The spliced leader RNA gene array in phloem-restricted plant trypanosomatids (*Phytomonas*) partitions into two major groupings: epidemiological implications

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(*Received 19 April 2000; revised 24 August 2000; accepted 30 August 2000*)

SUMMARY

The arbitrary genus *Phytomonas* includes a biologically diverse group of kinetoplastids that live in a wide variety of plant environments. To understand better the subdivisions within the phytomonads and the variability within groups, the exon, intron and non-transcribed spacer sequences of the spliced leader RNA gene were compared among isolates of the phloem-restricted members. A total of 29 isolates associated with disease in coconut, oil palm and red ginger (*Alpinia purpurata*, Zingibreaceae) were examined, all originating from plantations in South America and the Caribbean over a 12-year period. Analysis of non-transcribed spacer sequences revealed 2 main groups, I and II; group II could be further subdivided into 2 subgroups, IIa and IIb. Three classes of spliced leader (SL) RNA gene were seen, with SLI corresponding to group I, SLIIa to group IIa, and SLIIb to group IIb. Two isolates showed some characteristics of both major groups. Group-specific oligonucleotide probes for hybridization studies were tested, and a multiplex amplification scheme was devised to allow direct differentiation between the 2 major groups of phloem-restricted *Phytomonas*. These results provide tools for diagnostic and molecular epidemiology of plant trypanosomes that are pathogenic for commercially important flowers and palms.

Key words: red ginger, coconut, oil palm, mini-exon, PCR, tandem array.

INTRODUCTION

Among plant trypanosomatids, phloem-restricted isolates form a noteworthy group because of their specific association with wilts of cultivated plants such as coconut, oil palm and red ginger in Latin America and the Caribbean (Dollet, 1984, 1994; Camargo, 1999). The palm diseases, hartrot of coconut and marchitez sorpresiva of oil palm, occur everywhere these plants are grown north of an hypothetical line between Lima, Peru, and Salvador, Brazil, and up to Trinidad and Honduras. In most cases the flagellated organisms are transmitted through pentatomid bugs (Heteroptera) of the genus *Lincus* and in at least 1 case through *Ochlerus* (Louise, Dollet & Mariau, 1986; Camargo & Wallace, 1994; Mariau, 1999). No insect vector has been identified for the red ginger wilt in Grenada, a Windward island in the Caribbean.

Following the proposal by Donovan (Donovan, 1909) for distinguishing plant trypanosomatids from animal or human isolates, the parasites associated with these wilts were called *Phytomonas*. The *Phytomonas* genus does not include all trypanosomatids isolated from plants, but provides a common genus for the 3 main groups: phloem-restricted, latex and fruit isolates. Diverse trypanosomatids have been isolated from plants or propagated in fruit, including members of *Leptomonas*, *Herpetomonas* and *Crithidia* (Conchon *et al*. 1989), in a transient state as a result of recent insect feeding activity and not as a chronic true infection or symbiosis. The different groups of phytomonads are biologically distinct from each other based on biochemistry, genetic markers and host/vector characteristics (Dollet, 1994). The use of a single genus for all plant trypanosomatids has led to mis-generalizations for the entire group based on study of fruit (specifically tomato) isolates, all called *Phytomonas serpens*. At least 4 genera of trypanosomatids have been propagated in or isolated from tomato (Conchon *et al*. 1989; Fernandez-Ramos *et al*. 1999; V. Jankevicius, personal communication) that are non-pathogenic and are more readily cultivated in the laboratory than, for instance, the phloem-restricted phytomonads.

There are 2 main groups of phloem-restricted phytomonads that are distinct from the latex and fruit isolates by a variety of criteria: isoenzyme analysis (Guerrini *et al*. 1992; Muller *et al*. 1994), randomly amplified polymorphic DNA (RAPD; Muller *et al*. 1997), and kDNA minicircle length, restriction fragment length polymorphisms (RFLP) or molecular hybridization (Ahomadegbe, Dollet &

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Riou, 1992; Muller *et al*. 1995). It is not possible to assign a level by which these groups are separated (line, species, subspecies, subgenus) due to inconsistencies in the standards for classification of other trypanosomatids. For example, the unweighted pair-group method with arithmetic averages dendrograms obtained after RAPD studies indicate the same level of separation for *Leishmania* sp., *Trypanosoma cruzi* or subgenera *Nannomonas* and Trypanozoon corresponding to different taxonomic levels (Banuls, 1998; Tibayrenc, 1998); the standards for level of significance vary depending on the group being examined.

