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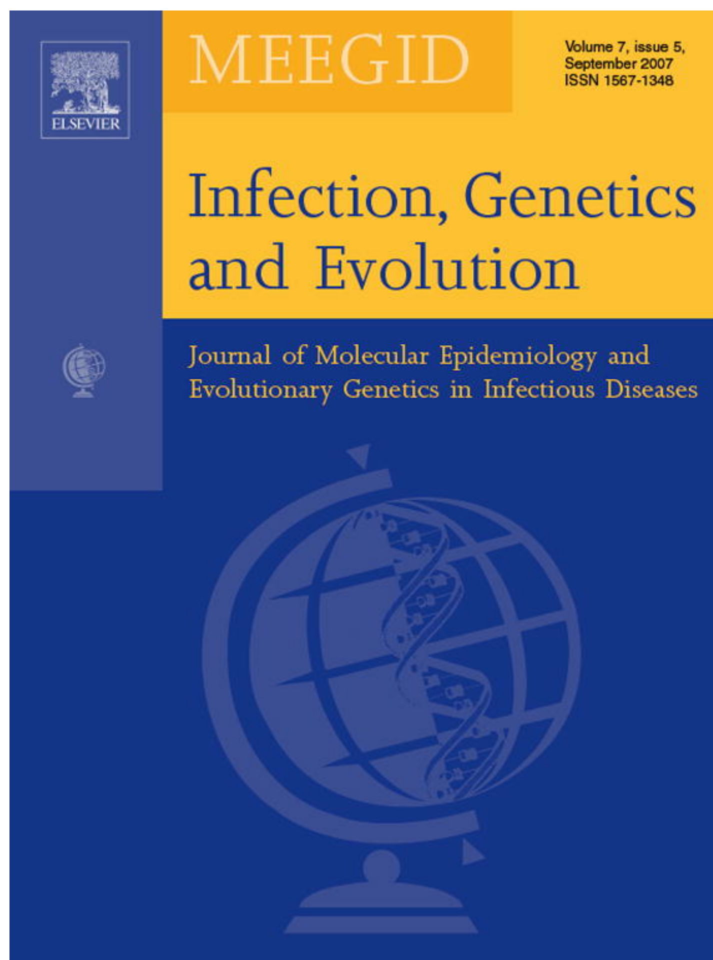

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Rational sub-division of plant trypanosomes (*Phytomonas* spp.) based on minicircle conserved region analysis ☆

Nancy R. Sturm [a,1], Michel Dollet [a,*], Julius Lukeš [b], David A. Campbell [a,1]

[a] *CIRAD,UPR29 Etiologie Dépérissements, Département Systèmes Biologiques, TA A-29/F, Campus International de Baillarguet, 34398 Montpellier Cedex 5, France*

[b] *Biology Centre, Institute of Parasitology, Czech Academy of Sciences and Faculty of Biology, University of South Bohemia, 370 05 České Budějovice, Czech Republic*

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Abstract

The sequences of minicircle conserved regions from various plant trypanosomatids have been determined and analyzed. The goal of this study was to add another tool to the arsenal of molecular probes for distinguishing between the different trypanosomatids occuring in plants: systemic trypanosomatids multiplying in the sap, those from the laticiferous tubes, and those developing in fruits, seeds or flowers but not in the plant itself and that are frequently considered as opportunistic insect trypanosomatids. As some plant intraphloemic trypanosomatids are the causative agents of important diseases, a clear definition of the different types of trypanosomatids is critical. The conserved region of the mitochondrial minicircle provides several specific features in a small sequence region containing three functionally elements required for minicircle replication. Trees generated from the analysis recapitulated trees drawn from analyses of isoenzymes, RAPD, and particular gene sequences, supporting the validity of the small region used in this work. Three groups of isolates were significant and in accordance with previous work. The peculiarity of phloem-restricted trypanosomatids associated with wilts of coconut and oil palm in Latin America – group H – is confirmed. In agreement with previous studies on their biological and serological properties the results highlighted this group called 'phloemicola'. It always differentiated from all other latex and fruit isolates or opportunistic trypanosomatids, like insect trypanosomatids. We can assert that phloemicola is the only well-defined taxon among all plant trypanosomatids. A group of non-pathogenic latex isolates from South American euphorbs (G), and a heterogenous group (A) including one fruit, one possible latex and one insect isolate are clearly distinct groups. The group of Mediterranean isolates from latex (D), even with a low boostrap, stood out well from other groups. The remainder of the isolates fell into a heterogeneous cluster. At least eight different groups in the plant trypanosomatids were identified.
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1. Introduction

