction patterns (Fig. 3C).

The restriction patterns distinguished minicircles of the same size (Fig. 2). In the 1.6-kb group, HART 1, 3, 4, and 10 all had different restriction cleavage patterns ([1]; Fig. 3A and B). The apparently homogeneous class of minicircles seen in the HART 1 restriction patterns exists for isolates HART 3, 4, and 10. The isolates from *L. esculentum* and *Annona cherimolia* (TOMA and CHERI, respectively) had patterns identical to each other (results confirmed by hybridization profiles) and clearly differed from those of HART 1, 3, 4, and 10 (Fig. 2). Because the restriction pattern did not always enable a clear comparison between isolates, analyses were confirmed by examination of hybridization patterns. Using this approach, it was possible to distinguish all isolates in the 1.8-kb group; isolates differed at between one and four enzymes (Fig. 4; HART 6 and 9 had different restriction

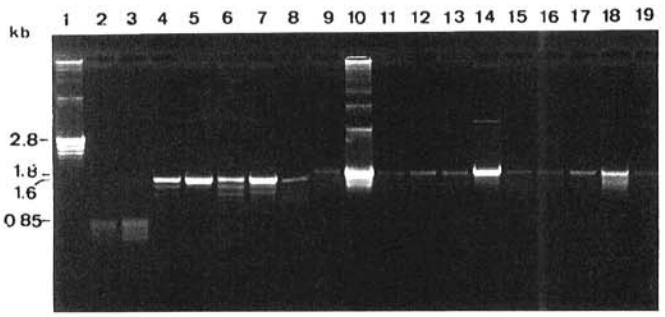

Fig. 1. Decatenation of the kinetoplast DNA networks from different *Phytomonas* isolates by topoisomerase II. Lane 1, EM 1 (2.8 kb); lane 2, ASCL 1 (850 bp); lane 3, ASCL 3 (850 bp); lane 4, HART 1 (1.6 kb); lane 5, HART 3 (1.6 kb); lane 6, HART 4 (1.6 kb); lane 7, HART 10 (1.6 kb); lane 8, TOMA (1.6 kb); lane 9, HART 9 (1.8 kb); lane 10, HART 11 (1.8 kb); lane 11, HART 12 (1.8 kb); lane 12, HART 13 (1.8 kb); lane 13, MAR 1 (1.8 kb); lane 14, MAR 2 (1.8 kb); lane 15, MAR 4 (1.8 kb); lane 16, MAR 5 (1.8 kb); lane 17, MAR 6 (1.8 kb); lane 18, MAR 7 (1.8 kb); and lane 19, ALP I (1.8 kb). Sizes are given in kilobase pairs (kb). Electrophoresis was done in 1.5% agarose slab gel. Approximately 0.2 µg of DNA per well was used, except in wells 1, 10, and 14 in which a higher amount was used.

TABLE 2. Sizes of kinetoplast DNA (kDNA) minicircles

Phytomonas isolate	Minicircle size (SD)[a] (kb)	Size class
EM 1	2.8	1
HART 6, 9, 11, 12 MAR 1, 2, 4, 5, 6, 7 ALP I	1.8	2
HART 13	1.788* (±0.044)	2
HART 1, 3, 4, 10 CHERI	1.6	3
TOMA	1.641* (±0.038)	3
ASCL 1	0.85	4
ASCL 3	0.844* (±0.038)	4

[a] * = the size of these minicircles was measured by electron microscopy.

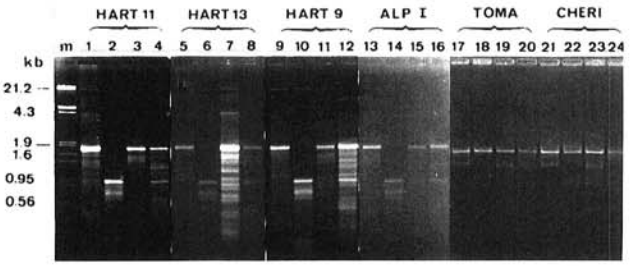

Fig. 2. Restriction endonuclease analysis of *Phytomonas* isolates. Kinetoplast DNA (kDNA), 0.2 µg per assay, usually was used, except in lane 7 in which a higher amount was used. m = size markers (given in kilobase pairs [kb]) of λ-phage DNA cleaved with *Eco*RI and *Hin*dIII. Lanes 1, 5, 9, 13, 17, and 21: kDNAs + *Hin*fI; lanes 2, 6, 10, 14, 18, and 22: kDNAs + *Hae*III; lanes 3, 7, 11, 15, 19, and 23: kDNAs + *Hpa*II; and lanes 4, 8, 12, 16, 20, and 24: kDNAs + *Taq*I.