The spliced leader (SL) RNA gene repeat has proved a useful marker for the characterization of several genera of kinetoplastid protozoa (Murthy, Dibbern & Campbell, 1992; Ramos *et al*. 1996; Fernandes *et al*. 1997) and their near relatives (Campbell, Fernandes & Sturm, 1997). The sequence of the SL RNA gene itself is of interest as it encodes a vital RNA for kinetoplastid gene expression that is highly conserved in primary sequence (the exon) and structure (the intron); the intergenic spacer is highly variable in length and content from species to species. Thus, the SL RNA gene repeat provides defined sequences with differential selection pressures that are likely to follow 3 distinct molecular clocks. Preliminary results indicated that the SL RNA gene repeat from plant trypanosomatids could be amplified by PCR using universal primers designed for *T. cruzi* (Sturm, Fernandes & Campbell, 1995). Similarities in the DNA sequence of the SL RNA have been proposed to define the genus *Phytomonas* (Sturm *et al*. 1995; Serrano *et al*. 1999*b*) while differences in the spacer region are consistent with multiple groups within the genus (Nunes *et al*. 1995; Sturm *et al*. 1995). In this paper, we report the comparison of phloem-restricted trypanosomatid SL RNA gene repeat sequences from 29 isolates. Analysis of these sequences demonstrated high similarity among the phloem-restricted isolates and confirmed the existence of 2 main groups.

MATERIALS AND METHODS

Parasites

The phloem-restricted *Phytomonas* isolates used in this study are listed in Table 1. Their geographical distribution is shown in Fig. 1. Primary cultures of these isolates were obtained in lepidopterous hemocyte cells in Grace's Insect cell medium supplemented with 10% foetal calf serum (Menara *et al*. 1988). Axenic cultures were obtained after several passages (4–8) with progressively decreasing concentrations of insect cells up to the point of no insect cells in the same medium.

DNA extraction, PCR amplification and cloning

Genomic DNA was phenol extracted from mass cultures of $0{\cdot}5–1{\cdot}0 \times 10^9$ organisms (Dollet *et al*. 2000). Approximately 10 ng was used per PCR reaction with the oligonucleotides ME-L and ME-R (Fig. 2) and the amplification profile detailed in Murthy *et al*. (1992) with an extended initial denaturation of 10 min at 94 °C. Optimal buffer conditions were: 10 mM Tris–HCl, pH 9·2, 15 mM $MgCl_2$, 25 mM KCl. Resultant products were analysed initially by ethidium bromide staining after separation on 1% agarose gels and monomer and dimer products were cloned directly from the PCR reaction using the TOPO™ TA Cloning Kit (Invitrogen). Amplification products were analysed by hybridization and sequencing.

Hybridization analysis

Agarose gel electrophoresis-resolved PCR products were blotted onto nylon membranes (Magnagraph, MSI) and probed with various oligonucleotides 5′-labelled with [γ-^{32}P]ATP by polynucleotide kinase (New England Biolabs). The following oligonucleotide probes were used: 41/58 (nts 58 to 41; equivalent to SL3′ (Nunes *et al*. 1995) = 5′-TTCTGGAAGTTTCTCATA; −56/−39 (nts 56 to 39; (Sturm *et al*. 1995) = 5′-TGCCATATCTCGCACTAT. The oligonucleotide positions are shown in Fig. 2. Hybridization was carried out in aqueous buffers as reported previously (Wood *et al*. 1985) and washed in 2 × SSC, 0·1% SDS at 48 °C.

Sequencing and sequence analysis

Plasmids were prepared using the QIAquick PCR Purification Kit (Qiagen) for DNA sequencing by Davis Sequencing (Davis, CA). DNA sequences were entered into the University of Wisconsin GCG Package (Devereux, Haeberli & Smithies, 1984) and were aligned using the PILEUP program (version 9.1) using the parameters Gap Weight = 1 and Gap Length Weight = 0·3. GENBANK sequence accession numbers are included in Table 1.

Phloem-restricted Phytomonas *multiplex PCR*

Specific primers were designed for a multiplex assay as follows: Phy-com (nts −2 to +21 'common' to all groups and an additional *Bam*HI site in lower case) = 5′-ggatccTCAACTAACGCTATTCTAGATAC; Phy GI (nts −5 to −30 from Group I) = 5′-ATCCGCCTACCCCTTGGAGCAACGAC; Phy GII (nts 129 to 148 from Group II) = 5′-CCCATTTTGGGTCGTGCCGGG. Amplification conditions are described above.