The genus *Phytomonas* (Donovan, 1909) was created the same year as the first trypanosomatids were discovered in plants by Lafont (1909) just to distinguish them from the trypanosomatid parasites from animals or plants. This arbitrary genus does not reflect the huge diversity of trypanosomatids mutiplying in plants (Guerrini et al., 1992; Muller et al., 1997). However, it has become an easy habit to call every trypanosomatid found in plants a "*Phytomonas*", specially for those who have never studied trypanosomatids from plants. Within this digenetic group of plant trypanosomatids transmitted by insects, some trypanosomatids are isolated from phloem, from latex, from fruits, or from flowers and seeds. However, some previously described "monogenetic, insect-specific", trypanosomatids (of the genera *Leptomonas*, *Herpetomonas*, and *Crithidia*) can replicate in fruits, seeds and flowers, in the vicinity of the feeding sites of various insects, with the absence of subsequent systemic plant colonization (Conchon et al., 1989; Jankevicius et al., 1989;

☆ Nucleotide sequence data reported in this paper are available in the GenBank database under the accession numbers: AF440572-AF440588.

* Corresponding author. Tel.: +33 4 67 59 39 22; fax: +33 4 67 59 38 19.
E-mail address: michel.dollet@cirad.fr (M. Dollet).

[1] Permanent address: Department of Microbiology, Immunology & Molecular Genetics, University of California, 609 Charles E. Young Drive East, Los Angeles, CA 90095-1489, USA.

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Serrano et al., 1999a; Fiorini et al., 2001). Even mixed infection of a single fruit with two different genera of ''insect trypanosomatids'' can occur (Catarino et al., 2001). In that case, the distinction between the ''insect-specific'' trypanosomatids of the three genera cited above and ''plant trypanosomatids'' can be obscured.

In vitro culture of plant trypanosomatids is not easy. It was even considered impossible until the first success in 1982 (Dollet et al., 1982), which led to new cultures in the following years. But it remains a very long and difficult challenge for some groups, such as the phloem-restricted group, to obtain a primoculture (Menara et al., 1988; Dollet, 2001). Conversely, ''insect trypanosomatids'' can be easily cultured from fruits or seeds, with high yields (Marin et al., 2007). The phloem-restricted trypanosomatids, transmitted by pentatomid bugs, are systemic and are specifically associated with very severe pathological syndromes such as ''hartrot'' of coconut and ''marchitez sorpresiva'' of oil palm, resulting in considerable economic consequences (Dollet, 1984). They form the ''phloemicola'' group, a very distinct group, among plant trypanosomatids (Dollet, 2001). They multiply in the phloem and are translocated throughout the plant because there is anastomosis between the sieve tubes of phloem tissues. In laticiferous plants, trypanosomatids live in the latex vessels that often do not anastomose, and infection remains localized in the branch of the plant fed upon by the insect. These standards cannot be applied broadly to fruit, seed or flower isolates, which might actually be previously identified monoxenous flagellates deposited by phytophagous insects. For instance, in the case of one tangerine isolate, from *Citrus reticulata*, presumed to be a *Leptomonas* sp., Conchon et al. (1989) considered that their occurrence in fruits could simply result from an accidental or opportunistic infection. Their presence might induce some fruit damage, such as yellow-brown blotches or necrosis but not a plant disease. The fruits finish by dropping or are collected, and the plant itself stays healthy.

