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Classification of trypanosomatids from fruits and seeds using morphological, biochemical and molecular markers revealed several genera among fruit isolates

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Abstract

Trypanosomatids are widespread in several plant families and although most isolates have been classified as *Phytomonas*, other trypanosomatid genera can also infect plants. In order to assess the natural occurrence of non-*Phytomonas* trypanosomatids in plants we characterized 21 new trypanosomatid cultures, 18 from fruits and three from seeds of 17 plant species. The trypanosomatids from fruit and seeds were compared in terms of morphological, growth, biochemical and molecular features. The high diversity among the isolates permitted the classification of the new flagellates into the genera *Crithidia* and *Leptomonas* as well as *Phytomonas*. The data showed that natural fruit infection with non-*Phytomonas* trypanosomatids is more common than usually thought, being detected in 43% of the fruit isolates.

Keywords: Plant trypanosomatid; Taxonomy; Mini-exon gene; Ribosomal gene

1. Introduction

Infection of plants with trypanosomatids has been known since 1909, when Lafont [1] described these flagellate protozoa in laticiferous plants. Based on host tissue tropism, plant trypanosomatids can be classified into four major groups: (a) lacticola, which include flagellates that live in latex of various families of laticiferous plants; (b) phloemicola, consisting of flagellates preferentially living in the phloem vessels of palms, although infecting coffee trees and other plants; (c) fructicola, consisting of flagellates found in fruit sap and seeds of several plant families; (d) floricola, flower flagellates so far limited to two plant species [2–4].

Plant flagellates are transmitted to their plant hosts by phytophagous insects, mainly piercing hemipterans. Donovan [5] created the genus *Phytomonas* to distinguish plant trypanosomatids from those infecting insects. Members of this genus have been defined as flagellates present only as promastigote forms in plants and insects. However, this definition is not sufficient as a taxonomic parameter to classify plant trypanosomatids as *Phytomonas*. Isolation of a trypanosomatid from plants or from phytophagous insects is not sufficient evidence in itself that it belongs to the genus *Phytomonas* because both host plants and insects can harbor, in addition to *Crithidia* species, trypanosomatids of the genera *Herpetomonas*, *Leptomonas* and *Phytomonas*, which share indistinguishable promastigote forms [4].

Various approaches have been proposed to classify plant trypanosomatids, such as reactivity with monoclonal antibodies (mAbs) [6,7], enzymatic activities [8–10] and molecular markers based on ribosomal genes [11–13]. In addition, we developed two methods based on spliced-leader (SL) genes for generic identification of *Phytomonas*: (a) hybridization of genomic DNA with a SL (SL3′)-based

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probe [7], and (b) PCR amplification (Phy-SLPCR) of an SL sequence able to detect *Phytomonas* from different plant tissues and phytophagous insects, using either culture flagellates [14] or crude preparations of field-collected plants and insects [15,16].

Trypanosomatids are widespread in fruits and have been found in more than 27 species of 11 plant families [4]. Besides infecting several fruit saps, trypanosomatids have also been detected in seeds of maize [17], beans and soybeans [18] and annato [19]. With the use of molecular approaches it has been possible to show that there is a wide range of genera of trypanosomatids infecting plants, mainly fruits. So far, all isolates from phloem have been classified as *Phytomonas*, whereas the few isolates from latex and fruits were classified as *Leptomonas* and *Crithidia* [7,11,14]. The only available culture of trypanosomatids isolated from flowers was classified as *Herpetomonas* [20]. However, despite the generic polymorphism of plant trypanosomatids, most plant isolates continue to be classified as *Phytomonas* exclusively on the basis of host origin, with only five plant isolates classified into other genera thus far [4,7,14,20]. In order to assess the occurrence of different trypanosomatid genera in plants, we evaluated the generic diversity of flagellates from fruits and seeds. For this purpose we compared 21 isolates not yet classified on the basis of the following taxonomic parameters: morphology and growth features, enzymatic profiles of the urea cycle, reactivity with *Phytomonas*-specific mAbs, and assessment of ribosomal, SL and randomly amplified polymorphic DNA (RAPD)-derived markers.