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Table 1. List of intraphloemic trypanosomatid isolates used in this study

Isolate	Plant/Vector	Country/Plantation/Year	SL RNA	Accession no.
Alp 1	*Alpinia purpurata*/N.D.	Grenada, West Indies/N.A./1991	IIb	AF250967
Alp 3	*Alpinia purpurata*/N.D.	Grenada, West Indies/N.A./1992	IIa	AF250968
Hart 1	Coconut/*L. croupius**	French Guiana/Saut-Sabbat/1986	I	AF250969
Hart 2	Coconut/*L. lamelliger*	French Guiana/RN1, km 16/1987	I	AF250970
Hart 3	Coconut/*Ochlerus* sp.	Para, Brazil/Moju/1987	I	AF250971
Hart 4	Coconut/*L. croupius**	French Guiana/Saut-Sabbat/1987	I	AF250972
Hart 5	Coconut/*L. croupius**	French Guiana/Saut-Sabbat/1990	IIb	AF250973
Hart 6	Coconut/*Lincus* sp.*	French Guiana/Combi/1990	IIb	AF250974
Hart 9	Coconut/*L. croupius**	French Guiana/Organabo/1990	IIb	AF250975
Hart 11	Coconut/*Lincus* sp.*	Sucre, Venezuela/Irapa/1990	IIb	AF250976
Hart 13	Coconut/*Ochlerus* sp*	Para, Brazil/Moju/1992	IIb	AF250977
Hart 14	Coconut/*Lincus* sp.*	Sucre, Venezuela/Irapa/1993	IIb	AF250978
Hart 15	Coconut/*L. croupius**	French Guiana/Saut-Sabbat/1996	I	AF250979
Hart 17	Coconut/*L. croupius**	French Guiana/Saut-Sabbat/1996	I	AF250980
Hart 18	Coconut/*L. croupius**	French Guiana/Saut-Sabbat/1996	I	AF250981
Hart 19	Coconut/*L. croupius**	French Guiana/Saut-Sabbat/1996	I	AF250982
Hart 20	Coconut/*L. croupius**	French Guiana/Saut-Sabbat/1997	I	AF250983
Hart 21	Coconut/*L. croupius**	French Guiana/Saut-Sabbat/1997	I	AF250984
Hart 22	Coconut/*L. croupius**	French Guiana/Saut-Sabbat/1997	I	AF250985
Mar 1	Oil palm/*L. tumidifrons*	Meta, Colombia/La Cabana/1989	IIa	AF250986
Mar 2	Oil palm/*L. lethifer*	Oriente, Ecuador/Shushusfindi/1989	IIa	AF250987
Mar 3	Oil palm/*L. tumidifrons**	Meta, Colombia/La Cabana/1990	IIa	AF250988
Mar 4	Oil palm/*L. tumidifrons*	Meta, Colombia/Palma de Llano/1991	IIa	AF250989
Mar 5	Oil palm/*Lincus* sp.*	Meta, Colombia/Manuelita/1992	IIa	AF250990
Mar 6	Oil palm/*L. tumidifrons**	Zulia, Venezuela/Casigua el Cubo/1992	IIb	AF250991
Mar 7	Oil palm/N.D.	Monogas, Venezuela/Palmonagas/1993	IIb	AF250992
Mar 8	Oil palm/ *Lincus* sp.*	Meta, Colombia/Manuelita/1994	IIb	AF250993
Mar 9	Oil palm/N.D.	Santander, Colombia/Monterey/1994	IIb	AF250994
Mar 10	Oil palm/N.D.	Santander, Colombia/Monterey/1995	IIb	AF250995

* Designates insects regularly found in the locale but not obtained from an experimentally infected tree under controlled conditions.
N.A., Not available.
N.D., Not determined.

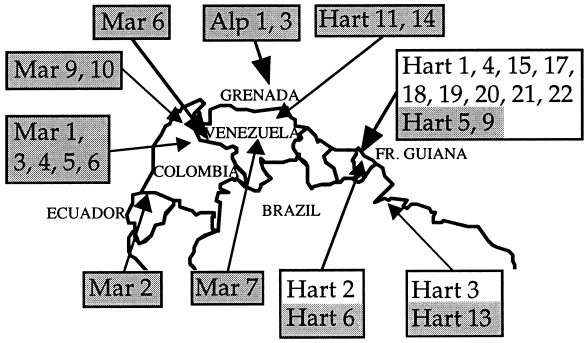

Fig. 1. Geographical distribution of phloem-restricted *Phytomonas* isolates from South America and the Caribbean. Arrows indicate the physical site of location of the 29 isolates used in this study. Multiple samples were collected from the same site at different times, as detailed in Table 1. Non-shaded isolates are from SL RNA gene group I and shading indicates those isolates that were classified as group II.