Further classification of these kinetoplastids is necessary to differentiate between what appear to be obligate plant trypanosomatid inhabitants, be they intraphloemic or intralaticiferous, from some transient or accidental opportunists. A useful molecular marker for that purpose is the spliced leader (SL) RNA gene (Murthy et al., 1992). SL RNA-gene PCR assays to detect and identify *Phytomonas* have been developed (Sturm et al., 1995; Nunes et al., 1995; Serrano et al., 1999b) and features unique to plant trypanosomatids have been identified, including a C residue at position 14 of the SL RNA exon (Sturm et al., 1995; Dollet et al., 2001b). Classification by RAPD (Serrano et al., 1999a) and 5S ribosomal RNA gene sequences (Dollet et al., 2000) indicates that the spectrum of plant trypanosomatids is composed of at least eight groups.

In addition to distinguishing between plant and insect trypanosomatids, we are searching for markers to detect and differentiate between the eight described groups and other possible groups, when new isolates are obtained, within the plant trypanosomatids.

Kinetoplast minicircles contain a relatively short but conserved region that includes three highly conserved sequence blocks (CSB) believed to be important for DNA replication (Ray, 1989; Shapiro and Englund, 1995). These conserved regions (CR) have been used previously for parasite detection (Sturm et al., 1989; Avila et al., 1993) and to examine the relationship between a broad selection of trypanosomatids (Yurchenko et al., 2000). As variability has been previously reported in the size of plant trypanosomatid minicircles (Ahomadegbe et al., 1990; Muller et al., 1995) we felt these minicircle sequences could be valuable comparative molecular tools.

To generate more molecular differentiators for plant trypanosomatids we designed a set of oligonucleotide primers to amplify *Phytomonas* minicircles. Those primers did not amplify minicircles from insect trypanosomes such as *Crithidia* spp., *Leptomonas* spp., and *Herpetomonas* spp., but yielded products from 19 isolates from plants and one from a phytophagous insect (Dollet et al., 2001a). We used minicircle CR sequences from 19 isolates to establish phylogenetic relationships with and without an outgroup. The trees generated from these analyses of mitochondrial minicircle DNA were consistent with data from nuclear and total genomic DNA markers, and provided a clear definition for four of the eight groups defined thus far among plant trypanosomatids.

2. Material and methods

2.1. Trypanosomatids

The trypanosomatid isolates used in these analyses are described in Table 1, along with the accession numbers of new sequences. For the record, in vitro culture of phloem-restricted trypanosomatids – phloemicola group – is a long and difficult process (Menara et al., 1988; Dollet, 2001), which is why we are so far the only laboratory with a phloem trypanosomatid cryobank. But even latex isolates such as E.hi.Vz, E.hi.Sur or E.het.Vz are very laborious to grow, which is why we only tested a part of our plant trypanosomatid collection. New unstudied isolates are: E.pro.Vz, isolated by M.D. in 1992 in Puerto Chama, Estado Zulia, Venezuela, from *Euphorbia prostata*; E.het.Vz, isolated by M.D. in 1991 in San Augustin, Municipio Yaguaraparo, Estado Sucre, Venezuela from *E. heterophila*. The isolate 'Trifolium' was obtained from Manuel Sánchez-Moreno, University of Granada, Spain (Uttaro et al., 1997; Sanchez-Moreno et al., 1998). The Ps1G sequence (Maslov et al., 1998) was retrieved from Genbank (Accession number AF034625). We sequenced the Hart 1 minicircle (Accession number AF397906) in its entirety (Dollet et al., 2001a). The *C. fasciculata* CR sequence (Sugisaki and Ray, 1987) used as an outgroup was obtained from Genbank (Accession number M19266).