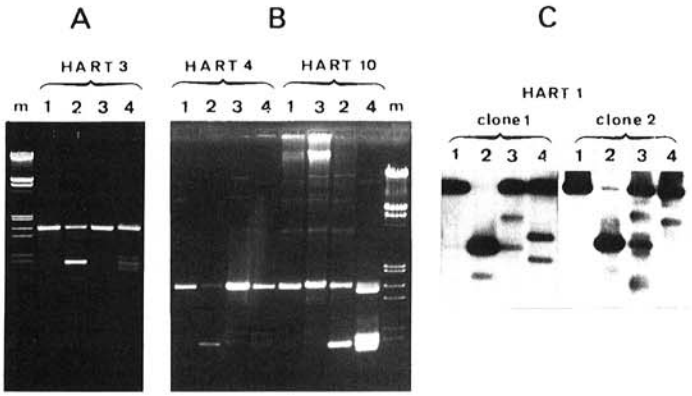

Fig. 3. Restriction endonuclease analysis of kinetoplast DNA from *Phytomonas* isolates. **A and B,** Agarose gels stained with ethidium bromide. Kinetoplast DNA was incubated with lane 1, *Hin*fI; lane 2, *Hae*III; lane 3, *Hpa*II; and lane 4, *Taq*I. Kinetoplast DNA (0.2 µg per well) usually was used. m = size markers of λ-phage DNA cleaved with *Eco*RI and *Hin*dIII. **C,** kinetoplast DNA from two clones of HART 1 was cleaved with lane 1, *Hin*fI; lane 2, *Hae*III; lane 3, *Hpa*II; and lane 4, *Taq*I. DNA fragments were analyzed by Southern blot hybridization using free minicircles from a clone of HART 3 as probes. Filter was exposed to Hyperfilm MP (Amersham) for 6 h.

patterns for enzyme *Hpa*II; HART 9 differed from MAR 4 at least for enzymes *Hpa*II and *Taq*I). The enzyme *Hae*III cut all the minicircles of the intraphloemic isolates in the 1.8- or 1.6-kb groups into two opposite sites, thereby releasing fragments of size L/2 and smaller (where L is the length of a minicircle). Isolates ASCL 1 and 3 had very similar if not identical restriction patterns (data not shown).

The restriction patterns revealed very faint bands corresponding to the presence of molecules with a molecular weight of around 20 kb (Fig. 2, lanes 1, 7, 9, 10, and 11). These large molecules probably were cleaved maxicircles and were only clearly detected if a large quantity of DNA was loaded on the gel (Fig. 2). Other, more intense staining bands with a high molecular weight seemed to be minicircle oligomers with no restriction site for the enzyme used, since they hybridized substantially with the minicircles, which is not the case with bands thought to be maxicircles.

Cross-hybridization of *Phytomonas* minicircles. The kDNA networks, either decatenated with Topo II or cleaved by restriction enzymes, were analyzed by Southern blot hybridization. The groups formed according to minicircle size also corresponded to groups with high sequence similarity, apart from the isolates from *L. esculentum* and *Annona cherimolia*, which had minicircles of 1.6 kb with only very slight sequence similarity with the 1.6-kb minicircles of intraphloemic isolates HART 1, 3, 4, and 10 (Fig. 5A and E).

Table 3 summarizes the results obtained: four hybridization groups were detected. One group contained the intraphloemic isolates of 1.8-kb minicircles that hybridized strongly with the labeled minicircles of HART 13, MAR 1 and 2, and ALP I. The minicircles of the isolates in this first group had low similarity with the minicircles of HART 1 and 10, which belonged to a second hybridization group (Fig. 5A, B, C, and D). This second group contained four intraphloemic isolates of 1.6-kb minicircles: HART 1, 3, 4, and 10. In contrast, sequence similarity was very low between the minicircles of the isolates in these two intraphloemic groups and the minicircles of the isolates from latex or fruits: EM 1, ASCL 1 and 3, TOMA, and CHERI (Fig. 5D).