RESULTS

The oligonucleotide pairs used for PCR were designed to anneal to the exon/intron portion (nts 21–42) of the SL RNA gene of *T. cruzi* (Murthy *et al*. 1992) and to amplify the entire intron and non-transcribed spacer region (Fig. 2). Representative PCR amplification products using the ME-L and ME-R oligonucleotide pair are shown (Fig. 3). The PCR product sizes varied from 280 to 550 bp, the smaller corresponding to the 'monomer' size and the larger to the 'dimer' products (see Fig. 2). Two similar-sized dimer products were resolved in some samples, as seen previously in studies of human-pathogenic *Leishmania* (Fernandes *et al*. 1994), reflecting polymorphisms within the SL RNA gene arrays in the form of small deletions between direct repeats in the non-transcribed spacer region. However, in this case a distinct secondary SL RNA gene array was amplified that showed variant exon and intron sequences; this array could not be used for comparison because it was not shared by all isolates (M. Dollet, N. R. Sturm and D. A. Campbell, unpublished results). Monomer and dimer classes hybridized with the 41/58 probe (Fig. 3), corresponding to the beginning of the well-conserved intron sequence of plant trypanosomatids (Nunes *et al*. 1995; Sturm *et al*. 1995).

Sequence analysis of PCR products from all isolates revealed that the overall length of the repeat unit ranged from 224 bp (Hart 13) to 233 bp (Alp 3).

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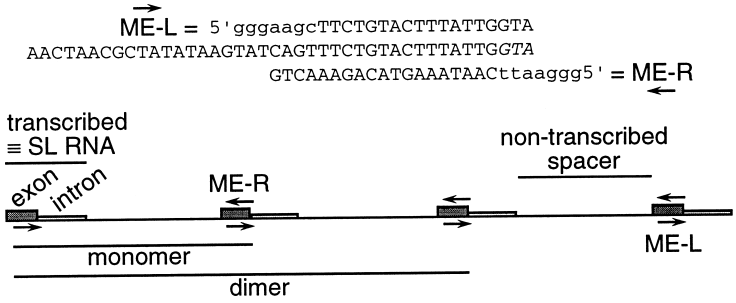

Fig. 2. SL RNA gene array PCR amplification scheme. The primary sequence of the exon and intron (italics) are shown with the sequence and positions of the general amplification primers above (ME-L) and below (ME-R). Extra nucleotides on the primers are indicated in lower case. In the schematic of the amplification, the SL RNA gene is represented by a large box for the exon and a slender box for the intron. Monomer and dimer products are indicated.

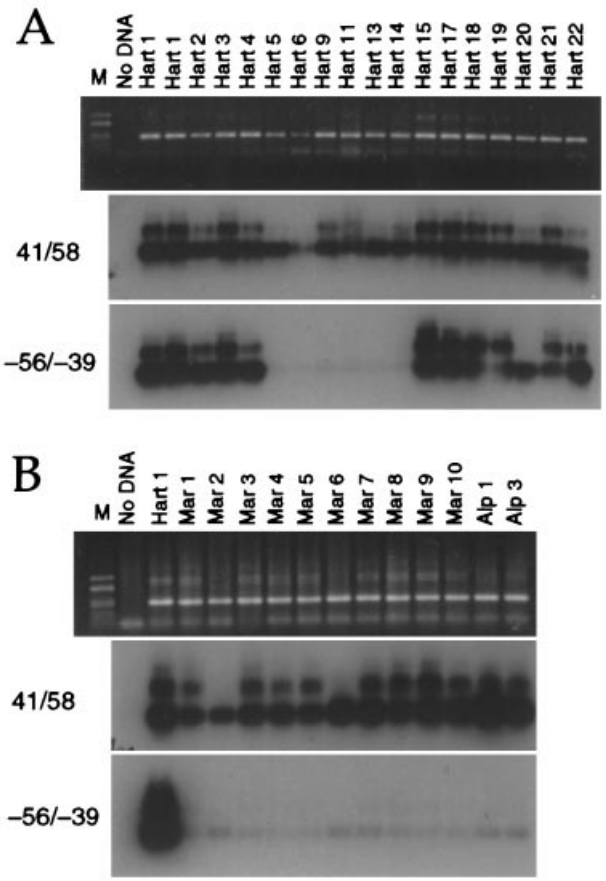

Fig. 3. Phloem-restricted *Phytomonas* SL RNA genes are amplified by primers ME-L and ME-R. Amplification products from 29 strains resolved by electrophoresis through a 1% agarose gel and visualized by ethidium bromide staining. (A) Isolates from hartrot. The marker lane is the plasmid pUC digested with *Hae*III. DNA blot hybridizations with the *Phytomonas*-specific probe 41/59 (Serrano, Camargo & Teixeira, 1999*a*) and a non-transcribed spacer probe (−56/−39) based on the Hart 1 sequence (Sturm *et al.* 1995) are shown. (B) Isolates from marchitez sorpresiva and red ginger wilt. DNA blots probed as in (A) are shown below.