2.2. PCR amplification, cloning and sequencing

The *Phytomonas* spp. minicircles were amplified using the Qiagen PCR Core Kit and oligonucleotides directed against the CSB-3 motif: mcCSB3a, 5′-GGTTT TTTAG GGGTT GGTAT AAT; mcCSB3b, 5′-TACCA ACCCC TAAAA AACCC C. A

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Table 1
List of *Phytomonas* spp. isolates used in this study

Group	Isolate	Genbank no.	Host	Tissue[a]	Country
A	Berg	AF440573	*Citrus bergamia*	F	Brazil[b]
	Lima	AF440581	*Jatropha macrantha*	L	Peru
	Ps1G[c]	AF034625	*Phthia picta*[c]	I	Brazil[b]
B	Aca	AF440572	*Allamanda cathartica*	L	Surinam[d]
	E.hi.Sur	AF440576	*Euphorbia hirta*	L	Surinam[d]
	Rhabdadenia	AF440586	*Rhabdadenia biflora*	L	Surinam[d]
C	Blepharodon	AF440574	*Blepharodon nitidus*	L	Surinam[d]
	Mand.sc.Br	AF440582	*Mandevilla scabra*	L	Surinam[d]
	Tom.Sp	AF440587	*Lycopersicon esculentum*	F	Spain
	Trifolium	AF440588	*Trifolium repens*	S	Spain
D	E.M.1	AF440579	*Euphorbia pinea*	L	France
F	Mani.Br	AF440583	*Manihot esculenta*	L	Brazil[b]
G	E.hi.Vz	AF440577	*Euphorbia hirta*	L	Venezuela
	E.hy.Gu	AF440578	*E. hyssopifolia*	L	Guiana
H	Hart1	AF397906[e]	*Cocos nucifera*	P	Guiana
	Mar1	AF440584	*Elaeis guineensis*	P	Colombia
	Mar6	AF440585	*E. guineensis*	P	Venezuela
New	E.pro.Vz	AF440580	*Euphorbia prostata*	L	Venezuela
	E.het.Vz	AF440575	*E. heterophylla*	L	Venezuela

[a] Nature of the tissue. F: fruit; I: insect; L: latex; P: phloem; S: crushed stem.
[b] Isolates originating from E. Camargo's laboratory, Sao Paulo, Brazil. Berg = TCC 233; Mani.Br = TCC 064.
[c] Isolate from the salivary glands of *Phthia picta* (Hemiptera, Coreidae) pest of tomato fruits (Sã-Carvalho et al., 1993). Sequence, from Maslov et al. (1998).
[d] Isolates originating from P. Kastelein, Surinam.
[e] Dollet et al. (2001a).

range of 0.05–10 ng of total cell DNA or 1:1000 dilutions of guanodine isothiocyanate cell lysates (4×10^8 cells/ml) in H_2O were used successfully as template material. The thermal cycle profile was 94 °C for 2 min; 30 cycles of 94 °C for 15 s, 60 °C for 15 s, 72 °C for 1 min; 72 °C for 9 min. Reaction products were separated on 1.0% agarose gels and evaluated for appropriate size by ethidium bromide staining and UV–light transillumination in the cases for which such information was available (Dollet et al., 2001a).

The minicircle products, representing approximately half of a single minicircle in sequence content due to the presence of two conserved regions per minicircle, was cloned directly from the PCR reaction using the TOPO-TA Cloning Kit (Invitrogen). A single clone from each isolate was subjected to dideoxy sequencing (MGW Biotech, Germany).

2.3. *Computer analyses*

The initial alignment was made using the PILEUP program (GapWeight: 1, GapLengthWeight: 0.1) in the University of Wisconsin's GCG program (Devereux et al., 1984). Phylogenetic analyses were performed using maximum parsimony, maximum likelihood (model HKY85) and distance (minimum evolution with distance measure program HKY85 and LogDet-paralinear) as optimality criteria implemented in the PAUP program (Swofford, 1998). Generated trees were either unrooted or were rooted using the *C. fasciculata* minicircle sequence. Bootstrap support was calculated from 1000 replicates.