2. Materials and methods

2.1. Isolation, growth features and morphology of trypanosomatids

Twenty-one trypanosomatids isolated from fruit saps (18) and seeds (three) were characterized in this study. Five plant trypanosomatids, including the reference species *Phytomonas serpens* and *Phytomonas macgheei*, plus three previously characterized isolates (Cbe1, Cre, Mor) [14] were used for comparative purposes (Table 1). The new flagellates were isolated from fruits and grains showing signs of insect bite, obtained on the market or field-collected in Southern Brazil from 1983 to 1997. For this purpose, small fragments of several parts of fruits and grains were mixed with saline and examined for the presence of trypanosomatids under a phase-contrast microscope. Seeds were crushed with a mortar and pestle in saline before microscopic investigation (MI). MI positive samples were inoculated into tubes containing biphasic

Table 1
Trypanosomatids isolated from fruits and seeds

Organism	Plant host			Plant tissue	Isolation year
		Family	Species		
Tom10	tomato	Solanaceae	*Lycopersicon esculentum*	fruit	1983
Tom15	tomato	Solanaceae	*L. esculentum*	fruit	1983
Tom30	tomato	Solanaceae	*L. esculentum*	fruit	1983
Tom268	tomato	Solanaceae	*L. esculentum*	fruit	1989
Tom269	tomato	Solanaceae	*L. esculentum*	fruit	1989
Tom270	tomato	Solanaceae	*L. esculentum*	fruit	1989
Tom274a	tomato	Solanaceae	*L. esculentum*	fruit	1989
Tom274c	tomato	Solanaceae	*L. esculentum*	fruit	1989
Tom489	tomato	Solanaceae	*L. esculentum*	fruit	1991
App717	apple	Rosaceae	*Malus* sp.	fruit	1995
App771	apple	Rosaceae	*Malus* sp.	fruit	1997
Plu750	plum	Rosaceae	*Prunus persica*	fruit	1997
Pom421	pomegranate	Punicaceae	*Punica granatum*	fruit	1990
Cbe1	bergamot	Rutaceae	*Citrus bergamia*	fruit	1988
Cre	orange	Rutaceae	*Citrus reticulata*	fruit	1988
Ora485	orange	Rutaceae	*Citrus aurantum*	fruit	1991
Gra488	grape	Vitaceae	*Vitis vinifera*	fruit	1991
Mul490	mulberry	Moraceae	*Morus* sp.	fruit	1993
Mor	mulberry	Moraceae	*Morus* sp.	fruit	1995
Sur743	Surinam cherry	Myrtaceae	*Eugenia uniflora*	fruit	1996
Pin772	pineapple	Bromeliaceae	*Ananas ananas*	fruit	1997
Mai251	maize	Gramineae	*Zea mays*	seed	1989
Ann412	annato	Bixaceae	*Bixa orellana*	seed	1989
Bea492	bean	Fabaceae	*Cajanus flavus*	seed	1993
Reference species					
P. serpens	tomato	Solanaceae	*L. esculentum*	fruit	1983
P. macgheei (163)	maize	Gramineae	*Z. mays*	seed	1989

GYPMY medium [17] and smeared on glass slides which were fixed with methanol and stained with Giemsa. The optimum growth and temperature were determined in GYPM medium by incubation at 21, 25, 28, 31, 34, and 37°C, for 72 h. Growth and generation time (GT) were determined in the exponential phase of cultures at 28°C [21].

2.2. Enzymatic tests and indirect immunofluorescence assay (IFA) with mAbs

Crude extracts of cells grown in GYPMI medium were assayed for the presence of arginase (ARG), citrulline hydrolase (CH), arginine deiminase (AD), and ornithine carbamoyltransferase (OCT), as previously described [8,17]. Formaldehyde-fixed culture forms of flagellates were used as antigen for IFA tests using a pool of *Phytomonas*-specific mAbs as described [6].

2.3. Hybridization with SL and ribosomal (rSSU) gene-derived probes

Extraction of genomic DNA and preparation of slot blots of total genomic DNA (3.0 μg) and of whole cells were performed as described by Teixeira et al. [7]. Two oligonucleotides based on the SL gene sequences SL3′, which is *Phytomonas*-specific, and SL201, which is conserved in all trypanosomatids, were labeled and used for hybridization of slot blots of genomic DNA as described elsewhere [7]. The ribosomal gene-derived oligonucleotide probe SSU3 was labeled and used for slot blot hybridization of whole cells as described by Teixeira et al. [22].

