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The internal transcribed spacer of ribosomal RNA genes in plant trypanosomes (*Phytomonas* spp.) resolves 10 groups

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ABSTRACT

The distinction between plant trypanosomatids and opportunistic monoxenous insect trypanosomatids has not been demarcated clearly due to the mass placement of all trypanosomatids isolated from plants into the arbitrary genus *Phytomonas* spp. The advent of molecular markers has been useful in distinguishing plant trypanosomatids from the rest of the Trypanosomatidae family. Here we have examined the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) locus for classification purposes. This region contains two distinct ITSs flanked by the small subunit and large subunit of ribosomal RNA genes and separated by the 5.8S ribosomal RNA gene. Sequences within the 5.8S ribosomal RNA gene and in the ITS sequences can serve as specific markers for several of the *Phytomonas* groups. Microsatellite sequences were identified in *Phytomonas* spp. in both ITS regions. Several classes of microsatellites were seen, with inter-isolate variation that has potential for future use. Maximum Likelihood analysis of the ITS sequences of 20 *Phytomonas* isolates representing the eight defined groups and a few unclassified isolates revealed a total of 10 distinct subgroups within our collection, of which two are new. The ITS region, which includes the 5.8S sequence, is a robust marker for the subdivisions within the genus *Phytomonas* spp.

1. Introduction

Trypanosomatids isolated from plants have been arbitrarily classified under the cover-all genus *Phytomonas* by sole virtue of the host sampled (Donovan, 1909). Within this class of protozoa one well-defined group is an obligate plant parasite responsible for systemic infection, the Phloemicola group, containing the phloem-restricted pathogens specifically associated with wilts of cultivated crops including marchitez of oil palm, hartrot of coconut palm and yellow wilt of decorative red ginger (*Alpinia purpurata*), in Latin America and the Caribbean (Dollet, 2001). A second well-defined group (Old World Euphorbia) includes the intra-laticiferous isolates from *Euphorbia* species from France, Sénégal and India. This group of latex-associated trypanosomatids possess shared serological and molecular markers (Dollet et al., 2000; Guerrini et al., 1992; Marché et al., 1995; Petry et al., 1986; Serrano et al., 1999a) and appear to be geographically restricted to the Old World (Europe, Africa and India).

The remaining isolates from latex or fruit are distributed among at least six to eight different clusters by different molecular markers (Dollet et al., 2000; Sturm et al., 2007). The position of these last isolates may change from one group to another depending on the marker used, for example the tomato isolate from Spain which falls in Group C according to its 5S ribosomal RNA (rRNA) gene (Dollet et al., 2000) is closer to Group B according to its kinetoplast DNA (kDNA) minicircle sequence (Sturm et al., 2007). In the context of epidemiological studies of trypanosomatid diseases and disorders of agriculturally-important crops, it is necessary to understand the relationships among the trypanosomatids found in different plant species, plant tissues (latex, fruits, seeds, phloem sap) and in phytophagous insects (Batistoti et al., 2001; Fuxa et al., 2000; Godoi et al., 2002; Sbravate et al., 1989). Furthermore it is important for taxonomic and phylogenetic studies to define if there are specific plant trypanosomatids, in contrast to the so-called "monoxenous" kinetoplastid from a phytophagous insect that is a fortuitous dweller in or on the plant or its fruits or seeds. Other trypanosome genera like *Herpetomonas, Leptomonas*, and *Crithidia* can be isolated from plants, particularly from fruit, but also from seeds or flowers (Conchon et al., 1989; Fiorini et al., 2001; Kastelein and Camargo, 1990). Frequently a trypanosomatid isolated from tomato fruit, "*Phytomonas serpens*", is used in comparative studies. *P. serpens* was the name given by (Gibbs, 1957) to a trypanosomatid found in a tomato fruit in South Africa. Since this time any non-*Herpetomonas*, non-*Leptomonas*, or non-*Crithidia* trypanosomatid isolated from a tomato fruit anywhere in the world has been

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called *P. serpens*. There are no additional criteria to characterize *P. serpens* other than its isolation from a tomato fruit. However it has been shown that *Herpetomonas*, *Leptomonas*, *Crithidia* and other genera of trypanosomatids can be isolated from tomato (Conchon et al., 1989). The problems associated with studying a single isolate as representative of all plant trypanosomes is that there is great heterogeneity among isolates in a given geographical region and that two tomato isolates from two different regions can belong to different groups, as exemplified by the tomato isolate obtained in Southern Spain, Tom.Sp and isolate 9T from Brazil (Fernandez-Ramos et al., 1999; Serrano et al., 1999a). Even in a restricted region two isolates from the same host can be different, as was the case of trypanosomatids cultured from *Euphorbia hirta* in French Guiana and Surinam (Guerrini et al., 1992; Muller et al., 1997). The significance of the conclusions from such studies is dependent on the plant isolate chosen.