3. Results and discussion

It has been shown previously that kinetoplast minicircle CRs are conserved sufficiently in 14 latex and phloem isolates, 3 insect/fruit (tomato) isolates, and 2 isolates from *Amaranthus* and *Trifolium* to allow amplification of half-minicircles by PCR (Dollet et al., 2001a). To gain a better understanding of the basis for this specificity and to investigate relationships among the different types of trypanosomatids isolated from plants, we sequenced and cloned minicircles from 17 isolates (data not shown). Curiously, the minicircle fragments were all cloned in the same orientation relative to the CR. In each case, CR sequences were obtained upstream from CSB3, which was the annealing site of one of the primers (GenBank Accession numbers for all sequences used are listed in Table 1). Based on the minicircle structure proposed, the region between CRs is predicted to contain either the template for a guide RNA or to be GT-rich (Maslov et al., 1998). Attempts to identify guide RNA sequences in the absence of edited mRNA counterparts would be speculative and beyond the scope of our goals, thus the sequence between the CRs was not analyzed further.

Previous analysis of CR sequences from 29 trypanosomatids and one bodonid revealed a phylogeny that was consistent for closely related taxonomic groupings such as the genus *Leishmania* (Yurchenko et al., 2000). Unrooted phylogenetic trees consisting solely of 18 trypanosomatids originating from a plant and one from the salivary glands of the propagator insect of a Brazilian tomato fruit trypanosomatid were generated. Maximum parsimony (Fig. 1) and maximum likelihood

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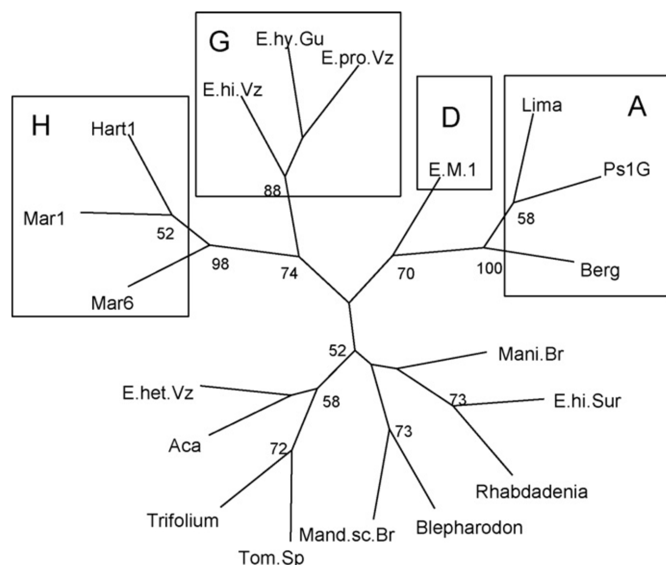

Fig. 1. An unrooted parsimony bootstrap (1000 replicates) tree based on 270 characters (gaps included). Number of informative characters was 146. The best tree was 609 steps long. Bootstrap support lower than 50 not shown.

analyses generated trees with very similar topology. Bootstrap values for both are shown in Table 2. Four previously defined groups were recognized in this work, further supporting their original classification into different groups (Guerrini et al., 1992; Dollet et al., 2000; Dollet, 2001). Three major branches, corresponding to groups A, G, H, were well-supported (bootstrap >80) in both trees; isolate E.M.1, from group D was distinct from those branches. The groups were defined by 5S rRNA array sequences (Dollet et al., 2000) and RAPD analyses (Serrano et al., 1999a). The only group F isolate defined by our 5S rRNA analysis, Mani.Br, branched as a single isolate.

In this minicircle analysis, some members of previously defined groups were mismatched. From group B of Serrano et al. (1999a), two isolates were on one branch (E.hi.Sur = ‘‘Ehi1’’ and Rhabdadenia = ‘‘Rbi’’) and two (Aca and Blepharodon = ‘‘Bni’’) were on two other separate branches, both of which had low bootstrap values. Two isolates (Tom.Sp and Mand.sc.Br) that fell in group C according to the 5S rRNA study (Dollet et al., 2000), were on different branches. These last two isolates were placed in group C based on the transcribed sequence, with 99.9% homology (Dollet et al., 2000).