Multiple sequence alignment of the complete SL RNA gene repeats showed major divisions among the isolates. As anticipated, within the exon portion of the SL RNAs (Fig. 4A) all isolates showed identical sequence and contained the *Phytomonas*-specific C residue at position 14 (Nunes *et al.* 1995; Sturm *et al.* 1995). Positions 21–39 for most of these isolates are representative of the amplification primers; however, the inferred primary sequence was confirmed in 6 isolates (Hart 1 from group I (Sturm *et al.* 1995); Mar 3, Mar 5, Mar 8, Mar 9 from group IIa; Alp 1 from group IIb) by the central SL RNA gene present in dimer product clones. The intron alignments (Fig. 4B) were highly similar, but showed specific changes at positions 63 and 64, suggestive of subgroupings within the phloem-restricted phytomonads. These small but conserved differences define the SLI, SLIIa and SLIIb transcribed regions.

Specific motifs were evident in an alignment of the intergenic spacer regions (Fig. 5). In general, the 8 to 10 nt T tract, the transcription terminator for the SL RNA gene (Sturm, Yu & Campbell, 1999), was followed by homopolymeric C (from position 13) and homopolymeric G (from position 50) stretches ranging from 8 to 13 nt. A strong region of nucleotide identity was evident from 71 to 87 (consensus: AAAGTGTGCSYGCCATA where S = G or C; Y = C or T, T at 90% in this data set), as expected relative to the predicted start point of transcription. By analogy to the *Leishmania*, *Leptomonas* and *Trypanosoma* promoters (Campbell, Sturm & Yu, 2000), this region likely contains a transcription promoter element for the SL RNA gene. Comparison of the adjacent non-transcribed spacer regions of the cloned dimers or multiply cloned monomers revealed a low frequency of intra-array sequence heterogeneity, notably in the number of repeated residues in the tracts.

The intergenic alignment confirmed the presence of 2 distinct sequence classes, which we designate groups I and II. Groups I and II are distinguished from one another by sequence between the C and G tracts, and by motifs downstream of the presumptive promoter region. Group I contained only Hart isolates (1, 2, 3, 4, 17, 18, 19, 20, 21, 22) and showed nearly homogenous SL RNA transcribed regions

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A Exon sequence

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1          10          20          30       39
AACTAACGCTATTCTAGATACAGTTTCTGTACTTTATTG
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B Intron sequence (40-100)

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40         50         60         70         80         90         100
GTATGAGAAACTTCCAGAATTAATTTTCTGGGAAATTTTGGACTGGGGGCCTTCGGGCCCC – Hart 3, 15, 17, 18, 19, 20, 21, 22
..........................................................C..... – Hart 1, 2          -- SLI
..............G............................................... – Hart 4
.......................-C.................................... – Alp 3, Mar 1, 2, 3, 4, 5, 8, 9, 10   -- SLIIa
.......................-..................................... – Alp 1, Mar 6, 7, Hart 5, 6, 9, 11, 13, 14   -- SLIIb
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Fig. 4. Sequence analysis of the SL RNA transcribed region divides the isolates into 3 groups. (A) The identical exon of SLI, SLIIa and SLIIb, from position +1 to 39. This region includes the PCR primers from 21 to 39 and primary sequence from dimer product clones for Alp 1, Hart 1, Mar 3, Mar 5, Mar 8 and Mar 9. (B) Alignment of representative intron sequences for SLI, SLIIa and SLIIb from position +40 to the T tract located at approximately +100. This alignment is the product of a PILEUP analysis (UWGCG). Nucleotide identity is shown as dots (·), gaps introduced to maximize the alignment are shown as dashes (–), and positions of nucleotide change relative to the SLI reference sequence are shown explicitly. Shaded nucleotides represent diagnostic positions for SLIIa and SLIIb.

referred to as SLI. A subdivision within group II was supported by particular nucleotide changes in the non-transcribed spacer region, specifically an A to C differential at position 20, A to T at position 30, T to C at position 93, and T to C at position 115 (IIb to IIa, respectively).

Group IIa (Alp 3, Mar 1, 2, 3, 4, 5, 8, 9, 10) and IIb (Alp 1, Hart 5, 6, 9, 13, Mar 6, 7) were thus defined, yielding transcripts SLIIa and SLIIb, respectively. Two strains, Hart 11 and Hart 14, both from the north of Venezuela, appeared to bridge the subdivision boundary because they resemble group IIa at intergenic positions 20 and 30, but group IIb at positions 93 and 115. Since their intron sequence is SLIIb, we placed them in group IIb. The correlation of SLI and SLIIa/SLIIb with the 2 major groups of phloem-restricted *Phytomonas* inferred from isoenzyme, RAPD and other data suggests their value as specific genetic markers. On the basis of the combined results from multiple different techniques, the designated groupings appear to have real significance in differentiating the isolates of phloem-restricted phytomonads.