Different regions of the nuclear rRNA genes have been used for systematic and phylogenetic studies of parasites (Almeyda-Artigas et al., 2000; Cupolillo et al., 1995; Dollet et al., 2000; Fernandes et al., 1993; Haag et al., 1998; Marcilla et al., 2001; Olsen, 1988; Philippe and Laurent, 1998). The complete sequences of the small subunit (SSU) rRNA have been inferred for two plant trypanosomatids, one phloem-restricted isolate from coconut affected by hartrot disease in French Guiana, and the other from a latex plant (*E. hirta*) isolate from Senegal in West Africa (Marché et al., 1995). Short sequences from the V7 variable region of the SSU rRNA from other plant trypanosomatids have also been used to draw phylogenetic relationships among these isolates and other Trypanosomatidae (Marché et al., 1995). Despite the variability of the V7 region, the high conservation of the SSU rRNA did not allow resolution of some isolates that have subsequently been separated into different groups using other molecular markers (Dollet et al., 2000, 2001; Muller et al., 1997; Serrano et al., 1999a). The two internal transcribed spacers (ITS-1 and ITS-2) of rRNA are useful for resolving relationships between closely related taxa, and are useful markers for species distinction and identification (Almeyda-Artigas et al., 2000; Desquesnes et al., 2001; Hernandez et al., 1993; Marcilla et al., 2001).

In this work we present the sequence analysis obtained from the ITS-1/5.8S/ITS-2 region of 20 plant trypanosome isolates and three isolates of genera *Crithidia*, *Herpetomonas* and *Leptomonas* that may be associated either with phytophagous insects, or fruits, or seeds. In addition to the two previously well-defined groups (Phloemicola – Group H- and Old World Euphorbia – Group D-) Maximum Likelihood tree analysis indicated three further strong groupings. The first one is a large cluster of latex isolates from Latin America – embracing three previously described Groups B, C, F – that includes one tomato isolate from Spain. This tomato isolate could originate in Latin America (see Dollet et al., 2001). The second and third well distinct groups contain some latex isolates from Latin America. Group G, with two isolates is always distinct from the preceding cluster whatever the marker used. Group I contains only one isolate so far. This isolate is different from all the other latex isolates because of its biological properties and several markers (Muller et al., 1995, 1997; Dollet et al., 2000). It is the only latex isolate requiring feeder cells for its primoculture.

2. Materials and methods

2.1. Trypanosomatids

The *Phytomonas* spp. isolates used in these analyses are described in Table 1, along with Accession numbers for new sequences. The provenance of the isolates has been described previously (Dollet et al., 2000; Muller et al., 1997) and in Table 1.

2.2. PCR amplification, cloning and sequencing

The plant and insect trypanosomatid ITS-1/ITS-2 regions were amplified using the Qiagen PCR Core Kit and oligonucleotides similar, but not identical, to those described (Cupolillo et al., 1995) corresponding to the 3′-end sequence of the SSU rRNA gene, IAMWE: 5′-GCTGT AGGTG AACCT GCAGC TGGA, and the inverse complement of the 5′-end of the large subunit 1 (LSU1) rRNA gene: IRBAB: 5′-GCGGG TAGTC CTGCC ACACT CAG (Fig. 1). Where amplification was refractory with these primers (E.het.Vz and Rhab.Sur) the individual ITS regions were amplified separately. ITS-1 was obtained with IAMWE and Tc5.8-R (5′-CTCCC ATGCG CCGTT TGCGT); ITS-2 with Tc5.8-L (5′-ACGTG TCGCG ATGGA TGACT) and IRBAB. PCR reactions included 0.05–10 ng total cell DNA or cell lysate (diluted 1:1000 in H_2O) as template. Lysates were made by resuspending the pellet from 4×10^7 to 4×10^8 cultured cells in 0.1 ml 4 M guanidinium isothiocyanate. The thermal cycle profile was 94 °C for 2 min; 30 cycles of 94 °C for 15 s, 65 °C for 15 s, 75 °C for 2 min; 72 °C for 9 min. Reaction products were resolved by electrophoresis through 1.0% agarose gels.

The PCR products were cloned directly from the reaction mixtures 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). Grouping of isolates was performed using the Maximum Likelihood and Maximum Parsimony algorithms for phylogeny. *Crithidia oncopelti*, from the phytophagous bug (*Oncopeltus* sp., family Coreidae), *Herpetomonas muscarum* and *Leptomonas mirabilis* from blowflies were included as representatives of insect isolates. Bootstrap support was calculated from 100 replicates.

3. Results

3.1. Analysis of ribosomal RNA gene ITSs

The use of short DNA sequences from small RNA genes, such as the 5S rRNA gene, has allowed the designation of at least eight distinct groups within plant trypanosomatids (Dollet et al., 2000). To challenge these designations with longer sequences, we examined the rRNA ITS region that has been useful for classification of other kinetoplastids (Cupolillo et al., 1995; Desquesnes and Davila, 2002; Desquesnes et al., 2001; McLaughlin et al., 1996). The ITS-1 and ITS-2 regions were amplified in one reaction from most trypanosomatids tested, including 17 plant isolates, one from the salivary glands from a tomato-fruit-feeding insect, and from three insect isolates, yielding a range of PCR product sizes (Figs. 2A and B). The ITS-1 and ITS-2 regions from E.het.Vz and Rhab.Sur isolated were amplified separately (Fig. 2C) as described in Materials and methods. The PCR products were cloned and the DNA sequences obtained for comparison.

The shortest and longest ITS regions, 773 bp from *H. muscarum* and 1473 bp from *L. mirabilis*, respectively, were from isolates considered "insect trypanosomatids" even though some species of these genera can be isolated from plant (Fiorini et al., 1993). Among the plant trypanosomatids, the longest ITS regions (1349 bp) were found in the isolates Mand.sc.Br and Bleph.Sur, both representatives of Group C. The shortest ITS regions among plant trypanosomes, 976 and 978 bp, respectively, were found in two Group A isolates, "Lima", which is supposed to originate from a Peruvian *Euphorbia* (see (Dollet et al., 2000) and Ps1G, an isolate from the phytophagous insect *Phtia picta* (Coreidae).