The same data set was rooted using *C. fasciculata* as an outgroup. Three trees with very similar topologies were produced by maximum parsimony (Fig. 2), maximum likelihood and distance methods. Bootstrap values for all three rooted trees are shown in Table 2. Bootstrap values supported essentially the same branching order as determined in the unrooted analysis. Three branches, which corresponded to groups A, G and H were strongly supported (>80).

First of all, the results confirmed the peculiarity of phloem-restricted trypanosomatids (Dollet, 2001). The rooted and unrooted trees highlighted this very distinctive group of intraphloemic trypanosomatids –group H – associated with wilts of coconut (hartrot) and oil palm (marchitez sorpresiva) in Latin America. According to their biological and serological properties (Dollet, 1994, 2001), from the body of data collected on these phloem-restricted parasites, which always differentiated from all other latex or fruit isolates, we can assert that they form the only well-defined taxon among all trypanosomatids isolated from plants. This group was recognized as group H in our 5S rRNA gene study (Dollet et al., 2000) and the term ‘Phloemicola’ was proposed by Vickerman in 1995 to refer to them (Dollet, 2001). The internal variability within that group reinforced its identity. The study of SL RNA gene sequences from 29 phloem-restricted isolates resulted in the

Table 2
Bootstrap values for minicircle-CR groups determined by different methods

Group	5S	RAPD	Unrooted			Rooted	
			Maximum parsimony	Maximum likelihood	Maximum distance	Maximum parsimony	Maximum likelihood
Well-supported groups							
Phloem	H	nd[a]	98	98	95	93	97
OW Euphorbia (EM1[b])	D	D	70	70	72	56	52
NW Euphorbia	G	nd	88	88	100	92	88
Easy grow	A	A	100	100	99	97	99
Nebula of poorly, but consistently, defined groups							
E.hi.Sur	B	B	73	81	77	75	79
Rhabdedenia	B	B					
Blepharodon	C	B	73	60	80	62	61
Mand.sc.Br	C	nd					
Aca	B	B	29	45	45	31	35
E.het.Vz	nd	nd	29	45	45	61	35
Mani.Br[b]	F	F	28	37	53	29	38
Tom.Sp	C	C	72	34	81	75	31
Trifolium	C	C					

nd: Not determined; NW: new world; OW: old world.
[a] Not done by Serrano et al. (1999a,b), however a robust group by RAPD in Muller et al. (1997).
[b] Single isolate used to define the group in this study, resulting in lowered stability of bootstrap values for branch assignment.

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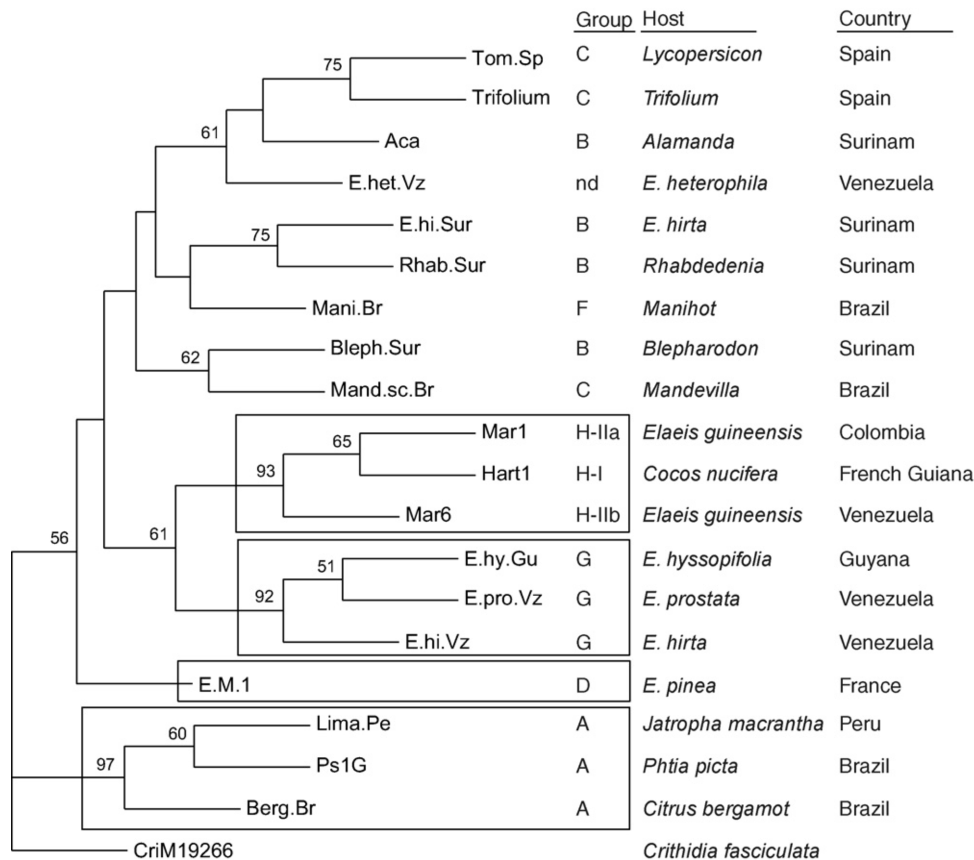