We pursued a multiplex PCR approach to distinguish between the 2 groups of phloem-restricted *Phytomonas*. With the design of 3 primers, a common exon and 2 specific non-transcribed spacer region oligonucleotides, we sought to differentiate between the 2 groups in a single PCR reaction without having to hybridize the products specifically (Fig. 6A). The group I representative was Hart 1 and the group II isolate was Mar 1. Group I amplification products were predicted to result in a band of 230–233 bp and group II products in a band of 148–150 bp; the anticipated products were visible and distinctive in agarose gel electrophoresis (Fig. 6B). The group I combination did not result in a product from group II isolate DNA, nor *visa versa*, demonstrating that the primers were specific for their targets. As with the exon-to-exon amplification scheme (Fig. 2), this assay could generate product ladders with a periodicity of 1 repeat unit; however, this would not diminish specificity of target recognition.

DISCUSSION

We have demonstrated that the phloem-restricted phytomonads segregate into 2 major groups using the SL RNA gene as a marker. The SL RNA gene sequences from phloem-restricted trypanosomatids are distinct from the latex or fruit isolates (Nunes *et al*. 1995; Sturm *et al*. 1995). The 2 groups conform to those derived from previous analyses using different markers and techniques (Muller *et al*. 1995, 1997). As part of our study we have developed a suite of primers for use in distinguishing between the two groups in a single PCR reaction based on the size of the amplification products. The sequence homogeneity of the SL RNA gene sequences indicate that these *Phytomonas* specifically associated with wilt syndromes of palms and red ginger in South America and the Caribbean are a true species. The latex and fruit isolates, by contrast, present a variety of SL RNA gene spacer sequences, consistent with multiple species; additional work is required to resolve the relationship among all the plant trypanosomatids.

Generally, the results presented here are in agreement with those obtained with isoenzymes, kDNA minicircles and RAPD studies (Muller *et al*. 1994, 1995, 1997), and provide nucleotide-level evidence supporting 2 main groups with a further subdivision within 1 group. Group I contained only coconut isolates. Group II is divided between

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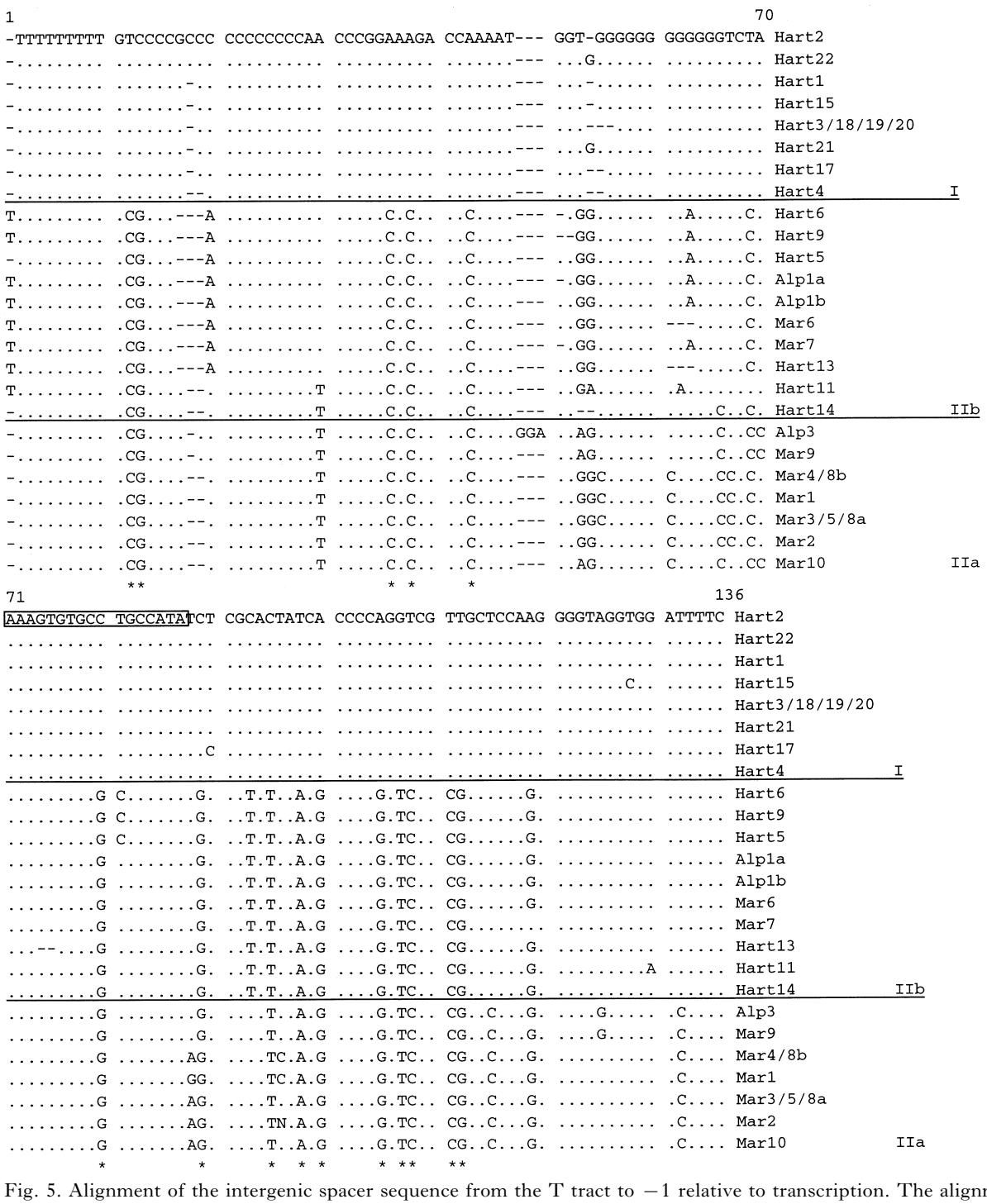