2.4. PCR amplification of the Phytomonas-specific SL sequence (Phy-SLPCR)

PCR amplification using as template DNA from culture

Table 2
Classification and characterization of trypanosomatids from fruits and seeds

Organism	Form[a]	GT[b]	Enzymatic profile[c]				mAbs *Phytomonas*-specific[d]	SL gene markers[e]		rRNA 360/SSU *Pvu*II site[f]	RAPD profile[g]	Classification
			ARG	CH	AD	OCT		SL3′	Phy-SLPCR			
Pse[h] (9T)	P	14.0	−	+	+	−	+	+	+	−	A	*Phytomonas*
Cbe[h]	P	ND	−	+	+	−	+	+	+	−	A	*Phytomonas*
Tom10	P	15.6	−	+	+	−	+	+	+	−	A	*Phytomonas*
Tom15	P	15.0	−	+	+	−	+	+	+	−	A	*Phytomonas*
Tom30	P	14.8	−	+	+	−	+	+	+	−	A	*Phytomonas*
Ann412	P	15.8	−	+	+	−	+	+	+	−	A	*Phytomonas*
Pom421	P	14.5	−	+	+	−	+	+	+	−	A	*Phytomonas*
Ora485	P	13.0	−	+	+	−	ND	+	+	−	A	*Phytomonas*
Gra488	P	11.0	−	+	+	−	ND	+	+	−	A	*Phytomonas*
Tom489	P	11.7	−	+	+	−	+	+	+	−	A	*Phytomonas*
Mul490	P	12.8	−	+	+	−	ND	+	+	−	A	*Phytomonas*
Bea492	P	12.7	−	+	+	−	+	+	+	−	A	*Phytomonas*
Plu750	P	13.0	−	+	+	−	ND	+	+	ND	A	*Phytomonas*
Pma[h] (163)	P	16.5	−	+	+	+	+	+	+	−	A	*Phytomonas*
Mai251	P	15.1	−	+	+	+	+	+	+	−	A	*Phytomonas*
Cre[h]	P	ND	+	+	−	−	−	−	−	+	NP	*Leptomonas*
App717	P	14.0	+	+	−	−	−	−	−	+	NP	*Leptomonas*
Sur743	P	13.0	+	+	−	−	ND	−	−	+	NP	*Leptomonas*
App771	P	13.0	+	+	−	−	−	−	−	+	NP	*Leptomonas*
Tom268	P	8.4	+	−	−	−	−	−	−	−	NP	*Leptomonas*
Tom269	P	8.5	+	−	−	−	−	−	−	−	NP	*Leptomonas*
Tom270	P	8.8	+	−	−	−	−	−	−	−	NP	*Leptomonas*
Tom274a	P	8.7	+	−	−	−	−	−	−	−	NP	*Leptomonas*
Mor[h]	P	ND	+	ND	ND	−	−	−	−	+	NP	*Crithidia*
Tom274c	C	7.0	+	+	−	−	−	−	−	−	NP	*Crithidia*
Pin772	P	13.5	−	+	+	+	−	−	−	+	NP	*Herpetomonas*

Pse (9T), *P. serpens*; Pma (163), *P. macgheei*. ND, not determined.

[a]Morphology of Giemsa-stained culture forms.

[b]Generation time (average of triplicates).

[c]ARG, arginase; CH, citrulline hydrolase; AD, arginine deiminase; OCT, ornithine carbamoyltransferase.

[d]IFA with a pool of *Phytomonas*-specific mAbs.

[e]Slot blot hybridization of genomic DNA with the SL3′ probe and amplification by Phy-SLPCR.

[f]Assessment of the rRNA SSU/360 *Pvu*II site by probing with SSU3 and restriction analysis of PCR-amplified SSU rRNA.

[g]RAPD patterns generated using primer 684: A, pattern corresponding to the previously described subgroup A and NP (non-*Phytomonas*), distinct RAPD patterns never observed in any *Phytomonas* spp. [24].

[h]Trypanosomatids previously classified used for comparative purpose [14].

flagellates was done using primers complementary to regions flanking the SL3′ sequence in order to amplify specifically a 100-bp DNA fragment of *Phytomonas* spp. [14].