Fig. 2. Maximum parsimony tree based on 270 characters (gaps included), from which 146 were informative. The tree was 663 steps long, and was rooted using *Crithidia fasciculata* minicircle sequence (M19266) as an outgroup. Bootstrap support (1000 replicates) are indicated. Bootstrap support lower than 50 not shown. E.: Euphorbia. Alphabetic groups are based on classifications by RAPD (Serrano et al., 1999a) and 5S rRNA genes (Dollet et al., 2000).

partitioning of this group into two subgroups: SLI and SLII, with SLII further subdivided into SLIIa and SLIIb (Dollet et al., 2001b). The sequence of minicircles obtained in this study from one representative of each subgroup, Hart1 for SLI, Mar1 for SLIIa and Mar6 for SLIIb, was consistent with the group H designation.

Two latex isolates from northern Latin America previously grouped together in group G form a robust branch. The uncharacterized E.pro.Vz isolate originated from the same region and from the same plant family, Euphorbiaceae, as the other group members, so it was not surprising to find it grouped with E.hi.Vz and E.hy.Gu. Nevertheless they were not identical. The three isolates shared the same sequence 18-bp upstream of CSB-1 but they showed several differences in the 80-bp farther upstream. That might reflect their collection at different times (E.pro.Vz in 1992, E.hi.Vz in 1990, and E.hy.Gu in 1986), from three different locations: French Guiana, Northeast and Northwest Venezuela, with more than 500 km separating the localities.

The third robust cluster brought together the isolate Ps1G, obtained from the salivary glands of *Phthia picta* (the Coreidae bug that propagates tomato fruit trypanosomatids) (Jankevicius et al., 1989; Sã-Carvalho et al., 1993), one isolate from bergamot fruit, Berg (Conchon et al., 1989), and one isolate called Lima, assumed to originate from *Jatropha macrantha* (Euphorbiaceae) (Burstein and Romero, 1956). In our 5S rRNA study Lima and Berg were together in group A, however Ps1G was not included in the comparison. In the RAPD study of Serrano et al. (1999a,b), Berg (=Cbe), Ps1G (=ppi1SG), and Lima (=Jma) were on three separate branches of a large cluster defining group A that was further divided in two subgroups A′ and A″ with very low boostrap values. Group A′ itself divided into three branches, one of them supporting the only isolate Jma. The question of origin of the Lima isolate, from latex or the surface, remains open (see discussion in Dollet et al., 2000). It should be noted that the pedigree of that isolate may not be accurate, as it was found by chance in a trypanosomatid collection in Germany in 1985 (Petry et al., 1989).

It should also be noted that a trypanosomatid isolated from the fruit of another citrus tree (*C. reticulata*) in Brazil, by Conchon et al. (1989) was finally identified as a possible *Leptomonas* spp., and at least a ‘‘non-*Phytomonas*’’ by the authors. Whether this Berg isolate from the fruit of *C. bergamia*, obtained from the same laboratory in Brazil could be a ‘‘non-Phytomonas’’ is an open question.