The minicircles of the TOMA and CHERI isolates had very strong sequence similarity, but hybridized very slightly with the minicircles of all the other isolates. The minicircles of EM 1 and ASCL 1 and 3 had only low sequence homology with the minicircles of the intraphloemic isolates and of the TOMA isolate. Moreover, based on the results already acquired, it can be assumed that the minicircles of EM 1 have no great sequence homology with the minicircles of ASCL 1 and 3, because they are very different in size. Ahomadegbe et al. (2) had already shown that the minicircles of HART 1 hybridized very slightly with those of EM 1 but had no significant homology with the trypanosome minicircles of other genera such as *Trypanosoma cruzi* and *Herpetomonas samuelpessoai*. The difference in minicircle se-

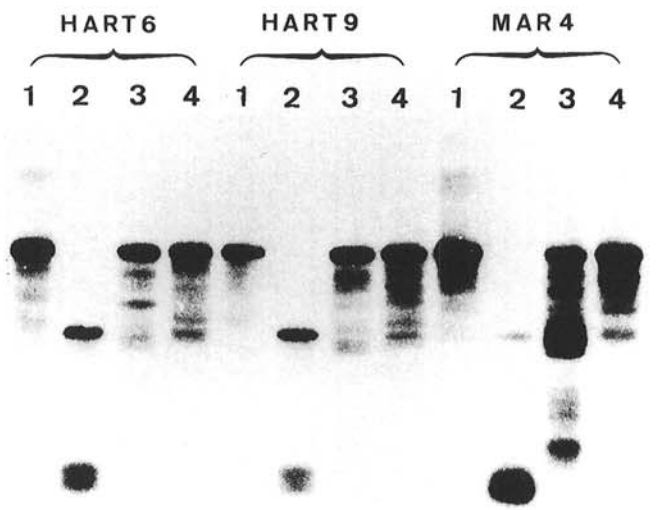

Fig. 4. Restriction endonuclease analysis of kinetoplast DNA from *Phytomonas* isolates. Kinetoplast DNA was cleaved with lane 1, *Hin*fI; lane 2, *Hae*III; lane 3, *Hpa*II; and lane 4, *Taq*I. DNA fragments were analyzed by Southern blot hybridization using free minicircles from isolate HART 13 as probes. Filter was exposed to Hyperfilm MP (Amersham) for 6 h.