Fig. 5. Alignment of the intergenic spacer sequence from the T tract to −1 relative to transcription. The alignment is the product of a PILEUP analysis (UWGCG), and the output follows the convention described in Fig. 4. Where dimers with differing sequences were found (Alp 1, Mar 8), the first and second spacers are shown and distinguished by suffixes 'a' and 'b'. Nucleotide positions that distinguish group I from group II are denoted by asterisks (*). The putative promoter region is boxed in Hart 2.

subgroup IIa, comprised of oil palm isolates and a red ginger isolate, and subgroup IIb, contained a mix of coconut, oil palm and the other red ginger isolate. Curiously, the 2 red ginger isolates did not fall into the same group II subdivision. Our previous RAPD and isoenzyme studies did not include Alp 3; however Alp 1, which was studied by these 2 techniques, fell into the same subgroup (IIb) as Hart 11 and Mar 6. In different studies the variable positions of the Hart 11 and Hart 14 isolates are particularly interesting, as both isolates were obtained from coconut trees in the same plantation in the state of Sucre in Venezuela separated in time by 3 years. Past analyses have placed Hart 11 in group IIa by RAPD and in group IIb by isoenzymes (Muller *et al*. 1997).

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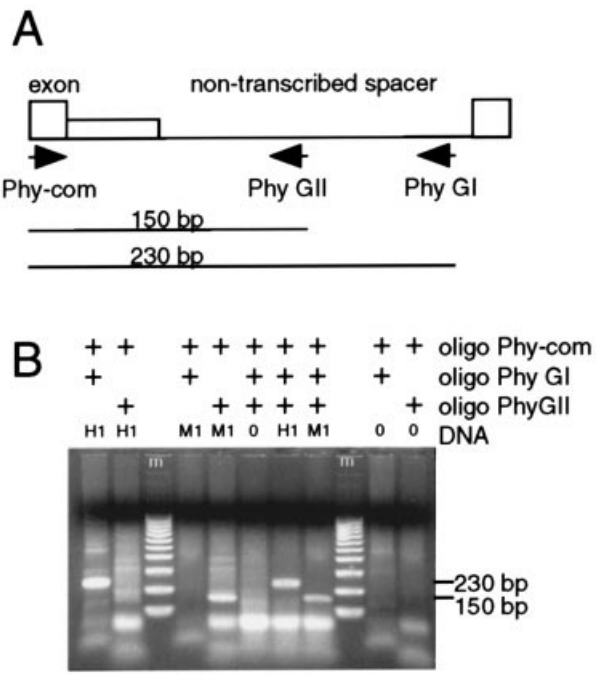

Fig. 6. A multiplex PCR assay distinguishes group I from II in a single reaction. (A) Schematic representation of the reaction and possible products for groups I and II. (B) A trial run of the multiplex system using known isolates is shown. The product sizes are determined by separation on a 1·5% agarose gel and visualized by ethidium bromide staining. m, 100-bp ladder size marker; H1, Hart 1 (group I) DNA; M1, Mar 1 (group II) DNA; 0, no DNA.