E.M.1, defining group D, appeared different from all others but did not have a robust bootstrap, indicating that its placement within the tree was arbitrary. This group D, contains latex trypanosomatids from the South of France, Africa and India. Latex trypanosomatids from around Montpellier isolated from *Euphorbia pinea* and *Euphorbia characias* (‘‘E.M.’’ and ‘‘E.C.’’ isolates), formed a well-defined group by isoenzyme

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analysis (Guerrini et al., 1992), RAPD (Muller et al., 1997), minicircle size (Ahomadegbe et al., 1990), and 5S rRNA sequence (Dollet et al., 2000). All isolates obtained from *E. pinea* in different locations around Montpellier between 1980 and 1987 shared the same characteristics, thus providing a strong biological validation of this grouping.

Two branches were moderately supported at 75%: the first branch contained two isolates from group B and the second branch contained two isolates from Spain that were members of group C, the same as observed in the unrooted tree. The remaining branches (Mani.Br; Mand.sc.Br and Bleph.Sur; E.het.Vz and Aca) were weakly or not supported. When gaps were removed from the alignment, the number of parsimony informative sites dropped from 146 to 42 sites, but groups A, G and H remained well-supported (data not shown).

The bottom half of the unrooted tree contained isolates from insect, fruit or latex, except those of group D. That result provided further evidence of the substantial heterogeneity and diversity of plant trypanosomatids. There were some groupings with two isolates: E.hi.Sur and Rhabdadenia (both from Surinam); Blepharodon (Surinam) and Mand.sc.Br (from Brazil); and Tom.Sp and Trifolium (both were isolated in the same area in southern Spain). Despite the low bootstrap values, the first two groups were consistent with data previously obtained by isoenzyme studies showing that E.hi.Sur and Rhabdadenia were in the same clearly defined group, and that Blepharodon and Mand.sc.Br were in another recognizable group (Guerrini et al., 1992). Contrary to our 5S rRNA results, Mand.sc.Br was not in the same group as the Spanish isolates (Tom.Sp and Trifolium). We are reticent to create spurious groups at this time. The 5S rRNA gene of Mand.sc.Br differed from the Tom.Sp by a single nucleotide at the 3′ end of the transcribed sequence; but each of their spacers was distinctive: only 81.7% identity. Thus, they could be placed in two different groups or at least two different subgroups, a decision that will rely on new molecular markers.

Tom.Sp and Trifolium from Spain were in the same RAPD group C with other isolates from fruit in Spain (Serrano et al., 1999a). In fact, all the trypanosomatids isolated by the Sánchez-Moreno team in Spain were very close whether they were obtained from fruits (tomato, mango, cherimolia), or by crushing the stems of *Trifolium* or *Amaranthus* plants (Fernandez-Becerra et al., 1996; Sanchez-Moreno et al., 1998; Serrano et al., 1999a,b). These results also confirmed that Mani.Br (=Pfr) was a peculiar isolate as it was the only isolate of group F in the 5S rRNA analysis. The new isolate E.het.Vz also stood alone (close to the isolate Aca from Surinam) consistent with its peculiar behaviour in culture (M.D., unpublished data). However, the position of Aca was questionable: it was strongly associated with E.hi.Sur and Rhabdadenia, both by isoenzyme analysis (Guerrini et al., 1992) and RAPD (Serrano et al., 1999b), but it was isolated when defined by the minicircle sequence.

With the exception of Aca, the conclusion of this work is that the kDNA minicircle sequences reflected most of the previously obtained groupings and strengthened the confidence level for at least eight different groups in the plant trypanosomatids. Furthermore, as the number of minicircles in trypanosomatid cells is large (about 7000 in E.M.1; Riou et al., 1987) these specific sequences from well-characterized groups, at least four in this work, are a valuable molecular diagnosis tool.

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