2.5. PCR amplification and restriction analysis of the small subunit of rRNA (SSU rRNA)

The SSU rRNA genes were amplified using primers complementary to conserved sequences from the 5′ and 3′ ends [23]. PCR amplification was performed in 50-μl reaction mixtures, each containing 25 ng of purified DNA, 0.3 mM each dNTP, 2.0 μM each primer, PCR buffer containing 1.5 mM $MgCl_2$, and 2.5 U of *Taq* DNA polymerase (MBI Fermentas). Reactions were submitted to the following cycles 30 times: 1 min at 94°C, 1 min at 45°C, and 2 min at 72°C, with an initial denaturation for 3 min and a final extension of 10 min at 72°C. Amplification products were digested with *Pvu*II restriction enzyme, separated on 1.5% agarose gel and stained with ethidium bromide.

2.6. RAPD fingerprinting

Amplifications of genomic DNA were performed using the primer 684 as described [24]. The amplified DNA fragments were separated on 2.0% agarose gels and stained with ethidium bromide.

3. Results

3.1. Detection, isolation and growth features of trypanosomatids

Seventeen species of 12 plant families were found to be infected with trypanosomatids (Table 1). Fruits and seeds obtained on the market or in the field were selected on the basis of the presence of discolored spots on the surface indicating insect bites or the presence of phytophagous Hemiptera under field conditions. Although we could not isolate the flagellates in culture, we also detected trypanosomatids in fruits of papaya (*Papaya* sp.) and guava (*Psidium guayava*) and in grains of beans (*Phaseolus vulgaris*) and soybeans (*Glycine max*) (Table 1). The established cultures were cloned and are cryopreserved in liquid N_2 at the University of Londrina and in the Trypanosomatid Culture Collection of the University of São Paulo (TCC-USP). All isolates can be grown in GYPMY [17], LIT [25] and Grace (Gibco) media with optimum pH between 6.5 and 7.0. The cultures showed wide growth and GT variability: the Tom274c choanomastigote culture showed the shortest GT (7.0 h) and the highest cell density (above 10^8 cells ml^{-1}) whereas the GT varied from 8.4 to 15.8 h for promastigote cultures, which presented growth ranging from 2 to 6×10^7 cells ml^{-1}. All flagellates had temperature optima for growth between 25 and 28°C, and only the Tom274c isolate was able to grow at 37°C (Tables 1 and 2).

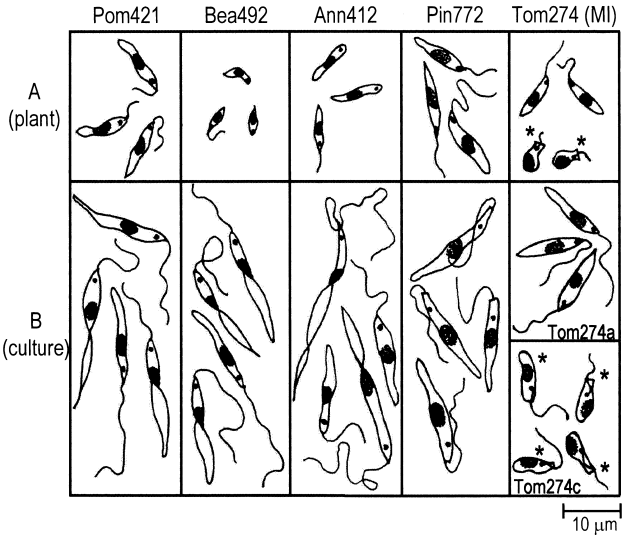

Fig. 1. Camera lucida drawings of trypanosomatids from fruits and seeds recovered from: A: naturally infected plants; B: exponential-phase LIT culture. Most plant and cultures presented only promastigote forms of different size and shape, except the Tom274c isolate which also showed choanomastigote forms (*). MI, mixed infection.

3.2. Morphology of fruit trypanosomatids before and after isolation in culture

Morphology of Giemsa-stained flagellates from the same plant sample before and after culturing showed strong differences in flagellate size and shape. Plant smears revealed choanomastigotes and highly pleomorphic promastigote forms, with several dividing flagellates. Apparently, the level of humidity of plant samples influences the size and motility of the organisms. Flagellates from seeds were small and immobile without an external flagellum, whereas those infecting fruit sap and fresh grains were longer and mobile with a short external flagellum (Fig. 1). In general, trypanosomatids on naturally infected fruits were smaller and presented reduced motility compared to their corresponding cultures, as previously described for *P. serpens* [21]. In contrast to the forms detected on their natural host, cultured flagellates possess promastigote forms with long free flagella, varying in body and flagellum size and length. Stationary-phase cultures presented several forms with body torsions. Only the Tom274c culture presented choanomastigote forms. Morphological differences were detected between clones Tom274a (promastigote) and Tom274c (choanomastigote), both from the same tomato sample, indicating mixed infections in the original culture (Fig. 1; Table 1).