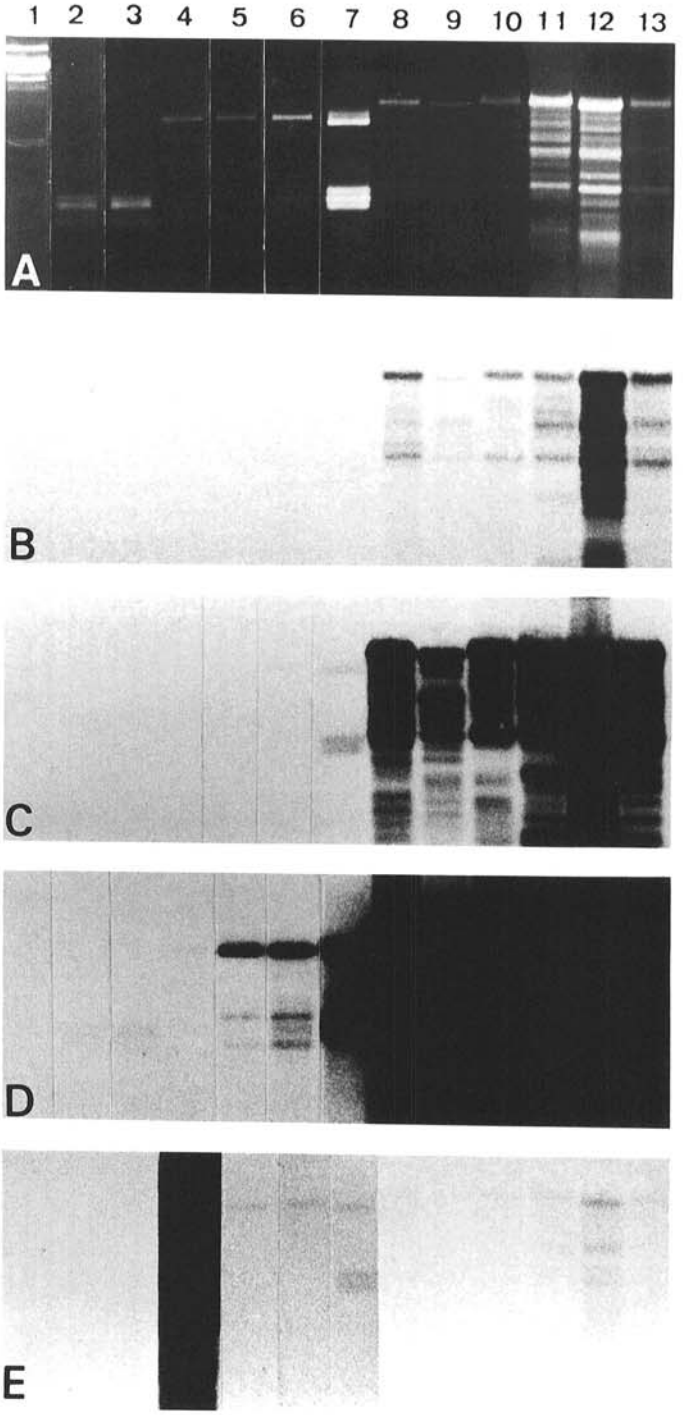

Fig. 5. Southern blot hybridization of DNA minicircles from *Phytomonas* isolates. **A,** Agarose gel electrophoresis of kinetoplast DNA cleaved with *Taq*I. Lane 1, EM 1; lane 2, ASCL 1; lane 3, ASCL 3; lane 4, TOMA; lane 5, HART 3; lane 6, HART 4; lane 7, HART 10; lane 8, HART 9; lane 9, HART 12; lane 10, HART 13; lane 11, MAR 2; lane 12, MAR 6; and lane 13, ALP I. **B–E,** DNA was transferred onto a Biodyne B nylon membrane (Pall Industries), and DNA was hybridized under stringent conditions using free minicircles from ALP I (**B–D**) or from TOMA (**E**) as a probe. Filter was exposed to Hyperfilm MP (Amersham) for **B,** 2 , **C,** 8 , **D,** 24, and **E,** 24 h.

quence level confirmed the differences in minicircle size and enabled a more discriminant separation of our isolates.

DISCUSSION

Trypanosomes isolated from phloem are linked specifically to several types of decay in South America and the Caribbean: Marchitez and Hartrot diseases of oil palm and coconut, coffee phloem necrosis, and decay in *Alpinia purpurata*. The flagellates responsible for these diseases morphologically are virtually identical to flagellates isolated from the latex of healthy plants (Euphorbiaceae or Asclepiadaceae) or from fruits taken from plants (*L. esculentum* and *Annona cherimolia*) affected by fundamentally different symptoms from those seen in coconut and oil palm. One of these plants, *Annona cherimolia,* was examined under an electron microscope, but unlike *Alpinia purpurata,* no flagellates were detected in either the phloem or any other leaf or stem cell (M. Dollet and D. Gargani, *unpublished data*). The arbitrary *Phytomonas* genus, therefore, contains organisms that are very different biologically with regard to both their location in the plant and their pathogenicity (7). These isolates also reacted differently when grown in vitro, since only the intraphloemic isolates required insect cells for culture (22). However, there is one exception for isolates from the latex of *Asclepias curassavica,* which also require insect cells for adaptation to cultivation (7).

The biological role of kDNA minicircles, which were discovered in 1968 (28), has yet to be elucidated. Studies by Simpson's group (33) revealed the existence of small RNA molecules, called guide RNA, which play a part in mRNA editing and apparently are coded for by the maxicircles and minicircles of kDNA. Whatever the case, even though their function has not been clearly defined, the characterization of kDNA minicircles can be used to distinguish species of Trypanosomatidae and strains within the same species (23,35).

Our results first of all confirm the substantial variability of plant trypanosomes and show the variability of intraphloemic trypanosomes. In fact, two size classes of 1.8 and 1.6 kb were obtained for the minicircles of intraphloemic isolates. Camargo et al. (4) observed, using kDNA restriction pattern analysis, that intraspecific variability was nonexistent for insect trypanosomes (monoxenic) compared to the variability observed in a heteroxenic species such as *T. cruzi*. For plant trypanosomes, which also are heteroxenic, substantial variability can be seen in kDNA restriction patterns, even within subgroups formed according to minicircle size.

The separation of intraphloemic isolates into two groups and the close relationship between the intraphloemic isolate from *Alpinia* and the other intraphloemic isolates confirmed the results obtained previously in an isoenzymatic study with the same isolates (24). However, the sequence homology between kDNA minicircles of *Phytomonas* isolates from very different geographic origins is particularly noteworthy. Moreover, it is interesting to see that the variability in kDNA, which has a different replication mode from nuclear DNA (30), can be linked to genetic variability (total DNA) studied through isoenzymatic variability. A correlation between the two characterization methods had already been observed by Tibayrenc and Ayala (37) for *T. cruzi* isolates. However, these new results do not enable separation of intraphloemic isolates, with regard to either geographic origin or host, and the possibility remains that the three decay diseases affecting coconut, oil palm, and *Alpinia purpurata* may correspond to one and the same disease. It suggests that *Alpinia purpurata* may serve as secondary host or as reservoir for the *Phytomonas* that infect palm trees.