The diseases of hartrot and marchitez wilt co-exist in the locations from which our samples were originally isolated. Insects collected from infected oil palms in the field can be used subsequently to infect coconut in the laboratory (isolates Mar 2 and Mar 4; Dollet *et al*. 1993). Thus, there is no biological boundary to infection by these phloem-restricted phytomonads, except perhaps for natural exposure to the vector and/or distance and geographical barriers. Hartrot isolates are more numerous due to the practical fact that the parasites are easier to culture. The Hart isolates are harvested from coconut inflorescences, a relatively clean starting material, while oil palm isolates are taken from the roots and compete in culture with fungi, bacteria and other protozoa that are difficult to eliminate specifically. A further bias was introduced by the sources of funding during these years of collection. Thus, had coconut isolates been gathered concurrently with the marchitez isolates from Colombia, or oil palm isolates gathered with hartrot isolates in Guiana and Brazil, they may have shared the same grouping.

Most of the members of group I are from the Saut Sabbat plantation, which is the only location surrounded by primary forest (Louise *et al*. 1986). The Hart 5 isolate is an exception to this generality, falling into group IIb. The Saut Sabbat trees were exposed to several waves of infection, separated by periods of sustained drought during which the insect vector disappeared from the plantation and consequently different 'strains' could parasitize the area at different times.

The Brazilian isolates Hart 3 and Hart 13 also display a difference in group consistent with previous results from RAPD and isoenzyme analyses (Muller *et al*. 1997). Both are associated with an unusual vector insect. Instead of the more common *Lincus* vector, only *Ochlerus* is found in the Moju plantation. The two pentatomids have distinctly different behaviours; whereas *Lincus* is reclusive, *Ochlerus* can be seen at night on the undersides of the leaves from which they feed and during the day they disappear into the plantation floor. Thus, we can envisage that *Ochlerus* is significantly more mobile than *Lincus* and may be responsible for the infection of many trees within the same area, while *Lincus* stays on a particular tree until that tree becomes too diseased to be a good food source.

Two disparate plantations in our study have undergone a group typing switch. Because of the location of the Saut Sabbat plantation several new populations of *Lincus* spp. could have arrived at different times bringing both phytomonad groups. We know for instance, that in 1984 there were 3 different *Lincus* species in this plantation (Louise *et al*. 1986). Although the region surrounding the Moju plantation is developed, multiple genetic variants may have been present within the area.

What does the group II subdivision reveal about phloem-restricted *Phytomonas* infection? Group IIa separates the oil palm from the coconut isolates; however, no coconut isolates were obtained from the equivalent geographical region, although hartrot does exist in Colombia and Ecuador. Group IIb is comprised of oil palm and coconut isolates, and both subgroups contain red ginger isolates. Perhaps the partitioning of the geographically remote red ginger isolates is the most informative within this group, providing a clue as to how different groups of phytomonads travel over large distances. Phloem-restricted *Phytomonas* was first seen in red ginger, a popular ornamental flower propagated by rhizomes, in 1989 (P. Hunt and M. Dollet, unpublished observation). Although no insect vector has been identified for red ginger, *Lincus* sp. has been found in Venezuela close to the coconut plantation where Hart 11 and Hart 14 were obtained on *Heliconia* sp. (A. Diaz and M. Dollet, unpublished observation), another rhizome-propagated plant producing a commercially important flower. While coconut and oil palms are not mobile and are seed propagated, the plants producing ornamental flowers are transported as rhizomes with ease and relative freedom, despite existing agricultural regulations. Thus, a mode of *Phytomonas* dissemination may have been identified, and this could explain why red ginger isolates are related to coconut and oil palm isolates from

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Venezuela and Colombia. Farmers introduced red ginger rhizomes from Venezuela into the Caribbean to start new horticultural programs at the end of the eighties.

The SL RNA gene repeat is a robust marker for phloem-restricted *Phytomonas*, to the extent of reflecting the ambiguity of isolates Hart 11 and Hart 14 that we speculate could represent hybrid isolates between groups IIa and IIb. An important advance offered by these data is the availability of a molecular test that can be used on potential reservoir plants, especially palms, or on potential insect vectors found in the field, avoiding long and fastidious *in vitro* culture. This aspect of the collection and study of pathogenic *Phytomonas* has been problematic because of the losses that can occur when culturing attempts fail or result in differential outgrowth of mixed populations within infected plants, thus a PCR based test has great appeal.

The authors thank D. Gargani for technical assistance with the mass cell culture and A. Diaz, P. Hunt and V. Jankevicius for communication of unpublished work. This study was supported in part by a donation from Margaret H. Moffat to D. A. C. M. D. was a grantee of CIRAD.

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https://doi.org/10.1017/S0031182001007417 Published online by Cambridge University Press

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