3.3. Enzymatic assays and IFA with Phytomonas-specific mAbs

Among the 21 new isolates, ARG enzyme was found in

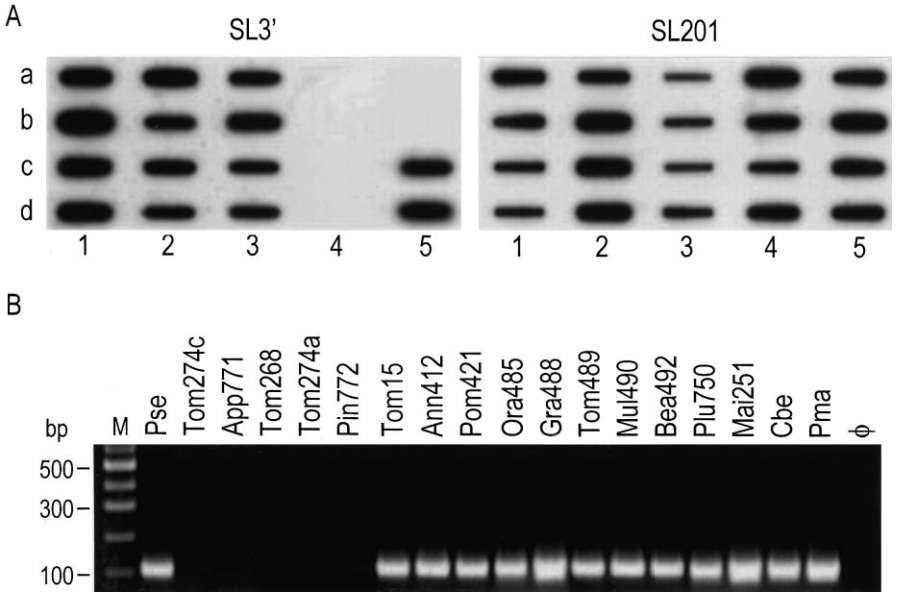

Fig. 2. Analysis of SL gene markers of trypanosomatids from fruits and seeds. A: The same slot blot membrane of genomic DNA was hybridized with SL3′ and SL201 probes. 1a, Tom10; 1b, Tom15; 1c, Tom30; 1d, Mai251; 2a, Ann412; 2b, Pom421; 2c, Ora485; 2d, Gra488; 3a, Tom489; 3b, Mul490; 3c, Bea492; 3d, Plu750; 4a, App771; 4b, Tom268; 4c, Tom274c; 4d, Pin772; 5a, App717; 5b, Sur743; 5c, *P. macgheei*; 5d, *P. serpens*. B: Agarose gel (2%) stained with ethidium bromide of Phy-SLPCR-amplified DNA fragments. ϕ, control without DNA; M, molecular marker.

seven promastigote cultures and in one choanomastigote culture. CH was found in all isolates examined, except Tom268, Tom269, Tom270 and Tom274a. AD was detected in 16 isolates and OCT was found in only two isolates. Among the isolates tested by IFA, only those further classified as *Phytomonas* on the basis of all molecular markers were recognized by the pool of mAbs (Table 2).

3.4. Hybridization with SL3′ probe and Phy-SLPCR

Twelve of 21 new isolates hybridized with the SL3′ probe which is specific for *Phytomonas* (Table 2), as exemplified by the slot blot hybridization shown in Fig. 2A. To confirm that lack of hybridization was not due to insufficient amounts of DNA the same membrane was hybridized with probe SL201. As expected, only the isolates positive for SL3′ probing generated the *Phytomonas*-specific DNA band in the Phy-SLPCR assay (Fig. 2B; Table 2).