Restriction pattern analysis is more discriminant than isoenzymatic banding pattern analysis. Nevertheless, great caution is required when using minicircle restriction patterns for precise characterization of isolates, since the restriction patterns of HART 3 minicircles (Fig. 3A) obtained in this study were not identical to those obtained by Ahomadegbe et al.(2): the major class seems to be maintained, but differences were observed in the lower intensity bands. Likewise, in the very weakly represented minicircle classes of HART 1, there are differences between two clones that had not had the same number of transfers (25 or 159 transfers): these variations are seen only in the minicircle hybridization patterns of HART 1 with homologous minicircles since only the major class is visible on restriction pattern gels (Fig. 3C). This set of observations is similar to the weak differences observed by Leon et al. (18) in the minicircle restriction patterns for certain *T. cruzi* isolates and can be attributed to the rapid evolution of these DNA molecules (3,32).

The different hybridization groups obtained confirmed the separation between fruit, latex, and phytopathogenic trypanosomes isolated from phloem. For most other Trypanosomatidae genera, each species seems to be associated with a single minicircle size (3); however, several minicircle size classes can be found within

TABLE 3. Southern blot hybridization analysis of kinetoplast DNA sequence homology between minicircles from *Phytomonas* isolates

Minicircle used for hybridization	Minicircle probe[a]						
	HART 1	HART 10	HART 13	MAR 1	MAR 2	ALP I	TOMA
HART 1	+++	+++	+	+	+	+	±
HART 3	+++	+++	+	+	+	+	±
HART 4	+++	+++	+	+	+	+	±
HART 10	+++	+++	+	+	+	+	±
HART 6	+	+	+++	+++	+++	+++	±
HART 9	+	+	+++	+++	+++	+++	±
HART 11	+	+	+++	+++	+++	+++	±
HART 12	+	+	+++	+++	+++	+++	±
HART 13	+	+	+++	+++	+++	+++	±
MAR 1	+	+	+++	+++	+++	+++	±
MAR 2	+	+	+++	+++	+++	+++	±
MAR 4	+	+	+++	+++	+++	+++	±
MAR 5	+	+	+++	+++	+++	+++	±
MAR 6	+	+	+++	+++	+++	+++	±
MAR 7	+	+	+++	+++	+++	+++	±
ALP I	+	+	+++	+++	+++	+++	±
ASCL 1	±	±	±	±	±	±	±
ASCL 3	±	±	±	±	±	±	±
EM 1	±	±	±	±	±	±	±
CHERI	±	±	±	±	±	±	+++
TOMA	±	±	±	±	±	±	+++

[a] +++ = very strong homology; + = low homology; and ± = very low homology.

Crithidia oncopelti for the same isolate (26). Moreover, classification of the lower Trypanosomatidae (Trypanosomatidae other than *Leishmania* and *Trypanosoma*, commonly called higher Trypanosomatidae) appears to be somewhat more difficult since the groups defined by Kolesnikov et al. (16) on the basis of kDNA minicircle and maxicircle restriction patterns do not entirely correspond to the groupings into genera, which are themselves defined according to morphological criteria. This fact can be explained by the misidentification of isolates at the generic level. It can be assumed that it would be possible, for the arbitrary *Phytomonas* genus, to separate isolates that are different with regard to minicircle size, but particularly with regard to their sequence, into clearly distinct groups corresponding to one or more species. The biological differences are confirmed by molecular differences; in fact, the intraphloemic isolates, which are alone in being specifically associated with pathological syndromes, are clearly separated from other plant trypanosomes by the sequence of their minicircles. Sequence studies for the small subunit ribosomal RNA genes confirmed this separation, but the intraphloemic group seemed to be more homogeneous with this technique (20) than by a kDNA minicircle study. Moreover, the possibility exists that the molecular differences observed between the groups of intraphloemic isolates may have some biological or epidemiological correspondence. It also seems probable that once a protocol for the inoculation of pathogenic trypanosomes into plants has been fully tested, the isolates of the two intraphloemic groups will be able to be tested for their ability to induce symptoms in healthy coconut and oil palms.

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