3.5. Investigation of the SSU/360 PvuII site of rRNA by riboprinting

To test for the presence of the *Pvu*II site located 360 bp downstream of the SSU rRNA 5′ end (SSU/360 *Pvu*II), we used the oligonucleotide SSU3, which is complementary to

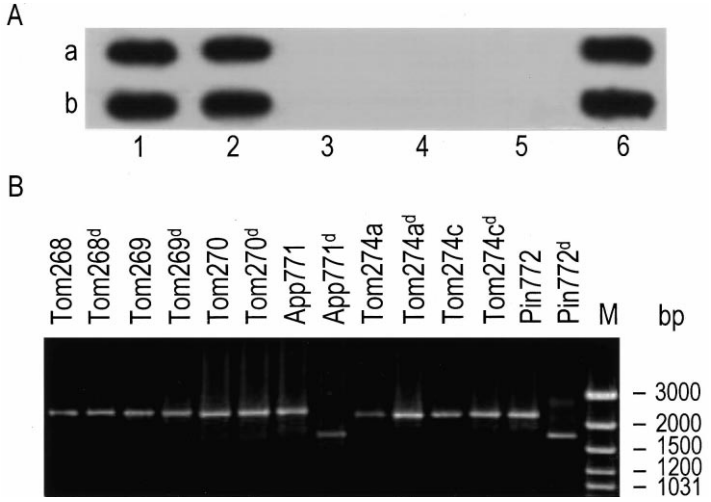

Fig. 3. Evaluation of the existence of the rRNA SSU/360 *Pvu*II restriction site. A: Slot blot hybridization of whole cells of trypanosomatids with the SSU3 probe: 1a, Pin772; 1b, App717; 2a, Sur743; 2b, Tom274c; 3a, Pom421; 3b, Mai251; 4a, Mul490; 4b, Tom489; 5a, Ora485; 5b, *P. macgheei*; 6a, *C. fasciculata*; 6b, *H. samuelpessoai*. B: Agarose gel (2%) stained with ethidium bromide showing DNA fragments resulting from *Pvu*II digestion (^{d}) of PCR-amplified SSU rRNA (PCR-RFLP). M, molecular marker.

the sequence flanking this site of *Herpetomonas samuelpessoai* [7,22], as a probe for slot blot hybridization of whole flagellates. Positive signals were detected for only four isolates (Fig. 3A; Table 2). However, although containing the SSU/360 *Pvu*II site, some trypanosomatids did not hybridize with this probe due to variability in the sequences flanking this *Pvu*II site [22]. Thus, to ensure the absence of this site, SSU rDNAs from all isolates that did not hybridize with the SSU3 probe were PCR-amplified and digested with *Pvu*II. Besides the four isolates positive that hybridize to the SSU3 probe, the results showed a band smaller than 2200 bp, indicating the presence of this site in isolate App771 (Fig. 3B; Table 2).

3.6. Analysis of Phytomonas spp. by RAPD fingerprints

RAPD patterns generated by primer 684 were previously used to classify *Phytomonas* spp. from different plant tissues and of different geographic origins into several subgroups according to synapomorphic fragments. Evaluation of these markers revealed a 300-bp RAPD-amplified fragment (data not shown), thus allowing all new *Phytomonas* spp. to be classified into the previously defined subgroup A [24] of *Phytomonas* (Table 2).

4. Discussion

Several studies have shown that plants can harbor trypanosomatids of various genera besides *Phytomonas* [7,14,15,20,26]. In contrast to *Phytomonas*, for which specific molecular taxonomic markers are available [7,14,15], classification of members of *Herpetomonas*, *Leptomonas* and *Crithidia* still requires the development of specific methods. However, despite the lack of genus-specific markers, these flagellates can be classified at the genus level using a combination of criteria which, taken together, indicate a reliable taxonomic position. In addition, despite the availability of SL-based genus-specific markers for *Phytomonas*, new species may not necessarily be detected by these markers. Thus, to avoid misclassification, we have considered as *Phytomonas* only isolates that shared the following features: exclusive presence of promastigotes, lack of ARG activity, absence of the rRNA SSU/360 *Pvu*II restriction site and positive results for both Phy-SLPCR and hybridization with SL3′ probe [4,7,14].

In this study we classified 21 new isolates from fruit saps and seeds at the genus level by analysis of morphology, growth features and biochemical and molecular markers. Most isolates presented promastigote forms except Tom274c, which showed choanomastigotes. This isolate exhibited the shortest GT, highest cell density and ARG activity, hybridized with the SSU3 probe and was found to be negative when probed with SL3′ and tested by Phy-SLPCR. Thus, the Tom274c isolate possesses all the taxonomic markers indicative of a *Crithidia* species. The Pin772 isolate from pineapple, as well as the previously characterized maize isolate Mai251, showed OCT activity. This enzymatic activity, although previously defined as a characteristic of *Herpetomonas*, has already been detected in *Phytomonas* [4,8,17]. Although typical opisthomastigotes were not detected in the Pin772 culture, this isolate may be a member of *Herpetomonas*, given that it lacks ARG and presents OCT activity which was never detected in *Leptomonas* and, in addition, lacks all DNA markers of *Phytomonas* (Table 2). However, further studies are needed for its definitive generic classification.

Seven isolates with promastigote forms showed ARG activity, lacked OCT and did not present any molecular marker of *Phytomonas*, suggesting that they can be classified as *Leptomonas*. These isolates were divided into two groups on the basis of biochemical and molecular variability: (A) consisting of three isolates with the longest GT (average GT of 13.3 h), CH activity and presence of the SSU/360 *Pvu*II site, and (B) consisting of four tomato isolates exhibiting an average GT of 8.6 h and lacking both CH activity and the SSU/360 *Pvu*II site. Thus, as observed by Camargo et al. [11], our findings again showed high genetic variability among isolates classified as *Leptomonas* using the available taxonomic parameters, indicating that this taxon is probably constituted by isolates not very closely related.

However, most promastigote cultures were negative for ARG and thus need to be distinguished as *Leptomonas*, *Herpetomonas* or *Phytomonas*. Thus, generic classification of promastigote isolates requires additional taxonomic criteria besides morphology and enzymatic profiles of the urea cycle.

Among the 20 new promastigote isolates, 13 lack ARG activity. Nine isolates from sap of fruits (tomato, plum, pomegranate, orange, grape and mulberry) and three from seeds (maize, beans and annato) failed to hybridize with the SSU3 probe, were amplified by Phy-SLPCR and were recognized by the *Phytomonas*-specific mAbs. Thus, all taxonomic parameters allowed us to consider them as belonging to the genus *Phytomonas*. We previously demonstrated by RAPD analysis that the genus *Phytomonas* is a complex taxon with different subgroups consisting of isolates clustered according to both plant tissue tropism and geographic origin. In this study, isolates from Southern Brazil were segregated into two clusters, with all those of fruits and insects from Paraná State located in cluster A [24]. In agreement with this study, all new isolates identified as *Phytomonas*, which were all isolated in Paraná, were classified into subgroup A by analysis of RAPD patterns.

Comparison of our data with those obtained by isoenzyme analysis [10] revealed that in both studies isolates Tom9, Tom10, Mai251 and Ann412 were classified as *Phytomonas* and were found to be genetically related to *P. serpens*. In contrast, our data did not show any characteristic that enables us to classify the isolates Tom270

and Tom274a as *Phytomonas*, whereas isolate Bea492 showed all the requisites for classification into this genus.

Experimental infections of tomato fruits [26] and maize grains [27] showed intense multiplication of trypanosomatids of different genera with long-term infections, indicating that they are able to develop in these hosts. The flagellates are restricted to fruit saps and seeds and thus do not cause a permanent plant infection. However, the infection seems to make fruits and grains more susceptible to secondary infections by other microorganisms and may reduce the viability of seeds and grains. Experimental infections also demonstrated that distinct trypanosomatid genera could be transmitted between maize and phytophagous insects, where they also develop, suggesting that the monoxenic genera *Leptomonas*, *Herpetomonas* and *Crithidia* can also undergo heteroxenic cycles in plants [27].

In this study we demonstrated that trypanosomatids of genera other than *Phytomonas* are very common in fruits, to which they could be transmitted by phytophagous insects. Only 57% of fruit isolates belonged to the genus *Phytomonas*. By contrast, in seeds we detected exclusively species of this genus. Together, morphological, biochemical and molecular markers showed high generic diversity, permitting the classification of the 21 new isolates as *Phytomonas* (13), *Leptomonas* (six), *Crithidia* (one) and, eventually, *Herpetomonas* (one). The diversity at the species level among these isolates is currently being examined by SL and SSU rRNA gene sequencing.

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